EFFECTS OF NOVEL FOOD PROCESSING METHODS ON ALLERGENIC AND NUTRITIONAL ATTRIBUTES OF KIWIFRUIT COMPONENTS

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ABSTRACT

Kiwifruit is rich in bioactive components, including dietary fibers, carbohydrates, vitamins, minerals, omega-3 fatty acids, and antioxidants. These components are beneficial to boost the human immune system contributing to the prevention of cancers and heart diseases. However, kiwifruit is becoming one of the most common plant-based food allergens worldwide after soy and peanut. Kiwifruit allergy can trigger a series of symptoms ranging from oral allergy syndrome to life-threatening anaphylaxis. Many factors such as variety, maturity, and storage conditions of fruit affect the allergenicity of kiwifruit. In this thesis, we have reported that major kiwifruit allergen, Act d 2, is mainly detected in the green kiwifruit, while no detectable level of Act d 2 was observed in golden kiwifruit. The Act d 2 allergen content of kiwifruit seeds is 2-fold and 4-fold higher when compared with that of pulp and peel, respectively. The Act d 2 content reached to its highest level in the ripe kiwifruit from the unripe stages to the overripe stages. Further, the results found that Act d 2 content showed a negative correlation with the ascorbic acid content and total antioxidant activity at various conditions. Thus, antioxidants are potentially important factors involved in the regulation of the expression of the Act d 2 allergen in kiwifruits.

To reduce the allergenicity of kiwifruit, microwave treatments (75 °C, 0-5 min) were applied to evaluate their effects on the nutritional properties and allergenicity of kiwifruit. The results found that the total antioxidant activity of kiwifruit, the digestibility, and peptide content of kiwifruit protein were significantly improved under the microwave processing (high temperatures and electromagnetic field). Whereas, a negative effect on the color attributes and sugar content of kiwifruit juice was obtained after microwave processing. Further, 5-min microwave treatment modified the secondary structures of kiwifruit proteins which resulted in an 80% reduction in the Act d 2 allergen content compared with the untreated samples. Thus, microwave processing showed a potential application in the reduction of kiwifruit allergenicity. Further, a novel non-thermal processing, high-intensity ultrasound (400 W, 25 kHz frequency,

0-16 min) has been analyzed in this thesis. The results have reported that ultrasound processing significantly increased phenolics and flavonoids content and total antioxidant capacity compared to the untreated kiwifruit juice. Also, high-intensity ultrasound processing significantly improved the color attributes (a*, b*, and YI), digestibility, peptide content, and rheological characteristics (flow and viscoelastic behavior) of kiwifruit juice. Further, the major kiwifruit allergen, Act d 2 content was significantly decreased (up to 50%) after ultrasound processing. Thus, high-intensity ultrasonication could be considered as an alternative processing method not only in improving bioactive compounds of kiwifruit juice, but also in reducing the allergenicity of kiwifruit.

Apart from the experimental work, we have also used Molecular Dynamics (MD) simulations to evaluate the structural changes of kiwifruit allergen, Act d 2 when the external physical stresses (high temperature and electric field) are applied. The results showed that Act d 2 is a heat-stable protein, while the combination of thermal and electric field (0.05 V/nm, 2450 MHz) significantly changed its secondary structure, especially when the processing temperature reached 375 K. Further, the surface area and molecular structure of Act d 2 showed significant changes under this combination treatment. Therefore, the combination of a thermal and electric field might be considered as a novel processing method in the reduction of food allergenicity.

RÉSUMÉ

Le kiwi est riche en composants bioactifs, notamment des fibres alimentaires, des glucides, des vitamines, des minéraux, des acides gras oméga-3 et des antioxydants. Ces composants sont bénéfiques pour renforcer le système immunitaire humain en contribuant à la prévention des cancers et des maladies cardiaques. Cependant, le kiwi devient l'une des sources d'allergènes alimentaires d'origine végétale les plus courantes, après le soja et l'arachide. L'allergie au kiwi peut déclencher une série de symptômes allant du syndrome d'allergie orale à l'anaphylaxie menaçant le pronostic vital. De nombreux facteurs tels que la variété, la maturité et les conditions de stockage du fruit affectent l'allergénicité du kiwi. Dans cette thèse, nous avons rapporté que l'allergène majeur du kiwi, Act d 2, est principalement détecté dans le kiwi vert, alors qu'aucun niveau détectable d'Act d 2 n'a été observé dans le kiwi doré. La teneur en allergènes Act d 2 des graines de kiwi est de deux fois et de quatre fois plus élevée que celle de la pulpe et de la peau, respectivement. La teneur en Act d 2 a atteint son plus haut niveau chez les kiwis mûrs, lorsque comparée à la teneur des stades non mûrs et des stades trop mûrs. Les résultats ont aussi démontré que la teneur en Act d 2 était négativement corrélée à la teneur en acide ascorbique et à l'activité antioxydante totale. Ainsi, les antioxydants sont des facteurs potentiellement importants dans la régulation de l'expression de l'allergène Act d 2 chez les kiwis.

Afin de réduire l'allergénicité des kiwis, des traitements par micro-ondes (75 ° C, 0-5 min) ont été appliqués pour évaluer leurs effets sur les propriétés nutritionnelles et l'allergénicité des kiwis. Les résultats ont montré que l'activité antioxydante totale du kiwi, la digestibilité et la teneur en peptides de la protéine du kiwi, étaient considérablement améliorées lors du traitement par micro-ondes (températures élevées et champ électromagnétique). Cependant, le traitement par micro-ondes a eu un effet négatif sur la couleur et la teneur en sucres du jus de kiwi. En outre, un traitement par micro-ondes de 5 minutes a modifié les structures secondaires des protéines de kiwi, ce qui a entraîné une réduction de 80% de la teneur en allergènes Act d 2 par rapport aux échantillons non traités. Ainsi, le traitement par micro-ondes a montré une application potentielle pour la réduction de l'allergénicité du kiwi.

Un nouveau traitement, non thermique, par ultrasons de haute intensité (400W, 25 kHz, 0-16 min) a été utilisé dans cette étude. Les résultats ont montré que le traitement par ultrasons augmentait significativement la teneur en composés phénoliques et en flavonoïdes, et la capacité antioxydante totale par rapport au jus de kiwi non traité. Le traitement par ultrasons à haute intensité a considérablement amélioré les attributs de couleur (a *, b * et YI), la digestibilité, le contenu en peptides et les caractéristiques rhéologiques (écoulement et comportement viscoélastique) du jus de kiwi. De plus, le principal allergène du kiwi, l'Act d 2, était significativement réduit (50%) après le traitement par ultrasons. Ainsi, les ultrasons à haute intensité pourraient être considérés comme une méthode de traitement alternative non seulement pour améliorer les composés bioactifs du jus de kiwi, mais également pour réduire le pouvoir allergène des kiwis.

Des simulations de la dynamique moléculaire (DM) ont été utilisées pour évaluer les modifications structurelles de l'allergène du kiwi lorsqu'il était soumis à des contraintes physiques externes (température élevée et champ électrique). Les résultats ont montré que l'exposition à la combinaison des champs thermique et électrique (0,05 V / nm, 2450 MHz) modifiait de manière significative la structure secondaire de l'Act d 2, en particulier lorsque la température de traitement atteignait 375 K. En outre, la surface et la structure moléculaire de Act d 2 ont montré des changements significatifs sous ce traitement combiné. Par conséquent, la combinaison des champs thermique et électrique devrait être considérée comme une nouvelle méthode de traitement permettant la réduction de l'allergénicité des aliments.

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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:

"As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis).

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. 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 conclusion and summary;

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers".

CONTRIBUTION OF AUTHORS

The following are the manuscripts (published/submitted/being prepared for submission) whose content is used for completing this thesis:

1. **Wang J**., Vanga, S. K., Raghavan, V. Effect of pre-harvest and post-harvest conditions on the fruit allergenicity: A review. (2017). Critical Reviews in Food Science and Nutrition.59(7):1027-1043. [Chapter I & Chapter II]
2. **Wang J**., Vanga, S. K., McCusker, C., Raghavan, V. A Comprehensive Review on Kiwifruit Allergy: Pathogenesis, Diagnosis, Management and Potential Modification of Allergens Through Processing. (2019). Comprehensive Review in Food Science and Food Safety.18(2), 500-513. [Chapter I & Chapter III]
3. **Wang J**., Raghavan, V. Effect of variety, maturity and storage conditions on the allergenic potential of kiwifruit and its relationship with antioxidant activity. (2019). LWT-Food Science and Technology. (Under review). [Chapter IV]
4. **Wang J**., Raghavan, V. Effect of microwave treatment on the nutritional properties and allergenic potential of kiwifruit. (2019). (to be submitted). [Chapter V]
5. **Wang J**., Vanga, S. K., Raghavan, V. High-intensity ultrasound processing of kiwifruit juice: Effects on the ascorbic acid, total phenols, flavonoids and antioxidant capacity. (2019) LWT-Food Science and Technology.107: 299-307. [Chapter VI]
6. **Wang J**., Vanga, S. K., Raghavan, V. High-intensity ultrasound treatment of kiwifruit juice: Effects on the microstructure, pectin, carbohydrates and hydrodynamic properties. (2019). Food Chemistry. (Accepted) [Chapter VI]
7. **Wang J**., Raghavan, V. Effect of ultrasound processing on the digestibility, secondary structure and allergenic potential of kiwifruit proteins. (to be submitted). [Chapter VI]
8. **Wang J**., Vanga, S. K., Raghavan, V. Potential utility of thermal and electric field modification on the secondary structure of Act d 2 kiwifruit allergen. (2019). Food & Function. (Accepted) [Chapter VII]

The work reported above was designed and performed by Jin Wang. Jin Wang performed preliminary data analysis and composed the manuscripts. Dr. Vijaya Raghavan as the supervisor provided all the suggestions for the project and edited the final version of the manuscripts. Dr. Sai Kranthi Vanga reviewed the manuscripts and provided suggestions for the final version of the text. Also, he has provided technical help with regard to the molecular modeling software, GROMACS. Dr. Christine McCusker, working at Montreal Children Hospital, contributed critical feedback to the concept, design, and language of the manuscripts related to food allergies based on her medical research background.

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ABBREVIATION

IgE	Immunoglobulin E
OAS	Oral Allergy Syndrome
nsLTPs	non-specific Lipid Transfer Proteins
LTPs	Lipid Transfer Proteins
MAP	Modified Atmosphere Packaging
Bp	Base Pair
PPO	Polyphenol Oxidase
SPT	Skin Prick Test
DCs	Dendritic Cells
IP	Integrated Production
OC	Organic Cultivation/Organic Production
DM	Dry matter
WHO/IUIS	World Health Organization and International Union of Immunological Societies
AAFA	Asthma and Allergy Foundation of America
DBPCFC	Double Blind Placebo Controlled Food Challenge
ELISA	Enzyme-linked Immunosorbent Assay
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FPS	Food Pollen Syndrome
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PR-protein	Pathogenesis-Related Protein
Rt-PCR	Real-time Polymerase Chain Reaction
USDA	United States Department of Agriculture

MD	Molecular Dynamics
US	Ultrasonication/Ultrasound processing
MW	Microwave
SASA	Solvent Accessible Surface Area
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuations
FTIR	Fourier Transform Infrared Spectroscopy
IVPD	*In vitro* Protein Digestibility
CD	Circular Dichroism
VMD	Visual Molecular Dynamics
POD	Peroxidase
PPO	Polyphenol oxidase
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
ELISA	Enzyme-Linked Immunosorbent Assay
FRAP	Ferric-reducing/antioxidant power
TPTZ	2,4,6-Tripyridyl-S-triazine
TCD	Total Color Difference
YI	Yellow Index
TP	Total Protein
TAC	Total antioxidant capacity
AA	Ascorbic Acid

CHAPTER I

INTRODUCTION

Kiwifruit (*Actinidia*) is a wild plant that was first found in China and hence named 'Chinese gooseberry'. There are around 55 species cultured including green kiwifruit (A. *deliciosa*), golden kiwifruit (A. *chinensis*), hardy kiwifruit (A. *arguta*), Chinese egg gooseberry (A. *coriacea*), and hearty red kiwifruit (A. *purpurea*) [1,2]. At their commercial harvesting stage, the fruits have a soft texture and a sweet but unique flavor. Kiwifruit is an excellent source of dietary fibers, carbohydrates, vitamins (e.g., Vitamins C and E), minerals (e.g., calcium, magnesium, potassium, and phosphorus), omega-3 fatty acids and antioxidants (e.g., phenols). Recent studies have demonstrated that these bioactive components are beneficial to boost the human immune system and can prevent cancers and cardiovascular diseases [3,4]. The demand for kiwifruit and its derived products has been increasing rapidly due to its health-related benefits. Furthermore, kiwifruit has considerable economic value. In 2016, the Food and Agriculture Organization of the United Nations (FAO) reported that global production of kiwifruit amounted to 4.3 million tonnes, which is valued at 12.3 billion US dollars (www.FAO.org).

However, kiwifruit is a common elicitor of food allergies all over the world. Kiwifruit allergy represents the fourth most common food allergy in France, after milk, egg, and peanuts [5,6]. In a survey, among 182 children studied with a history of hypersensitivity to one or more foods suggested that 9% of the population has reported allergic reactions to kiwifruit [7]. In Sweden and Denmark, nearly half of food allergic patients self-reported allergy to kiwifruit [8]. In Spain, approximately 1.8% of the total population is allergic to kiwifruit [9]. The percentage of the population who is allergic to kiwifruit reaches up to 2% in North America [10].

Further, many studies also have reported that the sensitivity associated with kiwifruit ingestion may be related to pollen protein cross-reactivity due to the sharing of similar protein structures between the fruit and the pollens [11]. To date, estimates show that 20-40% of the population around the world is allergic to pollen [12]. Recently, in a study, 55 patients (female: male=39:16)

with kiwifruit allergy diagnosed by oral food challenge test were recruited. Of these subjects, 78% had an allergic cross-reactivity to other plant foods, especially to the foods belonging to the Rosaceae family (50.1%), tree nuts (41.8%), and peanuts (30.9%) [13]. Based on the data related to pollen and latex allergy cross-reactivity, we can conclude that the number of people susceptible to kiwifruit allergy is rising.

A wide range of factors such as variety, maturity, storage conditions and components of fruit could have an impact on the final allergenicity level at the time of consumption. López-Matas et al., reported that the protein profile and allergen quantification of six varieties of tomatoes (Rama, Rambo, Canario, Kumato, Pera and Raf) were different [14]. The highest allergen content (Lyc e 3) was shown in the 'Rambo', followed by 'Pera', and 'Canario'. The 'Rama' showed the lowest value of allergen content. A study found that apples showed a higher Mal d 1 content when they were ripe than that of unripe apples [15]. Similarly, Sancho et al., reported that the apples that were picked at later stages of maturity showed 3-4 times higher allergen content (Mal d 1) [16]. Further, research found that one of major apple allergens, Mal d 1 had a significant increase during modified atmosphere packaging (MAP) use for storage [16]. Moreover, many researchers stated that allergic components were determined at present not only in the peel but also in the pulp of fruits [17,18]. In the cultivar 'Tommy Atkins', Knödler et al., found that main mango allergen, alk(en)ylresorcinol content in the peel was 419.30 mg/kg which is 16 times higher than that in the pulp (26.33 mg/kg) [18]. In apples and peaches, the allergic reaction to peel was found to be higher than that of the pulp [19,20]. Also, recent research results found that apple allergen content had a relationship with antioxidant activity and enzyme activity. Higher polyphenol oxidase (PPO) activities and polyphenolic content resulted in less Mal d 1 whereas, higher antioxidant activity inhibited the interaction between oxidized phenols and Mal d 1, resulting in lower allergenicity (Mal d 1 content) [15].

Among many investigations, there are few studies that report on kiwifruit allergy linking allergenicity among different varieties, maturities, and components of fruit (e.g. peel, pulp and seeds). Applications of processing techniques on the reduction in kiwifruit allergenicity are also very limited. Ultrasound treatment, especially high-intensity (10-1000 W cm^{-2}) ultrasound has gained significant attention in the food processing industry due to its production of high-power, high-intensity, and low-frequency ultrasonic waves. It could result in altering material

properties such as physical disruption and chemical reactions through the generation of immense pressure, shear force and temperature [21]. This innovative emerging technology could be considered as an alternative food processing technique in reducing kiwifruit allergenicity without affecting its nutritional properties. In addition, microwave treatment was applied in the study as a thermal processing method to compare its effects with ultrasound processing.

1.1. HYPOTHESIS AND IMPLICATIONS

In spite of the increasing demand for kiwifruit and /or its related products, there are very limited studies reported on kiwifruit allergy, especially on the changes in allergenicity as a function of different varieties, maturities, storage conditions and fruit components. The effects of thermal (microwave) or non-thermal processing (ultrasound) on the nutritional properties and allergenicity of kiwifruit are also scarce. The hypotheses of this study are described as follows:

- Green kiwifruit has a higher allergen (Act d 2) content compared to golden kiwifruit.
- Seeds of kiwifruit contain the highest allergen (Act d 2) content followed by its peel and pulp samples.
- Non-thermal processing like ultrasound not only can improve the fruit juice quality but also can reduce the kiwifruit allergenicity.

This thesis attempts to provide a deeper understanding of ultrasonication and microwave applications on the nutritional properties and allergenicity of kiwifruit. The results of the study would help in evaluating the relationship between kiwifruit allergenicity and its variety, maturity, storage conditions, and fruit components. Further, it also can help to assess whether or not these processing techniques (microwave and ultrasound treatments) can be an alternative method for the reduction of kiwifruit allergenicity in its derived products.

1.2. OVERALL OBJECTIVE

The overall objective of this study is the evaluation of physiological characteristics (e.g., variety, maturity, fruit components), storage conditions, and processing techniques (e.g. microwave and ultrasonication) on the nutritional properties and allergenicity of kiwifruit.

1.3. SPECIFIC OBJECTIVES

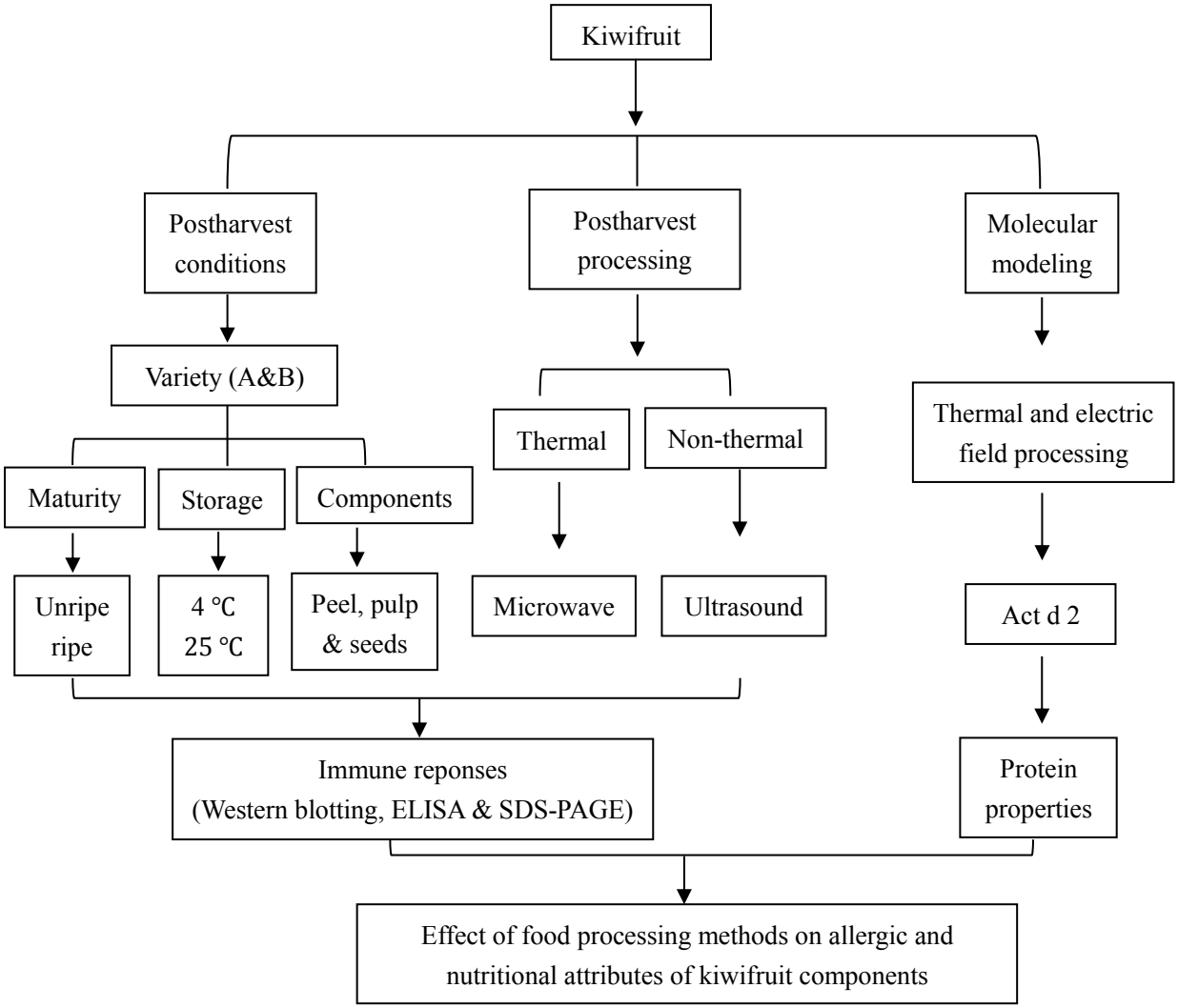


Figure 1.1. The schematic representation of study objectives and its use in experimental designs.

The specific objectives of the study are as follows:

i) To reveal the changes of kiwifruit allergen (Act d 2) content among different varieties, maturities, fruit components (peel, pulp and seeds), and storage conditions.

ii) To investigate the impacts of microwave treatment on the nutritional properties and allergenicity of the kiwifruit.

iii) To evaluate the influences of ultrasonic treatment on the nutritional properties and allergenicity of the kiwifruit.

iv) To evaluate the changes of protein (kiwifruit allergen, Act d 2) structures during food processing through molecular dynamics simulation.

CHAPTER II

LITERATURE REVIEW-PART 1

EFFECTS OF PRE-HARVEST AND POST-HARVEST CONDITIONS ON THE FRUIT ALLERGENICITY

2.1. ABSTRACT

Fruits are an essential source of vitamins and antioxidants that can effectively delay aging and contribute to the health and well-being of humankind. However, they are growing to be one of the primary elicitors of food allergies around the world. Fruit allergens can induce an IgE-mediated (Immunoglobulin E) reaction, presenting with a symptom like localized oral allergy syndrome (OAS). Numerous studies showed that varying environmental and cultivation conditions can influence the fruit allergen content during flowering and ripening stages. Further, the variety, harvesting maturity, and storage conditions can also significantly affect the allergenicity potential. For example, unripe apples and tomatoes have lower levels of allergens compared to ripened fruits. Researchers have also reported that modified atmosphere packaging (MAP) can help reduce Mal d 3 content present in apples during storage. Post-harvest processing like peeling is also considered an excellent method to help reduce the overall allergenicity in few fruits whose peel might contain the majority of the allergens. This review will discuss the overall influence of both -harvest and post-harvest factors on the fruit allergens. We will also discuss the progress regarding the cause, symptoms and diagnostic methods of fruit-based allergies.

KEYWORDS: Fruit allergy; cross-reactivity; storage; pollen allergy; harvesting maturity; modified atmosphere package

2.2. INTRODUCTION

Food allergy is a widespread phenomenon: An estimated 2-5% of the adult population and up to 8% of the children and infants suffer from some types of food allergy[22,23]. Fruits are considered to be among the primary elicitors of food allergy in humans [24]. Recent data suggested that pollen allergies affect approximately 40% of the population all over the world [12]. Due to the similarity between the allergens present in various pollens and fruits, the individuals sensitive to pollen can have cross-reactivity to various fruits and latex allergens. It has been reported that up to 70% of birch pollen allergic people had allergenic reactions after consuming raw fruits [25]. Fruit allergy is an IgE-mediated (Immunoglobulin E) reaction and is the result of a sensitization process occurring in the gastrointestinal tracts [26]. Many reports show that fruit allergies cause a wide range of symptoms, mostly limited to localized oral allergy syndrome (OAS) [27,28]. Many of the consumed fruits from around the world, are known to trigger allergic reactions in sensitive individuals [29]. A survey was conducted in France, containing a total of 182 children varying in the ages from 2-14 years, who are reported to be allergic to one or more fruits. Of these children, 12% were reported to be hypersensitive to kiwis, 5.5% to tomatoes, 4.4% to strawberries, 3.8% to pineapples, 2.7% to oranges and about 1.2% to apples [7].

However, fruits are a vital part of our daily life due to the presence of rich essential nutrients and health-promoting bioactive compounds. For example, strawberry fruit is a source of vitamins, phenolic compounds (i.e. flavonols, flavones, and anthocyanins) and various biofactors, which in turn can delay aging, resist cancer, and prevent infections[30,31]. Also, strawberry is appreciated worldwide for its unique flavor [24,32]. However, various studies reported that allergenic protein, Fra a 1 present in strawberries could cause discomfort and allergic symptoms [33,34].

Many studies have shown that a wide range of factors such as storage conditions, variety, and harvest maturity of fruit could have an impact on the final allergenicity level at the time of consumption. Studies found that one of major apple allergens, Mal d 1 had a significant increase during modified atmosphere packaging (MAP) use for storage [16]. While the content of another major allergen like Mal d 3 present in apples was lower when stored under the

controlled atmosphere conditions (0.5% O_2, 1.5% CO_2 at 3.8℃) compared to those were kept under room temperature (Sancho et al., 2006b). Moreover, López-Matas et al. (2011) reported that the protein profile and allergen quantification of six varieties of tomatoes (Rama, Rambo, Canario, Kumato, Pera and Raf) were totally different. The highest allergen content (Lyc e 3) was shown in the 'Rambo', followed by 'Pera', and 'Canario'. The 'Rama' showed the lowest value of allergen content. Also, Schmitz-Eiberger and Matthes (2011) reported that the Mal d 1 content was the highest in 'Topaz' apples, followed by 'Golden Delicious' and 'Braeburn'. Studies were also done to evaluate the impact of harvest maturity on fruit allergen content. A study found that apples showed a higher Mal d 1 content when they were ripe than that of unripe apples (Schmitz-Eiberger and Matthes, 2011). Similarly, Sancho et al., (2006b) reported that the apples that were picked at later stages of maturity showed 3-4 times higher allergen content (Mal d 1). Further, research found that apple allergen content had a relationship with the antioxidant activity and enzyme activity. Higher polyphenol oxidase (PPO) activities and polyphenolic content resulted in less Mal d 1 whereas, higher antioxidant activity inhibited the interaction between oxidized phenols and Mal d 1, resulting in lower allergenicity (Mal d 1 content) (Schmitz-Eiberger and Matthes, 2011). Similarly, Tulipani et al., (2011) found that strawberry allergen Fra a 1 content decreased with an increase in the total antioxidant capacity, total phenolic content and total flavonoid content in 'Adria', 'Sveva', 'AN94.414.52' and 'AN00.239.55', varieties of strawberry.

This review will report the available information about the cause and symptoms of various fruit allergies and also detail the diagnostic methods and related proteins responsible for causing allergic reactions. Finally, this review will discuss the influence of various factors including cultivars, harvesting maturity, the difference in the peel and pulp of the fruit, storage conditions, and different environmental cultivation conditions i.e. nitrogen, water shortage and climatic factors on the fruit allergenicity. Hopefully, it can provide new ideas for the growers, breeding industry, and consumers who are suffering from one or more types of fruit allergies.

2.3. CAUSES, SYMPTOMS AND DIAGNOSIS OF FRUIT ALLERGIES

2.3.1. Causes

Fruit allergies are often caused by profilins present in a wide range of fruits and can trigger hypersensitivity because of conflicting immune feedback to dietary antigens which are often difficult to avoid [35,36]. These allergens are kinds of specific antigens presenting with the capacity to induce IgE-mediated reactions. The allergenic proteins have certain site(s) which can combine with the specific antibodies; these sites are called epitopes with linear or conformational features [37]. After the first consumption of fruit, the specific allergens are recognized by certain antigen presenting in cells especially dendritic cells (DCs) of lamina propria in the intestine [38]. The allergens are incorporated by DCs because of receptor-mediated endocytosis process, macropinocytosis or phagocytosis [39]. When an allergy-prone person has a secondary exposure to the same allergen, a hypersensitive reaction is presented with a series of symptoms, and can even lead to anaphylactic shock due to allergen exposure [37].

The profilins, small proteins causing allergies are usually found in pollens of grass (ex: mugwort), trees (ex: birch) and weeds (ex: ragweed) other than in fruits and vegetables [40]. These allergens from various sources mentioned above have a similar structure to the profilins found in various fruits. Hence, in the case of patients with tree-pollen allergies, their immune systems cannot recognize the difference between allergens present in birch pollen and apples because of their similar three-dimensional structures [41]. Ebner et al., (1991) reported that proteins present in birch pollen and apples share the common allergenic structures (i.e. Epitopes) by using western and northern blotting techniques. For example, apple allergen (Mal d 1), pear allergen (Pyr c 1) and cherry allergen (Pru av 1) can induce birch pollen-related fruit allergies i.e. their structural conformation is similar to that of allergens present in birch pollen and hence can have similar epitopes leading to cross-reactivity in hypersensitive patients [42].

In patients who suffer from hay fever, the immune system would recognize profilins present in pollens as harmful proteins which can trigger an allergic reaction. This condition is referred to as OAS which is considered a form of contact allergy that is confined almost solely to the oropharynx and hardly affects other target organs [43]. In about 35% of people suffering from hay fever might be due to an allergy to profilins. They may find that they experience mild

allergy symptoms after eating fruits to which they are sensitive or that contain allergens similar to their counterparts in pollen. Some people with OAS only reacts to one or two fruits, while others are allergic to a wide range of products. Several of the most common culprits include apples, strawberries, pears, tomatoes, cherries, peaches, plum, kiwifruit, and melon [29].

2.3.2. Symptoms

Table 2.1. Allergic symptoms on consumption of different fruits.

Allergen sources	Symptoms	References
Mango	A two-day history of patchy pruritic erythema of the face, neck, and arms with periorbital edema, systemic shock occurs according to the level of severity.	49 50
Kiwifruit (Act d 1, Act d 2)	Causes allergic asthma, urticaria, allergic purpura, allergic dermatitis, itching and difficulty in breathing.	51 52
Apple (Mal d 1, Mal d 3)	Causes OAS, which is generally restricted to the lips, tongue, and throat.	16 82
Strawberry (Fra a 1)	Allergic symptoms presenting with tightness of the throat, swelling, burning or prickling sensation in the lips or tongue and other parts of the oral cavity.	34 33

Symptoms of allergy usually arise just several minutes after eating the fruit, although they can sometimes take up to two hours to manifest itself. These symptoms often can be settled down within one hour. Fortunately, profilins are easily inactivated during the cooking process and by digestion, meaning the immune system can no longer recognize them. Common fruits allergy symptoms can be divided into mild oral and systemic reactions. The former is referred to as OAS which is considered a form of contact allergy and its symptoms include mild itching and swelling [43-45]. The symptoms of systemic reaction can be observed in certain organs, such as

skin (local or systemic urticaria, hereditary allergic eczema), gastrointestinal tract (abdominal cramps, diarrhea, vomiting), nose and lung (rhinitis and asthma), cardiovascular system (anaphylactic shock) [46-48].

Many studies have reported where these symptoms vary among different varieties of fruits. As shown in **Table 2.1**, the patients showed an allergic contact dermatitis and presented a two-day history of patchy pruritic erythema of the face, neck, and arms with periorbital edema after eating mango [49,50], and the symptom of systemic shock occurs according to the level of severity. Kiwifruit can cause allergic asthma, urticaria, allergic purpura, allergic dermatitis, itching and difficulty in breathing [51,52]. After eating raw or fresh apples, the main allergen (i.e. Mal d 1) present in apples causes OAS, which is generally restricted to the lips, tongue, and throat [16]. In strawberry, Fra a 1 (a Bet v 1-homologous allergen), was identified as a major allergen [33,34] which causes allergic symptoms presenting with the tightness of the throat, swelling, burning or prickling sensation in the lips or tongue and other parts of the oral cavity after consuming the fruit.

2.3.3. Diagnosis of allergy

Nowadays, there are several common methods for the diagnosis of fruit allergies such as skin prick tests and blood tests. The skin prick test (SPT) is one of the primary diagnostic methods and can be taken through using various solutions of suspected allergens during the first consultation. SPT produces reactions for an immediate type of allergies leads to determination. Hence, it determines allergy by the reaction of a patient's skin to different substances based on medical history and clinical symptoms. The principle of SPT is when certain allergens are contacted into the skin, specific IgE combining with the surface receptors present in mast cells are cross-linked resulting in mast cell degranulation, and mediators (i.e., histamine) being released [53], which leads to corcles looking like a wheal. The subjects of the skin prick test are mostly children compared to adults when the total numbers are considered. The processing time of a test is generally short and evaluated within 15-20 minutes. Also, it is very sensitive and low risk with no reported fatalities as reported in a study of five years in the USA [54,55].

Table 2.2. Common allergenic fruits and their main allergens (www.allergen.org).

Species	Major allergen	MW(SDS-PAGE)	Year	Biochemical name
Apple	Mal d 1	17 kDa	2003	Pathogenesis-related protein (PR-10)
(*Malus domestica*)	Mal d 2	23 kDa	2003	Thaumatin-like protein
	Mal d 3	9 kDa	2003	Non-specific lipid transfer protein
Peach	Pru p 1	18 kDa	2007	Pathogenesis-related protein (PR-10)
(*Prunus persica*)	Pru p 2	25 kDa	2010	Thaumatin-like protein
	Pru p 3	10 kDa	2003	Non-specific lipid transfer protein
	Pru p 4	14 kDa	2003	Profilin
Strawberry	Fra a 1	18 kDa	2006	Pathogenesis-related protein (PR-10)
(*Fragaria ananassa*)	Fra a 3	9 kDa	2006	Non-specific lipid transfer protein
	Fra a 4	13 kDa	2006	Profilin
Sweet cherry	Pru av 1	9 kDa	2003	Pathogenesis-related protein (PR-10)
(*Prunus avium*)	Pru av 2	30 kDa	2003	Thaumatin-like protein
	Pru av 3	10 kDa	2003	Non-specific lipid transfer protein
	Pru av 4	15 kDa	2003	Profilin
Kiwifruit	Act d 1	30 kDa	2007	Cysteine protease (actinidin)
(*Actinidia deliciosa*)	Act d 2	24 kDa	2007	Thaumatin-like protein
	Act d 4	11 kDa	2007	Phytocystatin
	Act d 5	26 kDa	2007	Kiwellin
	Act d 11	17 kDa	2009	Bet v 1 family member

However, during the skin prick test, it would cause a slight discomfort because of itching; and it is difficult to perform in patients with eczema or dermatographism. Blood test detects specific IgE antibodies by using a radioimmunoassay test. A single needle is used to obtain blood samples, and the needle stick is often more gentle than other skin tests. After allergy testing, an elimination diet is usually designed by medical professionals. It would remove one or more food items from the patient's diet for several days. Then, this eliminated food group are determined if certain food allergic reactions develop again. Therefore, an elimination diet may also be used as one of the diagnostic methods to help identify if a person has food allergies or not. An allergist or dietician must be involved in designing the diet to prevent imbalances in nutrient intake. It is necessary to read food labels carefully for people who are allergenic to a certain food. In some countries, they already enacted the related law to protect those people with food allergy histories. For examples, in the year of 2004, the Food Allergen Labeling and Consumer Protection Act (United States) mandated that nutritional labels on food packages should plainly identify the eight specified food allergen sources including milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans if present.

2.4. FRUIT ALLERGENS

2.4.1. Common allergenic fruits and their allergens

A wide variety of fruits have been reported to cause allergic reactions which are listed in the database (www.allergen.org) of World Health Organization and International Union of Immunological Societies (WHO/IUIS). However, the most prevalent and widely studied are reactions to apple, peach, strawberry and kiwifruit. As shown in **Table 2.2**, several fruit allergens are present in each fruit and their size (molecular weights) are widely different.

2.4.2. Fruit allergen families

To date, many researchers have identified various plant-derived allergens which have been classified into specific groups, such as PR proteins due to their similar sequences and biological function [56]. PR proteins are regulatory proteins that are produced in the plant in response to a pathogenic bacterial attack [57]. Some of these proteins are antimicrobial and can protect the

plant through attacking the cell wall of a bacteria or fungi [58]. As shown in **Table 2.3**, researchers found that five members of the 14 member PR protein family can trigger allergic reactions in humans [56,59]. These five members are: β-1,3-glucanases (PR-2), class I chitinases (PR-3), thaumatin-like proteins (TLPS, PR-5), Bet v 1 homologous protein (PR-10) and nonspecific lipid transfer proteins (nsLTPs, PR-14).

Table 2.3. Allergens of pathogenesis-related proteins (Breiteneder, 2004; Hoffmann-Sommergruber, 2002).

Pathogenesis-related protein	Protein families	Examples
PR-2	β-1，3-glucanases	Grape (Vit v Glucanase)
PR-3	class I chitinases	Avocados (Pers a 1)
PR-5	TLPs	Apple (Mal d 2), cherry (Pru av2)
PR-10	Bet v 1 homologous	Apple (Mal d 1), strawberry (Fra a 1)
PR-14	ns-LTPs	Peach (Pru p 3), pear (Pyr c 3)

PR proteins play an major role in fruit allergy and can induce reactions in a wide variety of fruits, such as allergy to grapes (Vit v Glucanase), avocados (Pers a 1), apples (Mal d 1 and Mal d 2), cherries (Pru av 2), peach (Pru p 3), pear (Pyrc 3) and strawberries (Fra a 1) (**Table 2.3**). As the main function of these proteins is to protect the plant from external predators including pathogens, the development of disease and pathogen-resistant varieties for enhancing the yield in modern farming could have increased the plant's ability to produce these PR proteins in higher quantities. With this increased rate in production, the higher residual levels of these proteins could be responsible for causing hypersensitive reactions in humans. As mentioned earlier, main fruit allergens are from four different protein families, pathogenesis-related protein (i.e. PR-10), thaumatin-like protein, non-specific lipid transfer protein, and profilins. They have different characters and cause various fruit allergies (**Table 2.4**).

2.4.3. Pathogenesis-related (PR-10) proteins

The PR-10 proteins are widely present in seed plants. Most genes of the PR-10 proteins have

an 456-489 bp open reading frame which can encode a polypeptide with 151-162 amino acids [58]. PR-10 proteins are identified as intracellular pathogenesis-related proteins with homology to ribonucleases, including allergens from tree pollen and food [60]. Also, they are generally heat-labile proteins and hence are denatured when cooked or processed thermally for pasteurization and/or sterilization. They belong to the Bet v 1 homologues associated with local symptoms (e.g. OAS) and are usually found in strawberry (Fra a 1), peach (Pru p 1), apple (Mal d 1) and sweet cherry (Pru av 1) (**Table 2.4**).

Table 2.4. Main allergen protein family in fruits (www. allergen.org).

Protein family	Characteristics	Examples
Pathogenesis-related protein (PR-10)	Heat-labile proteins and cooked foods are therefore often tolerated. They are Bet v 1 homologues and often associated with local symptoms such as OAS.	Strawberry (Fra a 1) Peach (Pru p 1) Apple (Mal d 1)
Thaumatin-like proteins (TLPS)	Polypeptides of about 200 amino acid residues, defence-related PR proteins. It's unstable to heat and are easily destroyed by cooking.	Kiwifruit (Act d 2) Apple (Mal d 2) Peach (Pru p 2)
Non-specific lipid transfer proteins (ns-LTPs)	Stable to heat and digestion, causing reactions also to cooked fruit. Often associated with systemic and more severereactions in addition to OAS.	Strawberry (Fra a 3) Peach (Pru p 3) Apple (Mal d 3)
Profilin	Actin-binding proteins showing great homology and cross-reactivity even between distantrelated species. Recognized as a minor allergen in plants and plant-related foods. Profilinsare seldom associated withclinical symptoms.	Kiwifruit (Act d 9) Mango (Man i 3) Apple (Mal d 4) Strawberry (Fra a 4)

2.4.4. Thaumatin-like proteins (TLPs)

The thaumatin-like proteins (TLPs) are polypeptides with 200 amino acid residues and are named based on the similar sequences to the sweet-tasting protein in *Thaumatococcus daniellii* [61]. There are three types TLPs that are included those produced due to pathogen infection, and osmotins produced in response to osmotic stress, and antifungal proteins present in cereal seeds [59]. In some species, TLPs are expressed in flowers and fruits that are susceptible to pathogen infection and it is hypothesized that they play a primary function in the defense mechanism against infections [62,63]. Recently, TLPs are becoming a rich source of allergens and are usually found in peach, apple, grape and kiwifruit [64]. For example, one of the major apple allergens, Mal d 2 which is a kind of TLP with a molecular mass of 23 kDa. It can cause IgE-mediated reactions in patients allergic to apples. Similarly, Pastorello et al. (2003) found that a 24 kDa TLP which is classified as a minor allergen in grapes (Pru av 2) have a highly similar amino acid sequence to the apple allergen (Mal d 2) (**Table 2.4**).

2.4.5. Non-specific lipid transfer proteins (ns-LTPs)

The non-specific lipid transfer proteins (ns-LTPs) are basic, 7-9 kDa proteins which are widely distributed throughout the plant kingdom, comprising over 100 potential sources from 50 different species of plants. They are also able to bind and transfer various lipids among membranes *in vitro* due to their three-dimensional structure and affinity towards lipids [65,66]. Douliez et al., (2000) identified that there are two main types of plant nsLTPs; nsLTP1 of the molecular mass of 9 kDa and nsLTP2 of 7 kDa. The disulfide bond linkages are located in between Cys_1-Cys_6 and Cys_5-Cys_8 of nsLTP1, wheal these disulfide bond linkages are at Cys_1-Cys_5 and Cys_6-Cys_8 present in nsLTP2. Another main structural difference is the size of the hydrophobic cavity which is usually larger in nsLTP1compared to nsLTP2 protein [67]. Many researchers have stated that nsLTPs show an essential biological function which includes mediating phospholipid transfer in plant defense system [68]. nsLTPs are also associated with maintenance of hydrophobic cell wall and their absence can disrupt the basic structure and functioning of the cell wall. They also facilitate the extension of cell walls when required depending on the surrounding environmental conditions [69].

Table 2.5. Properties of allergens in different fruits. # A, F, G, I, L, M, P, V and W are categorised as hydrophobic amino acids, C, N, Q, S, T and Y are categorised as polar amino acids and D, E, H, K and R are categorised as charged amino acids. A: Alanine; C: Cysteine; D: Aspartic acid; E: Glutamic acid; F: Phenylalanine; G: Glycine; H: Histidine; I: Isoleucine; K: Lysine; L: Leucine; M: Methionine; N: Asparagine; P: Proline; Q: Glutamine; R: Arginine; S: Serine; T: Threonine; V: Valine; W: Tryptophan; Y: Tyrosine.

Fruit	Allergen	MW (kD)	Ig-E banding Epitopes	References
Strawberry	Fra a 1	18	47GDGGPGTIK55[#]	Muñoz et al., 2010
Cherry	Pru a 2	30	157ANVNAVCPSELQ168	Schein et al., 2007
Tomato	Lyc e 2	50	148ANINGECPRALK159	Schein et al., 2007
Kiwifruit	Act c 2	24	56CNFDGAGRGKCQTG69	Sharma et al., 2013
			127TADINGQCPNELRAPGGCN146	
Apple	Mal d 3	9	16YVRSGGAVP24	Borges et al., 2008
			35INGLARTADRQ46	
			76NVPYKISTS84	
Peach	Pru p 3	10	11APCIPYVRGGGAVPP25	García-Casado et al.,
			31IRNVNNLARTTPDRQ45	2003; Zuidmeer
			71GKCGVSTPYK80	et al.,2006
Plum	Pru d 3	9	16YVKGGGAVP24	Borges et al., 2008
			37LARTTADRRAACNCLHQL54	
			76NVPYKISASTNCATVK91	
Apricot	Pru ar 3	9	16YVRGGGAVP24	Borges et al., 2008
			37LARTTPDRRTACNCL51	
			76NIPYKISASTNCATVK91	

However, nsLTPs are also involved in triggering food allergies, especially in fruits and vegetables. These proteins are stable to heat and digestion and hence can trigger allergenic reactions in cooked foods (**Table 2.4**). In addition to OAS, it maybe induces systemic reactions and more severe symptoms. They are usually found in strawberry, peach, apple, and sweet cherry.

2.4.6. Profilin

The profilin is a 12-15 kDa protein which is generally found in all eukaryotic organisms [70]. It plays a major role in controlling the growth of actin microfilaments, which is a crucial step in cellular locomotion [71]. However, Ebner et al., (1995) reported that profilin is one of the major causes to induce the cross-reactive allergies between pollen and fruits (**Table 2.4**). It is considered as a clinically relevant fruit allergen as it induces profilin hypersensitivity. Various fruits that can trigger allergic reactions due to profilin include melon, watermelon, tomato, banana, pineapple and orange [72]. For example, peach allergen (Pru p 4) is a 14 kDa allergenic profilin that induces IgE-mediated symptoms in allergic patients after consuming this fruit (**Table 2.4**).

2.5. IgE-BINDING EPITOPES OF ALLERGENS IN VARIOUS FRUITS

Jerne (1960) was first in defining the term of epitope and in charactering the epitopes as "surface configurations, single determinants, structural themes, immunogenic elements, haptenic groups, antigenic patterns, specific areas" of an antigen. His definition of the epitope is focused on the contacting relationship between the antibody and the antigen [73]. The IgE recognition sites i.e. IgE-binding epitopes are classified into linear and conformational epitopes. Linear epitopes consist of continuous amino acid sequences, while the conformational ones are formed by spatially adjacent amino acids (that constitute the secondary and tertiary structures) but are distantly located in the amino acid primary sequence of the proteins [29,74]. There are also many non-peptidic epitopes such as glycan epitopes and classical haptens. For example, IgE-binding glycan epitopes are the cross-reactive carbohydrate determinants [75] and galactose alpha-1, 3-galactose [76]. Therefore, it is important to identify the epitope structure for the

development of new strategies for accurate diagnosis and allergen-specific immunotherapy of fruit allergy as well as the production of hypoallergenic foods [77].

As shown in **Table 2.5**, Fra a 1 is a 15 kDa fragment with accession no. of DQ385511 (Fra 1.0102) and it has already been identified as the major allergen present in strawberries. One of the epitope in Fra a 1, 47GDGGPGTIK55 has been mapped by Muñoz et al., (2010)**.** Similarly, Mal d 3 (accession no: AY572500) is one of the main allergens present in apples with a 9 kDa molecular weight. The three main IgE-binding epitopes of Mal d 3, 16YVRSGGAVP24, 35INGLARTADRQ46 and 76NVPYKISTS84 have been mapped by Borges et al. (2008) using spot membranes. The physicochemical properties of allergens from other Rosaceae fruit are well characterized (**Table 2.5**). 10 kDa Pru p 3 is one of the main allergens of peach and belongs to the nsLTP family. Its accession no. is P81402 (Pru p 3.0101). Garcia-Casado et al., (2003) reported three possible IgE binding peptides present in Pru p 3 which are 11APCIPYVRGGGAVPP25, 31IRNVNNLARTTPDRQ45 and 71GKCGVSTPYK80. Also, Borges et al., (2008) reported epitope sites from another two Rosaceae family members, plum, and apricot with 9 kDa molecular weights. Their possible IgE binding peptides are 16YVKGGGAVP24, 37LARTTADRRAACNCLHQL54 and 76NVPYKISASTNCATVK91 in plum and 16YVRGGGAVP24, 37LARTTPDRRTACNCL51 and 76NIPYKISASTNCATVK91 in apricot. The two fruits share similar epitope sites, which easily induce an allergic reaction in patients with plum or apricot allergy. Therefore, patients with fruit allergies are vulnerable in showing allergic potential to different fruits at the same time.

2.5.1. Cross-reactive fruit allergy

The definition of cross-reactivity is when the antibodies exposure to a specific allergen are also shown to react against allergens from other sources even when the person is not allergic to that particular food [78]. Cross-reactions in the case of fruits, nuts, and vegetables are generally limited to OAS with pollen allergy [79]. For example, some people are allergic to birch pollen may react to apples as well [80,81]. This cross-reactivity is due to the homologues structure leading to similar epitopes in allergens from multiple sources. For example, antibodies released when exposed to birch pollen allergens can also be triggered when exposed to apples because of their

homologous structure resulting in similar epitope configuration. The cross-reactivity also can cause various reactions including swelling, redness, and itching restricted to the lips, tongue, and throat [82].

Table 2.6. Lists of common fruits cross-reacting with pollen allergy.

Species	Pollen allergy			Latex allergy	References
	Tree (e.g. birch)	Grass (e.g. ragweed)	Mugwort		
Apple	Yes				Mauro et al., 2011
Kiwi	Yes		Yes		Gall et al., 1994
Peach	Yes		Yes		Lauer et al., 2007
Cherry	Yes				Neudecker et al., 2003
Pear	Yes				Karamloo et al., 2001
Mango	Yes	Yes	Yes		Paschke et al., 2001
Tomato	Yes	Yes		Yes	Werfel et al., 2015
Melon		Yes	Yes		Patel et al., 2015
Orange	Yes	Yes			Takamatsu et al., 2016
Banana				Yes	Werfel et al., 2015
Pineapple				Yes	Werfel et al., 2015
Avocado				Yes	Poley and Slater, 2000

As mentioned earlier, various allergens similar to the ones from fruits are mainly found in pollens of grass (e.g., mugwort), trees (e.g., birch) and weeds (e.g., ragweed). As shown in the **Table 2.6**, many studies have been reported where the major allergen, Bet v 1 from birch pollen has shown cross-reactivity to homologous protein allergens existing in a wide range of fruits, such as apple (Mal d 1), cherry (Pru av 1), pear (Pyr c 1), and kiwi (Act d 8) [80,81,83]. Pollen present in the grass (e.g., ragweed) can also induce cross-reactivity to various fruit allergies sourcing from mango, tomato, melon and orange due to their similar protein (epitope) structure

[42,84-86]. Similarly, allergens from peach, mango, kiwifruit and melon share their epitope structure with pollens from mugwort [42,84,87].

Some proteins present in natural rubber latex or related products made from latex rubber trees can trigger latex allergy with allergic symptoms i.e. skin irritation, or even a potentially life-threatening situation. Allergy to latex proteins has been known since the 1980s and is currently well-recognized health trouble among using products made of natural latex such as protective gloves or other goods [88]. As shown in **Table 2.6**, many studies have reported the clinical and immunochemical cross-reactivity between latex and banana, tomato, pineapple and chestnut, avocado and other fruits [85,89]. People sensitive to pollen and latex can suffer from cross-reactivity to various fruit allergens due to the similarity in the protein structures [25].

2.6. FRUIT ALLERGY IN DIFFERENT COUNTRIES

Fruits belonging to the Rosaceae family are responsible for most of the fruit-related allergenic reactions around the world [90]. The first clinical report was from northern and central Europe, where apple allergy which is the primary fruit allergy, is associated with birch pollens due to cross-reactivity [19]. However, in Spain, the country which is free of birch trees, peach from the Rosaceae family was the primary fruit inducing allergic reactions, followed by apple. Up to 70% of peach-allergic patients also showed an allergic potential to pollen which is mainly from grasses, mugwort, olive and cypress [91]. The fruit allergens, Pru p 1, Pru p 2, Pru p 3 and Pru p 4 have been identified as the major allergens present in peaches (www.allergen.org).

It has been reported that about 2% of the North American population is allergic to apples [92]. Consumption of fresh apples can trigger allergic reactions because of the allergen, Mal d 1 present in apples [15]. In addition, the apple allergen Mal d 1 shares similar allergenic epitopes with Bet v 1 which is a major birch pollen allergen, resulting in IgE cross-reactivity [81]. In Asian countries (such as Korea and Japan) kiwifruit is the main fruit that can easily induce allergic reactions, followed by peach and apple [93]. In recent years, it has been widely reported that fruit allergy has a strong association with pollen and latex. Many studies found that kiwi allergy has cross-reactivity with birch pollen, latex, avocado and banana [83,94]. To be exact, the patients suffering from aforementioned allergies are prone to experience allergic reaction upon consumption of Kiwi. This is because the protein contained in Kiwi is similar to those contained

in the birch pollen as well as latex i.e. they share similar epitopes and hence can trigger cross-reactivity in spite of the patient not being allergic to all the proteins. Five major allergens including Act d 1, Act d 2, Act d 4, Act d 5 and Act d 11 responsible for kiwi allergy have been extracted and characterized (www.allergen.org).

2.7. GLOBAL STATISTICS OF PUBLICATIONS ON FRUIT ALLERGIES

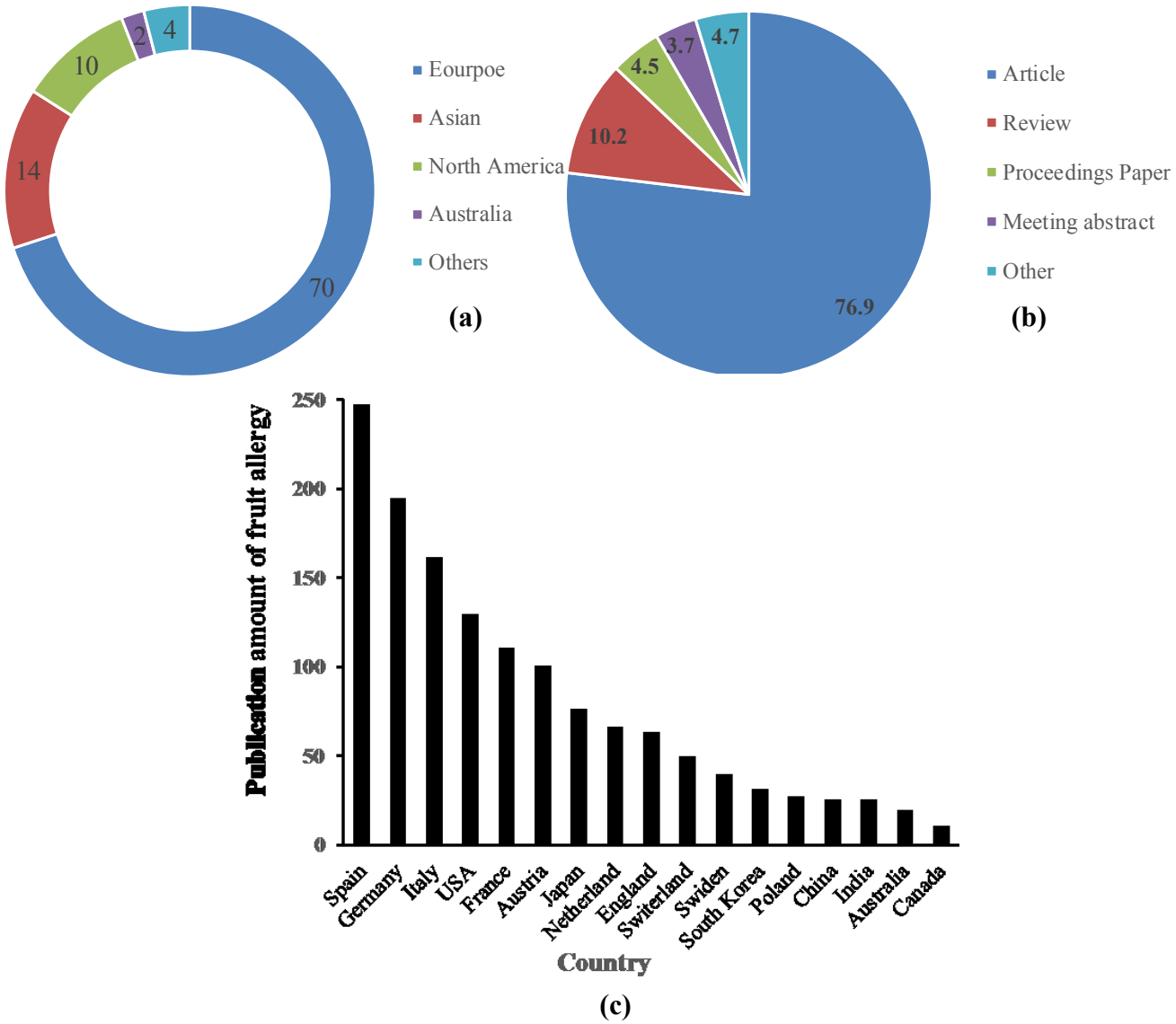


Figure 2.1. Percentage of published articles on fruit allergy based on geographical location (a), percentage of different article types (b) and the number of publications in different counties (c) since February 2017 (the data is collected from the Web of Science).

As of February 2017, the data from Web of Science showed that a total of 1265 manuscripts are published all over the world that deal with fruit allergies. As shown in **Figure 2.1a**, most of the publications come from Europe (70%) followed by Asia (14%), North America (10%) and others (6%). Most of these publications are research articles accounting to 76.9%, followed by reviews (10.2%) (**Figure 2.1b**). Conference proceedings account for 4.5% of the total manuscripts, followed by meeting abstract (3.7%) and others (4.7%). In terms of countries contributing to fruit allergy research, 247 papers were published by researchers from Spain, followed by Germany with 194 and Italy with 161 (**Figure 2.1c**). Other European countries like France (110) and Austria (100) have a similar number of publications. From North America, there were a total of 129 publications from the USA and 10 from Canada. South Korea (31) published the most articles between 1991-2017 in Asia followed by China (27) and India (25).

2.8. INFLUENCE OF VARIOUS FACTORS ON FRUIT ALLERGENICITY

2.8.1. Environmental cultivation conditions

During the plant growth, environmental cultivation conditions (e.g., nitrogen, water and climatic changes) have a significant influence not only on the plant growth and well-being, but also on the overall quality of the fruits which include various nutritional aspects such as sugar content, organic acids and health-related carotenoids [95,96]. Nitrogen is a necessary element for all plants in appropriate quantities for their growth and maintenance. Plants need nitrogen in combination with the carbon absorbed from the air to synthesize proteins for the production of enzymes and structural components such as 'rubisco' which is a photosynthetic protein and lignin in cell walls. Further, nitrogen intake can significantly influence fruit quality and yield. George and Nissen (1992) found that when peach trees were treated by 30 g nitrogen in the form of nitram before the flowering stage, increased peach yields by 48% and improved brix by about 14% when compared with untreated trees. On apples, researchers reported that nitrogen applied during the summer period can increase the number of fertile apple blossoms for the next year [97], which indicates fruit trees might generally starve due to lack of nitrogen before the development of the flowering stage. Nitrogen application during this growth period is beneficial and can increase the yield and overall quality of fruits.

Further studies reported that mature fruits of tomato plants after water stress have excellent taste and flavor. For example, Veit−Köhle et al. (1999) found lower water treatment (50% water supply) could increase the sugars, titratable acids, aroma volatiles and vitamin C contents in tomatoes, compared to that of higher water treatment (70% water supply). In peach, George and Nissen (1992) stated that water stress improved fruit size by 37% when compared with untreated trees. Climatic changes during the growing season are also one of the major factors that could influence fruit quality. Studies have been reported temperatures, carbon dioxide, and ozone levels could directly or indirectly affect the yield and quality of fresh fruits. For example, Moretti et al. (2010) found that temperature increase could directly affect photosynthesis, which results in alterations in sugars, organic acids, flavonoids contents, firmness, and antioxidant activity.

Furthermore, studies have shown that environmental cultivation conditions have an impact on fruit allergen content. Klockenbring et al. (2001) reported that patients showed a higher sensitivity to allergens that are organically cultivated 'Boskoop' fruits compared to those apples produced using integrated production (IP) techniques (using chemical fertilizers and pesticides) through skin prick test. As mentioned earlier, Mal d 1 is one of the major apple allergens and belongs to the PR-10 protein family. The environmental stress factors such as fungi, viruses, and bacterial attacks can induce PR-10 proteins which leads to the synthesis of various components including Mal d 1. Fruit trees under the organic cultivation method are more susceptible to the stress factors that synthesize higher Mal d 1 compared to fruits that are under the IP method with pesticide treatments. Similarly, Matthes and Schmitz-Eiberger (2009) found that 'Jonagold' apple fruit organically cultivated at Bonn University, Germany showed significantly higher level of Mal d 1 when compared to those apples produced through IP technique using the test of Sandwich-ELISA which is similar to the results obtained few years earlier as reported by Klockenbring et al. (2001). However, the apples cultivated under IP method at Hohenheim University, Germany under the German IP guideline QS-Gap 9, showed higher apple allergen (Mal d 1) level in comparison to that of the fruits cultivated using OC in 'Jonagold' cultivar. The same cultivar (i.e. 'Jonagold') showed a different Mal d 1 trend at two locations, which might be due to various OC standards. The OC was performed differently according to EU directive 2092/91 at Bonn University and 'Bioland' guidelines at Hohenheim

University, respectively. Further, the growing conditions of apple trees were different in these two places. The orchard in Bonn University provided a growing period of 170 days, 596 h sunshine and a rainfall of 1534 nm. In contrast, the apple trees at Hohenheim University had a 185-day growing period, 1678 h of sunshine and 861 nm rainfall. This can also be due to the influence of external triggers which could have led to the excess production of Mal d 1 in the apples cultivated in Hohenheim University, Germany. Pesticide treatment can be also responsible for inducing stronger responses than any biotic factors (such as fungi, viruses, and bacteria) for the accumulation of PR-10 (e.g. Mal d 1) in apples. The mechanism leading to the synthesis of Mal d 1 and the metabolic pathways are not clear, and more research about the influence of environmental conditions on fruit allergen should be conducted in detail.

Tulipani et al. (2011) reported that in the year of 2007, strawberry allergen, Fra a 1 content present in all the cultivars was lower than that of the strawberries cultivated in 2008. In the experiment, the daily minimum and maximum temperatures were all higher in 2007 compared to 2008, especially during the development of fruits i.e. in the months of April and May. Further, the weather in the month of April 2007 was drier when compared to April 2008. It indicated a possible inverse correlation between stressful environmental factors (such as higher temperature and drier weather) and the strawberry allergen Fra a 1 content. It is a conflicting result when compared to the research on the apple cultivation, where water shortage (completely deprived of the irrigation from June) strongly up-regulated the transcription and expression of genes in Mal d 1.04 and 4.01 present in apples [98]. Fra a 1 and Mal d 1 all belong to PR-10 proteins, while the mechanism of synthesis is influenced differently by various stressful environmental conditions, such as water shortage or higher temperature. Also, maybe the mechanism involved in the synthesis and degradation of the above-mentioned fruit allergens are different in apples and strawberries and hence the discrepancy. Further research is warranted to understand the mechanism involved in the synthesis of various allergens in spite of them belonging to the same PR-10 protein family.

2.8.2. Variety

Variety is one of the most important factors influencing the fruit allergenic potential. López-

Matas et al. (2011) determined the allergenic profile of six commonly known varieties of tomatoes (Rama, Rambo, Canario, Kumato, Pera, and Raf) and found that their protein profile and allergen quantification showed differences in the allergen composition. The highest allergen content (Lyc e 3) present in the tomato peel was shown in the 'Rambo', followed by 'Pera', 'Canario', 'Raf' and 'Kumato'. The 'Rama' showed the lowest value of allergen content. The 'Kumato' variety belongs to black tomatoes and is not generally consumed. It has an intense taste, higher sweetness, and juiciness than others, which might have resulted in its high allergenic potential compared to 'Rama'. Other components (e.g. type of carbohydrates), present in the pulp of 'Kumato' tomatoes are also considered as possible reasons [14]. Similarly, in another study, two different tomato varieties were used. The 'Reisetomate' induced significantly less positive skin reactions through skin prick test technique and caused fewer symptoms, compared to the cultivar 'Matina' [99]. 'Matina' is a variety selected for organic farming which can effectively resist environmental stress and diseases [99]. As mentioned early, plants growing in stressful environmental conditions could induce the production of higher levels of certain PR proteins. The main tomato allergen (Lyc e 3) belongs to this protein group and hence the higher quantities of PR proteins leading to synthesis of higher allergen content.

In addition, four strawberry varieties were considered by Tulipani et al. (2011) for allergy analysis and they found that the highest strawberry allergen (Fra a 1) concentration was shown in the variety of 'AN94.414.52', followed by the 'Adria' and 'AN00.239.55', whereas the 'Sveva' variety showed the lowest Fra a 1 content. Further, this study showed that the lower level of Fra a 1 in the 'Sveva', 'Adria' and 'AN00.239.55' varieties had a strong negative correlation with higher content of total antioxidant capacity and total anthocyanin contents [33]. These results showed a relationship between the antioxidant compounds and the expression of allergen (Fra a 1) in strawberries. The high level of antioxidants present in fruits possibly benefits in reducing the biosynthesis of fruit allergens. Similarly, Schmitz-Eiberger and Matthes (2011) reported that apple allergen content had a relationship with antioxidant activities and related enzyme activities. The lower level of apple allergen (Mal d 1) might be due to the higher PPO activity and polyphenols contents, which in turn can inhibit the interaction between oxidized phenols and Mal d 1, resulting in lower allergenicity.

Moreover, many researchers stated that allergenic differences between various apple varieties

are mainly due to the variation in expression of Mal d 1 [100,101]. In fruits cultivated at the farm in Germany, lowest amounts of Mal d 1 were found in 'Jonagold', 'Kanzi', 'Greenstar', 'Pinova', 'Topaz', and 'Golden Delicious' fruits. The highest amounts of Mal d 1 levels were found in cultivars 'Rubens' and 'Gala' [101]. In another research, among twenty-one cultivars selected for the assessment of allergenicity, 'Golden Delicious' apples showed the highest level of allergenicity, followed by Gala apples. The lowest level of allergenicity was observed in the 'Santana' apples [102]. Similar results were observed in mango, depending on different varieties, the main allergen, 5-alk(en)ylresorcinols content ranged from 79 to 1850 mg/kg of dry matter in samples of mango peels [18].

As discussed above, the fruit allergen level is different in various fruit varieties due to many reasons. The relation between fruit allergen concentration and the variety is not clear and more research is warranted. As far as practical implications are concerned, all available information should be used in breeding projects addressed in the selection of hypoallergenic fruit varieties.

2.8.3. Harvest maturity

Maturity at harvest is one of the essential factors that influence the shelf-life and fruit quality [103]. During the maturity stage, the overall fruit quality including various sensory attributes (such as color, glossiness, size, shape, flavor, texture and taste) and nutrient content (acids, sugars, vitamins, polysaccharides, polyphenols and valuable minerals) change distinctly [104]. For example, the 'golden' papaya was harvested at four different maturity stages, and the results showed a significant difference in fruit quality [105]. During the ripening period, the ascorbic acid content increased 20-30%, and fruit harvested at late maturity stages reported excellent scores for sensory evaluation, including flavor and appearance, compared to that fruit harvested at an early stage.

Ethylene is one of the primary plant hormones that regulates fruit ripening. Further, studies found the level of apple allergens might be affected through the regulation of ethylene levels. Sancho et al. (2006b) found that apple allergen, Mal d 3 increased when fruits reached their physiological maturity, which coincided with the increase in ethylene. Similarly, in three apple cultivars, 'Braeburn', 'Topaz' and 'Golden Delicious', Schmitz-Eiberger and Matthes (2011)

found that apples had a higher Mal d 1 content when they were ripe than that of unripe apple fruits. In the apples that were picked at a later stage, the allergen, Mal d 1 was 3-4 times higher, compared to those picked at an earlier stage [82]. Other relevant physiological factors (e.g. anthocyanin content, related to the color change) and environmental conditions (e.g. illumination intensity) could also influence the allergen content present in fruits. Therefore, consuming ripe and dark color fruit can easily induce allergic reactions, compared to unripe fruit.

2.8.4. Differences in allergenicity between the peel and pulp of the fruit

Most of the fruits have peel, pulp, and seeds. For some fruit, such as orange and banana, the pulp is the primary part used for consumption, and in other fruits, the entire fruit is consumed (such as tomatoes and strawberries). Many researchers stated that allergic components were determined at present not only in the peel but also in the pulp of fruit [17,106,107]. Fruit allergen contents vary within the different sections of fruit. The peel contains the majority of allergens in mango. In the cultivar 'Tommy Atkins', Knödler et al. (2009) found that main mango allergen, alk(en)ylresorcinol content in the peel was 419.30 mg/kg which is 16 times higher than that in the pulp (26.33 mg/kg). In Rosaceae family fruits, Borges et al. (2006) reported that the lipid transfer protein (allergen) was mainly concentrated in the peel, with lower detectable amounts present in the pulp. In apples and peaches, the allergic reaction to peel was found to be higher than that of the pulp [19,20]. Similarly, among 113 patients with sensitivity to tomato, Larramentdi et al. (2008) found that 110 patients showed positive reaction to the tomato peel extract whereas 47 patients were allergic to the pulp extract. López-Matas et al. (2011) found that the tomato allergen (Lyc e 3 and PG2A) content in peel extract was higher than that of in the pulp extract when six commonly ingested varieties of tomato fruits were tested. Furthermore, the protein content in the peel extract was approximately double that of the pulp extract. The difference in the allergen content between peel and pulp extracts is due to the lower protein concentration in the matrix composition leading to lower reactivity in fruits upon removal of the peel [108]. It is possible that the pulp which is the outer layer of the fruit is exposed to various environmental stresses resulting in higher allergen levels compared to the pulp.

Therefore, peeling might be a solution to reduce the overall allergenicity in few fruits whose peel contains the majority of allergens. For example, the peeled-off apples significantly reduced their fruit allergenicity, compared to those apples with peel [109]. Similarly, in the peaches and tomatoes, peeled-off fruit had a lower allergen content than that of fruit with peel [17,110]. Therefore, peeling the fruits can be a potential treatment in the processing industries to drastically reduce their allergenicity [111].

2.8.5. Storage conditions

Storage conditions are major factors for maintaining a fresh and good quality of post-harvest attributes of fruits. The application of several storage technologies including cold storage, modified atmosphere packaging, and ozonation are beneficial to inhibit decay, extend shelf life, and maintain the nutritional quality of fresh fruits [112-115].

During the storage of fruits, many studies found that a considerable change in the level of allergens depending on environmental stress and/or storage conditions [116]. Bolhaar et al. (2005) reported that five apple cultivars under controlled atmosphere storage at 3℃ (i.e. 2.5% O_2 and 1% CO_2) showed a significant reduction amounting to 15% in allergic reactivity（P<0.001） compared to that of fruits under cold storage at 2℃. Similarly, Sancho et al. (2006b) indicated that under room temperature, cold storage (2℃) and controlled atmosphere conditions (0.5% O_2, 1.5% CO_2 at 3.8℃), the levels of Mal d 3 decreased in three apple varieties (i.e. Cox, Jonagored and Gala). The rate of overall reduction was greatest when fruits were stored at controlled atmosphere conditions [102]. In contrast, Hsieh et al. (1995) reported that another major apple allergen, Mal d 1 present in the three apple varieties (i.e. McIntosh, Red Delicious, and Granny Smith) increased during cold storage at 4℃. Similar results were reported by Sancho et al. (2006a) that apple allergen, Mal d 1 gene expression showed a significant increase under modified atmosphere packaging during storage. It suggests a difference in the regulation of gene expression between the two major apple allergens (Mal d 1 and Mal d 3). Mal d 1 is a pathogenesis related protein belonging to PR-10 family which can be concluded by stress factors (ex, cold temperature). Apples stored under modified atmosphere packaging at cold temperature could induce more Mal d 1 synthesis because of cold stress or CO_2 and O_2

concentration changes present in packages. Whereas, Mal d 3 belongs to the PR-14 family which responds to various external stimulus when compared to the PR-10 family [82,117]. The reduction in Mal d 3 under controlled atmosphere conditions or cold storage was due to better fruit quality (firmness, moisture content and antioxidant compounds content). The synthesis and degradation mechanism of Mal d 1 and Mal d 3 are not clear, and hence requires further research.

2.8.6. Difference in the structural stability of fruit allergens

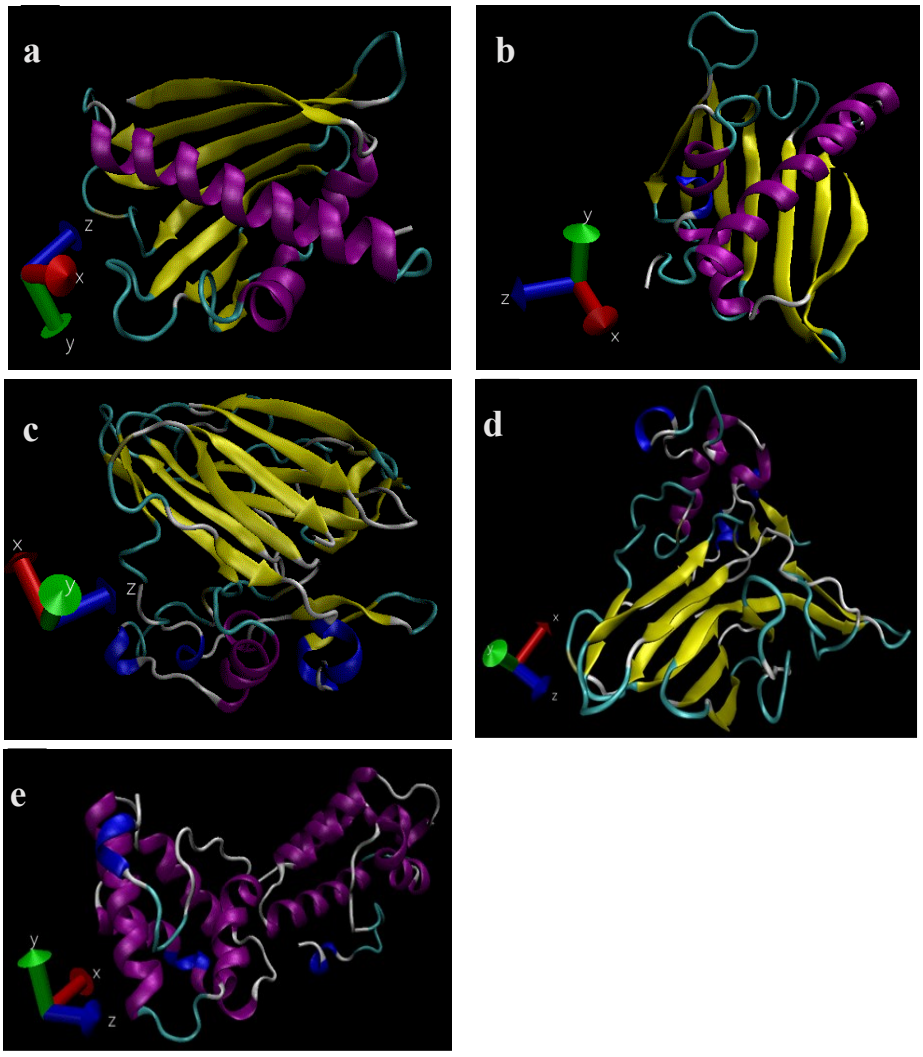


Figure 2.2. Secondary structures of birch pollen allergen Bet v 1(a), apple allergen Mal d 1 (b), and Mel d 2 (c) allergens, cherry allergen Pru av 2 (d), and peach allergen Pru p 3 (e). [α-helix: Purple, 3/10 helix: Blue, β-sheets: Yellow, Turns: Cyan, coils: White].

Most of the fruits are not only consumed fresh but are also used as the ingredient in various related products such as drink, dry powder, jam, yogurt, and cereals. Many reports stated that the allergenicity of fruits has a strong relationship to the form in which the fruits are consumed. It is well known that cooking substantially can help reduce the allergenicity of fresh fruit because heating destroys the pollen-related fruit allergens through changing their protein structures (secondary or tertiary structure) or conformational epitopes [29,118,119]. Therefore, consuming cooked fruits can be beneficial to help patients with birch pollen allergy to avoid cross-reactivity allergy [120]. Fruits share a similar allergen structure with birch pollen allergen which belongs to the PR-10 family of pathogenesis-related proteins and is easily destroyed by the cooking process due to its relatively unstable secondary structure (**Figure 2.2a**). Relevant examples are Pru av 1 allergen from cherries [121], Mal d 1 allergen in apples (**Figure 2.2b**) [122], Pru a 1 allergen present in peaches, Fra a 1 allergen in strawberries (**Figure 2.3a**) and Act d 11 allergen in kiwifruit (**Figure 2.4c**). Similarly, studies also reported that a commercial processing method, steam cooking at 100℃ or heating at 80-90℃ for several minutes can effectively eliminate the allergenic potential of patients to kiwifruit [123,124].

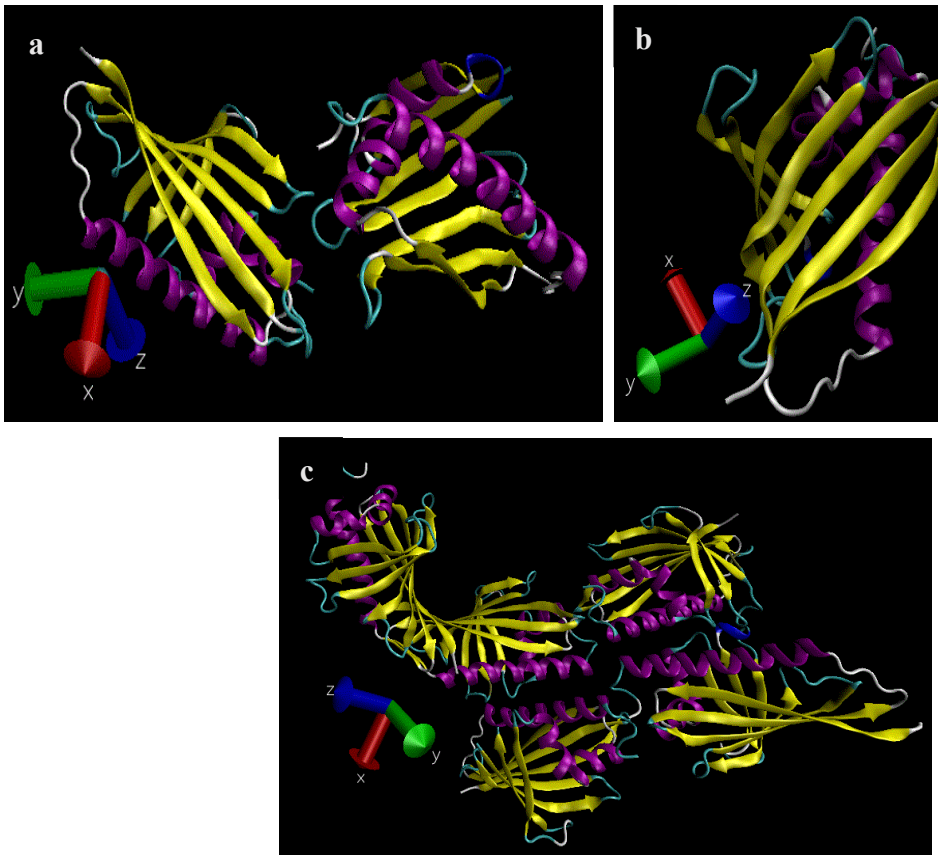


Figure 2.3. Secondary structures of Fra a 1 (a), Fra a 2 (b), Fra a 3(c) allergen (Source: protein data bank; PDB code: 4C9C, 5AMW, and 4C94, respectively). [α-helix: Purple, 3/10 helix: Blue, π-helix: Red, β-sheets: Yellow, Turns: Cyan, coils: White].

However, few allergens have a stable structure in some fruits. For example, the peach allergen (Pru p 3) is heat stable and therefore can also be found in the juice and other thermally processed products. In an experiment, fresh peaches were heat-treated at 121 ℃ for 30min. This treatment was not beneficial to decrease the allergenicity of the main allergen, Pru p 3 due to its stable secondary structure (**Figure 2.2e**) [125]. Similarly, Cit s 1 and Cit s 2 were still found in orange juice after thermal processing [126]. Many studies reported that the allergens Mal d 2 and Mal d 3 in apple (**Figure 2.2b-c**), the strawberry allergen, Fra a 3 (**Figure 2.3c**) from the non-specific lipid transfer protein family, the kiwifruit allergen, Act d 2 (**Figure 2.4a**) and cherry allergen, Pru av 2 (**Figure 2.2d**) all have the heat-stable secondary structures [127].

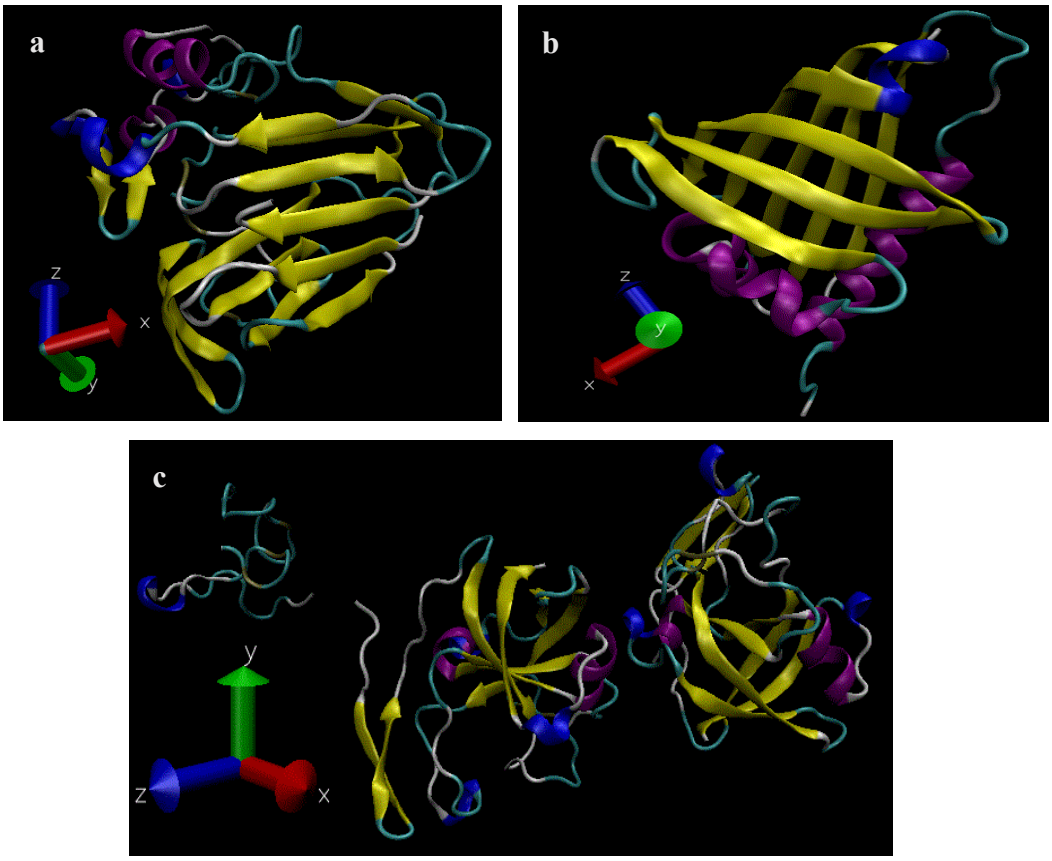


Figure 2.4. Secondary structures of Act d 2 (a), Act d 5 (b) and Act d 11 (c) allergen in kiwifruits (Source: protein data bank; PDB code: 4BCT, 4V9U and 4IGV, respectively). [α-helix: Purple, 3/10 helix: Blue, π-helix: Red, β-sheets: Yellow, Turns: Cyan, coils: White].

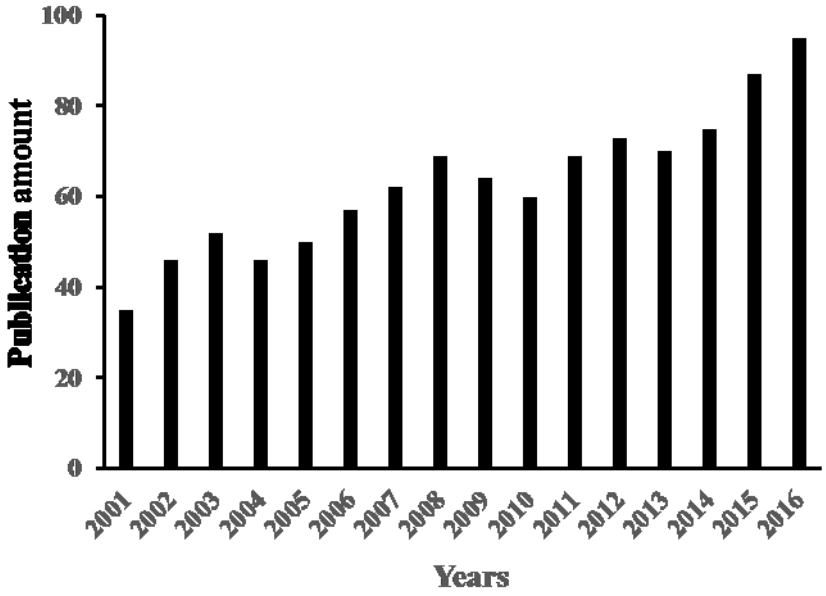


Figure 2.5. Number of published journal articles on fruit allergy from 2001 to 2016 (The data is collected from Web of Science).

2.9. FUTURE TRENDS OF FRUIT ALLERGIES

As mentioned earlier, many factors including environmental conditions, variety, maturity, parts of a fruit i.e. peel, pulp and seeds, storage conditions, different forms of processing, can affect the allergenicity in various fruits. As suggested in **Figure 2.5**, a gradual increase in publications was observed between the years 2001 and 2016 (the related data is from Web of Science). Understandably, various researchers may have different views about fruit allergy resulting in varied subfields of research that are being developed around the world. The future trends and challenges in the field of fruit allergies are outlined below:

2.9.1. Breeding

As discussed earlier, environmental conditions such as nitrogen, water and climatic changes have a significant influence on the fruit quality and fruit allergen content during the growth of fruit trees. Hence, the proper application of specific cultivation techniques and conditions (organic cultivation) can be beneficial in reducing the allergic potential of few fruits during their growth. Many studies have reported that the allergenicity level was different in various fruit varieties. For example, apple varieties ‘Jonagold’, ‘Kanzi’, ‘Greenstar’, ‘Pinova’, ‘Topaz’,

and ‘Golden Delicious’ had lower quantities of Mal d 1 than in varieties ‘Rubens’ and ‘Gala’ [101]. Therefore, breeding techniques can be used in the development of new varieties of apple presenting with a lower level of allergens [128,129]. Many biotechnological methods have been applied to the field of plant breeding, such as molecular plant breeding, which is a widely accepted technique. The transgenic technology is a common application in molecular plant breeding where transgenic events can be designed to facilitate the molecular stacking of transgenes that control a trait or suite of traits into a single locus haplotype [130]. For example, recently released ‘Yield-Guard VT’ triple transgenic maize hybrids where herbicide-tolerant and multiple insect resistance traits were integrated as one genomic locus (position of the chromosome) that simultaneously increases the synthesis and decreases the catabolism of Lysine in maize seeds [131]. Therefore, molecular plant breeding can provide a possible solution to reduce the level of allergen present in fruits by transgenic technique to regulate the expression of target genes (allergen synthesis related genes). Specific cultivation standards and varieties developed through breeding can lead to development of innovative fruit cultivation techniques for hypoallergenic fruits and fruit-based products.

2.9.2. Food processing industry

Fruit allergens are not only present in the peel but also in the pulp of the fruit. Many studies found that the lipid transfer protein (allergen) was mainly contained in the peel, with lower but detectable concentrations in the pulp of fruits from Rosaceae family [132]. For example, in apples and peaches, researchers reported that the reactivity of patients to the peel was higher than to the pulp when tested using skin-prick test [19]. In addition, some fruit allergens belong to the PR-10 family of pathogenesis-related proteins which are easily destroyed by thermal processing, such as Pru av 1 present in cherries [121] and Mal d 1 present in apples [122]. Therefore, peeling or heating is an effective method to reduce the overall allergenicity in few fruits whose peel contains the majority of allergens for the food processing industry. More novel processing methods like high-pressure processing [133], high voltage electric fields [134], EHD processing [135], microwave [29], pulsed ultraviolet light [136] and ultrasonication [137] should be explored to help reduce the allergen level present in fruit or vegetables.

2.10. CONCLUSION

At present, a considerable amount of the population around the world is allergic to various fruits and their only option is complete avoidance of such fruits. This can dramatically reduce their intake of natural vitamins, antioxidant compounds and other bio-factors which can effectively resist diseases. Further research with regard to the cause, symptoms and diagnostic methods for fruit allergies are needed to understand its detrimental effects on allergenicity. Many researchers found that a wide range of factors including pre-harvest and post-harvest methods can influence the allergen potential to fruits. For example, water stress can reduce the allergenicity level in pre-harvested fruits whereas, during the post-harvest storage, MAP can help reduce allergen (Mal d 3) content in apples. Therefore, research should be carried out to explore the relationship between allergen content in fruits to their pre-harvest and post-harvest conditions.

CONNECTING TEXT

The first part of the literature review (Chapter II) has emphasized understanding the effect of pre-harvest and post-harvest conditions on fruit allergenicity. The review mainly reported that varying environmental and cultivation conditions can influence the fruit allergen content during flowering and ripening stages. Further, the variety, harvesting maturity, and storage conditions can also significantly influence the allergenic potential. Potential post-harvest processing like peeling is also considered a good method to help reduce the overall allergenicity in few fruits whose peel might contain the majority of the allergens. The cause, symptoms, diagnostic methods and management of fruit-based allergies were also described.

Although the general information on fruit allergies was mentioned in Chapter II, the detailed knowledge on specific kiwifruit allergy is very limited. In the next chapter, the pathogenesis, clinical characteristics, diagnostic methods (medical and laboratory protocols) of kiwifruit allergy are described in detail. Further, the secondary protein structures of kiwifruit allergens were analyzed. We have also discussed three potential processing methods: thermal processing (e.g. steam cooking), non-thermal processing (ultrasound treatment) which may be used to reduce the allergenicity of kiwifruit in the future.

CHAPTER III

LITERATURE REVIEW-PART 2

A COMPREHENSIVE REVIEW ON KIWIFRUIT ALLERGY: PATHOGENESIS, DIAGNOSIS, MANAGEMENT AND POTENTIAL MODIFICATION OF ALLERGENS THROUGH PROCESSING

3.1. ABSTRACT

Kiwifruit is rich in bioactive components including dietary fibers, carbohydrates, vitamins, minerals, omega-3 fatty acids, and antioxidants. These components are beneficial to boost the human immune system and prevent cancer and heart diseases. However, kiwifruit is emerging as one of the most common elicitors of food allergies worldwide. Kiwifruit allergy results from an abnormal immune response to kiwifruit proteins and occurs after consuming this fruit. Symptoms range from oral allergy syndrome (OAS) to life-threatening anaphylaxis. Thirteen different allergens have been identified in green kiwifruit and, amongst these allergens, Act d 1, Act d 2, Act d 8, Act d 11 and Act d 12 are defined as the major allergens. Act d 1 and Act d 2 are ripening-related allergens and are found in abundance in fully ripe kiwifruit. Structures of several kiwifruit allergens may be altered under high temperatures or strong acidic conditions. This review discusses the pathogenesis, clinical features, and diagnosis of kiwifruit allergy and evaluates food processing methods including thermal, ultrasound and chemical processing which may be used to reduce the allergenicity of kiwifruit. Management and medical treatments for kiwifruit allergy are also summarized.

KEYWORDS: kiwifruit allergy; cross-reactivity; thermal processing; non-thermal processing; secondary structure

3.2. INTRODUCTION

Kiwifruit (*Actinidia deliciosa*) are wild plants that were first found in China and named 'Chinese gooseberry'. There are approximately 55 species cultured including green kiwifruit (*A. deliciosa*), golden kiwifruit (*A. chinensis*), hardy kiwifruit (*A. arguta*), Chinese egg gooseberry (*A. coriacea*), and hearty red kiwifruit (*A. purpurea*) [1,2]. At their commercial harvesting stages, the fruits have a soft texture and a pleasant flavor. In 2018, United States Department of Agriculture (USDA) Nutrient Database reported that kiwifruit is an excellent source of dietary fiber, carbohydrates, vitamin C and E, minerals (calcium, magnesium, potassium, and phosphorus), omega-3 fatty acids and antioxidants (e.g., phenols) as outlined in **Table 3.1**. In 2016, the Food and Agriculture Organization of the United Nations (FAO) reported that the total worldwide kiwifruit production was 4,274,870 tons. Kiwifruit is generally consumed in fresh form. However, the shelf life of fresh kiwifruit is very short because of tissue softening and senescence [138]. To avoid the food loss, kiwifruit is made into different products including juice, dried kiwifruit slices, and frozen products; which can further be used as an ingredient in ice creams, baked goods and fruit jam [139].

Studies suggest that bioactive compounds such as vitamins and polyphenols present in kiwifruit may contribute to the prevention of cancer and cardiovascular diseases [3,4]. Immune activity may also be influenced by the ingestion of kiwifruit. Iwasawa et al. (2010) and Skinner et al. (2011) reported that the production of specific cytokines (e.g. IL-12) in mice was significantly increased after oral administration of kiwifruit juice when compared to the control group that received the same quantity of water. Studies have also observed that d consumption of 0.5 L of kiwifruit juice (about eight kiwifruits) per day was associated with decreased DNA damage in human lymphocytes compared to the cells from the water-consuming control group [140]. Oxidative DNA damage is positively correlated with some cancers [141]. Thus, consuming kiwifruit may potentially reduce the risk of cancers due to its antioxidant effect. Furthermore, in one study of 43 volunteers with hyperlipidemia, a significant decrease in high-density lipoprotein cholesterol content was observed after eight-week consumption of two kiwifruits per day. Vitamin C and vitamin E levels in the blood were also increased [142].

Table 3.1. Nutrients in 100 grams (g) of kiwifruit (Source: USDA Nutrient Database, 2018).

Compounds	Nutrients	Amount
Proximates	Energy	61 kcal
	Water	83%
	Carbohydrate	14.66 g
	Fiber	3.0 g
	Sugar	8.99 g
	Protein	1.14 g
Minerals	Calcium	34 mg
	Magnesium	17 mg
	Potassium	312 mg
	Phosphorus	34 mg
Vitamins	Vitamin C	92.7 mg
	Vitamin E	1.46 mg
Lipids	Fatty acids	363 mg

In 1981, the first report on kiwifruit allergy was published [143] and kiwifruit is increasingly recognized as a common elicitor causing allergic reactions worldwide. In Sweden and Denmark, nearly half of food-allergic individuals reported kiwifruit allergy in self-reported surveys [8]. In Spain, approximately 1.8% of the total population is allergic to kiwifruit [9]. In France, in a study of 182 children (2-14 years of age), 9% of the population reported hypersensitivity to kiwifruit [7]. For many patients, symptoms associated with kiwifruit ingestion may be a form of food pollen syndrome (FPS). Kiwifruit FPS occurs in patients with allergic sensitization to plant pollen proteins which are sharing similar protein structures to those found in the kiwifruit [11]. Pollen allergy is estimated to occur in 20-40% of the population worldwide [12]. In European

countries, approximately 40% of individuals reported hypersensitive to tree and/or weed pollens [144], while in North America, pollen allergies are reported in >20% of the population [145]. Thus, the potential for a high frequency of FPS after exposure to kiwifruit is significant. In one study of 55 patients (female: male=39:16) with oral food challenge diagnosed kiwifruit allergy, 22% had isolated kiwifruit allergy with no cross-reactivity to pollens, while the majority had demonstrable cross-reactivity with other plant proteins, especially from foods belonging to the Rosaceae family (50%), peanuts (31%), and tree nuts (42%) [13].

Studies have shown that conformational epitopes of some protein allergens can be altered by heating or enzymatic hydrolysis [146]. Modification of these secondary and tertiary structures can lead to the loss of their potential IgE-binding sites in these proteins as IgE-binding is essential for triggering allergic symptoms. This modification suggests that kiwifruit allergic individuals might safely consume appropriately altered proteins from kiwifruit [147]. Studies have reported that thermal processing (e.g. steam cooking) and chemical treatments could modify the structures of the proteins present in kiwifruit, which in turn results in a reduction of kiwifruit allergenicity [124,148]. Further application and validation of novel techniques in processing are needed to demonstrate reductions in the kiwifruit allergenicity in food industries.

In this review, we describe the pathogenesis and clinical characteristics of kiwifruit allergy. Diagnostic methods based on in vivo reactivity to protein (e.g. skin prick test) and DNA (e.g. polymerase chain reaction), their advantages and disadvantages are also described. Further, thirteen kiwifruit allergens are recognized, and some of their secondary protein structures are analyzed. Several ripening-related kiwifruit allergenic proteins (e.g. Act d 1, 2, 5, and 11) are reviewed. We also discuss three potential processing methods: thermal processing (e.g. steam cooking), non-thermal processing (ultrasound treatment) and chemical treatment (e.g. enzymatic hydrolysis) that may reduce the allergenicity of kiwifruit.

3.3. PATHOGENESIS, CLINICAL FEATURES, DIAGNOSIS, AND EPIDEMIOLOGY OF KIWIFRUIT ALLERGY

3.3.1. Pathogenesis

Kiwifruit allergy is an abnormal immune response following consumption. As shown in **Figure**

3.1, food allergies develop when proteins presented in kiwifruit or other foods are recognized by the immune system, and an inappropriate type of immune response is generated. This response leads to the formation of allergen-specific antibodies (e.g. IgE isotype), a process known as sensitization. The newly formed IgE antibodies then link to specific effector immune cells known as mast cells. When there is a subsequent exposure to kiwifruit or related products, the allergens attach to the specific IgE antibodies and activate the high-affinity IgE receptors on the surface of mast cells [149]. Mast cell activation results in the release of histamine and other inflammatory mediators which lead to the initial symptoms of kiwifruit allergy [150].

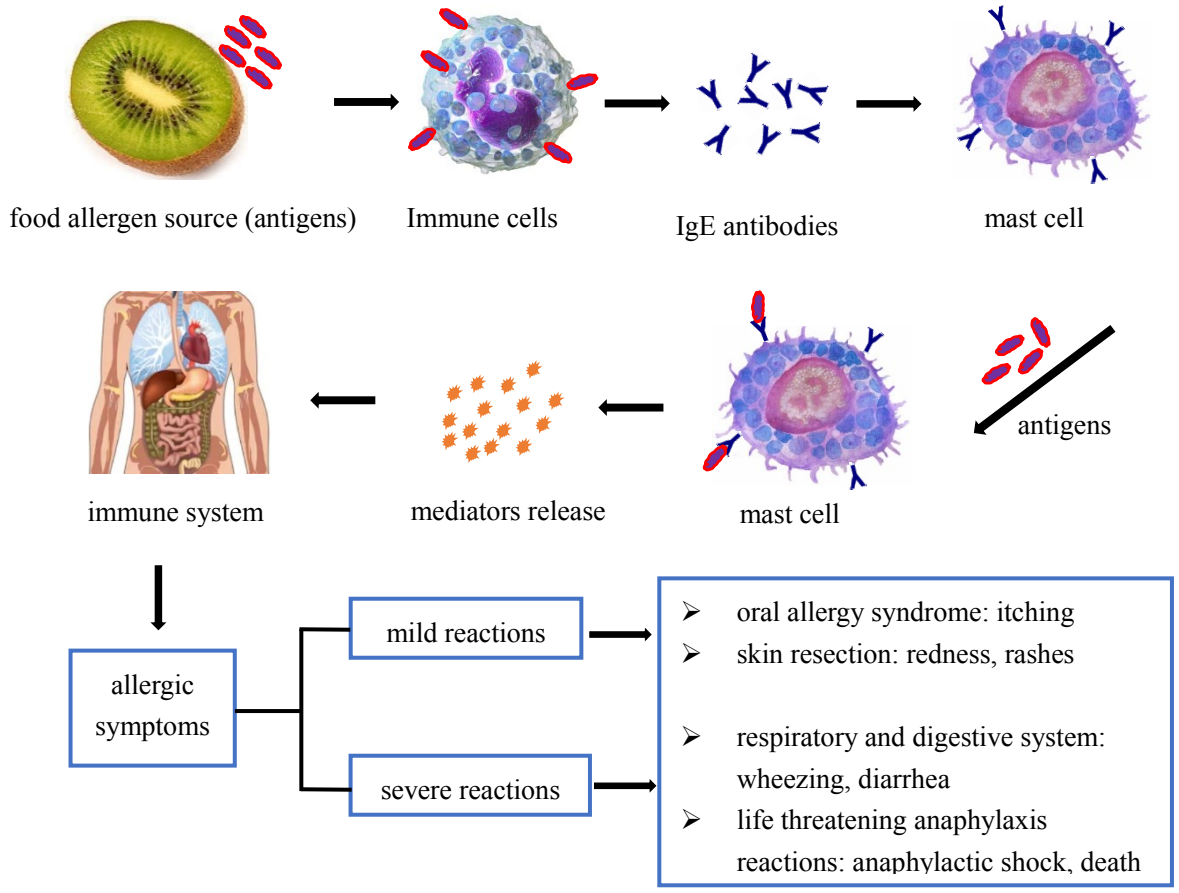


Figure 3.1. Overview of allergic sensitization to kiwifruit and related allergic reactions.

Kiwifruit, and pollen or plant-derived foods such as birch and grass pollen [151], avocado [152], banana [153], peanut and tree nuts [13] share homologous allergens and thus sensitization to one may result in symptoms following kiwifruit ingestion due to the presence of cross-reactive protein allergens. For example, individuals who are sensitized to tree pollen have pollen-specific IgE. This IgE molecule may also recognize proteins with similar structures present in kiwifruit leading to the activation of the IgE bound-mast cell, which causes symptoms of food

allergy [83]. Thus, clinically significant reactions to kiwifruit follow direct sensitization to the kiwifruit or through cross-sensitization with other allergens of similar structures. The severity of symptoms is, in part, dependent upon the route of sensitization with the highest risk of severe anaphylactic reactions in those patients sensitized directly to kiwifruit.

Table 3.2. Allergic symptoms on the consumption of kiwifruit.

Allergen sources	Symptoms		References
Kiwifruit (*Actinidia deliciosa*)	Oral allergy syndrome (OAS)	Swelling, itching, tingling or burning sensation in and around mouth, lips, tongue and throat	154
	Skin reactions	Itching and redness of skin, skin rashes, hives, eczema	143
	Respiratory and digestive system	Wheezing, nasal congestion, troubled breathing coupled with shortness of breath, abdominal pain, nausea or vomiting, diarrhea	155,156
	Life threatening anaphylaxis reactions	Shortness of breath, heart rate increase, blood pressure decreases, anaphylactic shock, dizziness, fainting or loss of consciousness	28

3.3.2. Clinical features

Kiwifruit allergy has been identified as the most common fruit-induced allergies and represents the fourth most common food allergy in France, after milk, egg, and peanuts [5,6]. Kiwifruit allergy is associated with a range of symptoms including oral allergy syndrome (OAS), which results from cross-sensitization to life-threatening anaphylaxis, a result of direct sensitization [28]. As shown in **Table 3.2**, mild kiwifruit allergic symptoms, related to the OAS, include swelling, itching, tingling or burning reactions in or around mouth, lips, tongue, and throat [154]. Skin symptoms include rashes, itching, hives and eczema, erythema and angioedema [143]. More significant systemic symptoms [156] include wheezing, nasal congestion, shortness of breath, abdominal pain, nausea or vomiting [155]. Severe, life-threatening reactions may lead to increasing heart rate, decreasing blood pressure, lightheadedness, respiratory failure, and

anaphylactic shock. Coma or death may ensue unless medical treatment is administered promptly [157]. In one study 55 kiwifruit-allergic volunteers (female: male=39:16) were challenged with kiwifruit. Sixty-two percent of the volunteers developed the local OAS, while 38% had systemic allergenic reactions after kiwifruit consumption [13].

Table 3.3. Common diagnosing methods for the detection of kiwifruit allergy.

Types	Method	Target analyte	Advantages	Disadvantages	References
Clinical diagnostic methods	DBPCFC	Proteins	Gold standard for a specific allergy	It needs several days to obtain the results	[158]
	SPT	Proteins	A brief time; Low cost; High sensitivity (93%)	High 'false positive' results (50%-60%) and low specificity (33-45%)	[51,53,159]
	ELISA	Proteins	A brief time; High specificity (99%) and sensitivity (71%)	Complex protocol; High cost with side effects (e.g. pain, bleeding)	[160-162]
	ImmunoCAP	Proteins	A brief time; High specificity (98%) and sensitivity (60%); Easy to operate	Side effects (e.g. pain, bleeding)	[160,161,163]
DNA-based detecting methods	PCR	DNA	High specificity; Stable DNA level	Equipment is expensive	[162,164]
	Rt-PCR	DNA	High specificity; Stable DNA level	Equipment is expensive	[162,164]

3.3.3. Diagnosis and detection

The major types of diagnostic and detection methods are shown in **Table 3.3**. Clinical diagnostic tools include double-blind placebo-controlled food challenge (DBPCFC), skin prick test (SPT), enzyme-linked immunosorbent assay (ELISA) and immunoCAP [158]. The DBPCFC is considered as the gold standard diagnostic tool. Patients are given foods to consume, and both the patient and the treating physician are blind as to whether to food consumed contains any relevant allergens or is a placebo. This procedure ensures an objective assessment of outcomes without operator-specific preconceptions or bias [165]. If patients develop clear objective signs and symptoms of an allergic response, the test is considered positive. However, the food challenge generally needs several days to complete because the allergic reactions can be only triggered after ingesting sufficient doses of allergen and placebo. SPT is one of the most common diagnostic methods used in the hospital. SPT involves placing small amounts of protein extract from kiwifruit pulp on the patient's skin (arm) which is scratched or pricked by using a needle [166]. In this way, small amounts of protein allergen are exposed to the mast cells of the patient's skin. In allergic individuals, allergen binding to kiwifruit-specific IgE on these cutaneous mast cells results in the release of histamine and a localized allergic reaction [53]. After 10-15 min, the skin is examined for redness (flare) and inflammation (wheal). The wheal sizes are 3 mm or bigger than the negative control (0.9% saline solution), which is considered as a positive test [53]. SPT is therefore quick and easy to perform. Further, it is also less expensive than DBPCFC, and multiple allergens can be tested at the same time [53,167]. This test is particularly useful in identifying IgE-specific sensitization to allergens (95%) [168]. The sensitivity (the true positive rate) of SPT ranges from 60- 93%, but there is an overall poor specificity (the true negative rate) ranging from 33-45%. Further, SPT has a significant false positive rate up to 50-60% [159], which limits the utility of SPT.

ELISA and immunoCAP (a commercial ELISA) are two similar blood test methods based on the detection of specific IgE present in the blood of patients. They can be used to diagnose if the individual is sensitized to kiwifruit or other commercial extracts (food products) and are used in various sectors including laboratories, food industry, and regulatory agencies [169]. The specific allergens are measured through a colorimetric reaction after binding to specific

enzyme-labeled antibodies and quantified using standard curves [162]. These IgE based diagnostic methods have a high sensitivity (60-71%) and specificity (98-99%) and are relatively rapid to perform compared to other methods [160,161]. However, similar to SPT, these diagnostic methods are characterized by a high rate of false positive or negative results when used to predict clinical disease, especially in patients with a high total serum IgE concentration [163,170]. These methods have a potential application in the detection of allergenicity of kiwifruit or related products. Le et al. (2011) evaluated the IgE-binding capacity of kiwifruit allergens present in six varieties by ELISA. The results showed that "Hayward" had the highest allergenicity (82%), followed by "Eriantha 96" of 55%, "Eriantha 114" of 55%, and "Summer 3373" of 45%. The lowest rate of allergenicity was observed in "Jintao" of 19%. However, this test is limited by cost to develop and perform the assay as the required biological reagents (e.g. primary and secondary detection antibodies) are derived from the animals (e.g. rabbit). Similar methods include western blotting and dot blotting of the IgE, but these have similar limitations.

DNA-based detecting methods including polymerase chain reaction (PCR) and real-time polymerase chain reaction (Rt-PCR) are used to detect specific allergen concentrations in food products in the research setting. In comparison to clinical diagnostic methods, DNA-based methods including PCR and Rt-PCR are used to test the concentrations of allergens present in food [164]. Detection of DNA has been shown to be more stable under different processing conditions (e.g. high temperature) compared to proteins that are potentially modified during processing [162]. When the protein content present in fruit (e.g. kiwifruit) is low, DNA-based methods can be used to detect minute contaminations from the fruit. PCR or Rt-PCR detection methods have been used to detect the concentrations of allergens present in citrus fruits [171], peaches [172] and strawberries [173], and is highly specific and sensitive compared to the traditional detection methods [174]. As kiwifruit allergy is not as common as the "Big Eight" allergies (peanut, tree nut, milk, egg, soy, sesame, fish, and wheat allergies), there are very limited reports regarding the detection of kiwifruit or related commercial products. Taguchi et al. (2007) developed a PCR method targeting the ITS-1 region in kiwifruit by designing two primer pairs (F123 and R178). The results showed that this method is able to detect amounts ranging from 5 to 50 fg of kiwifruit allergens. Further, Graziano et

al. (2018) have developed a quantitative real-time PCR to quantify the concentrations of kiwifruit allergens in complex food matrices. The results represented a specific and sensitive method for the rapid detection of allergens levels in mixed foods. However, the practical applications in food industries of these DNA-based methods like PCR are limited due to the complexity of the protocols and expense of related equipment. Therefore, development of novel diagnostic and detecting methods with improved sensitivity, specificity, ease of operation and low cost are needed.

3.3.4. Global epidemiology of kiwifruit allergy

Kiwifruit (*Actinidia*) is native to the Northeastern of China, specifically the Yangtze River valley, identified at the beginning of the 1900s [175]. It was first introduced in New Zealand in 1904 and exported to Europe and the United States started in 1962 [154]. Currently, kiwifruits are produced worldwide in countries including China, New Zealand, France, Italy, United States, Spain and Australia [176]. The first publication on kiwifruit allergy was in 1981 [143], and following that more patients living with kiwifruit allergy were reported. In the last decade, the data obtained from the Web of Science (April 2018) shows that the total number of publications on kiwifruit allergy has risen to 135, reflecting the increasing prevalence of kiwifruit allergy. In some European countries, for example, in Spain, approximately 1.8% of the total population are allergic to kiwifruit [9]. In France, among 182 children (2-14 years of age) with a history of allergies to one or more food, 9% were allergic to kiwifruit [7]. Currently, global statistics regarding the distribution of kiwifruit allergy is limited. The database of Web of Science shows that the majority of the publications (95%) on kiwifruit allergy are from Europe and USA. In contrast, only 5% of publications are from Asian countries (e.g. India and Japan). There is a higher concentration of researchers working on kiwifruit allergy in Europe possibly reflecting the prevalence of the disease in these regions [177,178]. Further, a large amount of birch trees located in European countries may be also contributing to the frequency of kiwifruit allergy through cross-reactivity between birch pollen and homologous structures found in kiwifruit proteins [123,179]

3.4. KIWIFRUIT ALLERGENS

The World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee have identified thirteen key allergenic proteins present in green kiwifruit (*Actinidia deliciosa*) and three in gold kiwifruit (*Actinidia chinensis*) (**Table 3.4**). Among these allergens, Act d 1 and Act d 2 are the first two kiwifruit allergens identified by Pastorello et al. (1998). Act d 1 is one of the main kiwifruit allergens with a 30 kDa molecular weight belonging to the papain-like family of cysteine proteases [151]. Act d 2 is a 20-26 kDa member of thaumatin-like proteins (TLP) with 200 amino acid residues [180]. These proteins are expressed in fruit tissues when the plants are stressed (e.g. by drought) or when infectious pathogens activate the defense system [181-183]. Act d 3 is a 40 kDa protein attached to complex glycans (cross-reactive carbohydrate determinants) [184]. Act d 4 is a 12-16 kDa phytocystatin and is considered a cysteine proteinase inhibitor [151,185].

Act d 5 (kiwellin) is a cysteine-rich 26-28 kDa protein which is abundantly presented in green and gold kiwifruit [186]. It consists of two domains belonging to the expansin, N-terminal domain family [191,194]. Generally, Act d 6 (18 kDa) is expressed in ripe fruits involving the synthesis of pectin methylesterase [187]. Fewer patients are allergic to Act d 7 which belongs to pectin methylesterase with a 50 kDa molecular weight [195]. Act d 8 (15-18 kDa) is one of pathogenesis-related (PR-10) proteins that is found to be homologous to birch pollen [83,189]. They are unstable proteins that can be eliminated through thermal processing (e.g. cooking or steaming) [196]. Act d 11 has been recognized as one of the main ripening-related proteins present in kiwifruit [197]. Both Act d 11 and Act d 8 belong to the Bet v 1 family and have been implicated in allergic reactions normally linked to the OAS [190]. Act d 9 (profilin) and Act d 10 (nsLTP) are two other minor allergens present in kiwifruit [151]. In 2014, two novel allergens: Act d 12 (a 50.2 kDa 11S globulin) and Act d 13 (a 12 kDa 2S albumin) were extracted from green kiwifruit seeds [13]. Studies found that these proteins have cross-reactivity with allergens present in peanuts and tree nuts [13,198].

Table 3.4. Main allergens present in kiwifruit (data source: WHO/IUIS Allergen Nomenclature Sub-Committee, 2010-2017).

Species	Allergen	MW	Biochemical name	Property	References
Green kiwifruit	Act d 1	30 kDa	Cysteine protease (actinidin)	Major allergen	[151]
(*Actinidia*	Act d 2	24 kDa	Thaumatin-like protein	Major allergen	[180]
deliciosa)	Act d 3	40 kDa	NA	Minor allergen	[184]
	Act d 4	11 kDa	Phytocystatin	Minor allergen	[151]
	Act d 5	26 kDa	Kiwellin	Minor allergen	[186]
	Act d 6	18 kDa	Pectin methylesterase inhibitor	Minor allergen	[187]
	Act d 7	50 kDa	Pectin methylesterase	Minor allergen	[188]
	Act d 8	17 kDa	Pathogenesis-related protein (PR-10)	Major allergen	[189]
	Act d 9	14 kDa	Profilin	Minor allergen	[151]
	Act d 10	10 kDa	Non-specific lipid transfer protein	Minor allergen	[151]
	Act d 11	17 kDa	Latex protein/ripening-related protein	Major allergen	[190]
	Act d 12	50.2 kDa	Cupin, 11S globulin	Major allergen	[13]
	Act d 13	113.6 kDa	2S albumin	Minor allergen	[13]
Golden kiwifruit	Act c 5	28 kDa	Kiwellin	Major allergen	[191]
(*Actinidia*)	Act c 8	17 kDa	PR-10	Minor allergen	[192]
chinensis)	Act c 10	10 kDa	nsLTP	Minor allergen	[193]

As shown in **Table 3.4**, there are also three allergens, Act c 5, Act c 8 and Act c 10 extracted from *Actinidia chinensis* (golden kiwifruit). Act c 5 (kiwellin) is the main allergen and is a 28-kDa protein representing approximately 30% of the total protein found in gold kiwifruit [191]. The 17-kDa Act c 8 belongs to the PR-10 family and is considerably less abundant in gold

kiwifruit [192]. This protein is encoded by open reading frames (ORFs) of 474 nucleotides, corresponding to 158 amino acid residues. There are six ORF sequences related to Act c 8 isoforms which were detected in gold kiwifruit [83]. Act c 10 is a 10-kDa protein belonging to the ns-LTP involved in the defense mechanisms of the plant [193].

3.4.1. Structural Properties of Kiwifruit Allergens

There are four main kiwifruit allergen families including PR-10, TLPs, nsLTPs, and profilins which can induce allergic reactions [11]. PR-10 (pathogenesis-related) proteins consist of 151-162 amino acids [199], and are presented in many fruits such as cherry, peach, and apple. These proteins are associated with symptoms of oral allergy syndrome reactions. LTPs are approximately 200 amino acid proteins and are significantly produced when the plant suffers from external stressors, like viral or bacterial infections [66]. The ns-LTPs are generally found in the plant-based food sources (e.g. strawberry, peach) and have a 7-9 kDa molecular mass [11]. Profilins are a 12-15 kDa protein family considered as functional proteins to regulate the polymerization of the actin filament [199]. Profilins are the most common allergens presented in many fruits including orange, mango, sweet cherry, strawberry, pineapple, and melon [72].

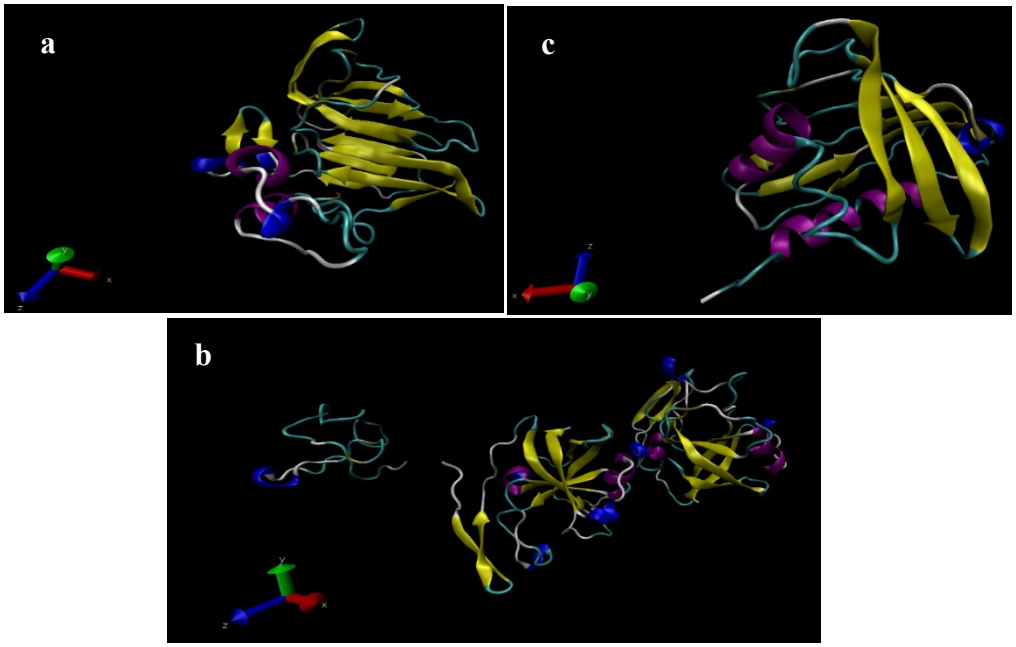


Figure 3.2. Secondary structure of Act d 2 (a), Act d 5 (b), and Act d 11 (c) allergens present in kiwifruit (Source: protein data bank, α-helix: Purple, 3/10 helix: Blue, η-helix: Red, β-sheets: Yellow, Turns: Cyan, coils: White).

The kiwifruit allergen, Act d 2 consists of a chain with 201 residues (PDB database, 2013). It is made up of 13% helical (6 helices; 28 residues) and 38% beta sheets (18 strands; 77 residues) in the form of the secondary structure (**Figure 3.2**). Act d 5 is a two-chain protein of 189 residues (PDB database, 2015). 10% helical (5 helices; 19 residues) and 26% beta sheets (12 strands; 50 residues) are observed in the secondary structure of this protein. Act d 11 is a 17-kDa protein that is found abundantly in ripe kiwifruit [200]. The secondary structure of Act d 11 is made up of 22% of helical (4 helices; 33 residues) and 46% of beta sheets (7 strands; 70 residues) containing 150 residues and 1299 atoms (PDB database, 2013). Studies have reported that 10% of all kiwifruit-allergic patients produce IgE specific to Act d 11 [197]. The related data regarding the secondary structure of other kiwifruit allergens are not available from PDB.

As shown in **Table 3.5**, Act d 8 present in kiwifruit is from PR-10 family and shows high sequence similarities to those proteins present in different fruits, vegetables, and pollens. These plant sources include cherry (Pru av 1), apricot (Pru ar 1), tomato (Sol al 4), pear (Pyr c 1), strawberry (Fra a 1), peach (Pru p 1), apple (Mal d 1), carrot (Dau c 1), celery (Api g 1), birch pollen (Bet v 1) and beech pollen (Fag s 1). These allergens share four similar amino acid sequences: FKAFVLD, LEGDGGVGTIK, TFGEGS, and VKHRIDG with kiwifruit allergen (data source: GenBank database). Many studies have reported that these sequence similarities in proteins may cause significant cross-reactivity [201,202]. Cross-reactivity between kiwifruit allergens and birch pollen was reported in 1994, where the cross-reactivity was evaluated through the use of immunoblot and radioallergosorbent techniques [123]. Clinical cross-reactivities of 55 kiwifruit allergic individuals were tested by an oral food challenge. Fifty percent of patients tested showed clinical cross-reactivity with the fruits from the Rosaceae family such as peach and pear [198].

Another kiwifruit allergen, Act d 10 belonging to nsLTP family also has a homologous sequences to the allergens present in apricot (Pru ar 3), mulberry (Mor n 3), peach (Pru p 3), orange (Cit s 3), peanut (Ara h 9) and hemp pollen (Can s 3) (as shown in **Table 3.5**). These similar sequences include KCGV, CGQV, PDRQAACNCLKQ, AAAL, and ISRSTDCSK, which are potentially cross-reactive. There are no studies reported to examine whether linear conformations exist in nsLTPs, and further studies are required. Recently, a new allergen, Act d 12 has been recognized in kiwifruit belonging to 11S globulin protein family.

Table 3.5. Amino acid sequence similarities between different allergens (data source: WHO/IUIS Allergen Nomenclature Sub-Committee, 2010-2017). Note: a. The amino sequences of Api g 1 are FQGFVID, IKGDGGPGTLK, MTLRIDG; b. IEGSGGPGTIK, VKHRIDE; c. FRCAGVAA, SGCPE, ESQQ; d. FECAGVA, QSQQ, PGCPR; e. AAGL, ISPSTDCKS; f. RCGV, ISTSTNCAT; g. RCGV, ISTSTNCAT.

Protein types	Amino acid sequences	Allergen sources
Pathogenesis-related protein (PR-10)	FKAFVLD LEGDGGVGTIK TFGEGS VKHRIDG	Kiwifruit (Act d 8); Apricot (Pru ar 1); Cherry (Pru av 1); Tomato (Sol al 4); Pear (Pyr c 1); Strawberry (Fra a 1); Apple (Mal d 1); Peach (Pru p 1); Celery[a] (Api g 1); Carrot (Dau c 1); Birch pollen (Bet v 1); Beech pollen[b] (Fag s 1);
11S globulin	FQCAGVA PGCPE OPQQ	Kiwifruit (Act d 12); Pistachio (Pis v 5); Peanut (Ara h 3); Soybean (Gly m 6); Walnut (Jug r 4); Hazelnut (Cor a 9); Brazil nut[c] (Ber e 2); Almond[d] (Pru du 6)
Non-specific lipid transfer protein (nsLTP)	KCGV CGQV PDRQAACNCLKQ AAAL/ISRSTDCSK	Kiwifruit (Act d 10); Apricot (Pru ar 3); Mulberry[e] (Mor n 3); Peach (Pru p 3); Orange (Cit s 3); Peanut[f] (Ara h 9); Hemp pollen[g] (Can s 3)

As shown in **Table 3.5**, Act d 12 shares significant similar sequences, including FQCAGVA, PGCPE, and OPQQ, with proteins present in plant seeds such as tree nuts, peanuts, and soybean. This plant-based protein is also found in walnut (Jug r 4), pistachio (Pis v 5), almond (Pru du 6), brazil nut (Ber e 2), and hazelnut (Cor a 9). Similar IgE-binding epitopes in kiwifruit seeds (e.g. storage protein, Act d 12) are also found in nuts (e.g. hazelnut, peanut, and walnut) and peanut proteins [198,203]. This spectrum of potentially cross-reactive proteins across a wide range of fruits, vegetables, nuts, and seeds may explain why many varied food sources induce allergic symptoms in highly sensitized individuals. One study of 59 adolescents and adults with peanut allergy showed 39% of these patients had allergic symptoms after consuming kiwifruit [204].

3.4.2. Ripening-related Allergens

Table 3.6. Ripening related allergens present in a variety of fruits.

Species	Ripening- related allergens	MW	Biochemical name	References
Kiwifruit	Act d 1	30 kDa	Cysteine protease (actinidin)	[157]
	Act d 2	24 kDa	Thaumatin-like protein	
	Act d 5	26 kDa	Kiwellin	
	Act d 11	17 kDa	Major latex protein/ripening-related protein (MLP/RRP)	
Peach	Pru p 3	9 kDa	Lipid transfer protein (LTP)	[111]
Apple	Mal d 1	17 kDa	Pathogenesis-related protein (PR-10)	[15]
	Mal d 3	9 kDa	Non-specific lipid transfer protein (nsLTP)	[16]

After harvest, many fruits need a period of ripening prior to consumption. This ripening improves color, flavor and nutrients content, enhancing the quality of the fruit [104,105]. However, studies have shown that maturity related allergens, present in kiwifruit, significantly increase during this storage period. During the ripening stages, total protein content has been shown to dramatically increase from 20 to 300 mg/100 grams in both green and gold kiwifruit [157]. The three allergenic proteins actinidin (Act d 1), TLP (Act d 2), and kiwellin (Act d 5), concentrations present in the kiwifruit, picked after commercial harvest (42 d), reached a higher level compared to those picked before commercial harvest (104 d) (**Table 3.6**). The WHO/IUIS Allergen Nomenclature Sub-committee has described that Act d 11 is also a ripening-related allergen present in kiwifruit [197]. Thus, there are four ripening-related allergenic proteins abundantly synthesized during the period of postharvest ripening.

Other fruit species such as peaches have similar-type ripening-related allergens like Pru p 3, a 9-kDa lipid transfer protein (LTP) [111]. During the ripening period, SDS-PAGE and immunoblotting analyses showed that the protein bands intensity corresponding to Pru p 3 in fully ripened peaches increased compared to the fruits harvested 15 days before commercial ripening dates. In the ripening stage, peaches undergo significant stressors such as high temperatures, pests, and diseases which may enhance the synthesis of Pru p 3 allergen as the first line of defense. In apples, Mal d 1 (17 kDa) and Mal d 3 (9kDa) are two major ripening-related apple allergens. Studies have reported that Mal d 1 belongs to pathogenesis-related proteins (PR-protein) and is present in unripe 'Golden Delicious' and 'Topaz' apples. This allergen sharply increased after an 8-week storage time [15]. Mal d 1 is upregulated by internal (e.g. polyphenol oxidase activity) and external factors (e.g. temperatures) resulting in its increased synthesis in stored apples and is specifically increased by 125% in 'Golden Delicious' apples and by 750% in 'Topaz' apples [15]. Similarly, the concentration of Mal d 3, a 9-kDa nsLTP is two times higher in fully matured apples compared to immature stages [82]. This significant increase is attributed to the increase of respiration rate and ethylene biosynthesis. These two factors are associated with the ripening and softening of apples. Altogether these data suggest that the expressions of fruit allergens differ significantly in various fruits during their ripening stages and highlights the need to fully elucidate the relationship between the concentrations of fruit allergens and ripening stages of fruits.

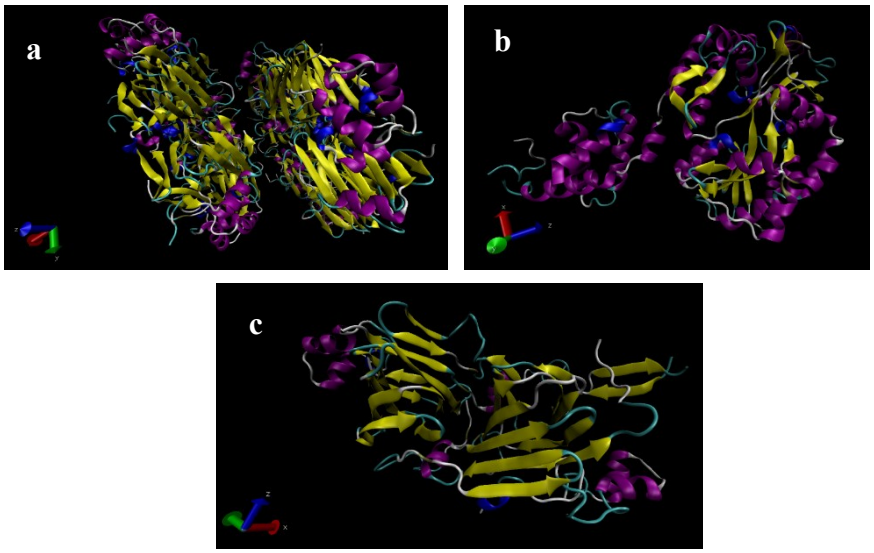


Figure 3.3. Secondary structure of Ara h 1 (a), Ara h 2 (b), and Ara h 3 (c) allergens in peanut (α-helix: Purple, 3/10 helix: Blue, η-helix: Red, β-sheets: Yellow, Turns: Cyan, coils: White).

3.5. PROCESSING TECHNIQUES AND KIWIFRUIT ALLERGY

3.5.1. Thermal processing

In the food industry, thermal processing is a primary technique widely used to destroy bacteria and enzymes through the combination of temperature and time [205]. The established processing conditions depend on the kind of food. Several studies have reported that some thermal processing methods including sterilization, microwave heating, boiling, and steam cooking showed potential applications in reducing food allergenicity [206]. For example, whey protein present in milk when treated with heat at 90-95°C resulted in a reduction in its IgE-binding capacity [207]. Moreover, several studies reported that boiling peanuts at 100°C for 20-30 mins in water effectively reduced the allergen content of Ara h 1, Ara h 2 and Ara h 3 [208,209] (**Figure 3.3**). These changes in IgE-binding capacity and concentrations of allergens mentioned may be due to the denaturation of allergenic proteins under the high-temperature condition for a longer duration [210].

Among kiwifruit allergens, Act d 2 is from the family of thaumatin-like proteins (TLPs). The structural integrity of these proteins is prone to change under stressful conditions (e.g. higher temperature). For example, in one study, F2/4JRU, a thaumatin-like protein in grape wine was shown to be temperature sensitive to temperature such that the protein structure was unfolded when is exposed to high temperatures (56°C) [211]. Denaturation of TLPs may be related to the unstable properties of some bonds (e.g. hydrogen bonds) [212]. There are three domains in the secondary structure of most TLPs. Domain I is located in the center of protein and consists of several β-strands; domain II consists of several α-helixes, while domain III is comprising of β-strands and small loops. S-S bonds present in each domain can stabilize the protein [213]. The S-S bonds present in soy milk proteins [214] and whey proteins [215] can be cleaved under specific stressful conditions such as high temperatures. The cleavage of S-S bonds results in the loss of secondary structure, which may lead to a reduction in the IgE binding capacity of protein allergens [216,217]. In kiwifruit, two major allergens, Act d 1 and 2 when treated with steam cooking at 100°C for 10 mins were lighter, when running on an SDS-Page gel compared to untreated controls [124]. These heated proteins also had reduced the IgE-binding capacity as demonstrated by immunoblotting. Together these data suggest that Act d 1 and 2 are thermally

unstable proteins and are prone to progressive loss of tertiary and secondary protein structures under specific temperature conditions. The effects of thermal processing on the structures of Act d 8 and Act d 11 have not been studied. In contrast, Act d 12 (50.2 kDa) is a novel relevant kiwifruit allergen from the globulin protein family. The secondary structure of Act d 12 shows slight reversible alterations when exposed to temperatures in the range of 20-80°C. It suggests that this allergen is highly resistant to thermal denaturation [198]. Overall data suggest that thermal processing can reduce the allergenic potential of kiwifruit.

However, thermal processing may also lead to chemical and physical changes, which in turn may decrease the sensory quality and amounts of nutritional compounds in the fruit, especially some heat-sensitive compounds such as ascorbic acid and polyphenols [205,218]. In apple juice, thermal processing at 90°C for only 30 seconds led to a 20-100% reduction of volatile flavor-related compounds [219]. The overall sensory properties of apple juice decreased significantly under thermal processing at 90°C for one minute compared to untreated apple juice [220]. Thermal treatment at 90°C for 100 seconds resulted in improving the turbidity of apple juice compared to untreated fresh apple juice [221]. Tomato puree has also been shown to be affected by thermal processing. The total phenolic content, vitamin concentration, and antioxidant activity significantly decreased after pasteurization processing at 98-128°C compared to untreated samples [218]. Currently, minimally processed food products with high nutrients content, natural flavor, and taste are increasing in popularity over the processed products. Thus, there is a need for novel thermal or non-thermal processing technologies that will potentially reduce the food allergenicity and also maintain or improve the food quality.

3.5.2. Non-thermal processing: high-intensity ultrasound treatment

A novel non-thermal application, ultrasound treatment has garnered attention as higher nutrients amounts, freshness, flavor, and color attributes are maintained during ultrasound processing [222]. This technique is an alternative processing method that is inexpensive, reliable, and environmentally friendly [223]. Several studies have shown that high-intensity ultrasound (20-100 kHz) treatment could reduce food allergenicity. In one study, the shrimp were treated with a sonicator at 30 kHz, 800 W for 30-180 min [224]. There was significantly decreased shrimp

allergenicity (IgE-binding capacity) by 74.7% after 180-min ultrasonic processing compared to untreated samples. A study of soybean protein demonstrated that high-intensity ultrasound treatment at 37 kHz for 10 min effectively disrupted the secondary structure of soy proteins resulting in a decrease of 24% in soy protein allergenicity [225]. In peanuts, the IgE-binding activity of peanut protein extracts was decreased by 10% after a high-intensity ultrasound processing at 50 Hz for one hour compared to untreated peanut samples [137].

These reductions in the IgE-binding activity of related food proteins may represent secondary structural changes in the proteins following ultrasound processing. Generally, during high-intensity ultrasound processing, cavitation effects are formed due to the rapid collapse of bubbles, leading to a significant increase in pressure (up to 70-100 MPa) and temperature (up to 5000 K) [226,227]. This provides enough energy to cause physical and chemical modifications of the proteins resulting in the changes of allergens conformation and their IgE banding activity. Further, high shear stress generated by ultrasound processing can produce micro streams resulting in the formation of a molten globule state of the protein [223,227]. These data suggest that ultrasound treatment may be a potential processing technology in the reduction of kiwifruit allergen, although this remains to be rigorously investigated.

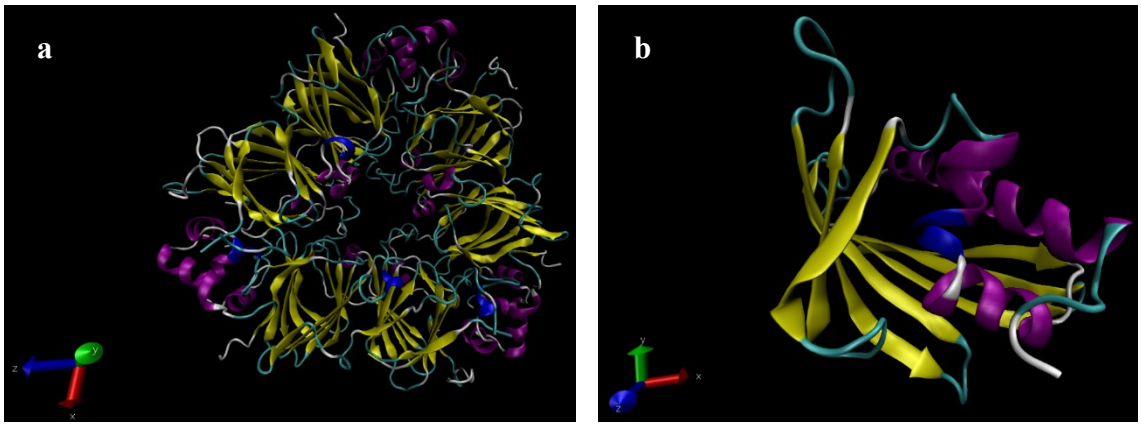


Figure 3.4. Secondary structure of glycinin (a) allergen present in soybean and Mal d 1 (b) allergens present in apple (Source: protein data bank, α-helix: Purple, 3/10 helix: Blue, η-helix: Red, β-sheets: Yellow, Turns: Cyan, coils: White).

3.5.3. Chemical processing: enzyme and ethylene treatment

3.5.3.1. Enzyme treatment

In addition to thermal processing, chemical treatments may also reduce food allergenicity.

Several studies have observed that specific allergy-related proteins are hydrolyzed by related enzymes. For example, when gliadin, a major wheat flour allergen, was treated with 0.01% alcalase combined with papain. The gliadin content significantly decreased, and lower IgE-binding was demonstrated compared to untreated samples [228]. In another study, there was a degradation of major soybean allergen (proglycinin) (**Figure 3.4a**) up to 100%, 100%, and 95.9% after alcalase, pepsin, and papain treatments, respectively [229]. In avocado, Pres a 1, a major avocado allergen was fully inactivated after pepsin treatment (0.32% (w/v) measured by an immunoblot inhibition analysis [230]. These reductions of food allergens by enzymatic hydrolysis may result from the changes in their structure, solubility and foaming characteristics. In kiwifruit, studies have reported that allergen properties are impacted by enzymes such as pepsin [231]. Specifically, when Act d 1 and Act d 2 were treated with pepsin in the ratio of 1:20 by weight [148]. There was a slight reduction in IgE-binding ability of Act d 1 after 120 min. Importantly, a 75% reduction in IgE binding capacity of Act d 2 was noted in these pepsin-treated samples. Act d 1 and Act d 2 differ in protein structures and molecular weight which may explain the differences in the effectiveness of pepsin treatment. The fragments of Act d 1 and Act d 2 remaining after pepsinolysis were then treated with trypsin and chymotrypsin in the ratio of 1:400:100 by weight. The remaining fragments were rapidly broken down by this additional treatment [148]. During these two enzymatic treatment stages, the allergenicity of these allergens was reduced drastically due to the disruption of disulphide bonds present in these allergens. The S-S bonds play a role in resisting digestion with proteases. Similar studies had reported that Pers a 1, an avocado allergen was highly sensitive to related enzymes (e.g. pepsin A) resulting in the elimination of allergic reactions when its structure was disrupted due to the fracture of disulphide bonds [148,230].

However, several studies have reported that enzymatic hydrolysis treatment shows a negative effect on food quality and taste. For example, soy protein isolates were treated with alcalase, pepsin or papain, respectively. Alcalase treatment increased the intensity of bitterness (8.2/10) when compared to all other treatment groups [229]. Pepsin treated samples were significantly more “sour” and “astringent” taste compared to other treatments and untreated samples. Similar results have been shown with pea protein hydrolysates, alcalase treated samples had the most bitter taste compared with that of papain and α-chymotrypsin treatment and untreated samples

[232]. This extra "bitter" or "astringent" flavor is strongly related to the production of hydrophobic bitter peptides during proteolytic reactions resulting from enzymatic hydrolysates [229,233]. These negative attributes limit the application of enzymatic hydrolysis processing in food systems. Therefore, it is necessary to evaluate the advantages and disadvantages before using this processing technology in the reduction of food allergens.

3.5.3.2. Ethylene treatment

During the growth, development, and senescence of kiwifruit, ethylene plays a primary role in the synthesis and metabolism of nutrients (e.g. sugars, vitamins or proteins), and physiological changes (e.g. color, flavor, texture), especially during the ripening stages [234,235]. Ethylene is considered as a multifunctional phytohormone that regulates the growth and senescence of plants depending on its concentration and mode of application. For example, strawberries when treated with ethylene accumulated higher amounts of sugar and titratable acidity compared to untreated controls [235]. While, fresh green in-husk walnut fruit treated with 500 mg/L ethephon had reductions in the decay index of fruit, increases of total phenol content and maintenance of a better kernel quality when compared to untreated walnuts [115]. Treatment of kiwifruit with 1000 μL/L ethephon significantly increased the soluble solid concentrations and total phenols content leading to maintenance of a better kiwifruit quality [236].

Ethylene has also been shown to influence the de novo synthesis of proteins in fruit. The total protein content of apples significantly increased by 13.5% following ethylene treatment, and gene expression of a major apple allergen (Mal d 1) (**Figure 3.4b**) was found to be up-regulated (Zheng et al., 2013). In kiwifruit, ethylene treatment slightly increased the total protein content. Interestingly, two kiwifruit allergens, pectin methylesterase (Act d 7) and pectin methylesterase inhibitor (Act d 8) are undetectable after ethylene treatment [157]. It is possible that, in kiwifruit, the process of ripening following ethylene treatment leads to degradation of these allergic proteins with a small molecular weight. Ethylene treatment for purposes of allergen reduction requires further study.

3.6. MANAGEMENT AND TREATMENT OF FOOD ALLERGY

3.6.1. Dietary management

Kiwifruit allergic individuals must maintain complete abstinence from the ingestion of the fruit and must learn to manage unexpected allergic reactions that may occur following accidental exposure to the kiwifruit or related products. Kiwifruit is a versatile ingredient which is found in various food products worldwide including cakes, juice, jam, ice cream, fruit wine or fruit salads. Avoidance is challenging especially for children. The International Life Sciences Institute (ILSI) has published official guidelines to provide accurate dietary limitation information, and a comprehensive list of allergens [237]. However, as there are multiple allergenic proteins in kiwifruit, the list of potential cross-reactive foods is significant, and many patients are not sensitized to all the potential allergens found in the fruit. Thus, while one patient should avoid all foods with cross-reactivity, another may only need to avoid kiwifruit in the fresh form. For kiwifruit, the potential allergen sources include pollens from birch trees, grass or ragweed, other kinds of fruit (e.g. banana) or vegetables (e.g. avocado), peanut or tree nuts and latex or related products [151,152,198,238]. Generally, apples, oranges, and pears or their combination can be used as substitutes for kiwifruit in the diet due to similar amounts of vitamin C and dietary fiber.

Unlike the common “Big Eight” major food allergens, the labeling of kiwifruit or related products is only recommended in Japanese legislation, while the labeling requirement is not presented in the EU Labelling Directive [239,240]. It is essential that the consumers allergic to kiwifruit read the detailed ingredients information of each product carefully. Furthermore, in some cases contacting the manufacturer to make sure there is no kiwifruit in a particular food product is required. Kiwifruit may be found as a “hidden ingredient” in various food products such as yogurt, juice, jams, and confections. Therefore, it is necessary to access the detailed ingredients lists of these food products before purchasing.

3.6.2. Emergency treatment

Kiwifruit can cause a wide range of symptoms from Oral allergy syndrome (OAS) to severe symptoms, life-threatening anaphylactic reactions which may include hypotension, tachycardia or loss of consciousness [157]. These reactions may lead to coma or even death if emergency treatments are not used in a timely manner. Antihistamines are prescribed to treat mild local symptoms including urticaria (hives), pruritus (itch), itchy eyes and runny nose. Antihistamines act by blocking the histamine receptor and preventing histamine released from activated mast cells [23]. When the allergic reaction extends to involve two organ systems, epinephrine injection is considered as the first treatment required [241]. Epinephrine also known as adrenaline is available as a self-injectable medication and was approved by the U.S. Food and Drug Administration in 1987 as its highly effective and safe for the management of anaphylaxis. Epinephrine primarily acts on alfa and beta-adrenergic receptors of the sympathetic nervous system. The insulin secretion is inhibited by regulating glycogenolysis when it combines with the alfa-adrenergic receptors. The combination of epinephrine with beta-adrenergic receptors increases the secretion of glucagon and adrenocorticotropic hormone, and also enhance lipolysis in the organs [242]. Together, these effects improve blood pressure and relax the smooth bronchial muscles improving airflow and respiration. Additional doses of epinephrine and intravenous fluids are also used to expand the blood volume in hypotensive patients.

3.6.3. Future immunotherapy

Currently, there are no immunotherapy options that consistently cure food allergies. However, several immunotherapy strategies are under investigation. The final goal of oral immunotherapy, or allergen-specific desensitization is to increase the level of tolerance to specific allergens by gradually increasing the amounts of the offending allergens ingested by allergic patients. In one study of 62 peanut-allergic children aged one to ten years, there was a decrease in the wheal size of the peanut-specific skin prick test following 18 months of oral peanut immunotherapy [243]. Many patients were able to tolerate ingestion of small amounts of peanut without clinical symptoms. However, there were significant side effects, and some patients experienced anaphylactic episodes during the process. While oral immunotherapy is

under development for other food allergens, this approach is time-consuming and carries significant risks of anaphylaxis during desensitization. Sublingual immunotherapy is also used for pollen allergies. In Canada there are now four sublingual pollen allergen immunotherapy products: Grastek, Oralair, Acarizax, and Ragwitek which are approved by Health Canada, for patients aged 10 to 65 years with grass or ragweed pollen and dust mite allergy [244]. Interestingly, while sublingual therapy does reduce the symptoms associated with inhalation of pollens during allergy seasons, there as yet appears to be no significant improvement in OAS in these patients. Taken together, these approaches may reduce the clinical symptoms in some patients but not for most. Thus, much work remains to be done in reducing the risks of allergic reactions to foods in individuals living with food allergies.

3.7. CONCLUSION

Kiwifruit can trigger a range of allergic symptoms from mild OAS to the severe, life-threatening anaphylaxis. Kiwifruit allergy affects a significant number of individuals and the frequency of the allergy appears to be increasing possibly due to an increased sensitization to other related allergens including pollen (e.g. birch pollen), other fruits (e.g. avocado or banana) or legumes (e.g. peanuts). Further studies regarding the pathogenesis, clinical features, and diagnosis of kiwifruit allergy will progress our understanding of the specific mechanisms of kiwifruit allergy. Complicating management of kiwifruit allergy is the variability of allergenic proteins during ripening stages. The four allergenic proteins Act d 1, Act d 2, Act d 5 and Act d 11 present in kiwifruit are ripening-related allergens. These findings provide a deep understanding of the relationship between the kiwifruit allergens concentrations and the ripening stages of kiwifruit. In food industries, many studies focus on the food processing impact on the reduction of kiwifruit allergens concentrations through changing of the secondary and tertiary protein structures. Although several processing methods such as steam cooking, enzyme, and ethylene treatment can be beneficial to reduce the allergenicity of kiwifruit, the efficacy of these processing methods need to be improved and well-studied. Novel processing techniques also need to be developed. Recognition of kiwifruit allergy, education regarding appropriate management and development of strategies for desensitization would improve outcomes and quality of life parameters for patients living with this food allergy.

CONNECTING TEXT

The literature review of part 2 (Chapter III) has reported the pathogenesis, clinical characteristics, diagnostic methods (medical and laboratory protocols) of kiwifruit allergy. Further, the secondary protein structures of kiwifruit allergens were analyzed and found that modification in the protein structures may cause a reduction in the IgE binding capacity of Act d 2, kiwifruit allergen. From the literature review, it is clear that variety, maturity, fruit components (peel, pulp, and seed) of fruit may be strongly associated with the allergenicity of kiwifruit. Further, non-thermal (e.g., ultrasound) and thermal processing (e.g., microwave) methods showed potential applications in the reduction of kiwifruit allergenicity through modifying the secondary structures of fruit proteins.

In the next chapter, the effect of physiological characteristics (variety, maturity, and fruit components) and storage conditions on the allergenic potential of kiwifruit were evaluated. Further, the antioxidants and their activities of kiwifruit under different conditions are determined. Hopefully, it can help us to shed some light on the possible physiological synthesis mechanism of kiwifruit allergens.

CHAPTER IV

EFFECT OF VARIETY, MATURITY, FRUIT COMPONENTS AND STORAGE CONDITIONS ON THE ALLERGENIC POTENTIAL OF KIWIFRUIT AND ITS RELATIONSHIP WITH ANTIOXIDANT ACTIVITY

4.1. ABSTRACT

The objective of the study is to explore the effect of physiological characteristics (variety, maturity, and fruit components) and storage conditions on the allergenic potential of kiwifruit. The relationship between kiwifruit allergen (Act d 2) and antioxidant compounds of fruit was also assessed. The results found that Act d 2 allergen was only measured in the green kiwifruit rather than golden kiwifruit. The Act d 2 allergen of seed was 2-fold and 4-fold higher than that of pulp and peel, respectively. The highest Act d 2 allergen content was determined in ripe kiwifruit, followed by overripe fruit and unripe fruit. During the 10-day storages at different temperatures, the results found a 50% of enhancement in Act d 2 content was observed when stored at 20 °C, compared with the initial level. Further, the Act d 2 content showed a negative relationship with the ascorbic acid content and total antioxidant activity at various conditions. It indicates antioxidants as an important factor that may be involved in the regulation of the expression of Act d 2 allergen in kiwifruit. These findings help us to understand the distribution of Act d 2 in different fruit components and its relationship with antioxidants.

KEYWORDS: Kiwifruit; allergenic potential; maturity; storage conditions; antioxidant activity

4.2. INTRODUCTION

Kiwifruit (*Actinidia deliciosa*) is one of the most popular fruit over the world due to its nutritional values and pleasant taste. In 2018, the United States Department of Agriculture (USDA) Nutrient Database has reported that kiwifruit is an excellent source of dietary fiber, carbohydrates, sugars, vitamin C and E, minerals, omega-3 fatty acids, and phenolic compounds. Many studies have reported that these nutrients present in kiwifruit are beneficial to the prevention of cancer and cardiovascular-related diseases [3,4]. For example, Collins et al. (2001) observed that the consumption of eight kiwifruits in the form of juice was strongly linked to the decrease of DNA damage in human cells compared to the control group (water) [140]. Oxidative DNA damage is positively correlated with some cancers [141]. Thus, consuming kiwifruit may potentially reduce the risk of cancers due to its antioxidant effect. Furthermore, in one study of 43 volunteers with hyperlipidemia, a significant decrease in high-density lipoprotein cholesterol content was observed after an eight-week consumption of two kiwifruits in the form of juice per day. Vitamin C and E levels in the blood were also increased [142].

However, kiwifruit is increasingly recognized as a common elicitor causing allergic reactions worldwide since the first case accrued in 1981. In Sweden and Denmark, nearly half of food-allergic individuals reported kiwifruit allergy in self-reported surveys [8]. In Spain, approximately 1.8% of the total population are allergic to kiwifruit [9]. In France, in a study of 182 children (2-14 years of age), 9% of the population reported hypersensitivity to kiwifruit [7]. Further, many studies have reported that there is a cross-reactivity between kiwifruit and pollen because of them sharing similar epitopes in protein [11]. Pollen allergy is estimated to occur in 20-40% of the population worldwide [12]. In European countries, approximately 40% of individuals were reported hypersensitive to the tree and/or weed pollens [144], while in North America, pollen allergies are reported in >20% of the population [145]. In one study of 55 patients (female: male=39:16) with oral food challenge diagnosed kiwifruit allergy, 22% had isolated kiwifruit allergy with no cross-reactivity to pollens, while the majority had demonstrable cross-reactivity with other plant proteins, especially from foods belonging to the Rosaceae family (50%), peanuts (31%), and tree nuts (42%) [13]. Thus, kiwifruit allergy is being considered as most common allergies among fruits and vegetables causing heath issues globally.

There are many factors such as maturity, variety, storage conditions, and various components of the fruit, affecting the allergenicity of fruit during their growth. Studies found that allergenicity levels of fruits are associated with their maturity stages. Pru p 3, a major peach allergen is related to its ripening attributes [111]. During the ripening period, the SDS-PAGE protein band intensities of Pru p 3 were higher as presented in fully ripened peaches compared to unripe peach. In apples, the concentration of allergen, Mal d 3 is two times higher when fully matured compared to immature stages. López-Matas et al. (2011) determined the allergenic profiles of six commonly known varieties of tomatoes (Rama, Rambo, Canario, Kumato, Pera and Raf) and found that their protein profile and allergen quantification showed differences [14]. The highest allergen content (Lyc e 3) was shown in the 'Rambo', followed by 'Pera', 'Canario', 'Raf' and 'Kumato'. The 'Rama' showed the lowest value of allergen. Sancho et al. (2006) reported that apple allergen, Mal d 1 gene expression showed a significant increase after an 8-week storage time [16,82]. Further, many researchers stated that allergic compounds were determined at present not only in the peel but also in the pulp of fruits [17]. The majority of allergens were determined in the peel of mango and the main mango allergen, alk(en)ylresorcinol content in the peel was 419.30 mg/kg which is 16 times higher than that in the pulp (26.33 mg/kg) [18]. In Rosaceae family fruits, Borges et al. (2006) reported that the lipid transfer protein (allergen) was mainly concentrated in the peel, with lower detectable amounts present in the pulp [132]. In apples and peaches, the allergic reaction to peel was found to be higher than that of the pulp [19].

However, few reports are published regarding the relationship between kiwifruit allergenicity and its physiological characteristics such as variety, maturity, storage conditions, and fruit components. The primary objective of this study was to explore the effect of physiological characteristics (e.g., variety, maturity, and fruit components) and storage conditions on the kiwifruit allergenicity and nutritional properties. The relationships between kiwifruit allergen (Act d 2) and antioxidant compounds of fruit were also assessed. Hopefully, it can help us to shed some light on the possible physiological synthesis mechanism of kiwifruit allergens.

4.3. MATERIALS AND METHODS

4.3.1. Plant material and treatment

As designed in **Figure 4.1**, two varieties of kiwifruit i.e. green, and golden kiwifruit were purchased from the local store (Zespri brand, Costco, Montreal, Quebec, Canada). Then, the size and weight-related parameters of all the samples were measured immediately. The fresh firm kiwifruits were selected as unripe samples (soluble solids: 6.2%-8%; firmness: 15-20 N) and the soft fruits selected as ripe stage (soluble solids:12-15%; firmness: 6-8 N). 30 fresh kiwifruits were stored at 4℃, 20℃, and -20℃ for 10 days, respectively, to explore the changes of nutritional properties and allergenicity of kiwifruit under different temperatures. Among these temperature conditions, -20℃ was set as an extreme condition to evaluate the response of allergen to this low temperature.

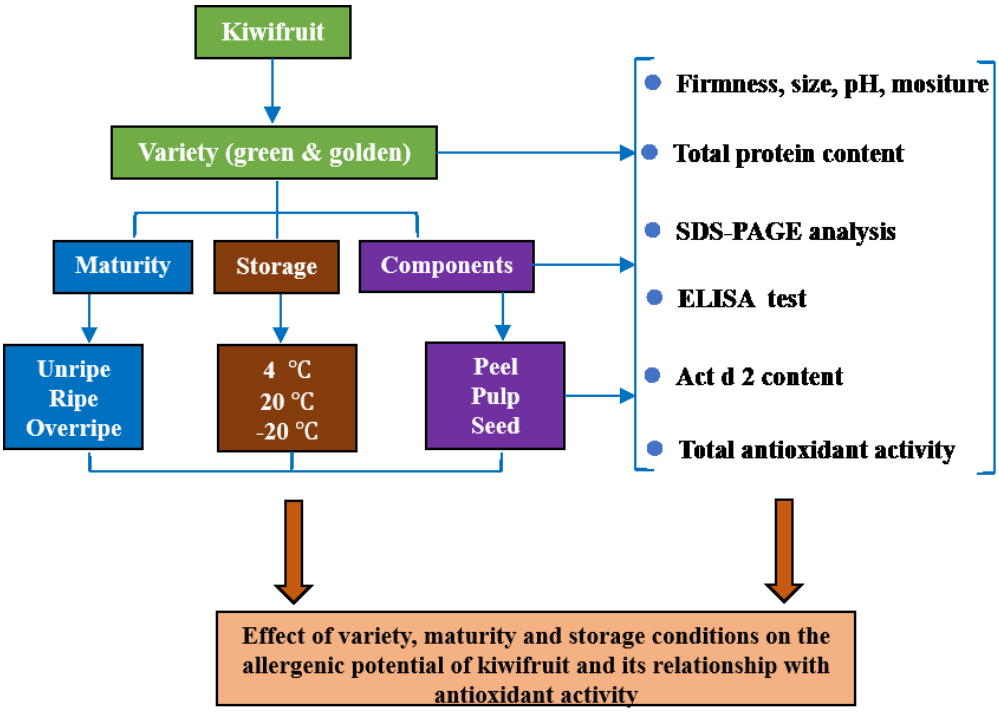


Figure 4.1. The schematic representation of study objectives and experimental designs.

For the different fruit components, the peel, pulp, and seeds of 20 kiwifruits were separated, and stored at -20℃. After finishing the collection of all the samples, they were dried using a freeze drier (7420020, Labconco Corporation, Kansas City, USA), and they were stored at -20℃ until further analysis. All treatments and analyses were performed in triplicates.

4.3.2. Chemicals and reagents

Sodium carbonate, sodium nitrite, aluminum chloride, ferric chloride, sodium hydroxide, 2,4,6-Tripyridyl-S-triazine (TPTZ), and ascorbic acid (≥99%) were obtained from Sigma-Aldrich, Canada. HPLC grade of water, methanol, acetic acid, and acetonitrile were purchased from Fisher Scientific, Canada.

4.3.3. Total soluble solids, pH, and moisture content determination

The total soluble solids (TSS) of kiwifruit samples were determined with a handheld refractometer (Cole-Parmer, QC, Canada) and results were expressed as °Brix at room temperature. The pH values were determined using a handheld pH meter (Fisher Scientific, USA) at 25°C. The moisture content of kiwifruit samples was measured using weight differences before and after drying using an oven at 75 °C until constant weight.

4.3.4. SDS-GAGE analysis

Kiwifruit samples are extracted with 0.01 M phosphate buffer (pH 7.0) for two hours at room temperature and centrifuged at 5000 × g for 10 min [245]. The supernatants were analyzed by SDS-PAGE. Electrophoresis was performed using Fisher brand™ FB-VE10-1 Vertical Electrophoresis System at 80 V for one hour.

4.3.5. Sandwich ELISA test

Immunodetection of protein (Act d 2) is performed by ELISA, using rabbit polyclonal primary antibody, goat-anti-rabbit secondary antibody, and recombinant protein (Act d 2) purchased from Elabscience (USA). After the reaction, the optical density (OD) was recorded at 450 nm according to the protocol provided with the ELISA test kit (Elabscience, USA). Recombinant protein will be used as a standard to quantify the Act d 2 content present in kiwifruit samples.

4.3.6. Total protein measurement

The total protein content of kiwifruit samples was analyzed using the Pierce BCA protein assay kit from Thermo Fisher Scientific. The samples and controls were tested according to the

protocol provided in the kit.

4.3.7. Total antioxidant activity

In the study, Ferric-reducing/antioxidant power (FRAP) assay was performed to test the total antioxidant capacity of kiwifruit juice samples [246]. Briefly, the FRAP working solution was prepared by mixing the 2,4,6-Tripyridyl-S-triazine (TPTZ), ferric chloride solution (20 mM) and acetic acid buffer (40 mM) by the ratio 10:1:1 (v/v/v) at room temperature. 100 µL of test samples extracted with methanol was added to the 1.5 mL of FRAP working solution. After 5-min incubation at 37°C in the dark, the absorbance changes were determined at 593 nm using a spectrophotometer. An external standard, 1000 µM of ferrous sulphate solution was added to make a calibration curve, and the results are presented as mmol Fe(II)/g DW kiwifruit samples.

4.3.8. Ascorbic acid

Ascorbic acid of test samples was extracted with a solvent containing 8% acetic acid and 3% meta-phosphoric acid [246]. After 30-min extraction in the dark at 4°C, the kiwifruit extract was centrifuged at 5000 ×g, 4°C for 10 min. The supernatant was used to quantify the ascorbic acid content in the test samples. Each test sample was filtered with a 0.45 µm filter (Fisher Scientific, Canada) before the determination of its components.

In this study, HPLC measurements were performed with an 1100 series HPLC system from Agilent Technologies equipped with a quaternary pump (G1311A), diode array detector (DAD, G131A), and an ALS auto-injector (G1313A). The separations were carried out on a C18 column (250 × 4.60 mm, 5 µm; Sigma, USA) at a flow rate of 0.5 mL/min. A gradient of mobile phase composed of two solvents, namely 0.17% acetic acid (A), and acetonitrile (B) was used following the timeline: from 0 to 9% B in 3 min, from 9 to 81% B in 4.5 min, from 81 to 76% B in 5.5 min, from 76 to 70% in 2 min, from 70 to 91% B in 10 min [246]. The column was balanced with the mobile phase for 5 min before each analysis and its temperature was maintained at 30 °C. The absorbance of ascorbic acid was recorded at 245 nm.

4.4. RESULTS AND DISCUSSION

4.4.1. Physiological parameters of ripe green and golden kiwifruit

The physiological parameters such as moisture content, firmness, pH, and total soluble solids of ripe green and golden kiwifruit are outlined in **Table 4.1**. The diameter of green kiwifruit (43.98 mm) is smaller than golden kiwifruit (51.12 mm), while a heavier fresh mass was observed in green kiwifruit (73.8 g per fruit) compared to the golden one (61.2 g per fruit). No significant differences in the firmness and pH value were noted between the green and golden kiwifruit. The moisture content of green kiwifruit was 83.24%, followed by the golden kiwifruit with 79.31%. A higher total soluble solids content was determined in green kiwifruit (16.17 Brix°) when compared with golden kiwifruit (14.13 Brix°). Thus, the higher moisture content and the higher sweetness of fruit may contribute to more consumption of green kiwifruit than the golden one.

Table 4.1. Physiological parameters of ripe green and golden kiwifruit. Note: values with different letters are significantly different ($p < 0.05$) from each other, and the following tables are the same.

Parameters		
Diameter (mm)	43.98 ± 1.08^{b}	51.12 ± 1.03^{a}
Fresh weight (g)	73.8 ± 1.64^{a}	61.2 ± 1.41^{b}
Moisture content (%)	83.24 ± 0.36^{a}	79.31 ± 0.42^{a}
Firmness (N)	7.4 ± 0.23^{a}	7.5 ± 0.18^{a}
pH	3.27 ± 0.02^{a}	3.25 ± 0.07^{a}
Brix°	16.17 ± 1.14^{a}	14.130.09^{b}

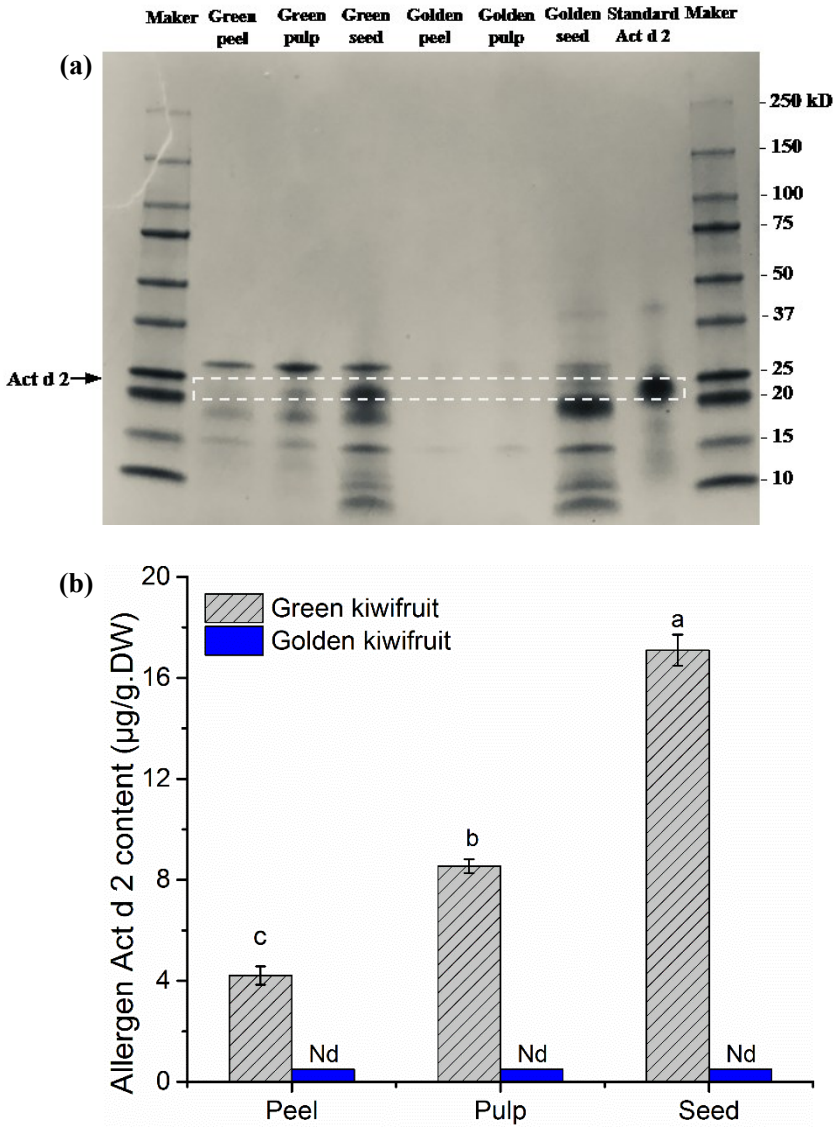


Figure 4.2. SDS-PAGE analysis (a) and ELISA test (b) of green and golden kiwifruit in different fruit components (peel, pulp, and seed). Note: Act d 2 band is located in 20-25 kD as highlighted in the box. Values with different letters in various columns are significantly different ($p < 0.05$) from each other, and the following figures are the same.

4.4.2. Kiwifruit allergenic potential of different varieties and fruit components

In the present study, SDS-PAGE and ELISA analyses were performed to evaluate the differences in allergen Act d 2 content between green and golden kiwifruit, and various fruit components (peel, pulp, and seed). As shown in **Figure 4.2a**, a recombined Act d 2 was used as a standard during the SDS-PAGE analysis. In green kiwifruit, the highest concentration of protein band at 24 KD was determined in seed, followed by pulp, and very limited intensity of band in peel samples. However, it's hard to observe the protein bands at 24 KD in golden

kiwifruit. ELISA analysis obtained similar results reported that Act d 2 allergen was mainly concentrated in the seed of green kiwifruit (17.1 μg/g. DW), which is two-fold and four-fold higher than that in pulp (8.5 μg/g. DW) and peel (4.2 μg/g. DW) samples, respectively (**Figure 4.2b**). In comparison, the concentration of Act d 2 allergen was hardly detected in the golden kiwifruit.

Many studies have reported that the allergenicity of fruit varies among different varieties. In tomatoes, López-Matas et al. (2011) compared the allergenic potential in six tomato varieties including Rama, Rambo, Canario, Kumato, Pera, and Raf [14]. The ranks of major tomato allergen, Lyc e 3, content was "Rambo > Pera > Canario > Raf > Kumato > Rama". In strawberries, Tulipani et al. (2011) determined the differences in the allergenicity of four varieties of strawberries [33]. The results found that a variety of 'AN94.414.52' showed the highest allergen content, followed by 'Adria' and 'AN00.239.55', whereas the variety of 'Sveva' contributed to the lowest allergen content. In apples, Bolhaar et al. (2005) determined the allergenicity of twenty-one cultivars, the highest allergenic potential was 'Golden Delicious' apples, followed by Gala apples. Whereas the lowest level of allergenicity was observed in the 'Santana' apples [102]. In the present study, the concentration of Act d 2 was hardly measured using the SDS-PAGE and ELISA test (**Figure 4.2**), which might be due to the lack of gene expression related to Act d 2 in golden kiwifruit. According to the data published by the World Health Organization and International Union of Immunological Societies (WHO/IUIS), four allergens including Act c1, Act c5, Act c8, and Act c10, have been recognized in golden kiwifruit [247]. Thus, patients with kiwifruit allergy history still may trigger allergenic reactions when consuming golden kiwifruit.

The obvious differences in the allergen content present in various fruit components (peel, pulp, and seed) have been observed in apples [19,20], peaches [248], tomatoes [249], and mangoes [18]. For example, studies found major mango allergen, alk(en)ylresorcinol, mainly concentrated in the peel (41.9 mg/100g) which is 16-fold higher compared to that of pulp (2.6 mg/100g) [18]. In one study, fifty-seven children (average age of 7.4) were hired to evaluate their allergic reactions to peach extracts from peel and pulp using a skin prick test [248]. The results found that 58% of the total volunteers showed a positive result with peach peel extract, 35% with peach pulp extract. However, in the present study, our results reported that kiwifruit allergen, Act d 2 was

mainly distributed in the seed, less amount of Act d 2 in the pulp, and very limited level in the peel (**Figure 4.2b**). This distribution of Act d 2 may be associated with total protein content. As shown in **Figure 4.3a**, the total protein content in seed extract of green kiwifruit (195.1 mg/g. DW) was significantly higher than that in peel (146.8 mg/g. DW) and pulp (159.9 mg/g. DW) due to the presence of storage proteins in seed. Thus, a higher total protein concentration in seed of kiwifruit possibly contributes to a higher allergenic potential to the patient with kiwifruit allergy [247].

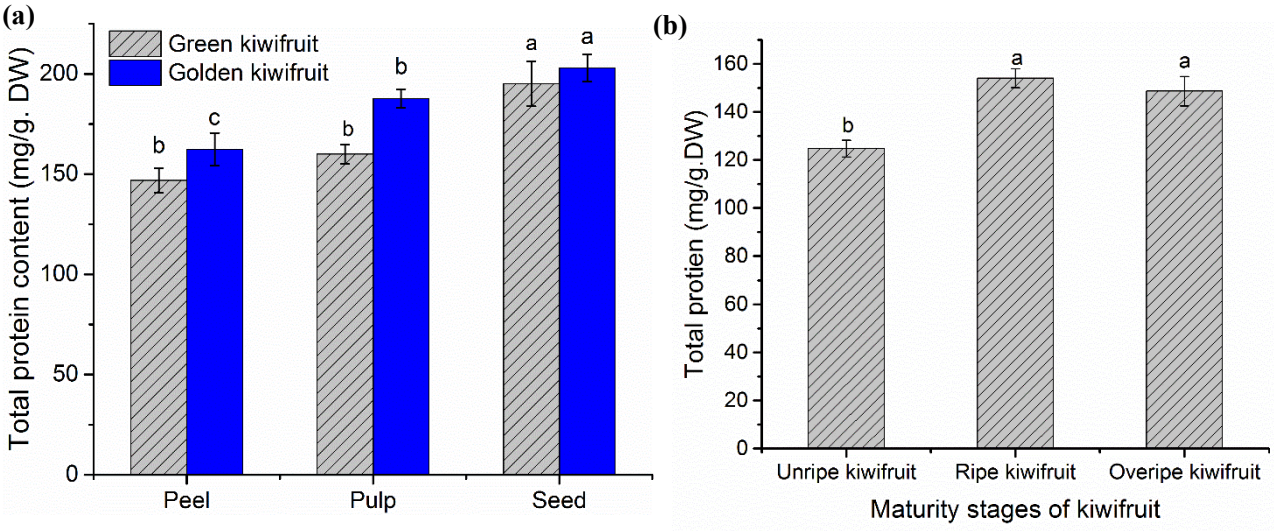


Figure 4.3. Total soluble protein content of kiwifruit in various components (a) and different maturity stages of green kiwifruit (b).

4.4.3. Changes of kiwifruit allergenic potential under different maturity stages

Maturity is one of the most important factors which affects the fruit quality parameters including sweetness, flavor, color attributes, and nutrients concentration [250,251]. Studies have reported that maturity plays an important role in affecting the allergenic potential of fruit. In apples, Sancho et al. (2006) observed that the expression of a major apple allergen, Mal d 3, significantly increased in ripe apples compared with the unripe fruit [82]. Another major apple allergen, Mal d 1, in ripe apples showed a similar trend of 4-fold higher than the apples picked at an early date (not reaching to the ripe stage). Similar results were obtained by Schmitz-Eiberger et al. (2011) in three different varieties of apple including 'Topaz', 'Golden Delicious' and 'Braeburn' [15]. In tomatoes, Kitagawa et al. (2006) found that increasing the expression of the ripening inhibitor gene could significantly reduce the syntheses of allergenic proteins [252].

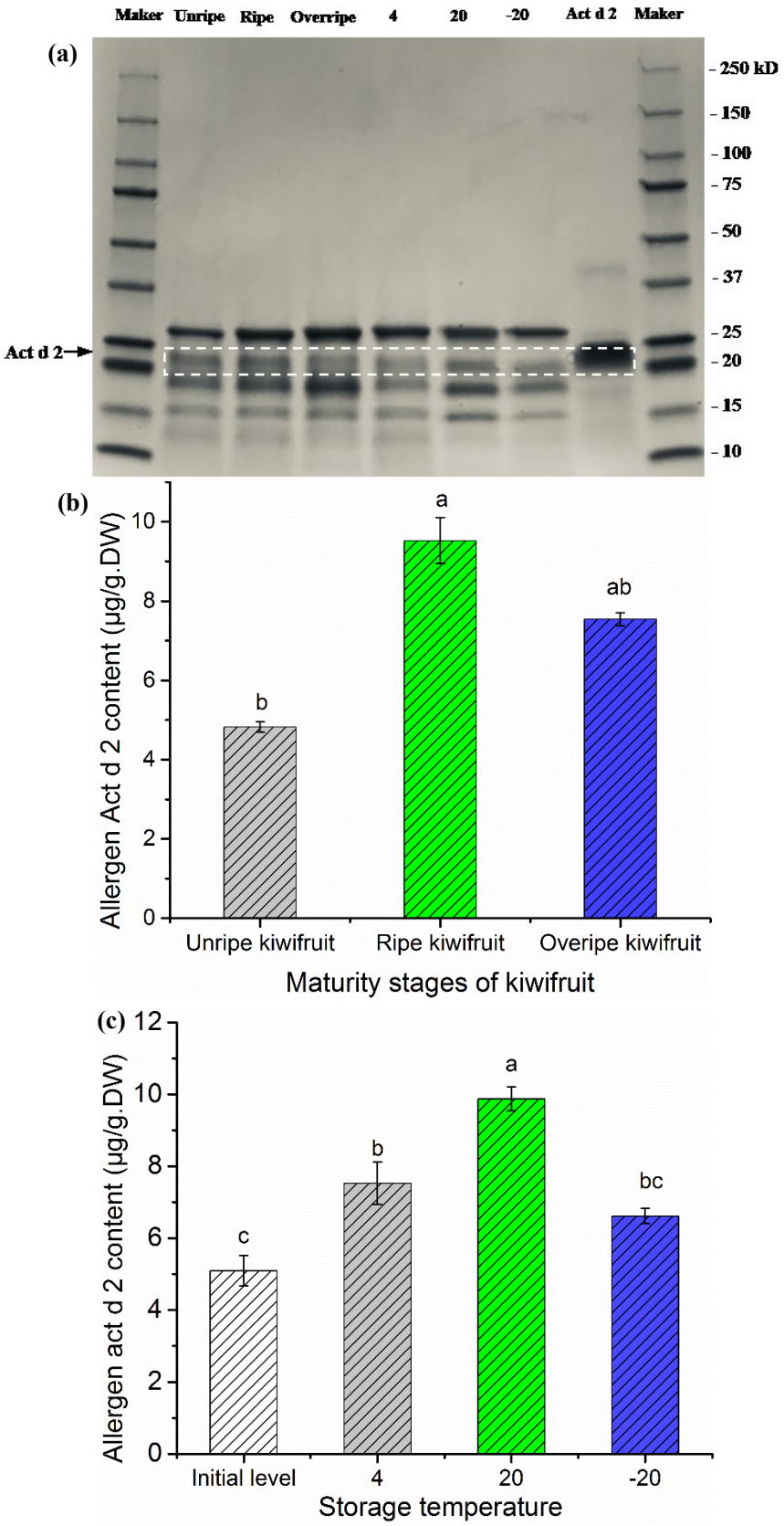


Figure 4.4. SDS-PAGE analysis (a) and ELISA test (b) of green kiwifruit at different maturity stages and storage conditions. Note: Note: Act d 2 band is located in 20-25 kD as highlighted in the box.

In the present study, our results observed that major kiwifruit allergen, Act d 2 was expressed differently at various maturity stages. The highest intensity of protein band at 24 KD was measured in extracts obtained from ripe kiwifruit using SDS-PAGE analysis, followed by overripe kiwifruit, and unripe kiwifruit (**Figure 4.4a**). The quantifying results obtained from the ELISA test proved the trend described above. The allergen content significantly enhanced by 50% from the unripe stage to the ripe stage (9.5 μg/g DW) of kiwifruit, while a slight decrease of Act d 2 in the overripe kiwifruit (7.5 μg/g DW) was observed (**Figure 4.4b**). The enhancement or decrease in the allergen content at various maturity stages is strongly associated with the presence of ethylene [253]. Yang et al. (2012) found that the expression of Mal d 1 (an apple major allergen) related genes were significantly up-regulated in apples during ripening and further obvious enhancement was measured after a certain concentration of ethylene treatment [253]. Whereas, the application of 1-Methylcyclopropene, an ethylene action inhibitor, significantly reduced the expression of Mal d 1 related genes. It provides evidence that ethylene plays a role in regulating the allergen related gene expression in fruit.

As mentioned above, higher total protein content may contribute to a higher allergenic potential. In the present study, ripe kiwifruit (154.0 mg/g DW) showed the highest total protein content, followed by the overripe kiwifruit (148.6 mg/g DW) and unripe kiwifruit (124.7 mg/g DW), which agrees with the trend of allergen, Act d 2, content in various maturity stages (**Figure 4.3b**). Further, many studies have reported bioactive components such as phenolics affected the synthesis of allergens during the growth of fruits. In sweet cherries, Schmitz-Eiberger et al. (2012) reported that cherries with higher phenolic compounds and anthocyanin demonstrated a lower allergen content [254]. In strawberries, the previous study found that a large amount of accumulation in total antioxidant capacity, total phenolics, and total flavonoids contributed to a reduction in synthesis of strawberry allergen Fra a 1 [33]. As shown in **Figure 4.5**, ripe kiwifruit presented the highest percentage of total antioxidant activity (42.9%), compared to unripe kiwifruit (31.35%) and overripe kiwifruit (25.75%), which is consistent with the trend of allergenicity of kiwifruit under different maturity stages.

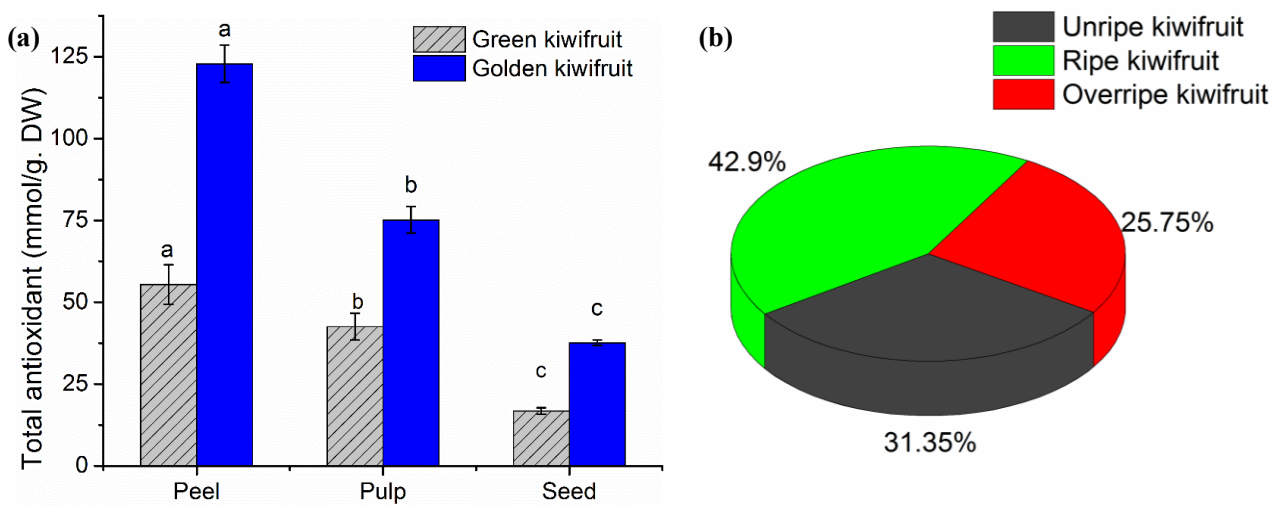


Figure 4.5. Total antioxidant activity of kiwifruit in various components and different maturity stages.

4.4.4. Changes of kiwifruit allergenic potential under different storage conditions

In the present study, unripe green kiwifruits were stored at 4 °C, 20 °C, and -20 °C for 10 days. As shown in **Figure 4.4a**, the SDS-PAGE analysis indicated that kiwifruit stored at 20 °C presented the highest intensity of Act d 2 protein band, followed by the kiwifruit stored at 4 °C and -20 °C. Similar results were obtained by the ELISA test. After 10-day storage at different temperatures, the Act d 2 allergen content was significantly enhanced compared with the initial level (**Figure 4.4c**). The highest concentration of Act d 2 allergen was obtained from the fruit stored at 20 °C for 10 days (9.8 µg/g DW), followed by kiwifruit stored at 4 °C (7.5 µg/g DW) and -20 °C (6.6 µg/g DW). Whereas, no significant differences in Act d 2 allergen content were observed in fruits stored at -20 °C when compared with the initial level (5.0 µg/g DW). Thus, the metabolisms of each compound are still being performed during the storages of kiwifruit even at a very low temperature (-20 °C). Similarly, previous study reported that a major apple allergen, Mal d 1, present in apples increased during cold storage at 4°C [255]. The increase of related fruit allergens may be due to the improvement of ripeness of fruit, which results in more syntheses of bioactive compounds in the fruit [247]. Further, the enhancement in total protein content after 10-day storage potential contributed to the increase of allergenic potential of kiwifruit (**Table 4.2**). However, Sancho et al. (2006) stored apples at room temperature and 2 °C for 0-5 months. The results found that the concentration of Mal d 3, a major apple allergen,

significantly decreased compared with the initial level [82]. Thus, the properties of fruit allergens are different among various fruit types.

Table 4.2. Total soluble protein content (mg/g DW) of green and golden kiwifruit after 10-day storage under different temperatures.

Temperature (°C)	Green 	Golden 
Initial content	125.09 ± 3.62^{b}	132.39 ± 5.20^{b}
4	128.02 ± 1.14^{b}	139.63 ± 3.40^{b}
20	149.01 ± 4.36^{a}	172.45 ± 3.62^{a}
-20	124.77 ± 3.29^{b}	134.15 ± 6.09^{b}

4.4.5. Ascorbic acid content of kiwifruit

Kiwifruit consistently ranks at the top of fruit category due to its essential nutrition density, especially high ascorbic acid content [256]. In the present study, we compared the distributional differences of ascorbic acid in various fruit components including peel, pulp, and seeds. As shown in **Figure 4.6a**, the ascorbic acid content in green and golden kiwifruit showed a decreasing trend from the peel to the seed. In green kiwifruit, the highest concentration of ascorbic acid was found in the peel (165.42 mg/100g. DW), followed by pulp (125.8 mg/100g DW) and seed samples (49.8 mg/100g DW). The ascorbic acid content in golden kiwifruit was up to 187.7 mg/100g DW, which is more than 2-fold higher than that in seed samples (81.8 mg/100g DW). Similar results are reported in pomegranate [257], peach tomato and pear apple [258]. Peel component contains a higher ascorbic acid compared with other fruit components, which is due to the receptibility of peel resisting the external stresses such as high temperature and drought [256].

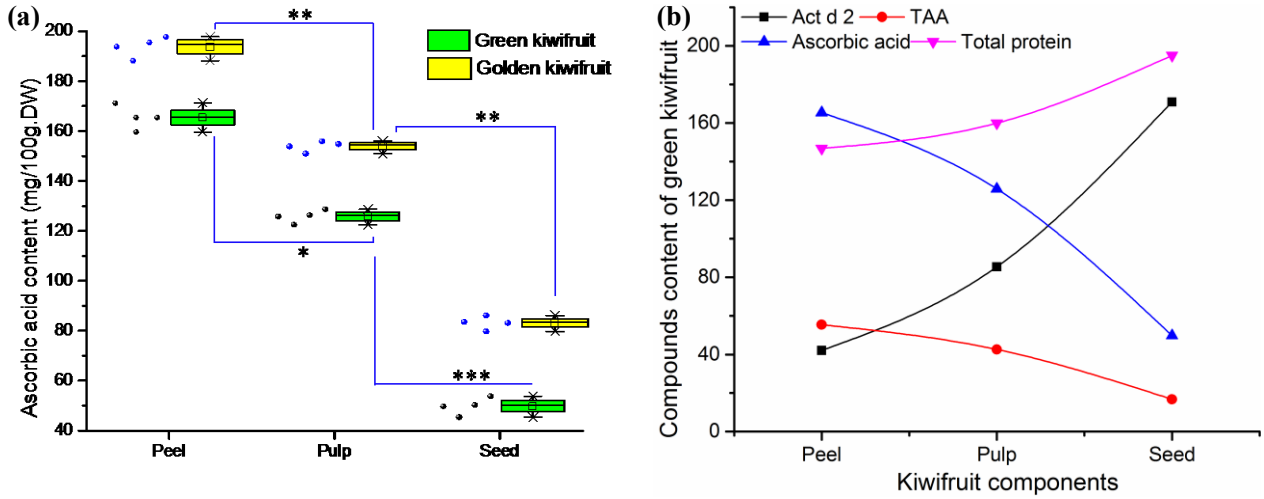


Figure 4.6. Ascorbic acid content of kiwifruit in various components (a) and the correlation between Act d 2 content, total protein, ascorbic acid, and total antioxidant activity (TAA) of green kiwifruit (b).

4.4.6. Correlation between Act d 2 content, ascorbic acid, and antioxidant activity

In the present study, the results found that Act d 2 allergen content showed a positive correlation with total protein content, while a negative correlation with the ascorbic acid content and total antioxidant activity were observed (**Figure 4.6b**). The correlation coefficient of Act d 2 with the total antioxidant, ascorbic acid, and total protein is -0.97, -0.93, and 0.99, respectively. Thus, antioxidants (e.g., ascorbic acid) may involve the metabolic regulation of Act d 2 allergen in various fruit components during the growth of kiwifruit. It leads to a lower Act d 2 allergen synthesis in the peel as a result of the higher antioxidants compared with pulp and seed component (**Figure 4.5a**). In contrast, the higher total protein concentration in the seed of green kiwifruit possibly contributed to a higher Act d 2 content present in seed compared to the peel and pulp. Therefore, culturing new varieties of kiwifruit without seeds through breeding technologies may provide a strategy in reducing the allergenicity of kiwifruit.

4.5. CONCLUSION

In this study, the influences of variety, fruit components, maturity and storage conditions on the allergenic potential were evaluated. The results found that Act d 2 allergen is mainly observed in the green kiwifruit, while no detectable level of Act d 2 was detected in golden kiwifruit. The Act d 2 allergen present in seeds is 2-fold and 4-fold higher when compared with pulp and peel, respectively. The highest Act d 2 allergen content was determined in ripe kiwifruit, followed by overripe fruit and unripe fruit. During the 10-day storages at different temperatures, the results found that a 50% enhancement in Act d 2 content was observed when stored at 20 °C compared with the initial level, while no significant increases in the allergen concentration were observed after 10-day storage at -20 °C. Further, the Act d 2 content showed a negative correlation with the ascorbic acid content and total antioxidant activity at various conditions. Therefore, antioxidants are considered as important factors which may involve the regulation of Act d 2 allergen in kiwifruit. Further studies regarding the mechanism of synthesis and regulation in Act d 2 allergen are to be explored.

CONNECTING TEXT

In this chapter, the effect of physiological characteristics of kiwifruit (variety, maturity, and fruit components) and storage conditions on the allergenic potential of kiwifruit have been evaluated. The results obtained from the analysis clearly showed that Act d 2 was only detected in the green kiwifruit. Act d 2 allergen content detected in the seeds is 2-fold and 4-fold higher when compared with pulp and peel, respectively. The highest Act d 2 allergen content was determined in ripe kiwifruit, followed by overripe fruit and unripe fruit. A negative correlation of Act d 2 with the ascorbic acid content and total antioxidant activity at various conditions of kiwifruit was observed. Therefore, green ripe kiwifruit was selected as samples in the next chapter to evaluate the effect of microwave processing on the allergenicity and nutritional properties of kiwifruit.

CHAPTER V

EFFECT OF MICROWAVE TREATMENT ON THE NUTRITIONAL PROPERTIES AND ALLERGENIC POTENTIAL OF KIWIFRUIT

5.1. ABSTRACT

Kiwifruit is one of the most popular fruits due to its essential nutrients and health benefits. However, kiwifruit can trigger allergenic reactions including oral allergy syndrome and life-threatening anaphylaxis, resulting in a major issue related to food safety and human health worldwide. The primary objective of the study was to evaluate the impact of microwave processing on the nutritional properties and allergenicity of kiwifruit. Kiwifruit samples were treated with microwave at 75℃ for 0-5 min. During the processing, physicochemical properties including microstructure, color attributes, total antioxidant activity, allergen (Act d 2) content, in vitro digestibility and secondary structure of kiwifruit protein were determined. The results found microwave processing significantly increased the total antioxidant activity through disruption of the microstructure of kiwifruit tissues. Also, the digestibility and peptide content of kiwifruit protein were improved. Whereas, a negative effect on the color attributes and sugars content of kiwifruit juice was obtained after microwave processing. Further, in comparison with the untreated samples, an 80% reduction in Act d 2 allergen content was observed after 5-min microwave treatment, accompanied with a 33% reduction of soluble protein content through decreasing the alpha-helix structures and increasing the beta-sheet structures. Therefore, microwave processing showed a potential application in the decrease of kiwifruit allergenicity.

KEYWORDS: kiwifruit allergy; microwave treatment; microstructure; nutritional properties; secondary structure; in-vitro digestibility

5.2. INTRODUCTION

Kiwifruit (*Actinidia deliciosa*) are wild plants that were first found in China and named 'Chinese gooseberry'. There are approximately 55 species cultured, including green kiwifruit (*A. deliciosa*), golden kiwifruit (*A. chinensis*), hardy kiwifruit (*A. arguta*), Chinese egg gooseberry (*A. coriacea*), and hearty red kiwifruit (*A. purpurea*) [1,2]. At their commercial harvesting stages, the fruits have a soft texture and a pleasant flavor. In 2018, the United States Department of Agriculture (USDA) Nutrient Database reported kiwifruit as an excellent source of dietary fiber, carbohydrates, vitamin C and E, minerals (calcium, magnesium, potassium, and phosphorus), omega-3 fatty acids and antioxidants (e.g. phenols). In 2016, the Food and Agriculture Organization of the United Nations (FAO) reported that the total worldwide kiwifruit production was 4,274,870 tons. Thus, kiwifruit is becoming one of the most popular fruits all over the world.

However, kiwifruit has been recognized as the fourth most common food source causing allergy after beef, milk, and cocoa, among young children (6-9 years old) in Turkey [259]. Up to 50% of patients with food allergy history are also allergic to kiwifruit due to cross-reactivity through a self-report analysis in Sweden and Denmark [8]. In Spain, there are approximately 1.8% of the total population allergic to kiwifruit [9]. Further, studies have reported that the prevalence of kiwifruit allergy is increasing worldwide because of the cross-reactivity between kiwifruit allergy and pollens (e.g., tree or grass) allergies or other plant-based food (e.g., avocado) allergies [11,151,153,154,198]. Kiwifruit allergy can trigger various symptoms classified into oral allergy syndrome (OAS) and life-threatening anaphylaxis [28]. The OAS is generally accompanied by some mild allergic reactions such as itching and tingling in the skin, which can be improved naturally or using antihistamines in a short time [154]. However, the severe, life-threatening anaphylaxis reactions including blood pressure decrease, heart rate increase, and loss of consciousness may even result in death if there is no immediate treatment [157]. Therefore, kiwifruit allergy is becoming a major issue related to food safety and human health.

In the food industry, thermal processing is a primary technique widely used to inactivate bacteria and enzymes through the combination of temperature and time [205]. Currently, some thermal processing methods such as, sterilization, microwave heating, boiling and steam

cooking are also generally applied in reducing food allergenicity [206]. During the heating processes, important structural and chemical changes in proteins occur, such as denaturation, aggregation and the Maillard reaction with other molecules. These alterations may have significant impacts on the antigenicity of food allergens [260]. For example, whey protein present in milk when treated by heat at 90-95°C resulted in a reduction of the IgE-binding capacity as evaluated using ELISA tests [207]. Moreover, several studies reported that boiling peanut at 100°C for 20-30 mins effectively reduced the allergen content of Ara h 1, Ara h 2 and Ara h 3 [208,209]. The reduction of allergen content may be due to the denaturation of allergenic proteins under the high-temperature condition [210]. These changes can lead to conformational changes of their secondary protein structures, which in turn may reduce the allergenicity of related food proteins. However, the application of microwave treatment in reducing the allergenicity of kiwifruit is rarely reported. According to the preliminary experiments, 75℃ was selected as the processing temperature. The kiwifruit samples were treated with microwave for one, three or five minutes at this temperature, respectively. During this study, color attributes, sugars content, antioxidant activity, total protein, protein digestibility, allergen (Act d 2) content, and protein structures were determined. These determinations would help in evaluating the impact of microwave processing as a novel thermal processing technique in the reduction of kiwifruit allergenicity.

5.3. MATERIALS AND METHODS

5.3.1. Chemicals and reagents

Sodium carbonate, ferric chloride, aluminum chloride, sodium hydroxide, and 2,4,6-tripyridyl-*S*-triazine (TPTZ) were obtained from Sigma-Aldrich. The acetonitrile, methanol, and water are HPLC grade which was obtained from Fisher Scientific. Mini-precast protein gel (11 wells) and Immun-Blot membrane were provided by Bio-Rad (Quebec, Canada). The rabbit polyclonal antibodies, goat-anti-rabbit secondary antibody, and recombinant protein (anti-Act d 2) were purchased from Elabscience (USA). The external standards including fructose (99.5%), sucrose (99%), and glucose (99.8%) were purchased from Sigma-Aldrich (Quebec, Canada).

5.3.2. Kiwifruit juice preparation and treatments

Unripe green kiwifruits (Hayward) were purchased from a local market of Baie-D'Urfe (Quebec, Canada). The measured radius of fruits was 40.6-45.6 mm, with firmness of 15-20 N and 6.2-8.0 (°Brix) of total soluble solids (TSS). These unripe green kiwifruits were stored at room temperature until reaching their ripe stage (soluble solids:12-15%; firmness: 6-8 N). As shown in **Figure 5.1**, the fresh kiwifruits were peeled and were crushed into the potable water (ratio 1:1) through a juice extractor. According to the preliminary experiments, 200 mL of kiwifruit juice samples were processed with microwave through a mini-wave reaction system (SCP Science, 60 Hz, 1000 W, Quebec, Canada) as follows: (a) untreated samples (MW0); (b) 1 min at 75 °C (MW1); (c) 3 min at 75 °C (MW3); (d) 5 min at 75 °C (MW5). After microwave treatment, all the samples were cooled down and were filtered using the double layers of nylon cloth. 100 ml of samples from each treatment were stored at 4℃ for further analysis. The leftover samples were dried by using a freeze drier (LyoLab 3000, Thermo Scientific Heto). All treatments and measurements were carried out in three replicates.

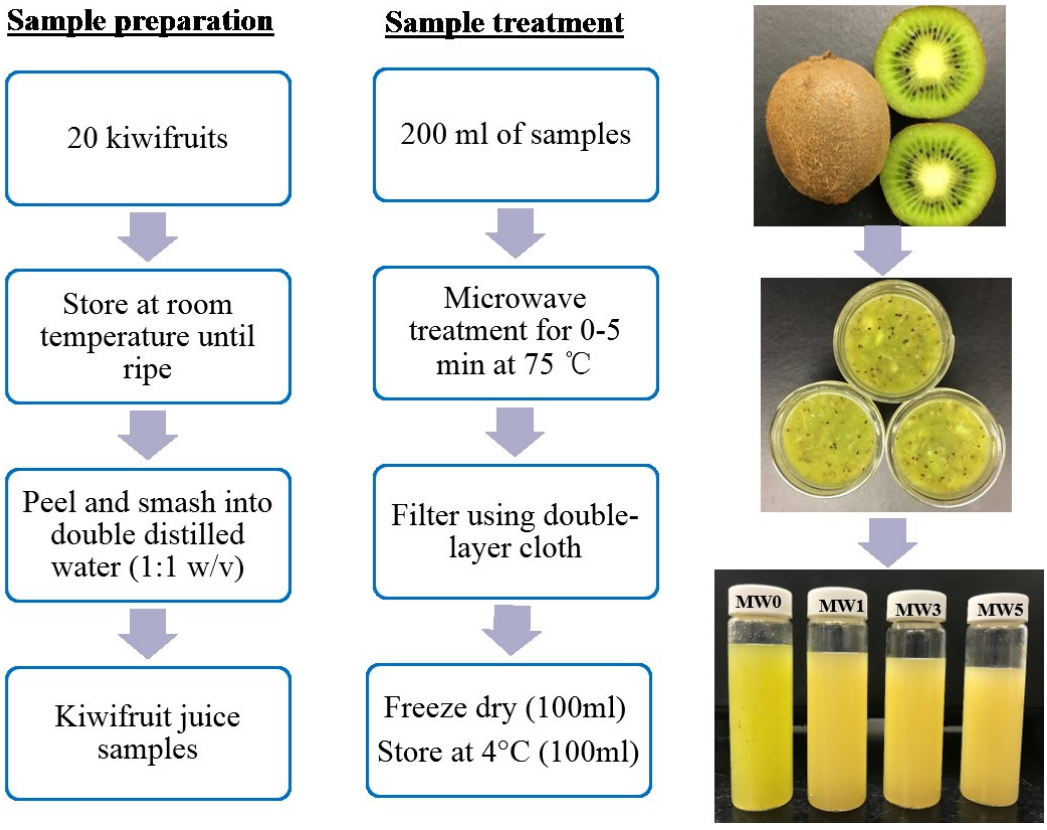


Figure 5.1. Sample preparation and treatment of kiwifruit.

5.3.3. Observation of optical microstructure

According to the method described by Stratakos et al. (2016), 20 μL of kiwifruit juice was transferred to the glass slide and stained using 0.1% toluidine blue solution for 2 min [261]. The mixture was observed under an optical microscope equipped with a digital camera (Leica DM500, Leica Microsystems Inc., Canada). The images were captured using imaging software (Leica LAS EZ, Leica Microsystems Inc., Canada) at a 10× objective.

5.3.4. Color determination and pH value measurement

The color of the treated kiwifruit juice was recorded through a portable colorimeter (CR-300 Chroma, Minolta, Japan). The L* (brightness\darkness), a* (redness\greeness), and b*(yellowness\blueness) values were obtained to express the color changes. The total color difference (TCD), chroma (C), and yellow index (YI) were expressed according to the following equation (1 to 3) [262]:

$$TCD = \sqrt{(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2} \quad (1)$$

$$C = \sqrt{a^{*2} + b^{*2}} \quad (2)$$

$$YI = (142.86b^*) / (L^*) \quad (3)$$

Where the values of parameters a_0, b_0 and L_0 were measured immediately after juicing and a*, b* and L* were determined after each ultrasonic processing. The pH values were determined using a digital pH meter (Fisher Scientific, USA) at room temperature.

5.3.5. HPLC analysis of fructose, glucose, and sucrose

Three grams of frozen kiwifruit samples were mixed with 10 mL of 50% acetonitrile and extracted for 30 min at room temperature. After 10 min centrifuge at 5000×g, the supernatant was collected. An 1100 HPLC (Agilent, QC, Canada) with a refractive index detector (30 °C) and carbohydrates column (30 °C) was used in the present study. The flow rate was set at 1 mL/ min, with 10 μL of sample injection, and 75% (v/v) acetonitrile was used as a mobile phase [263]. The related sugar standards were applied to make the standard curves.

5.3.6. FTIR analysis and total antioxidant activity

The total phenolics of freeze-dried kiwifruit was qualified by using a Fourier Transform Infrared (FTIR) assay. The FTIR consists of the deuterated triglycine sulfate detector and an IR spectra analysis software (Thermo Nicolet Analytical Instruments, Madison, WI, USA). An air background spectrum was performed to the spectra before each analysis [264]. And then, one milligram dried samples were transferred on the crystal, and each spectrum was scanned 32 times in the range of 4000-500 cm^{-1} at room temperature [264]. After each analysis, the crystal was cleaned with methanol. The further analysis was performed between 2990 and 3550 cm^{-1} because the targeted components with the hydroxyl groups (e.g., total phenolics) were present primarily in this range [265].

In the study, Ferric-reducing/antioxidant power (FRAP) assay was performed to test the total antioxidant activity of kiwifruit juice samples [266]. Briefly, the FRAP working solution was prepared by mixing the 2,4,6-Tripyridyl-S-triazine (TPTZ), ferric chloride solution (20 mM) and acetic acid buffer (40 mM) by the ratio 10:1:1 (v/v/v) at room temperature. 100 μL of test samples extracted with methanol was added to the 1.5 mL of FRAP solution. After a 5-min incubation at 37°C in the dark, the color changes were determined at 593 nm using a spectrophotometer. An external standard, 1000 μM ferrous sulphate solution was applied to make a standard curve, and the antioxidant activity of kiwifruit juice was represented as mmol Fe (II)/g DW of samples.

5.3.7. Total protein and *in-vitro* protein digestibility (IVPD)

The total protein content of fruit samples was analyzed using Pierce BCA protein assay kit from Thermo Fisher Scientific. The samples and controls were tested according to the protocol provided in the kit.

According to the method described by Chen et al. (2015) and Yao et al., (2018) with slight modification, two enzymes (pepsin and pancreatin) with three-stage digestion were applied to simulate the *in-vivo* digestion in the gastrointestinal tract [267,268]. During the first-stage digestion, 500 mg of dried kiwifruit samples were mixed with 20 mL of double distilled water and

incubated at room temperature for 30 min, and then the mixture was centrifuged at 5000×g for 10 min. The supernatant was considered as the initial protein extract solution. In the second-stage digestion, the pH of the initial protein extract (10 mL) was adjusted to 1.5 using 1 M HCl. Then, 100 μL of pepsin solution (10 mg pepsin/mL in 0.01 M HCl) was added and the mixture was incubated at 37 °C for 30 min. After incubation, 100 μL of 1.0 M NaOH solution was added in each sample to stop the second-stage digestion. The pH of the mixture was adjusted to 7.8 using 1.0 M NaOH, and 300 μL of pancreatin solution (10 mg/mL in sodium phosphate buffer, pH 7.0) added to start the third-stage digestion, and the mixture was incubated at 40 °C for one hour. Then, 100 μL of Na_2CO_3 solution (150 mM) was added to stop the overall digestion stages. The total protein content of samples during these three-stages digestions was measured using a BCA kit. The *in-vitro* digestibility of kiwifruit proteins was calculated using the following equation:

$$IVPD(\%) = \frac{P_0 - P_1}{P_0} \times 100 \quad (4)$$

Where P_0 is initial protein content, P_1 is the final undigested protein content.

5.3.8. Peptide content and circular dichroism (CD) spectroscopy analysis

During *in-vitro* protein digestion, one milliliter of initial protein extract solution, pepsin digested protein solution, and pancreatin digested protein solution was collected. Then, centrifuged them at 5000×g for 10 min, 500 μL of supernatant was obtained for the peptide measurement. According to the method described by the previous study, an o-phthalaldehyde (OPA) working solution including sodium tetraborate (100 mM), sodium dodecyl sulfate (20%, w/v), 40 mg/mL of OPA (methanol as solvent), and 100 μL of β-mercaptoethanol were prepared [269]. 10 μL of samples from those three digestion stages were mixed with 150 μL OPA reagent in a microplate and was incubated for 2 min, and then the absorbances were recorded at 340 nm using a plate reader (Spectra Max M2, Molecular Devices, USA). A standard curve (0-10000 μM) was obtained using leucine and glycine as a standard.

A J-815 spectropolarimeter (JASCO, Tokyo, Japan) with a temperature-controlled cuvette holder (cuvette path length: 0.1 cm) was used to determine the secondary structure (α-helix, β-

sheet, and turns) of Act d 2 [270]. The dried kiwifruit samples were extracted with 0.01 M phosphate buffer (pH 7.0). After centrifuging at 5000 ×g, the supernatant was scanned in the range of 260 nm to 190 nm with a bandwidth of 1 nm and a pitch of 0.2 nm. The scanning speed was set at 50 nm/min in a continuous model. Each spectrogram obtained is an average of five spectra accumulations. The CD analysis was performed using the CDPro Software.

5.3.9. SDS-PAGE, western blotting, and ELISA analysis

Kiwifruit samples were extracted with 0.01 M phosphate buffer (pH 7.0) for two hours at room temperature and centrifuged at 5000 × g for 10 min [245]. The supernatants were collected until further analysis. Electrophoresis was performed using Fisher brand™ FB-VE10-1 Vertical Electrophoresis System at 80 V for one hour. Western blotting was performed following the protocol from Bio-Rad through a Trans-Blot Turbo™ Transfer System (Bio-Rad, Quebec, Canada). The related gel figures were obtained using the image Lab™ software (Bio-Rad, Quebec, Canada). Biotin-conjugated rabbit polyclonal antibodies, goat anti-rabbit secondary antibody, and recombinant Act d 2 were purchased from Elabscience (USA). The immunodetection of kiwifruit samples was determined using a Sandwich ELISA test. After the reaction, the optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm according to the protocol of the kit [271]. The recombinant Act d 2 was used as a standard to obtain the standard curve.

5.3.10. Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test ($P < 0.05$) using an SPSS 22.0 analytical software (SPSS Inc., Chicago, USA). All treatments and experimental studies were conducted in triplicates.

5.4. RESULTS AND DISCUSSION

5.4.1. Microstructure observation

As shown in **Figure 5.2**, the microstructure of kiwifruit samples was measured when treated with microwave for 0-5 min at 75°C. A significant disruption in the cell structures of

microwave treated kiwifruit tissue was observed with the increase of processing duration. Specifically, the microstructure of untreated juice samples (MW0) showed in integral cells with intact walls. The intracellular components can be seen clearly within the cell structure, while a few disruptions occurred because of mechanical stress obtained from the juice extractor. After one-minute microwave treatment at 75°C, the cell structures started to tear when compared to the untreated samples, which results in a slight release of intracellular components into the juice. However, there were still integral cells as observed in the kiwifruit juice tissues. In comparison, more cell disruption and tearing in tissues were observed after MW3 treatment, which resulted in a higher release of intracellular compounds compared to that of MW1. Although it's hard to observe the intact cells in kiwifruit juice samples at this stage, large pieces of cell fragments were still present in the juice. In contrast, more obvious disruption in cell structures was observed with the longest processing duration (5 min). Specifically, MW5 treatment dramatically released the intracellular components and cut the large cell fragments into small pieces, which leads to the release of enormous amounts of small particles and nutritional compounds into the juice samples.

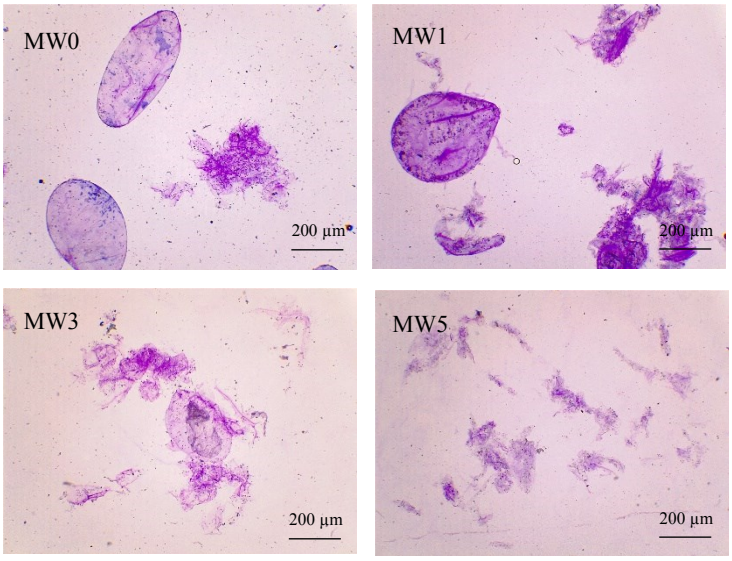


Figure 5.2. Optical microstructure (×10) of kiwifruit juice: untreated sample (MW0) and those treated by microwave at 75°C for 1 min (MW1), 3 min (MW3), 5 min (MW5).

Many studies have reported that microwave treatment caused different microstructural changes in relation to the increase of processing duration. In tomato juice, Stratakos et al. (2016) treated samples for 81.8 s at 85°C using a novel continuous microwave system. The results found that microwave treatment significantly disrupted the cell structures leading to increased intracellular components release compared with conventional processing [261]. In orange juice, Mapelli-Brahm et al. (2018) reported thermal microwave treatment to have caused the obvious rupture of the cell structures as determined using a transmission electron microscopy when compared with the untreated orange juice samples [272]. These significant disruptions in the microstructures of fruit juice tissues are attributed to the electric field effects during microwave treatment [273]. Due to the rapid changes of the electromagnetic field at high frequency, rotation and polarization of molecules occur at many million times per second resulting in a rise in the temperature and other physiochemical reactions (e.g., degradation and oxidation) [274].

5.4.2. Color attributes and pH

Color attributes are considered important to evaluate the quality of fruit juice or related products to satisfy the requirements of consumers [275]. Kiwifruit juice with a bright green or light yellow color is desired for the market [276]. The influences of microwave processing on the color attributes of kiwifruit juice are shown in **Table 5.1**. The results found no significant differences in the lightness (L*) values in all treatments, while greenness/redness (a*) and chroma (C) of juice was improved significantly in each treatment compared with the untreated samples. As shown in **Table 5.1**, the green color in the untreated kiwifruit juice turned into yellow after microwave treatment, which may be due to the degradation of the chlorophyll present in the pulp of green kiwifruit, which in turn could result in increases in a* and C values [277]. Also, oxidation of related compounds or enzymes presented in juice samples can contribute to this enhancement [278]. Further, the yellow index (YI) of kiwifruit juice decreased significantly from the initial level (4.39) to the final processing step (0.33), which is strongly associated with the degradation of carotenoid and oxidation of certain antioxidants (e.g., ascorbic acid) in the juice under thermal processing [278]. Moreover, the results found that the range of total color differences (ΔE) was 7.8-8.8 compared with untreated samples, which is over 2.0, reaching the

level of color variations perceived by human eyes [279]. Thus, microwave processing showed a negative effect on the color attributes of kiwifruit juice. pH value is related to the flavor quality of fruit juice. As shown in **Table 5.1**, there is no significant difference in the pH value of kiwifruit juice during all the processing conditions.

Table 5.1. Color attributes of kiwifruit juice under microwave treatment. Note: values with different letters in the same column are significantly different ($p < 0.05$) from each other.

Treatment	pH	*L*	*a**	ΔE	YI	C
MW0	3.65 ± 0.02^{a}	97.28 ± 0.06^{a}	0.03 ± 0.00^{b}	0	4.39 ± 0.15^{a}	3.09 ± 0.12^{b}
MW1	3.63 ± 0.01^{a}	100.27 ± 0.03^{a}	7.04 ± 0.06^{a}	8.75 ± 0.47^{a}	1.52 ± 0.02^{b}	7.14 ± 0.02^{a}
MW3	3.65 ± 0.01^{a}	99.98 ± 0.11^{a}	7.20 ± 0.25^{a}	8.88 ± 0.68^{a}	2.10 ± 0.01^{b}	7.33 ± 0.08^{a}
MW5	3.64 ± 0.01^{a}	99.41 ± 0.09^{a}	7.08 ± 0.03^{a}	7.87 ± 0.73^{a}	0.33 ± 0.00^{c}	7.09 ± 0.01^{a}

5.4.3. Fructose, glucose, and sucrose

Sugars are important compounds that can contribute to the sweetness of fruit juice or related fruit products. In the present study, we evaluated the influences of microwave treatment on the fructose, glucose, and sucrose in kiwifruit juice. As shown in **Table 5.2**, a decreasing trend in sugars content of kiwifruit juice was observed with the increase of processing duration. Specifically, a slight decrease but no significant difference in the fructose content was observed after one-minute microwave treatment at 75 °C (19.79 mg/mL) when compared with the untreated juice samples (23.47 mg/mL). After 3-min microwave processing, the fructose of kiwifruit juice was 18.70 mg/mL, which is lower than the control group ($P < 0.05$). A 43.3% reduction in the fructose was measured in the kiwifruit juice samples after 5-min microwave treatment. A similar result was obtained in the glucose of microwave treated kiwifruit juice samples. The lowest glucose content was determined in 5-min microwave treated samples (13.61 mg/mL), followed by MW3 (19.48 mg/mL) and MW1 (19.59 mg/mL). In comparison with fructose and glucose, the sucrose content in the kiwifruit juice was lower, which is in accordance with the results described by Turk et al. (2017) in strawberry juice [280]. The result

found that approximately 50% of degradation in sucrose was observed after MW1 and MW3 treatment, 89% of reduction in MW5, compared with the untreated samples. These significant reductions in the three sugars are mainly attributed to the degradation obtained from Maillard reaction and caramelization under a high temperature [281].

Table 5.2. Fructose, glucose, and sucrose content of kiwifruit juice under microwave treatment.

Treatment	Fructose (mg/ml)	Glucose (mg/ml)	Sucrose (mg/ml)
MW0	23.47 ± 0.96^{a}	21.03 ± 0.11^{a}	8.91 ± 0.17^{a}
MW1	19.79 ± 0.06^{ab}	19.59 ± 0.31^{ab}	4.48 ± 0.92^{b}
MW3	18.70 ± 0.79^{b}	19.48 ± 0.41^{b}	4.09 ± 0.03^{b}
MW5	13.30 ± 1.76^{c}	13.61 ± 0.69^{c}	0.96 ± 0.17^{c}

In plum juice, Cendres et al. (2012) evaluated the changes in sugars when treated with the microwave. The results found that there was an approximate 25-35% reduction in glucose, fructose, and sucrose of microwave treated plum juice compared with the pressed juice (untreated sample) [282]. In red date fruits, Gao et al. (2012) found that microwave treated fruit samples showed lower fructose and glucose compared with the untreated samples, while a 56% enhancement of sucrose was observed [283]. In carrot and grape juice, Petruzzi et al. (2017) reported that the total sugar content was significantly increased after 5-min microwave treatment at 80 °C [278]. Thus, the concentration of sugars in fruit or juice is different among various types of fruit.

5.4.4. FTIR analysis and total antioxidant activity

In the present study, FTIR analysis was used to evaluate the changes of compounds with hydroxyl (-OH) groups (e.g., phenolics), with peaks ranging from 2900 to 3550 cm^{-1}. The peak values of compounds with hydroxyl increased with the rise of processing time and reached the maximum level after a 3-min microwave treatment **(Figure 5.3a)**. The peak value of hydroxyl decreased to the initial level, which is strongly associated with long processing duration under high temperature. Overall, the enhancement of -OH groups could be attributed to the increase of phenolic compounds after microwave treatment. It agrees with the results of total antioxidant

activity obtained from the microwave treated kiwifruit juice. As shown in **Figure 5.3b**, the total antioxidant activity significantly increased ($P < 0.05$) in all microwave treated kiwifruit samples compared to the control. The highest total antioxidants activity of juice samples was observed in MW3 treatment (98.2 mmol/g), followed by MW5 (88.2 mmol/g) and MW1 (84.0 mmol/g). Similar results were reported by Benlloch-Tinoco in kiwifruit puree when treated with microwave at 300-900 W for 100-300 s [284]. In citrus mandarin pomace, Hayat et al. (2010) performed microwave processing at 250 W for 5-10 min and found that the total antioxidant activity of treated samples was significantly increased by 20-45% compared with the untreated samples. As mentioned above, "electric field effect" occurs during the microwave treatment, resulting in disruption of cell structures and release of intracellular compounds (**Figure 5.2**), which in turn could contribute to the increase of total antioxidant activity.

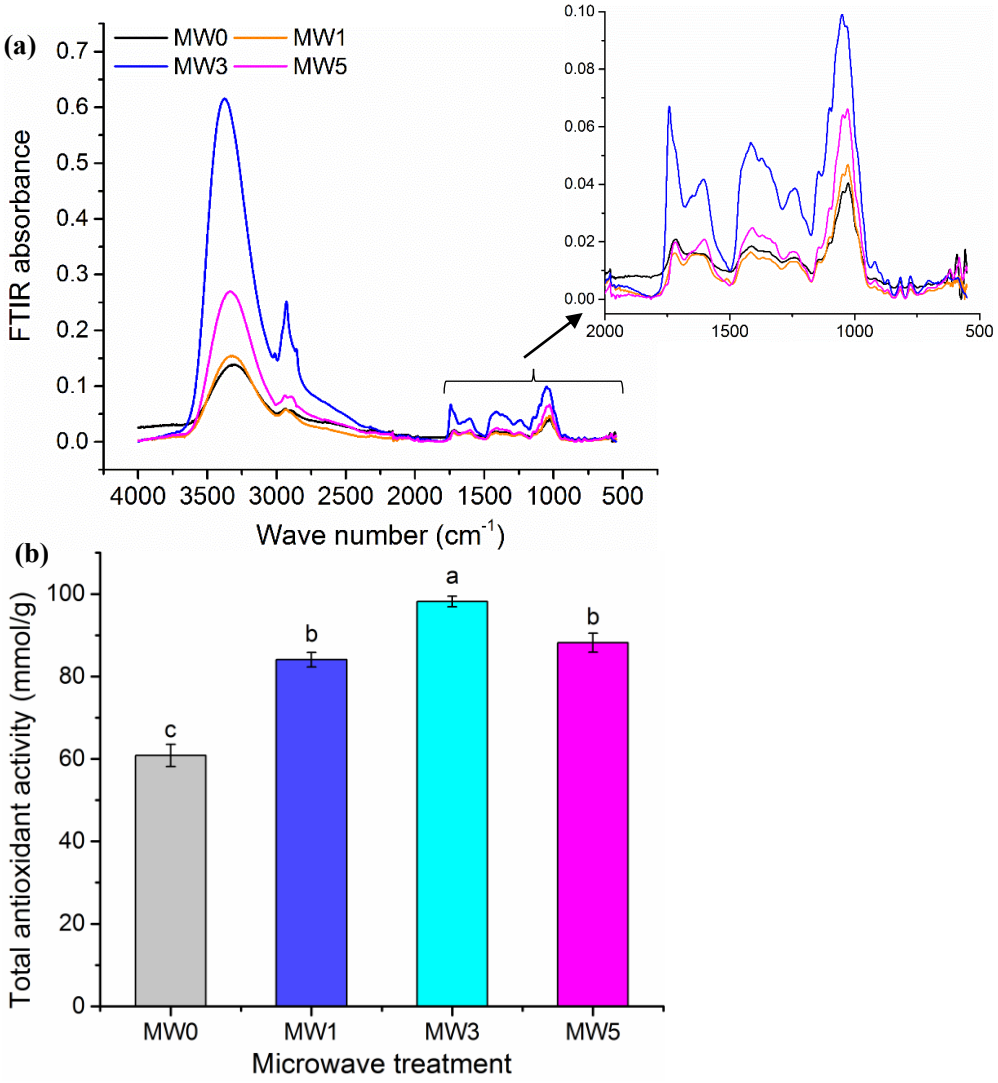


Figure 5.3. FTIR analysis (a) and total antioxidant activity (b) of kiwifruit samples treated with microwave.

5.4.5. Secondary structure of kiwifruit proteins

The secondary structure of the protein consists of alpha-helix, beta-sheet, turn structures, and unordered structures. Studies reported that alpha helix is a right-handed helical coil held together by hydrogen bonding between every fourth amino acid [268]. In the beta structures, two different regions of a polypeptide chain lie side by side and are bound by hydrogen bonds. It has been established that the alpha-helix and beta-sheet structures are playing an important role in maintaining the structural properties of a protein [217]. In the present study, the secondary structural changes in kiwifruit proteins were evaluated before and after microwave processing. As shown in **Figure 5.4**, secondary structures of kiwifruit proteins changed differently under various microwave processing conditions. It can be seen clearly that the beta-sheet is the major secondary structure present in kiwifruit protein, accounting for 35-45% of the total secondary structures. 25-35% of total secondary structures are unordered structures and turn structures, whereas the alpha helix only represents 3-10%.

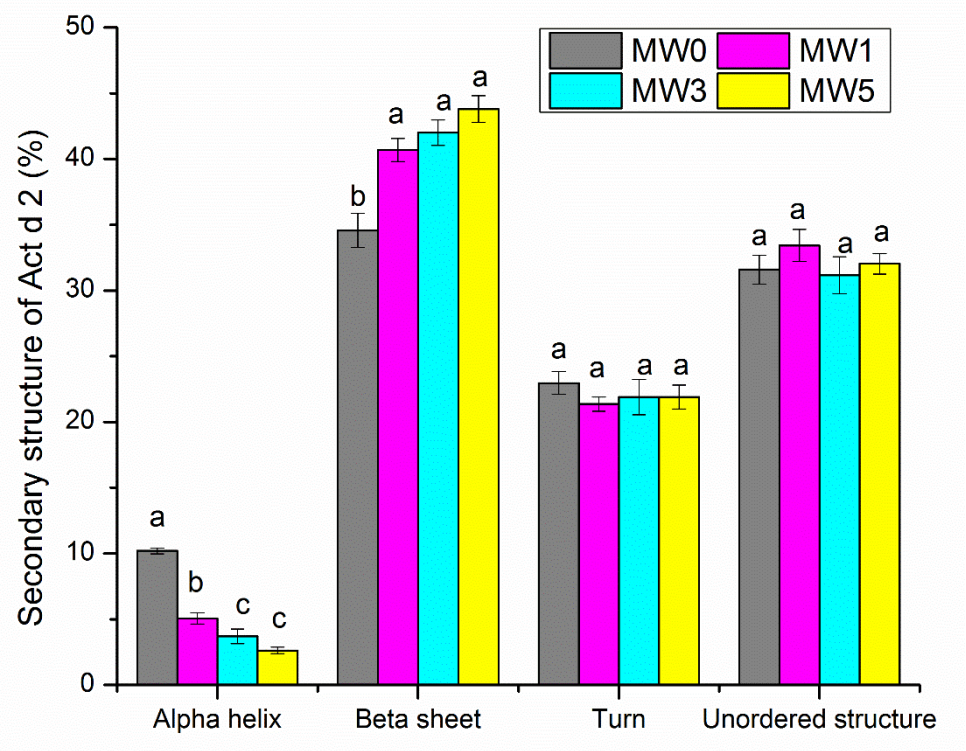


Figure 5.4. Secondary structure of kiwifruit protein extracts: untreated sample (MW0) and those treated by microwave at 75 °C for 1 min (MW1), 3 min (MW3), and 5 min (MW5).

Alpha helix content of protein significantly decreased with the increase of microwave processing time. The lowest percentage of alpha-helix was determined in the longest processing duration (MW5), followed by MW3 and MW1. In comparison with alpha-helix, the beta-sheet of kiwifruit proteins showed an increasing trend with the rise of microwave processing time. The highest percentage of beta-sheet structures was observed in MW5, while no significant differences were measured between each treatment. A slight increase or decrease was found in the turn and unordered structures of kiwifruit proteins but not reach a significant level. Similar results were reported by Zhu et al. (2018) in egg white proteins. In the study, they treated egg white proteins with microwave at 60-80 °C for 1-5 min [268]. The results found that 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. Turn and unordered structures remained constant during the microwave processing. Thus, alpha-helix and beta-sheet structures are sensitive to the thermal processing, while turn and unordered structures remain quite stable.

5.4.6. Total soluble protein content and in vitro digestibility of kiwifruit proteins

As shown in **Table 5.3**, the total soluble protein of kiwifruit showed a decreasing trend with the increase of processing duration. Specifically, the soluble protein content decreased by 15% approximately from the initial level (169.86 mg/g), during the first three-min microwave treatment. After 5-min microwave treatment, the soluble protein content in MW5 (113.81 mg/g) was decreased by 33% compared with the untreated samples. It agrees with the results obtained by Qin et al. (2015) on soybean protein isolate, and they found that the solubility of protein was significantly reduced with the rise of microwave power during processing [285]. The decrease of soluble protein content was possible due to the denaturation of kiwifruit protein under high temperature for a long duration under microwave processing, which can reduce the free S-H content of kiwifruit protein resulting in a low solubility of related proteins [285].

In comparison to animal or dairy proteins, plant-based proteins have very low digestibility. In the present study, we treated kiwifruit protein with microwave at 75 °C for 0-5 min. The results found that microwave treatment apparently improved the digestibility of kiwifruit proteins. The highest enhancement was measured in MW5 treatment with a 52% improvement in the

digestibility compared with the untreated samples (35.37%), followed by MW3 (50.33%) and MW1 (38.70), which is in accordance with the results obtained by Yao et al. (2018) in egg white proteins, and they reported that microwave treatment at 60-80 °C for 1-5 min could significantly improve the digestibility of egg white proteins [268]. Similarly, Vanga et al. (2016) reported that microwave processing increased the digestibility of peanut proteins by 10-15% compared with the untreated samples [286]. Increasing the digestibility of these proteins is strongly associated with conformational changes in their protein structures, especially secondary protein structures, including alpha-helix and beta-sheet (**Figure 5.4**), which results in changes in their functional properties [286]. In one study, the results found that treating peanut proteins over the temperature of 80°C leads to loss of secondary and tertiary structures and the formation of random coils with fully unfolded configuration [287]. Thus, microwave shows a potential application in improving the digestibility of kiwifruit proteins through modifying their secondary structures.

Table 5.3. Total soluble protein content and *in vitro* digestibility of microwave treated kiwifruit samples.

Treatment	Total soluble protein (mg/g)	*in vitro* digestibility (%)
MW0	169.86 ± 2.44^{a}	35.37 ± 1.25^{b}
MW1	148.81 ± 3.89^{b}	38.70 ± 0.89^{b}
MW3	147.29 ± 4.75^{b}	50.33 ± 2.02^{a}
MW5	113.81 ± 4.21^{c}	53.78 ± 1.65^{a}

5.4.7. Peptide content of kiwifruit proteins

In the present study, the free peptides content was determined during the three-stage *in vitro* digestion of kiwifruit proteins. In the initial level, the highest peptide content was observed in MW5 treatment (13.66 mg/g), followed by MW3 (12.22 mg/g) and MW1 (10.87 mg/g), which were significantly higher than the control group (8.94 mg/g) (**Figure 5.5**). Thus, the proteins started to denature and release the related peptides under high temperature and electromagnetic field provided by microwave processing. After pepsin digestion, the peptides content showed

a slight increase but no significant differences in all treatments were observed when compared with the initial level. Whereas, the peptides content of kiwifruit proteins was significantly enhanced in all treatments after pancreatin digestion. Specifically, the peptide content in MW5 treatment had a 3-fold enhancement compared with MW0 at the initial level. Similar results were measured in MW3 and MW1. However, MW0 treated samples in the third digestion stage still showed limited peptide content. These results were strongly associated with the digestibility of kiwifruit proteins following microwave processing (**Table 5.3**). Thus, microwave treatment could modify the protein structures under a certain temperature and electromagnetic field, which in turn can contribute to improving the digestibility of the plant-based proteins [268].

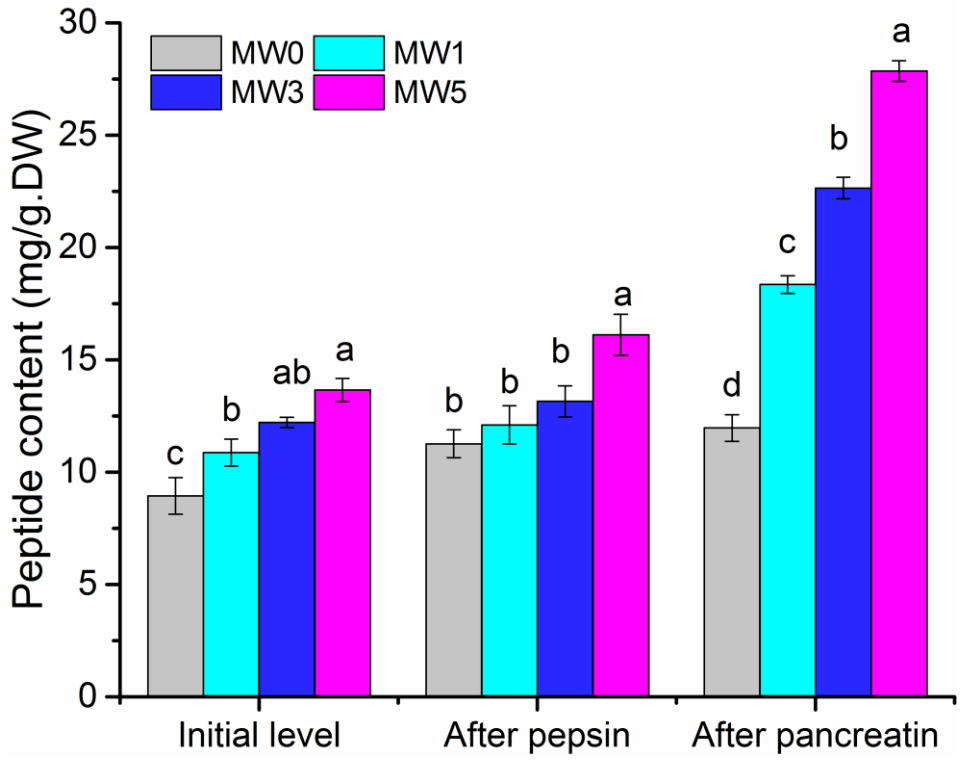


Figure 5.5. Peptide content of microwave treated kiwifruit protein during *in vitro* digestion.

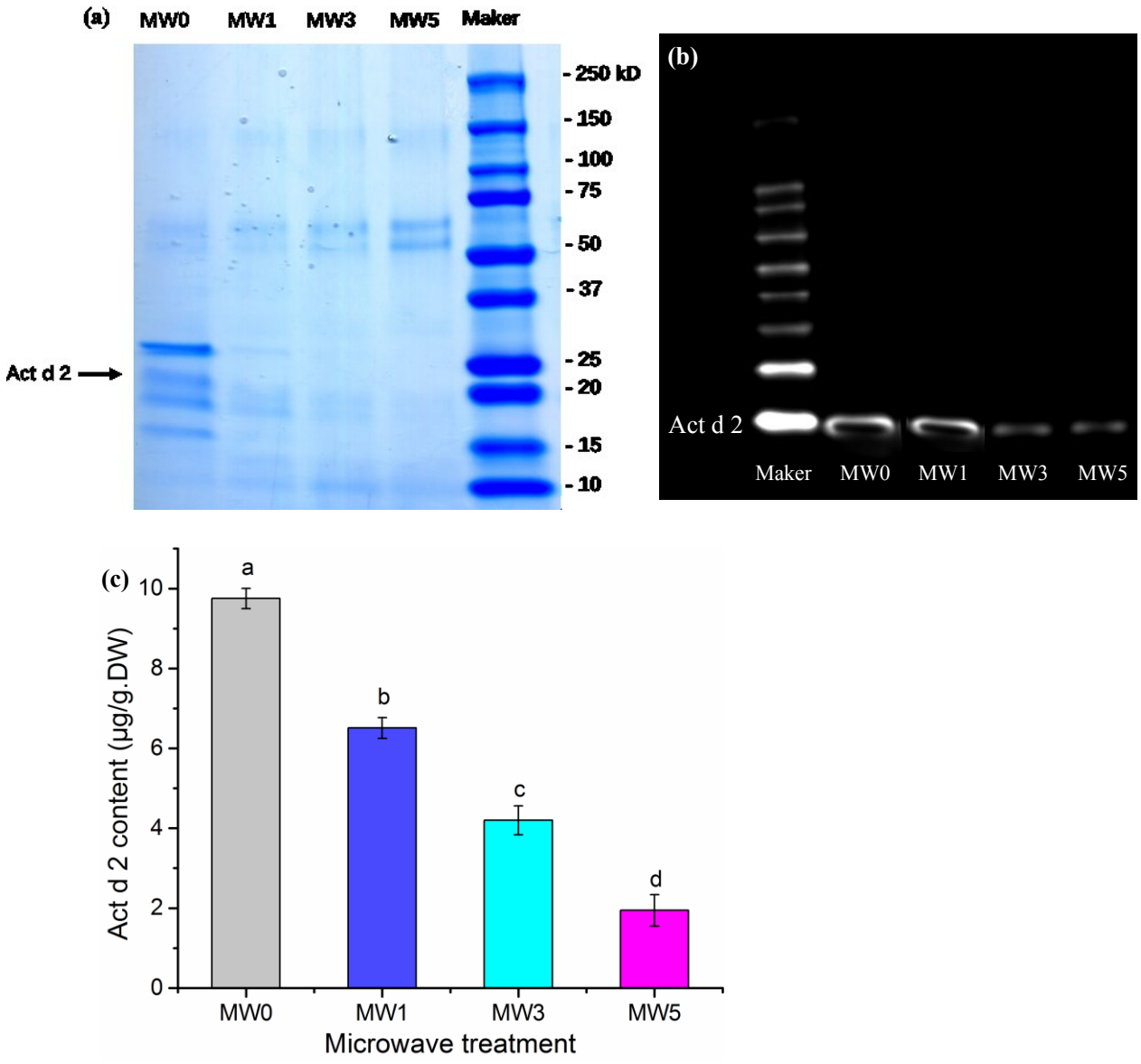


Figure 5.6. SDS-Page (a) and western blotting (b) of protein extract, and kiwifruit allergen Act d 2 content (c): untreated sample (MW0) and those treated by microwave at 75 °C for 1 min (MW1), 3 min (MW3), and 5 min (MW5).

5.4.8. SDS-PAGE, western blotting, and ELISA test

In the present study, the allergenic potential of kiwifruit was analyzed using SDS-PAGE, western blotting, and ELISA test. As shown in **Figure 5.6a**, the protein bands intensity of Act d 2 was decreased with the rise of microwave processing duration. The protein band of Act d 2 in the MW0 treatment can be seen clearly, while it is hard to be observed by human eyes after microwave processing, especially after 5-min microwave treatment. In order to detect the Ig E binding capacity of Act d 2 allergen in kiwifruit, the western blotting test was applied to provide

extra proof for the study. As described in **Figure 5.6b**, the IgE binding capacity of Act d 2 allergen was significantly decreased when the longer microwave processing time was applied. In comparison to the untreated samples (MW0), the combining band of antibody and allergen still can be observed after MW1 treatment, while the intensity of the band was very limited after MW5 treatment. It indicates that microwave processing showed a potential application in reducing the IgE binding capacity of kiwifruit or related products.

To prove this inference, a quantitative method, ELISA test was used to determine the Act d 2 content in the kiwifruit samples obtained from each treatment. As shown in **Figure 5.6c**, microwave treatment significantly decreased the Act d 2 allergen content in kiwifruit with the rise of processing duration. The initial level of Act d 2 in the raw kiwifruit was 9.75 μg/g DW, while a 33% reduction in Act d 2 content was observed after one-minute microwave treatment at 75 °C. The highest reduction in Act d 2 of kiwifruit was found in MW5 (80%), followed by MW3 (57%) compared with the untreated samples (MW0). Similarly, Ketnawa et al. (2017) treated fish frame protein with microwave at 55-90 °C for 2-10 min, the results reported that reduction of antigenicity (55-93 %) was obtained in all microwave-treated samples compared with untreated samples [288]. In soy proteins, Amponsah et al. (2016) evaluated the allergenicity changes of soy proteins extracted from soy samples (flour, isolate and milk) when processed with microwave at 60-100 °C for 5-10 min. The results observed that IgE binding capacity of microwaved treated soy proteins significantly reduced compared with untreated soy samples [289]. As we have mentioned above, microwave processing can provide high temperature and electromagnetic field, which causes the changes in the secondary structures (decrease of alpha-helix and increase of beta-sheet) of kiwifruit proteins, resulting in the reduction in the immunoreactivity of kiwifruit (**Figure 5.4 & 5.6**). However, Abbring et al. (2019) heated cow's milk at 80 °C for 10 min, and the mice model was used to evaluate the allergenicity of heat-processed cow's milk. The results found that mice sensitized to raw milk showed fewer acute allergic symptoms upon intradermal challenge than mice sensitized to heat-processed milk [290]. Thus, the properties of allergens present in different food sources are various, which leads to different responses to a thermal or other processing methods.

5.5. CONCLUSION

In this study, microwave treatment at 75 °C for 0-5 min was applied to evaluate the nutritional properties and allergenicity of kiwifruit. The results found that microwave processing significantly disrupted the microstructure of kiwifruit tissues with the rise of processing duration, resulting in more release of intracellular compounds, which contributes to the improvement of the total antioxidant activity. The digestibility and peptide content of kiwifruit protein was improved due to the changes of secondary structures under the high temperatures and electromagnetic field applied. Whereas, a negative effect on the color attributes and sugars content of kiwifruit juice was obtained after microwave processing. Further, in comparison with the untreated samples, an 80% reduction in Act d 2 allergen content was observed after a 5-min microwave by decreasing the alpha-helix structures and increasing the beta-sheet structures. Thus, microwave processing showed a potential application for reducing kiwifruit allergenicity. Although the allergenic potential of kiwifruit allergen (Act d 2) was decreased to a minimum level, the responses of patients with a kiwifruit allergy to these modified proteins are still unknown. Therefore, further research regarding clinical and immunology are needed.

CONNECTING TEXT

In Chapter V, the microwave treatment at 75 °C for 0-5 min has been applied to evaluate the nutritional properties and allergenicity of kiwifruit. The results found that microwave processing significantly decreased the allergen Act d 2 content by 80% after 5-min treatment through changing the secondary structures of protein (decreasing the alpha-helix structures and increasing the beta-sheet structures). Also, the digestibility, peptide content and total antioxidant activity of kiwifruit samples were improved markedly. However, a negative influence on the color attributes and sugars (sucrose, fructose, and glucose) content of kiwifruit samples were observed as a result of the high temperature (75 °C) applied. Thus, non-thermal processing techniques may show potential advantages in the maintenance of nutritional compounds and possible reduction in the allergenicity of kiwifruit compared with thermal processing.

In the following chapter (VI), a novel non-thermal processing method, high-intensity ultrasound, was applied to evaluate it influence on nutritional properties and allergenicity of kiwifruit samples.

CHAPTER VI

HIGH-INTENSITY ULTRASOUND PROCESSING OF KIWIFRUIT JUICE: EFFECTS ON THE MICROSTRUCTURE, ANTIOXIDANTS, RHEOLOGICAL PROPERTIES, AND ALLERGENICITY

6.1. ABSTRACT

The objective of the study was to investigate the effect of high-intensity ultrasound processing (0, 4, 8, 12 and 16 min at 400 W, 25 kHz frequency) on the microstructure, antioxidants, color attributes, rheological properties, and allergenicity of kiwifruit juice. The results reveal that ultrasound processing resulted in the rupture of cell walls causing the dispersion of the intracellular components into the kiwifruit juice, which contributed to the significant enhancement in the total phenolics (108.6%), flavonoids (105.5%) and antioxidant capacity (65.67%) after 16-min ultrasound processing compared to the control group. However, 16-min ultrasound treatment significantly decreased the total protein and ascorbic acid in kiwifruit juice. Further, ultrasound processing has an enormous potential to improve the physical properties including rheological properties, color attributes, and water-soluble pectin content of kiwifruit juice. Moreover, ultrasound treatment significantly increased the digestibility (55.0%) and decreased the allergenicity (50.3%) of kiwifruit samples which might be due to the secondary structural changes (decreasing of alpha-helix and increasing of beta-sheet). Therefore, high-intensity of ultrasonication shows potential applications in improving the rheological properties and bioactive compounds (e.g., phenolics), decreasing the allergenicity of kiwifruit samples.

KEYWORDS: High-intensity ultrasound, bioactive compounds, microstructures, rheological properties, secondary structure, allergenicity

6.2. INTRODUCTION

Kiwifruit (*Actinidia deliciosa*) is known as 'Chinese gooseberry' which originated from northeastern China [291]. More consumers, growers, and manufacturers are showing great attention to this horticultural produce due to its established nutritional and economical value, as well as its pleasant taste. It has been reported that per 100 g of raw green kiwifruit contains 14.66 g of carbohydrate, 8.99 g of sugar, 3.0 g of fiber, 1.14 g of protein, and 0.31 g of potassium [292]. Additionally, kiwifruit is rich in antioxidants such as polyphenols and flavonoids. These bioactive molecules are beneficial in boosting the human immune system and can prevent cancers and cardiovascular diseases [141]. Several studies also have reported that consumption of kiwifruit can protect cellular DNA against oxidation in human cells [140,293].

In the harvesting stage, kiwifruit generally is consumed in fresh. While the fresh kiwifruit contains high moisture causing rapid tissue softening and senescence at room temperature, which reduces its shelf life significantly [138]. To avoid post-harvest fruit loss, juice production can be considered as an alternative which can drastically extend its shelf life. Furthermore, juice as a product is more convenient for transport compared to the whole fruit. In 2016, the global sales volume of all fruit juice amounted to 20.54 billion liters, which is valued at $ 44.25 billion [294]. Generally, fruit juice is obtained by mechanical pressing machines or industrial blenders. However, these methods cannot cause a full breakdown of the cell wall, which leads to lots of bioactive compounds being left in the pomace [295,296]. By using novel processing techniques, extraction efficiencies of these phytochemicals can be improved which also could significantly add to the nutritional values of these juices. Generally, microwave processing is one of the novel processing methods that can be used. However, it has been reported that this technique could lead to a decrease in color attributes, flavor properties and nutritional value of fruit juice due to the higher temperatures [297,298]. Several studies have stated that the enzymatic treatments could contribute to improving the extraction rate of bioactive molecules (e.g., phenolics and anthocyanins) in fruit juice [295,299]. Conversely, customers are prone to select natural products without any additives due to health considerations. Therefore, non-thermal novel processing techniques are needed.

In comparison, ultrasonication is an innovative technology that could be applied as an

alternative food processing technique to enhance the extraction of bioactive molecules. Especially, high-intensity (10-1000 W cm^{-2}) ultrasound has obtained high attention in the food processing industry, for its low energy consumption, less processing time and being environmental friendly [300]. The process is also popular because the color properties, freshness, and aroma of juice samples could be maintained after processing with this technique [222]. Many studies have observed that high-intensity ultrasound treatment also can increase the content of bioactive compounds in juice products. In lime juice, the ascorbic acid was significantly increased to 40.2 mg/ 100mL from 37.6 mg/ mL after ultrasound processing at a frequency of 25 kHz for 60 min. The total phenolics and total flavonols contents in ultrasound treated lime juice were increased by 27.5% and 127.4%, respectively [301]. In grapefruit juice, the ascorbic acid, total phenolics, and total flavonoid contents were enhanced by 28.5%, 9.0%, and 30.5%, respectively after 90-min ultrasound processing at 28 kHz frequency [275]. In addition, studies found that ultrasound processing can improve the sugars (e.g., sucrose and fructose) content, minerals (e.g., Ca and K), and total carotenoid contents in apple juice [302] and protein content in pineapple juice [302]. However, reports regarding the influence of ultrasound processing on the bioactive compounds in kiwifruit juice are very limited. Additionally, few reports pay attention to the relationship between phytochemicals and the microstructural changes of fruit samples after ultrasound processing.

As we mentioned in previous chapters, kiwifruit allergy is becoming one of the major health-related public issues worldwide. Many studies have reported that high-intensity ultrasound has been applied in the reduction of food allergenicity. Li et al., (2006) found that high-intensity ultrasound treatment at 50 °C for 1.5 h showed an effect on the integrity and structure of shrimp proteins, which led to a reduction in the overall allergenicity [303]. Also, the inhibition value of IgE binding to immobilized Pen a 1 (major shrimp allergen) reached 20% when compared to the untreated samples. Similarly, a study has reported that ultrasonic pre-treatment (0-1.5 h at 50 Hz) alone increased the solubility of peanut protein, and significantly reduced Ara h 2 (one of major peanut allergens), compared to the raw peanut samples [137]. However, there is no report published about the effect of high-intensity ultrasound on kiwifruit allergenicity.

Thus, the present study was to measure the impact of ultrasound at various processing time on the total protein, total soluble solids, total phenolics, flavonoids, ascorbic acid, and antioxidant

capacity of kiwifruit juice. The changes of microstructure, rheological properties, allergenicity of kiwifruit were also analyzed. Hopefully, high-intensity ultrasound processing could be used as a novel technique in improving the physicochemical properties and decreasing the allergenicity of kiwifruit samples.

6.3. MATERIALS AND METHODS

6.3.1. Chemicals and reagents

Folin-Ciocalteu reagent, sodium carbonate, meta-phosphoric acid, sodium nitrite, ferric chloride, aluminum chloride, sodium hydroxide, 2,4-dinitrophenylhydrazine (DNPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,4,6-tripyridyl-*S*-triazine (TPTZ), toluidine blue and deuterated water (containing 0.75% sodium 3-trimethylsilyl-propionate-2,2,3,3-d4) were obtained from Sigma-Aldrich (Quebec, Canada). The acetic acid, acetonitrile, methanol, and water, all HPLC grade were obtained from Fisher Scientific. The external standards including ascorbic acid, catechin hydrate, gallic acid, and ferulic acid were purchased from Sigma, and their purity is up to 98%.

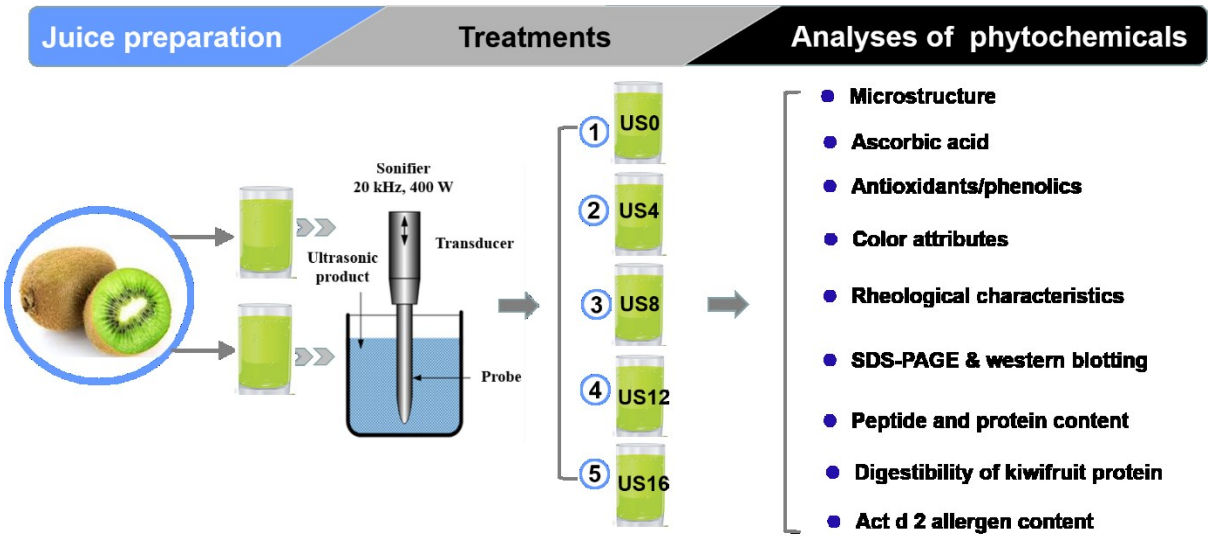


Figure 6.1. Sample preparation and treatment of kiwifruit.

6.3.2. Kiwifruit juice preparation and treatments

Fully ripe green kiwifruits were purchased from a local market of Baie-D'Urfe (Quebec, Canada). The radius value of fruits was 40.6-45.6 mm, with an 82-83% moisture content and

15.7-18.1 (°Brix) total soluble solids (TSS). As shown in **Figure 6.1**, the fresh kiwifruits were peeled and were crushed and mixed with water (ratio 1:1) through a juice extractor. 200 mL of kiwifruit juice samples were processed with a 400-W high-intensity ultrasonic processor (Model 450 Sonifier, CT, USA) at a frequency of 20 kHz, 50% duty cycle. During the ultrasound processing, an ice bath was applied to maintain the temperature of juice samples. The kiwifruit juice samples were treated for 4 min (US4), 8 min (US8), 12 min (US12) and 16min (US16), respectively. The untreated samples were considered as the control group. After the treatment, the kiwifruit samples were filtered with the double layers of nylon cloth. And then, 100 ml of samples from each ultrasound treatment were stored at 4℃ for further analysis. The leftover samples were dried by using a freeze drier (LyoLab 3000, Thermo Scientific Heto) for the Fourier Transform Infrared (FTIR) analysis. All treatments and measurements were carried out in three replicates.

6.3.3. Total phenolics and flavonoids measurement

Following the method reported by Larrauri et al. [304], phenolic compounds were extracted using methanol at a ratio 1:1 (v/v), and then the mixture was incubated at 25°C for 30 min. The supernatant was collected for the total phenolics and flavonoids analysis after centrifuging at 6000×g, 4°C for 15 min. In the study, the total phenolics content in kiwifruit juice samples was quantitatively analyzed by using the classical Folin-Ciocalteu assay with a slight modification [305]. 0.5 mL kiwifruit juice sample was mixed with the working solution which consists of double deionized water (1.5 mL), Folin-Ciocalteu reagent (0.25 mL) and sodium carbonate solution (7.5%, 0.5 mL). After a 30-min incubation in the dark at 25°C, the color changes were observed at 765 nm through a spectrophotometer (Ultrospec 2100 pro, Biochrom Ltd., Cambridge, England). The calibration curve was obtained from an external standard, gallic acid. The total phenolics of test samples was presented as per mg of gallic acid equivalents (GAE) per 100 mL of kiwifruit juice.

Aluminum chloride colorimetric assay was applied to measure the total flavonoids of each juice sample with some modifications [275]. 0.5 mL of kiwifruit extract was transferred to the 70 μL of sodium nitrite solution (5%). After a 5-min incubation, 0.15 mL of aluminum chloride (10%)

was added into the system. The mixture was incubated for 5 min at 20°C and then was mixed with 1.3 mL of double distilled water and 0.5 mL of sodium hydroxide (1 M). The color change was tested at 415 nm, and the (+)-catechin was applied as an external standard. The results were presented as per mg of catechin equivalents (CE) per 100 mL of kiwifruit juice sample.

6.3.4. Analysis of total antioxidant capacity

In the study, Ferric-reducing/antioxidant power (FRAP) assay was performed to test the total antioxidant activity of kiwifruit juice samples [266]. Briefly, the FRAP working solution was prepared by mixing the 2,4,6-Tripyridyl-S-triazine (TPTZ), ferric chloride solution (20 mM) and acetic acid buffer (40 mM) by the ratio 10:1:1 (v/v/v) at room temperature. 100 μL of test samples extracted with methanol was added to 1.5 mL of FRAP solution. After a 5-min incubation at 37°C in the dark, the color changes were determined at 593 nm using a spectrophotometer. An external standard, 1000 μM Ferrous sulphate solution was applied to make a standard curve, and the antioxidant activity of kiwifruit juice was presented as μmol Fe (II)/100 mL of samples.

The DPPH-RSA of each test sample was estimated as described by Brand-Williams (1995) with a slight modification. 0.1 mL of kiwifruit extract was transferred into 3 mL of DPPH solution (4.0 mg/100 mL methanol) [306]. The mixture was incubated at 25°C for 20 min. The color changes were analyzed at 517 nm using a spectrophotometer. The radical scavenging activity was analyzed by the equation:

$$DPPH(\%inhibition) = \left[(Abs_0 - Abs_1) / Abs_0 \right] \times 100 \quad (1)$$

where Abs_0 and Abs_1 represent the absorbance values of blank and test samples, respectively.

6.3.5. Measurement of ascorbic acid, catechin, gallic acid and ferulic acid by HPLC

Ascorbic acid of test samples was extracted with the solvent containing 8% acetic acid and 3% meta-phosphoric acid [307]. After 30-min extraction in the dark at 4°C, the kiwifruit extract was centrifuged at 5000 ×g, 4°C for 10 min. The supernatant was collected to quantify the ascorbic acid content in the test samples. For the analyses of the phenolic compounds, 2 mL of test samples were incubated with 5 mL of methanol (95%) in a 4°C refrigerator for 8 h [308]. After

centrifuging, the extracts were used to quantify the catechin, ferulic acid and gallic acid contents of kiwifruit samples. Each test sample was filtered with a 0.45 μm filter (Fisher Scientific, Canada) before the determination of its components.

In the study, HPLC measurements were performed with an 1100 series HPLC system from Agilent Technologies equipped with a quaternary pump (G1311A), diode array detector (DAD, G131A), and an ALS auto-injector (G1313A). The separations were carried out on a C18 column (250 × 4.60 mm, 5 μm; Sigma, USA) at a flow rate of 0.5 mL/min. A gradient of mobile phase composed of two solvents, namely 0.17% acetic acid (A), and acetonitrile (B) was used as the following program: from 0 to 9% B in 3 min, from 9 to 81% B in 4.5 min, from 81 to 76% B in 5.5 min, from 76 to 70% in 2 min, from 70 to 91% B in 10 min [309]. The column was balanced with the mobile phase for 5 min before each analysis, and its temperature was 30°C. Peaks of catechin, ferulic acid, and gallic acid were identified separately using UV/Vis detector at 280 nm, and the absorbance of ascorbic acid was recorded at 245 nm. 20 μL of the extract was injected for each analysis, and the peaks of ascorbic acid, catechin, ferulic acid, and gallic acid were identified by their retention times and quantified by using standards curves. The related standards concentrations of ascorbic acid, catechin, ferulic acid, and gallic acid ranged from 0 to 1.0 mg/mL with six gradients. The linearity range was plotted according to the peak area of each compound. The results were presented as mg/100 mL of kiwifruit samples.

6.3.6. Microstructure

According to the method described by Stratakos et al. (2016), 20 μL of kiwifruit juice was transferred on the glass slide and then were stained using 0.1% of the toluidine blue solution for 2 min [261]. The mixture was observed under an optical microscope equipped with a digital camera (Leica DM500, Leica Microsystems Inc., Canada). The images were captured using imaging software (Leica LAS EZ, Leica Microsystems Inc., Canada) at a 10× objective.

6.3.7. Color attributes of fruit samples

In this study, a portable colorimeter (CR-300 Chroma, Minolta, Japan) was applied to evaluate the color changes of samples after a calibration. CIELab parameters including L*, a*, and b*of

each sample were recorded. The total color difference (ΔE), chroma (C), hue angle (h), and yellow index (YI) was obtained from Eqs. 1-4, respectively [310]:

$$\triangle \quad \overline{*-L_0)^2+(a*-a_0)^2+(b*-b_0)^2} \tag{1}$$

$$C=\sqrt{a*^2+b*^2} \tag{2}$$

$$h=\tan^{-1}(b*/a*) \tag{3}$$

$$YI=142.86b^*/L^* \tag{4}$$

where a_0, b_0, and L_0 represent initial values of untreated samples, while a*, b* and L* represent values of ultrasound treated samples.

6.3.8. Rheological characteristics

Rheological analyses were performed using an AR2000 rheometer (TA Instruments, USA) with a cone-plate (40 mm diameter). 0.5 mL of sample was transferred on the bottom plate. The gap size and temperature were set at 0.056 mm and 25°C, respectively [311]. In the study of steady flow, the shear rate ranged from 0.1 to 100 s^{-1} [312]. Prior to each test, the kiwifruit sample was incubated in the plate for three min. After strain sweep tests, 2% of strain was selected to conduct the dynamic frequency sweep analysis. The frequency ranged from 0.1 to 10 Hz to evaluate the behavior of storage modulus(G′) and loss modulus (G″) of kiwifruit samples [312]. Rheological data analysis was performed using a rheology advantage software (TA Instruments, USA).

6.3.9. Total soluble protein and *in-vitro* protein digestibility (IVPD)

The total protein content of samples was analyzed using the Pierce BCA protein assay kit from Thermo Fisher Scientific. The samples and controls were tested according to the protocol provided by the kit.

According to the method described by Chen et al. (2015) and Yao et al., (2018) with slight modification, two enzymes (pepsin and pancreatin) with three-stage digestion were applied to simulate the *in-vivo* digestion in the gastrointestinal tract [267,268]. During the first-stage digestion, 500 mg of dried kiwifruit samples were mixed with 20 mL of double distilled water and

incubated at room temperature for 30 min, and then the mixture was centrifuged at 5000×g for 10 min. The supernatant was considered as the initial protein extract solution. In the second-stage digestion, the pH of the initial protein extract (10 mL) was adjusted to 1.5 using 1 M HCl. Then, 100 µL of pepsin solution (10 mg pepsin/mL in 0.01 M HCl) was added and the mixture was incubated at 37 °C for 30 min. After incubation, 100 µL of 1.0 M NaOH solution was added in each sample to stop the second-stage digestion. The pH of the mixture was adjusted to 7.8 using 1.0 M NaOH, and 300 µL of pancreatin solution (10 mg/mL in sodium phosphate buffer, pH 7.0) added to start the third-stage digestion and the mixture was incubated at 40 °C for one hour. Then, 100 µL of Na_2CO_3 solution (150 mM) was added to stop the overall digestion stages. The total protein content of samples during these three-stages digestions was measured using BCA kit. The *in-vitro* digestibility of kiwifruit proteins was calculated using the following equation:

$$IVPD(\%) = \frac{P_0 - P_1}{P_0} \times 100 \quad (4)$$

Where P_0 is initial protein content, P_1 is the final undigested protein content.

6.3.10. Peptide content and CD spectroscopy analysis

During in-vitro protein digestion, one milliliter of initial protein extract solution, pepsin digested protein solution, and pancreatin digested protein solution was collected. Then, they were centrifuged at 5000×g for 10 min, where 500 µL of supernatant was obtained for the peptide measurement. According to the method described by the previous study, an o-phthalaldehyde (OPA) working solution including sodium tetraborate (100 mM), sodium dodecyl sulfate (20%, w/v), 40 mg/mL of OPA (methanol as solvent), 100 µL of β-mercaptoethanol were prepared [269]. 10 µL of samples from those three digestion stages were mixed with 150 µL OPA reagent in a microplate and was incubated for 2 min, and then the absorbances were recorded at 340 nm using a plate reader (Spectra Max M2, Molecular Devices, USA). A standard curve (0-10000 µM) was obtained using leu-gly as a standard.

A J-815 spectropolarimeter (JASCO, Tokyo, Japan) with a temperature-controlled cuvette holder (cuvette path length: 0.1 cm) was used to determine the secondary structure (α-helix, β-

sheet, and turns) of Act d 2 [270]. Each sample is scanned in the range of 260 nm to 190 nm with a bandwidth of 1 nm and a pitch of 0.2 nm. The scanning speed is set at 50 nm/min in a continuous model. Each spectrogram obtained is an average of five spectra accumulations. The CD analysis is performed using the CDPro Software.

6.3.11. SDS-PAGE, western blotting, and ELISA analysis

Kiwifruit samples are extracted with 0.01 M phosphate buffer (pH 7.0) for two hours at room temperature and centrifuged at 5000 × g for 10 min [245]. The supernatants are analyzed by SDS-PAGE and western blotting analysis. Electrophoresis is performed using Fisher brand™ FB-VE10-1 Vertical Electrophoresis System at 80 V for one hour. Western blotting was performed following the protocol from Bio-Rad through a Trans-Blot Turbo™ Transfer System (Bio-Rad, Quebec, Canada). The related figures were obtained using the image Lab™ software (Bio-Rad, Quebec, Canada). Biotin-conjugated rabbit polyclonal antibodies, goat anti-rabbit secondary antibody, and recombinant Act d 2 were purchased from Elabscience (USA). The immunodetection of protein (Act d 2) is performed using the ELISA test. After the reaction, the optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm according to the protocol of the kit [271]. The recombinant Act d 2 was used as a standard to obtain the standard curve.

6.3.12. Statistical analysis

The data obtained from the control and treatments were analyzed by one-way analysis of variance (ANOVA). Duncan's multiple range test ($P < 0.05$) was performed to identify the possibility of significance between treatments operating with an SPSS 22.0 software (SPSS Inc., Chicago, USA). The OriginLab software (2018b, student version, OriginLab Corp., USA) was applied to draw the graphs. All the analyses were performed in triplicates.

6.4. RESULTS AND DISCUSSION

6.4.1. Total phenolics and flavonoids

In this study, Folin-Ciocalteau assay was used to qualitatively analyze the total phenolics

changes in kiwifruit samples before and after ultrasound processing. The results illustrated that ultrasound treatment significantly ($P < 0.05$) improved the total phenolics content in kiwifruit juice (**Table 6.1**). US16 treated samples showed the highest total phenolics content which was two-fold higher than that of the control. It was followed by US12 and US8. After US4 treatment, the total phenolics increased by 25.9% from the initial value (57.60 GAE mg/100 mL). Similar observations regarding the total phenolics were reported in ultrasound treated mango juice [313] and pear juice [314]. Generally, phenolic compounds are in the soluble form present in plant tissues. High-intensity ultrasound processing could result in the disruption of cell walls and vacuoles of plant tissues, which may contribute to the increase of total phenolics in kiwifruit juice [315]. Additionally, high-intensity ultrasound could lead to a cavitational collapse in the surroundings of colloidal particles, which is beneficial to release more phenolic compounds into the juice [316]. Further, high-intensity ultrasound processing might produce additional hydroxyl groups which can be connected to the aromatic ring to produce more phenolic compounds [275].

Table 6.1. Changes of bioactive compounds in ultrasonic-treated kiwifruit juice. Note: values with different letters in the same column are significantly different ($p < 0.05$) from each other.

Treatment	Total phenolics (GAE mg/100 mL)	Total flavonoids (CE mg/100 mL)	TAC (μmol/100 mL)	DPPH (% inhibition)
Control	57.60 ± 1.46^{d}	26.60 ± 0.36^{d}	185.60 ± 1.88^{e}	28.45 ± 0.11^{c}
US4	72.52 ± 0.79^{c}	31.83 ± 1.52^{c}	239.17 ± 1.60^{d}	30.50 ± 0.15^{c}
US8	83.04 ± 1.80^{b}	40.86 ± 0.73^{b}	268.33 ± 2.01^{c}	38.50 ± 0.08^{b}
US12	115.76 ± 1.02^{a}	50.74 ± 0.88^{a}	299.46 ± 0.88^{b}	43.00 ± 0.24^{a}
US16	120.18 ± 0.95^{a}	54.68 ± 0.92^{a}	307.48 ± 1.16^{a}	45.50 ± 0.88^{a}

The changes of total flavonoids content were similar in behavior to total phenolics present in kiwifruit juice. In **Table 6.1**, there was a significantly increasing trend in total flavonoids with the rise of ultrasound processing time. The total flavonoids content showed a significant increase by 16.43% in US4, 53.60% in US8, 90.75% in US12, and 105.56% in US16, as

compared to the control (26.60 CE mg/100 mL). The increase of total flavonoids in kiwifruit juice could bring benefits to the consumers, since these components (e.g., quercetin and kaempferol) have health-promoting properties. A study has reported that increased intake of flavonoids is linked to protection from chronic diseases (e.g., heart disease and cancer) [317]. Other studies performed by Aadil et al. (2013) also observed an enhancement in the total flavonoids of grape and apple juice under ultrasound treatment. This increase of total flavonoids might be attributed to the disruption of cell walls of kiwifruit tissues during ultrasound treatment, which leads to increased release of flavonoids into the juice samples [315].

6.4.2. Total antioxidant capacity and radical scavenging activity

FRAP assay is one of the most common determinations used by the food industry. As shown in **Table 6.1**, the influence of ultrasound treatment on the antioxidant capacity of kiwifruit samples was measured. Total antioxidant capacity of kiwifruit juice significantly increased in four ultrasound treatments compared to the untreated kiwifruit juice. The increase of total antioxidant capacity ranged from 53.57 up to 121.88 μmol/100 mL compared to control. Specifically, a minor increase was observed in US4 (28.86%) and US8 (44.57%), followed by US12 (61.35%) and US16 (65.67%).

DPPH radical scavenging assay was another method used to evaluate the antioxidant capacity of kiwifruit juice after ultrasound treatment. During the determination, the hydrogen supply ability of the antioxidants to the stable free radical DPPH was measured by quantifying the formation of diphenyl picrylhydrazine [306]. The results of DPPH in kiwifruit juice after ultrasound treatment were similar in behavior to that of total antioxidant activity. Among all the treatments and control, the highest percentage inhibition of DPPH was 45.50% in US16 sample, which is 1.6-fold higher than the control (28.45%) (**Table 6.1**). While there were no significant changes in US4 samples (30.50%). After 8 and 12-min ultrasound treatment, a significant increase in the percent inhibition of DPPH was observed in US8 (38.50%) and US12 (43.00%) samples when compared to the untreated group.

These results indicate that ultrasound processing not only increases the total antioxidant capacity but also enhances the radical scavenging activity of kiwifruit juice, which could be

beneficial to the health of consumers. Similarly, another study found that the total antioxidant capacity and percentage inhibition significantly increased by 34.6% and 249.07% in lime juice under ultrasound processing for 60 min at a frequency of 25 kHz [301]. These increases mainly could be explained because of the increase of antioxidants (e.g., phenolics) after ultrasound processing. Moreover, inactivation of some oxidation related enzymes like polyphenol oxidases resulted from the shear force formed during the processing period, which would also result in an increase of total antioxidant capacity in fruit juice [316].

6.4.3. Ascorbic acid content

The ascorbic acid content of kiwifruit juice significantly decreased ($P < 0.05$) after four ultrasound treatments when compared to the untreated samples (**Figure 6.2**). The highest reduction was 55.09%, which is from 18.59 mg/100 mL in control to 8.01 mg/100 mL in US16 samples, followed by US12 (54.47%), US 8 (46.42%), and US 4 (38.21%). A similar decrease of ascorbic acid has been observed in strawberry juice [318] and mango juice [313] treated with ultrasound. Studies found that lots of factors such as light, high temperatures, and pressures could affect the properties of ascorbic acids in fruit juice, which could result from degradation by the aerobic or anaerobic pathways [319]. During the treatment period, extra free radicals might be formed through some sonochemical reactions, which could enhance the oxidative process and lead to the degradation of ascorbic acid [313].

On the contrary, an increasing ascorbic acid content has been observed in grapefruit juice [275] and lime juice [301] treated using ultrasound. This enhancement might be due to the disruption of kiwifruit tissues or cell walls, which leads to more release of ascorbic acid [315]. The ascorbic acid may show different properties between various fruit tissues and further studies need to be done based on this point.

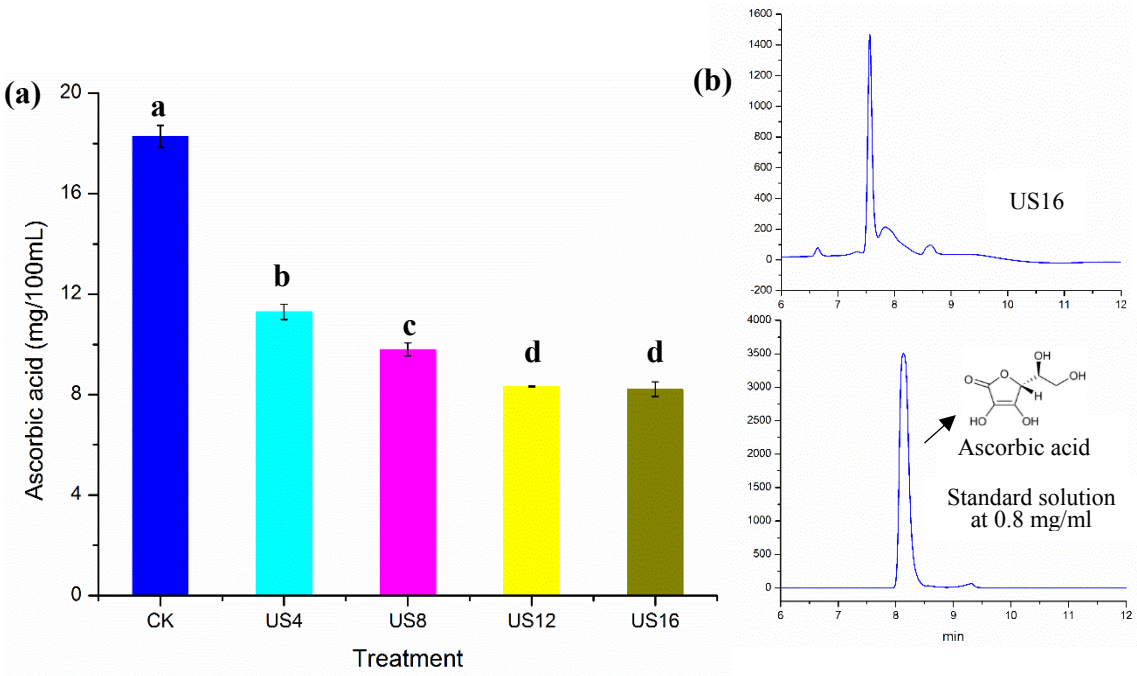


Figure 6.2. HPLC chromatogram for ascorbic acid present in ultrasound-treated kiwifruit juice (a) ascorbic acid content; (b) Absorbance of standard solution and kiwifruit juice samples at 245 nm. Note: values with different letters in various columns are significantly different ($p < 0.05$) from each other.

6.4.4. Catechin, ferulic acid, and gallic acid

In the study, the changes of three important phenolic compounds including catechin, ferulic acid and gallic acid in kiwifruit juice were evaluated before and after ultrasound processing. The effect of ultrasound treatment on their extraction was presented in **Table 6.2**. No significant differences in the ferulic acid of kiwifruit juice were observed after ultrasound treatment compared to the control. It may be due to its very minor content present in kiwifruit juice. On the contrary, a significant enhancement in catechin was measured with a further increase of processing time. The highest content was found in US16 sample (40.96 mg/ 100 mL) which is 2.35-fold higher than that of control (17.46 mg/ 100 mL), followed by US 4 (27.68 mg/ 100 mL), US8 (38.64 mg/ 100 mL) and US12 (40.27 mg/ 100 mL). Additionally, ultrasound treatment also enhanced gallic acid content in kiwifruit juice. As shown in **Table 6.2**, the increase in gallic acid ranged from 2.07 in US4 sample up to 3.35 mg/ 100 mL in US16 sample compared to the control group (1.79 mg/ 100 mL).

Table 6.2. Changes of phenolic compounds in ultrasonic-treated kiwifruit juice. Note: values with different letters in the same row are significantly different ($p < 0.05$) from each other.

Phenolic compounds	**Treatment**				
(mg/100 mL)	Control	US4	US8	US12	US16
Ferulic acid	0.10 ± 0.00^{a}	0.09 ± 0.00^{a}	0.12 ± 0.00^{a}	0.16 ± 0.00^{a}	0.08 ± 0.00^{a}
Catechin	17.46 ± 1.98^{c}	27.68 ± 2.65^{d}	38.64 ± 2.15^{b}	40.27 ± 2.89^{a}	40.96 ± 3.26^{a}
Gallic acid	1.79 ± 0.02^{d}	2.07 ± 0.04^{c}	3.08 ± 0.08^{b}	3.22 ± 0.04^{ab}	3.35 ± 0.02^{a}

6.4.5. Correlation between processing time, total protein, TSS and antioxidants

In the present study, we found that physicochemical properties such as total phenolics and ascorbic acid showed a positive or negative correlation with ultrasound processing time. As shown in **Figure 6.3**, a significantly negative correlation existed between the total protein content of kiwifruit juice and ultrasound processing time. The total protein present in samples decreased with the increase of processing time, which may be due to the rupture of hydrogen bonds of protein and the breakdown of peptide chains under high-intensity ultrasound treatment (São José et al., 2014). It indicates that ultrasound treatment has a potential application to improve the functional properties of proteins. Similarly, ascorbic acid of kiwifruit juice also showed a negative correlation with the processing time (**Figure 6.3**). During ultrasound processing, there was a degradation in ascorbic acid due to the stressful conditions such as intense light, higher temperatures or pressure. Therefore, in order to improve this issue, avoiding light and temperature increase is necessary during food processing. On the contrary, total phenolics, flavonoids, gallic acid, catechin, DPPH activity and antioxidant capacity of kiwifruit juice had a significantly positive correlation with the ultrasound processing time (**Figure 6.3**). It indicates that increasing ultrasound processing time is beneficial in enhancing the extraction of antioxidants in kiwifruit juice. Additionally, the TSS and ferulic acid were not impacted by ultrasound processing time due to their stable properties.

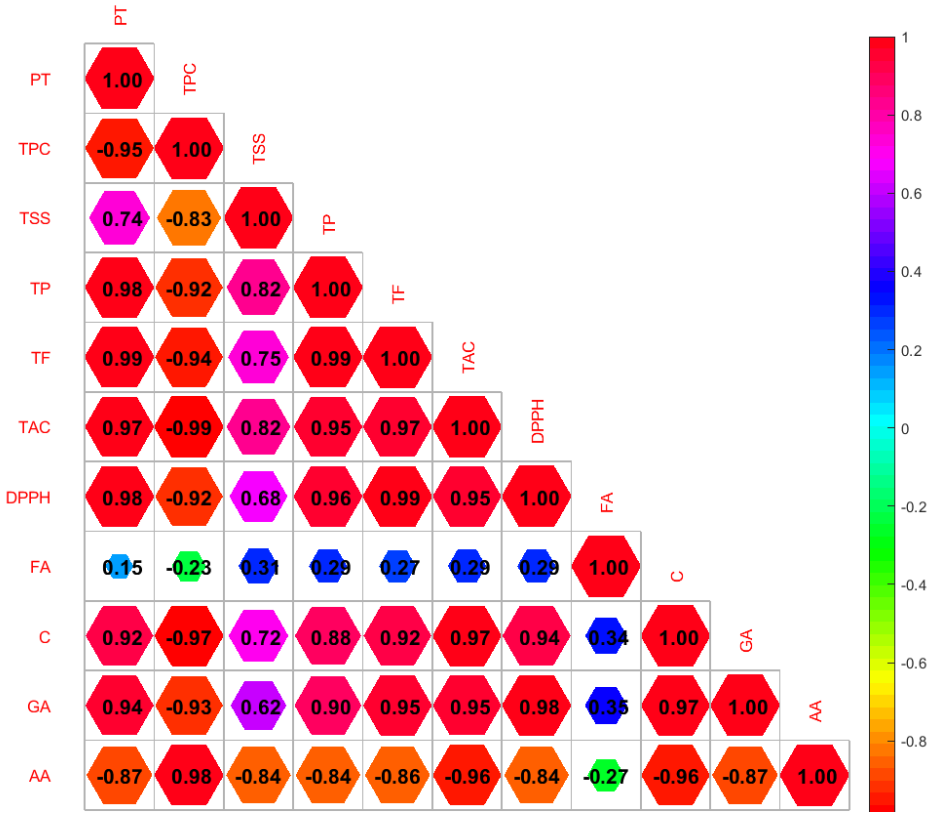


Figure 6.3. Correlation between processing time, phenolic compounds and total antioxidant capacity of kiwifruit juice. Note. PT: processing time; TPC: total protein content; TSS: total soluble solids; TP: total phenolics; TF: total flavonoids; TAC: total antioxidant capacity; DPPH: 1,1-diphenyl-2- picrylhydrazyl; FA: ferulic acid; C: Catechin; GA: gallic acid; AA: ascorbic acid.

Many studies have reported that the antioxidant capacity of fruit juice is mainly due to antioxidants content (e.g., phenolic compounds and flavonoids) in samples [320]. In this study, the total antioxidant capacity of kiwifruit juice showed the highest correlation coefficient with the total flavonoids (r=0.97) and catechin (r=0.97), followed by total phenolics (r=0.95), gallic acid (r=0.95) and DPPH (r=0.95) (**Figure 6.3**). The increasing antioxidant capacity might be attributable to the enhancing of phenolic components and flavonoids. However, ascorbic acid showed a negative correlation (r=-0.96) with the total antioxidant activity of kiwifruit juice. The finding states that ascorbic acid did not contribute to the total antioxidant activity of kiwifruit juice.

6.4.6. Microstructure

The microstructure of kiwifruit juice treated with 0, 4, 8, 12, and 16 min of high-intensity ultrasound processing was visually observed using optical microscopy (**Figure 6.4**). The results showed that high-intensity ultrasound processing significantly disrupted the cell walls of the tissue when increasing the duration of processing. Specifically, the microstructure of untreated juice samples (US0) presented in integral cells with intact walls. The intracellular components can be seen clearly within the cell structure. After 4 min of ultrasound processing, no significant differences in the structures of cells were observed when compared to the control. There were few cells of kiwifruit tissues that started to tear leading to a slight release of intracellular components into the juice. This result is similar to Campoli et al. (2018), the first several minutes of ultrasound processing can only affect the movement of these compounds inside of cells without obvious release into the juice.

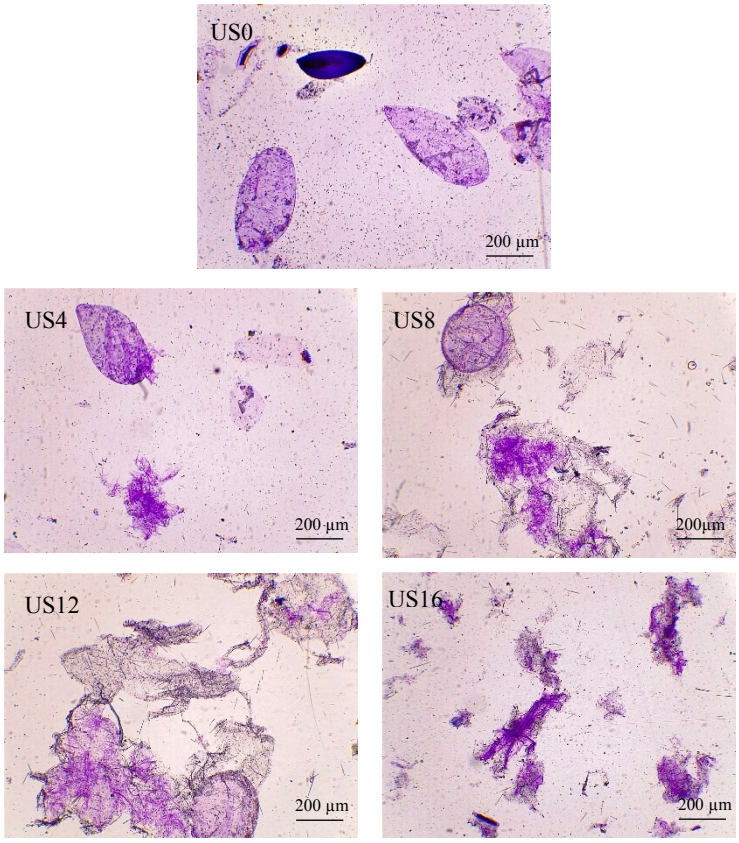


Figure 6.4. Optical microstructure (×10) of kiwifruit juice: untreated sample (US0) and those treated by ultrasound for 4 min (US4), 8 min (US8), 12 min (US12) and 16 min (US16).

In comparison, the microstructure of US8 and US12 processed juice samples presented a clear difference compared to the controls (US0) due to an increase in the processing time. Specifically, more cell disruption and tearing in tissues were observed after US8 treatment, which resulted in a higher release of intracellular compounds compared to that of US4. However, some intact kiwifruit cells still can be found in the juice samples. US12 treatment completely caused the breakdown of cell walls leading to the intracellular components transferring to the juice. The large pieces of cell fragments were still clearly present in the juice. In contrast, the clearest disruption in cell structures was observed when the longest duration (US16) was applied. Specifically, US16 treatment dramatically released the intracellular components and cut the large cells fragments into small pieces, which led to the release of enormous amounts of small particles into juice. Similar trends regarding microstructure changes responding to the processing time were observed in guava juice [321], peach juice [322], and strawberry juice [310] when processed with ultrasound at 20 kHz for 0-15 min.

The disruption, tearing and leakage of cell structures can be attributed to the cavitation effects resulted from ultrasound processing [323]. Studies have found that there are two types of cavitation generated during high-intensity ultrasound processing. The oscillations of ultrasound waves cause the formation of numerous small bubbles that rotationally travel through the sonic field leading to the generation of microstreaming. This stable cavitation is associated with certain small-scale effects such as movements and forces, which can be used to explain why US4 treatment caused slight damage on the cell tissues [323,324]. Further, transient cavitation occurs due to the rapid formation and collapse of big-size bubbles within a short time, resulting in a large amount of pressure and stress [323]. Altogether, these two cavitation effects provide enough energy to breakdown the cell walls of kiwifruit tissues causing cell torn, leakage, rupture, and loss of tissues [310,324]. The increase in degradation of microstructure with the increasing of processing duration also could be used to explain why the antioxidants (e.g., phenolic components and flavonoids) enhanced under ultrasound treatment (**Table 6.1 & 6.2**).

6.4.8. Color attributes

Color attributes are considered as an important standard to evaluate the quality of fruit juice or related products to satisfy the requirements of consumers [275]. Kiwifruit juice with a bright green

color is desired for the market [276]. The influences of high-intensity ultrasound processing on the color attributes of kiwifruit juice were shown in **Table 6.3**. No significant differences in the lightness (L*) values were investigated in all treatments, while a* and b* values of juice have significantly improved after processing. Specifically, the highest a* value (greenness) was observed in US12 (-7.64) treated samples, followed by US4 (-7.27) and US16 (-6.45), while no obvious differences were found between US0 (-5.22) and US8 (-5.94). Similarly, the yellowness (b*) of kiwifruit juice increased with the rise of processing time from 0 to 16 min. US16 significantly increased the yellowness of samples to 15.13 from an initial level of 4.99 (US0), followed by US12 (12.63), US4 (10.04), and US8 (8.50). The total color difference (ΔE) ranged from 3.59 to 10.70 after 4-16 min ultrasound treatment, which is greater than 2, and these noticeable changes can be seen by the naked eyes [325]. Thus, ultrasound processing showed a potential application to improve the quality of kiwifruit juice by increasing its yellowness and greenness. Similar results were observed in red grape juice [326] and pineapple juice [315]. These improvements are associated with the disruption observed in kiwifruit tissues under high-intensity ultrasound processing, which in turn could have led to a large amount of carotenoids and anthocyanins released into the juice [327].

Table 6.3. Color attributes changes in ultrasound treated kiwifruit juice. Note: values with different letters in the same column are significantly different ($p < 0.05$) from each other.

Treatment	*L**	*a**	ΔE	C	YI	h°
US0	107.10 ± 3.32^{a}	-5.22 ± 0.89^{d}	Na	7.22 ± 0.87^{d}	6.44 ± 0.77^{d}	44.66 ± 2.44^{e}
US4	110.10 ± 3.85^{a}	-7.27 ± 0.25^{ab}	6.22 ± 0.97^{c}	12.39 ± 0.62^{b}	11.91 ± 0.45^{c}	51.63 ± 0.50^{d}
US8	107.41 ± 1.92^{a}	-5.94 ± 0.29^{cd}	3.59 ± 0.88^{d}	10.36 ± 0.44^{c}	12.55 ± 1.20^{c}	55.78 ± 0.84^{c}
US12	106.50 ± 3.18^{a}	-7.64 ± 0.53^{a}	8.04 ± 1.10^{b}	14.76 ± 0.59^{ab}	17.08 ± 0.67^{b}	60.60 ± 1.33^{b}
US16	110.30 ± 2.84^{a}	-6.45 ± 0.79^{bc}	10.70 ± 2.23^{a}	16.45 ± 0.80^{a}	19.46 ± 1.81^{a}	66.45 ± 2.08^{a}

Chroma, yellow index, and hue angle of the ultrasound treated kiwifruit juice increased during 0-16 min processing (**Table 6.3**). Chroma level was enhanced by two folds in US16 (16.45) compared to untreated samples (7.22). The highest yellow index was measured in US16 (19.46), followed by US12 (17.08), US 8 (12.55), US4 (11.91), and US0 (6.44). The hue angle of juice

samples was increased to 66.45 from the initial level of 44.66 after 16-min ultrasound processing. These significant increases are due to the increase of a* and b*, the maintenance of L* during ultrasound processing. These findings agree with the results obtained by Abid et al. (2013) in apple juice and Bhat et al. (2011) in lime juice. However, the results reported by Ordóñez-Santos et al. (2017) found that the chroma level of kiwifruit juice significantly decreased during a 40-min ultrasound processing, which resulted from the oxidation reaction of juice under such a long-time exposure to air. Thus, the proper processing duration is strongly related to the color attributes of juice or products.

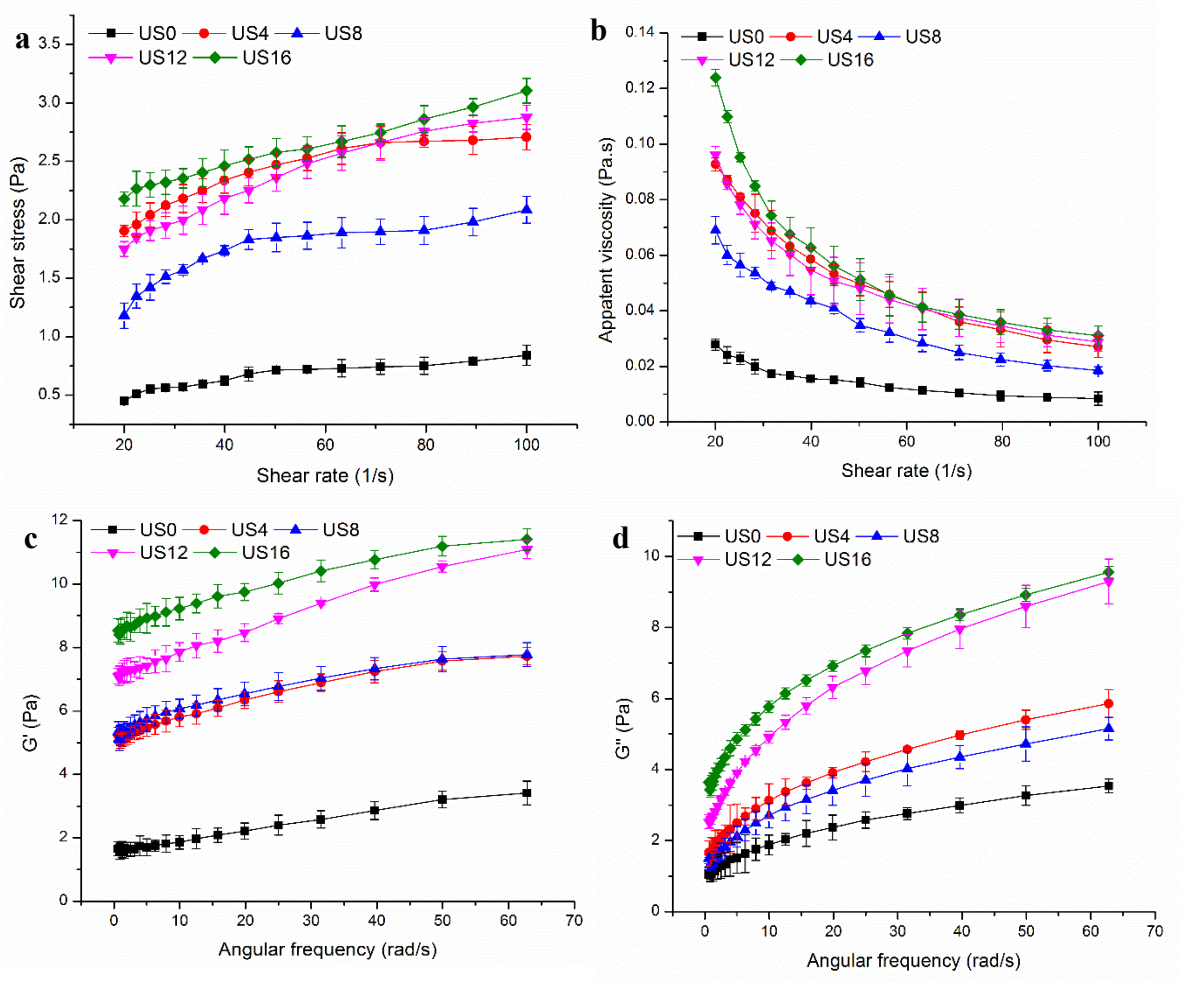


Figure 6.5. Rheological characteristics of ultrasound processed kiwifruit juice: (a) flow curves; (b) flow viscosity; (c) storage modulus; (d) loss modulus.

6.4.9. Rheological properties

6.4.9.1. Flow behavior of kiwifruit juice

The flow properties of ultrasound treated kiwifruit juice were illustrated in **Figure 6.5**. The

shear stress of kiwifruit juice increased gradually with the rise of the shear rate (**Figure 6.5a**). A significant increase in the shear stress of US8 was observed compared to the untreated samples, while no obvious differences were observed between US4, US12, and US16. In addition, a decreasing trend of apparent viscosity in all treatments was observed when the shear rate increased from 0.1 to 100 s^{-1} (**Figure 6.5b**). The ultrasound treated samples, especially in US16 showed a slower decrease in the viscosity when compared to the untreated samples (US0), which might be related to the structural changes in kiwifruit juice during ultrasound processing. Similar results were found in mango juice and peach juice [311,322].

The yield stress increased when treated with ultrasound from 0 to 16 min in both the models, while the flow behavior (n) of kiwifruit juice decreased after processing. The apparent viscosity showed a significant enhancement after ultrasound processing, especially in US16. These changes in the flow behavior of kiwifruit juice are influenced by a wide range of factors including the disruption of cell structures and breakdown of large molecules under high-intensity ultrasound treatment [311,322]. Furthermore, studies have reported that the particle size and particle size distribution of fruit juice decreased after processing and the smaller particle size in the fruit juice can provide a higher total surface area, which can explain the rise in yield stress and apparent viscosity of the juice [328]. However, there are a very limited number of studies that evaluated the effects of ultrasound processing on the flow behavior of kiwifruit juice and further studies are recommended.

6.4.9.2. Dynamic rheological characteristics of kiwifruit juice

As shown in **Figure 6.5c-d**, the behavior of storage modulus (G′) and loss modulus (G″) was determined at the frequency ranged from 0.1 to 10 Hz using the frequency sweeps model. In comparison to the untreated samples (US0), the kiwifruit samples in other treatments showed an increasing trend in the values of G′ and G″ with the rise of frequency. A significant increase was observed in ultrasound treated kiwifruit juice compared to untreated samples at the same frequency. The highest G′ and G″ were observed in US16, followed by US12, US8, US4, and US0. However, the differences in G′ and G″ found between US4 and US8 were not significant. The other significant increases in the values of G′ and G″ in kiwifruit juice were similar to the

flow behavior mentioned above. Furthermore, the results found that the value of G′ is higher than G″ at the frequency range of 0.1-10 Hz, which are similar to the results obtained in mango juice treated with ultrasound at 20 kHz, 400W for 0-40 min [311].

6.4.10. Total soluble protein content

Solubility is considered as one of the most practical measures of protein denaturation and aggregation, and hence a good index of protein functionality [329]. **Figure 6.6** shows the total soluble protein content of kiwifruit samples when treated with ultrasonication under different processing times. It was observed that the total soluble protein content of kiwifruit showed a decreasing trend with the rise of processing duration under ultrasonication. Specifically, the initial soluble protein concentration of kiwifruit samples (US0) was 165.09 mg/g DW, while a 10% reduction in the protein content was observed after 4-min ultrasound. In comparison with US4, the protein content of US8 treated samples (145.36 mg/g. DW) showed a slight decrease. After 12-min ultrasound treatment, the total soluble protein of kiwifruit samples decreased to 138.78 mg/g. DW. Among all the treatments, the lowest total soluble protein content was observed in US16 treated samples (132.33 mg/g. DW), which presents an approximate 20% of reduction when compared with the initial level. Thus, ultrasound resulted in a reduction in the solubility of kiwifruit proteins during processing.

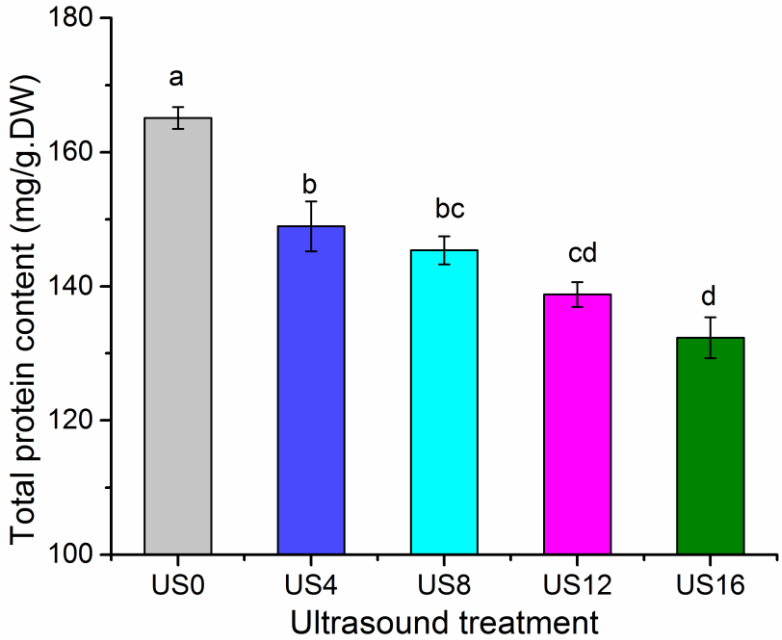


Figure 6.6. Total protein content of ultrasound treated kiwifruit samples.

Those results described above agree with the findings of previous studies, which reported that ultrasound processing with the power output level of 0-450 W, at 20 kHz for 12-24 min, resulted in a reduction of black bean protein [330]. Similarly, Costa et al. (2013) found that a significant decrease in the protein of pineapple juice was observed upon increasing the intensity and the time of ultrasound treatment. This decrease may be associated with the breakdown of hydrogen bonds and peptide chains presented in the proteins under ultrasound treatment, which in turn could alter the protein structure (e.g., secondary or tertiary structure) [323]. Another possibility is that the protein molecules partially unfolded and reformed, leading to the formation of macromolecular aggregates, which may be attributed to a reduction in solubility of related proteins [330]. It indicates ultrasound treatment has a potential application to modify the structure of the protein or rupture their peptide chains which in turn could affect the allergenicity of related allergens.

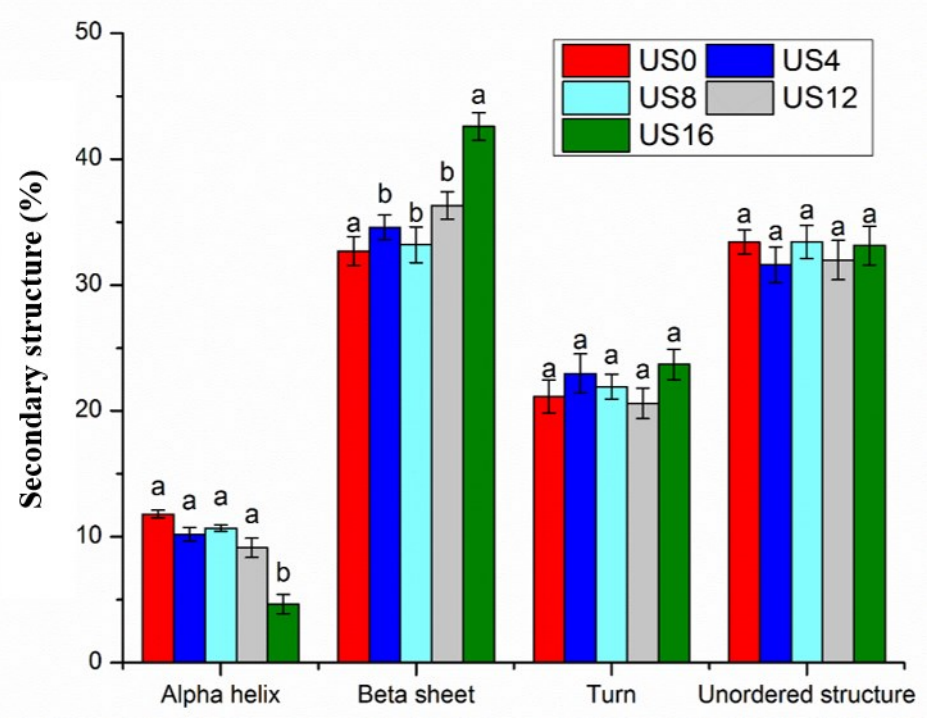


Figure 6.7. Secondary structure of protein extract using CD spectroscopy: untreated sample (US0) and those treated by ultrasound for 4 min (US4), 8 min (US8), 12 min (US12) and 16 min (US16).

6.4.11. Secondary structure of kiwifruit protein

In the present study, CD spectroscopy was used to determine the secondary structures before

and after the ultrasound processing. As shown in **Figure 6.7,** it can be seen clearly that the beta-sheet is the major structure accounting for 30-45% of the total secondary structures, followed by turn and unordered structures with the percentage of 20-25% and 30-35%, respectively. Whereas, the percentage of the alpha-helix structure only represented approximately 5-15%. Alpha helix and beta-sheet are considered as two major structures in the proteins, which is responsible for the maintenance of the protein structure and is also strongly associated with its functional properties [268]. During ultrasound processing, the alpha helix of kiwifruit proteins decreased with the further increase of processing time, especially after 16 min, while an enhancement in the beta-sheet of kiwifruit proteins was observed after ultrasound processing compared with the untreated samples (US0). However, no significant differences were determined in the turn and unordered structure of kiwifruit proteins. These changes in the secondary structure provided extra evidence that ultrasonication affected the solubility of kiwifruit proteins during the processing.

In egg white, Zhu et al. (2018) treated samples with ultrasonication at 400 W for 0-16 min. The results found that a one-fold reduction in the alpha-helix and a 2-fold increase in the beta-sheet of egg white proteins were observed after ultrasound processing when compared with the untreated samples [268]. Similarly, in blackberries, Dou et al. (2019) found that the alpha-helix content was decreased after the ultrasonication pretreatment (300 W, 8-24 h), while no significant differences were observed in the content of beta-turn and random structures [331]. These changes in the secondary structure of the proteins mentioned above might be due to the formation of shear forces, shock waves, and turbulence during the ultrasound processing. These cavitation effects obtained from the ultrasound can contribute to disrupting the interactions between the local sequences of amino acids and between different parts of the protein molecule, resulting in the secondary structures change of proteins [331]. This is supported by research conducted with soy protein isolate, where Ding et al. (2019) found ultrasonication decreased the contents of α-helix and β-sheet, increased the contents of random and coil β-turn structures [332]. In jackfruit seed proteins, Resendiz-Vazquez et al. (2019) reported that ultrasound processing (200-600 W, 15-30 min) significantly enhanced the content of alpha-helix and random coil, but decreased the beta-sheet content [333]. Thus, there is an obvious difference in the secondary structure of proteins when extracted from various food matrixes.

6.4.12. *In vitro* digestibility and peptide content of kiwifruit proteins

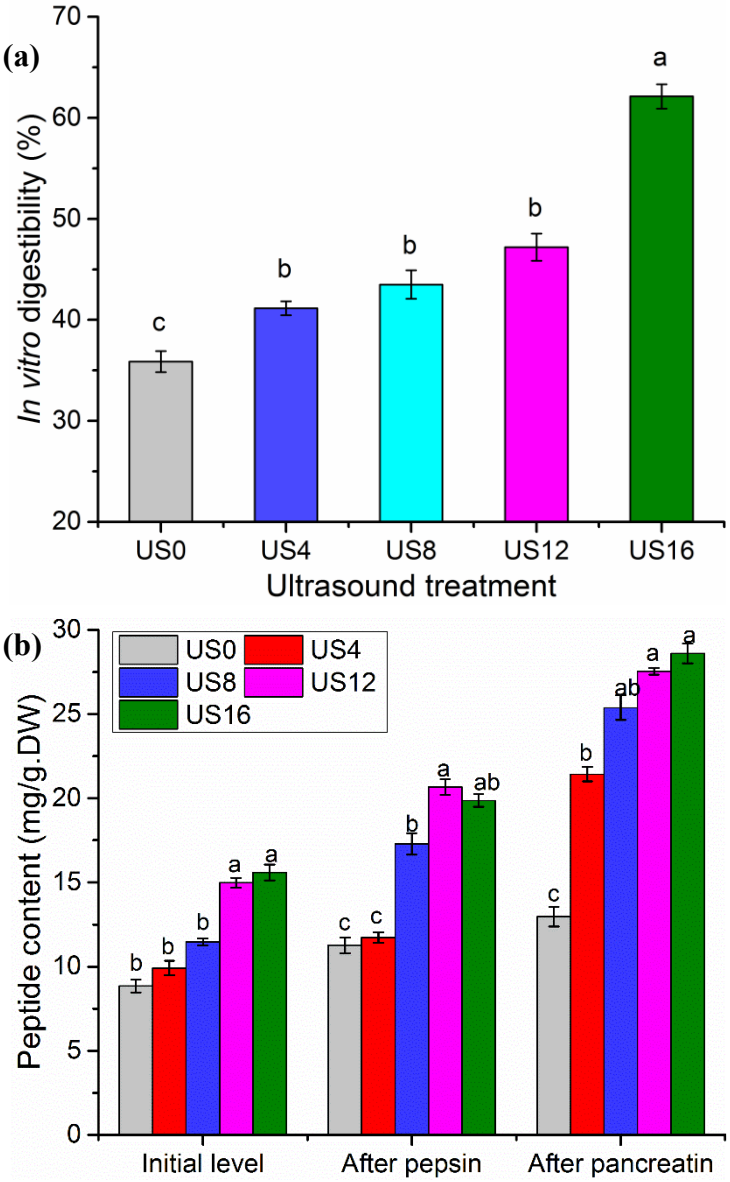


Figure 6.8. Digestibility (a) and peptide content (b) of kiwifruit proteins during digestion.

In comparison with animal proteins, plant-based proteins have a lower digestibility. The digestibility of raw soybean protein is approximate 40% [334], and 58.5% in mature pigeon pea seed [335]. In the present study, the *in vitro* digestibility of kiwifruit protein was determined after digestion with pepsin and pancreatin. The results reported that the *in vitro* digestibility of raw kiwifruit (US0) was 35.87%, increased to 41.14% after 4-min ultrasound processing (**Figure 6.8a**). A slight increase in the digestibility of kiwifruit proteins was determined in US8 (43.50%) and US12 treatment (47.19%) compared with US4. Among all the treatments, the highest

improvement in the digestibility of kiwifruit proteins was found in US16 (62%), which is approximate 2-fold higher than the untreated samples (US0). This result agrees with the findings reported in previous studies, which found that ultrasound processing at 400 W for 16 min could increase the digestibility of egg white proteins from 73.44% to 79.41% [268]. However, an opposite result was reported in faba bean protein isolate [336], which pointed ultrasonication caused a 4% reduction in the digestibility of ultrasound treated samples compared with the untreated protein samples.

During the three-stage of *in vitro* digestion of kiwifruit proteins, the peptide content was measured using a protein test kit. As shown in **Figure 6.8b**, ultrasonication increased the peptide content of kiwifruit proteins during all the three-stage of digestion. In the initial level, the peptide of raw kiwifruit protein was 8.85 mg/g. After 4-min and 8-min ultrasound processing, a slight improvement in the peptide content but no significant differences were observed compared with the control group (US0). Whereas, the peptide content was significantly increased in that US12 (14.98 mg/g) and US16 (15.59 mg/g). In the second stage of digestion (pepsin), the peptide content of pepsin digested samples was maintained at a similar level compared with the initial level. However, after the third stage of digestion (pancreatin), the peptide content was enhanced in all treatments. The highest concentration of peptide was observed in US16 (28.60 mg/g), followed by US12 (27.54 mg/g) and US8 (25.38 mg/g), which is approximately 3-4 times higher than the untreated samples. This result is consistent with the findings of *in vitro* digestibility of kiwifruit protein. These improvements in digestibility and peptide content are strongly associated with the secondary structures (decrease of alpha-helix and increase of beta-sheet) changes of kiwifruit proteins **(Figure 6.7**).

6.4.13. SDS-PAGE, western blotting, and ELISA test

As shown in **Figure 6.9a**, the intensity of each protein band showed a decreasing trend with the further increase of processing duration, especially after 16 min, which agrees with the changes of total soluble protein content during ultrasound processing (**Figure 6.6**). After 4-min and 8-min ultrasound treatment, no obvious changes in the intensity of Act d 2 band were obtained. Whereas, the Act d 2 protein band showed less intensity compared with the untreated

samples (US0), but it still can be observed. After 16-min ultrasonication, the Act d 2 protein band was hardly to be seen visually. To prove the protein band located in 24 kD, belonging to Act d 2, western blotting was applied to evaluate the IgE binding capacity of kiwifruit protein under ultrasound processing. As shown in **Figure 6.9b,** no apparent differences in the IgE binding capacity of US4 and US8 treated samples when compared with the untreated samples (US0), while the IgE binding capacity of Act d 2 was significantly inhibited after 12 and 16-min ultrasound processing.

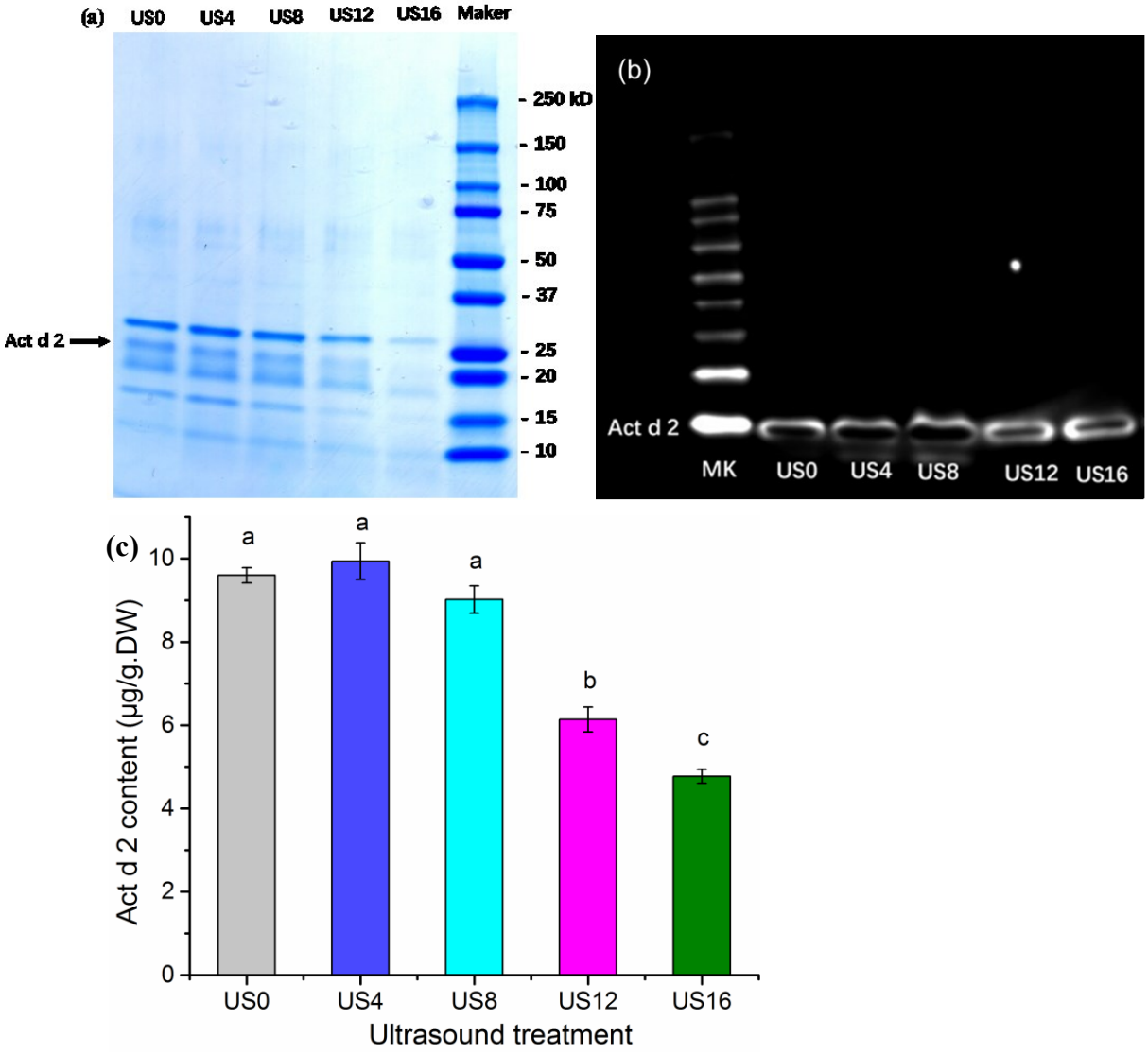


Figure 6.9 SDS-PAGE protein band (a) western blotting (b) of protein extract, and kiwifruit allergen Act d 2 content (c): untreated sample (US0) and those treated by ultrasound at 400 W, 20 kHz, for 4 min (US4), 8 min (US8), 12 min (US12), and 16 min (US16).

Further, a quantified method, Sandwich ELISA test was used to quantify the Act d 2 content in the kiwifruit samples. The initial concentration of Act d 2 in the raw kiwifruit samples was 9.6

μg/g. DW, and no significant differences were found in the samples during the first 8 min ultrasound processing (**Figure 6.9c**). After 12-min processing, Act d 2 content was decreased by 36%, compared with the untreated samples (US0). The highest reduction in the Act d 2 content was observed in the US16 treated samples (4.77 μg/g. DW) which is 2-fold lower than the untreated sample. Thus, ultrasound processing showed a potential application in the reduction of allergenicity of kiwifruit.

Li et al., (2006) treated shrimp samples with high-intensity ultrasound at 50 °C for 1.5 h and the results showed an effect on the integrity and structure of shrimp proteins, which led to a reduction in the overall allergenicity [303]. Also, the inhibition value of IgE binding to immobilized Pen a 1 (major shrimp allergen) reached 20% when compared to the untreated samples. Similarly, studies have reported that ultrasonic pre-treatment (0-1.5 h at 50 Hz) significantly reduced the Ara h 2 content (one of major peanut allergens), compared to the raw peanut samples [137]. These reductions in the above-mentioned allergens are strongly linked to their secondary structures. In the present study, ultrasonication significantly decreased the alpha helix and increased the beta-sheet percentage in the protein structures, as a result of "cavitation effects" formed during the ultrasound processing. However, further studies regarding on the mechanisms of ultrasonication causing a reduction in the allergenicity of proteins are needed.

6.5. CONCLUSION

In this study, we found that ultrasound processing significantly increased bioactive compounds (e.g., catechin, gallic acid), and total antioxidant capacity compared to the untreated samples, which is probably due to the disruption of cell structures in kiwifruit tissues under ultrasonication. Also, ultrasound processing significantly improved color attributes (a*, b*, and YI), digestibility, peptide content, and rheological characteristics (flow and viscoelastic behavior) of kiwifruit juice. However, Act d 2 was significantly decreased after ultrasound processing, which agrees with the results of the total soluble protein of kiwifruit samples. Thus, high-intensity ultrasound processing shows potential applications in improving healthy bioactive compounds and decreasing the allergenicity of kiwifruit samples. Further clinical studies regarding the allergenicity are needed.

CONNECTING TEXT

In Chapter VI, a non-thermal processing; high-intensity ultrasound was applied. The results found that the total phenolic compounds such as catechin, gallic acid and total antioxidant activity of kiwifruit juice were enhanced significantly after ultrasound processing when compared to the untreated kiwifruit juice. Also, high-intensity ultrasound processing significantly improved the color attributes, digestibility, peptide content, and rheological characteristics (flow and viscoelastic behavior) of kiwifruit samples due to the formation of cavitation effects (providing shear stresses and high pressure) during the ultrasound processing. Further, the Act d 2 was significantly decreased after ultrasound processing, which is strongly associated with the changes of secondary structures (decreasing of alpha-helix and increasing of beta-sheet). Thus, high-intensity ultrasonication could be considered as an alternative processing method not only in improving bioactive compounds of kiwifruit juice but also in reducing the allergenicity of kiwifruit.

In the following chapter, Molecular Dynamics (MD) simulations were used to evaluate the structural changes of kiwifruit allergen, Act d 2, when the external physical stresses (high temperature and electric field) are applied. This technique is used as a visual tool to predict the protein structural changes under different processing conditions (e.g., high temperatures).

CHAPTER VII

STRUCTURAL RESPONSES OF KIWIFRUIT ALLERGEN ACT D 2 TO THERMAL AND ELECTRIC FIELD STRESSES BASED ON MOLECULAR DYNAMICS SIMULATION

7.1. ABSTRACT

Kiwifruit is considered as one of the most common plant-based food sources causing an allergic immune response. The aim of this study is to explore the influence of thermal and electric field treatment on the secondary structure of Act d 2 kiwifruit allergen through using molecular dynamic (MD) simulations. The simulations were performed at four different temperatures (300K, 325K, 350K, and 375K) and their combinations with an external oscillating electric field (0.05 V/nm, 2450 MHz) by using GROMACS software. During the simulations, secondary structure, root mean square deviation (RMSD), solvent accessible surface area (SASA), dipole moment, root mean square fluctuations (RMSF), snapshot of surface properties of kiwifruit allergen, Act d 2 were analyzed. The results showed that the kiwifruit allergen, Act d 2 is a heat-stable protein without significant difference in RMSD and snapshot of surface properties after thermal treatments. However, electric field combined thermal treatments significantly affected the secondary structure, RMSD, RMSF and snapshot of surface properties resulted in conformational changes of Act d 2 kiwifruit allergen.

KEYWORD: Kiwifruit allergen, molecular dynamics, secondary structure, snapshot of surface properties.

7.2. INTRODUCTION

Food allergy is mainly caused by allergenic proteins in various food sources when patients are exposed to them [134]. In 2008, the American Disease Control and Prevention Center reported that an 18% increasing trend in the prevalence of food allergy from 1997 to 2007 among children was observed [337]. Nowadays, there are up to 10% of the total population suffering from food allergies worldwide [338,339]. Among all food allergies, kiwifruit is considered as the most common plant-based food source causing allergenic reactions after the big eight [166,177]. in Turkey, among young children (6-9 years old), kiwifruit has been recognized as the fourth most common food source causing allergy after beef, milk, and cocoa [259]. Up to 50% of patients with food allergy history are also allergic to kiwifruit due to cross-reactivity through a self-report analysis in Sweden and Denmark [8]. In Spain, there are approximately 1.8% of the total population allergic to kiwifruit [9]. Further, studies have reported that the prevalence of kiwifruit allergy is increasing worldwide because of the cross-reactivity between kiwifruit allergy and pollens (e.g., tree or grass) allergies or other plant-based food (e.g., avocado) allergies [11,151,153,154,198]. Kiwifruit allergy can trigger various symptoms classified into oral allergy syndrome (OAS) and life-threatening anaphylaxis [28]. The OAS is generally accompanied by some mild allergic reactions such as itching and tingling in the skin, which can be improved in a natural way or using antihistamines in a short time [154]. However, the severe, life-threatening anaphylaxis reactions including blood pressure decrease, heart rate increase, and loss of consciousness may even result in death if there is no immediate treatment [157]. Therefore, kiwifruit allergy is becoming a major issue related to food safety and human health.

To date, several studies have been reported regarding the modification of allergenic kiwifruit proteins through different food processing methods. For example, 10-min steam cooking at 100 °C decreased the concentration of two major kiwifruit allergens (Act d 1 and Act d 2) compared to the untreated samples [124]. This reduction in the allergens might be due to the changes in their tertiary and secondary protein structures under external thermal stress, which in turn could alter their ability to induce allergenic responses [340,341]. Moreover, the allergenicity of Act d 1 and Act d 2 can be alerted after two-stage enzymic treatments including pepsin and trypsin or chymotrypsin [148]. However, these processing methods including steam cooking and

enzymatic hydrolysis work inefficiently but also result in a high nutrient loss of the treated products during processing. Therefore, more novel and effective processing modifications on the reduction of kiwifruit allergenicity are in need.

Molecular dynamics (MD) simulation showed a potential application in visually evaluating the structural and dynamical changes of proteins under external stresses (e.g., high temperatures) at the molecular level. Recently, MD simulation technique has been used to observe the structural changes in peanut [134] and soybean [342,343] allergens under external stresses (e.g., high temperatures). This technique can contribute visually to understand the folding and unfolding of protein structure under different external stresses in the system. However, no related studies are performed on the modification of kiwifruit allergen Act d 2 under external stresses using MD simulation. In the present study, Act d 2 was selected as a simulation subject. During the MD simulation, Act d 2 was treated with thermal and electric field stresses. Hopefully, the results obtained from the simulations can provide a visual prediction in the structural changes of kiwifruit allergen Act d 2 and find a new strategy to solve kiwifruit allergy related issues.

7.3. MATERIALS AND METHODS

7.3.1. MD simulations

According to previous studies, MD simulations were performed using GROningen MAchine for Chemical Simulations (GROMACS) software (version 5.0.4, Stockholm Center for Biomembrane Research, Stockholm, Sweden) based on the classical MD algorithm [134]. Act d 2, a major allergen present in kiwifruit was used during the simulation. The protein data file was obtained from the Protein Data Bank (PDB) with an accession code, 4BCT [344] (**Figure 7.1**). The CHARMM27 and TIP3P water models were selected as the force held and solvent, respectively [345]. The protein, Act d 2 was transferred into a periodic water box with dimensions $7.799 \times 7.799 \times 7.799$ containing 15089 water molecules. The system was neutralized by using the TIP3P water model and then was minimized for energy by converging criterion at 10 kJ/nm/mol of force value using steepest descent for 50000 steps followed by 100 ps equilibrations to the constant pressure, constant temperature (NPT) and constant volume, constant temperature (NVT).

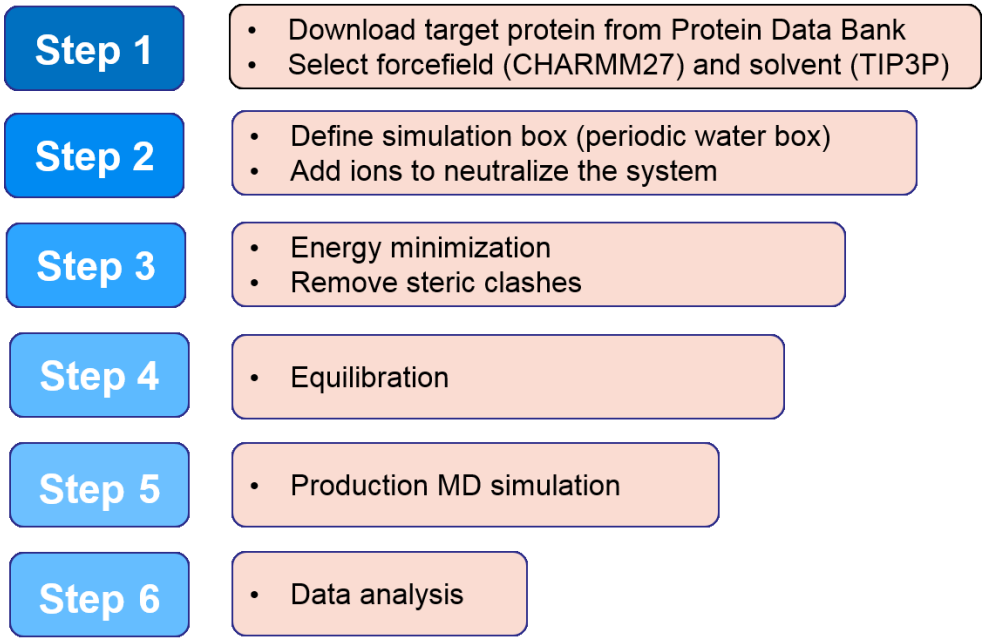


Figure 7.1. Experimental procedures of molecular dynamics modeling under the simulation of thermal and electric field.

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 one bar. The duration of MD simulations was set at 2000 picoseconds to evaluate the structural response of Act d 2 to the different temperatures (300K as control, 325K, 350K, and 375K) and external oscillating electric field with the intensity of 0.05 V/nm at a frequency of 2.45 GHz. Visual Molecular Dynamics (VMD) software (version:1.9.3, University of Illinois, Urbana-Champaign, USA) was used to obtain structural diagrams and STRIDE algorithm implemented inside was utilized to characterize the changes of Act d 2 after thermal processing and electric field treatment [346]. Several parameters such as root mean square deviation (RMSD) of backbone atoms, the root mean square fluctuations (RMSF) and surface hydrophobicity were evaluated by using GROMACS software [347]. VMD was also utilized to take snapshots of protein conformational changes [134].

7.3.2. Analysis of root mean square deviation (RMSD)

Recently, the RMSD has been applied on peanut protein (Ara h 6, peanut allergen) and soybean protein (1AVU, soybean trypsin inhibitor) to analyze and predict their arithmetical values of

the deviations in the molecule structure under different external stresses [134,342]. The following equation was used to calculate the RMSD of protein, Act d 2 present in kiwifruit [348]:

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

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

7.3.3. Analysis of root mean square fluctuations (RMSF)

The RMSF is a measurement of deviation between the position of atom i and some reference position in molecules of protein [349]. During MD simulation, the RMSF changes of Act d 2 were measured by the following equation [350]:

$$RMSF_i = \sqrt{\frac{1}{T}\sum_{t_i=1}^{T}\left|r_i(t_i) - r_i^{ref}\right|^2} \quad (2)$$

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

7.3.4. Measurement of solvent accessible surface area (SASA)

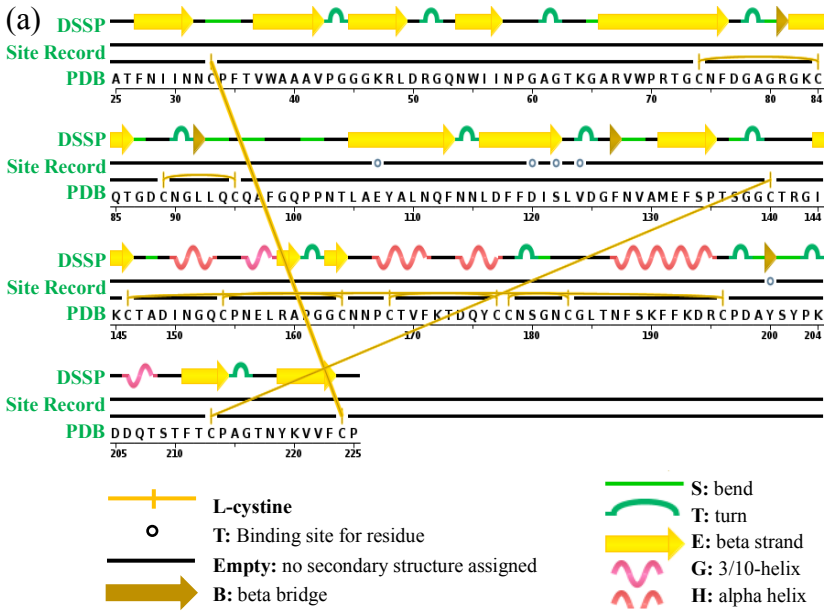
SASA is linked with the surface area which is available to interact with solvents and molecules. Generally, SASA is considered as a tool to evaluate the interactions between the protein and solvents, which in turn can be applied to obtain the protein characteristics and its functions [342]. Recently, studies have been reported where the surface properties are highly dependent on the structural conformations of the protein, and its functional properties change can be caused by a slight change of the conformations [134,342,348]. In this study, built-in commands present in the GROMACS software were used to evaluate SASA changes. The following equation was used to calculate the SASA of protein, Act d 2 [134]:

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

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

7.3.5. Statistical analysis

The data obtained from MD simulations under external thermal and electric field stresses were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test ($P < 0.05$) using an SPSS 22.0 analytical software (SPSS Inc., Chicago, USA).



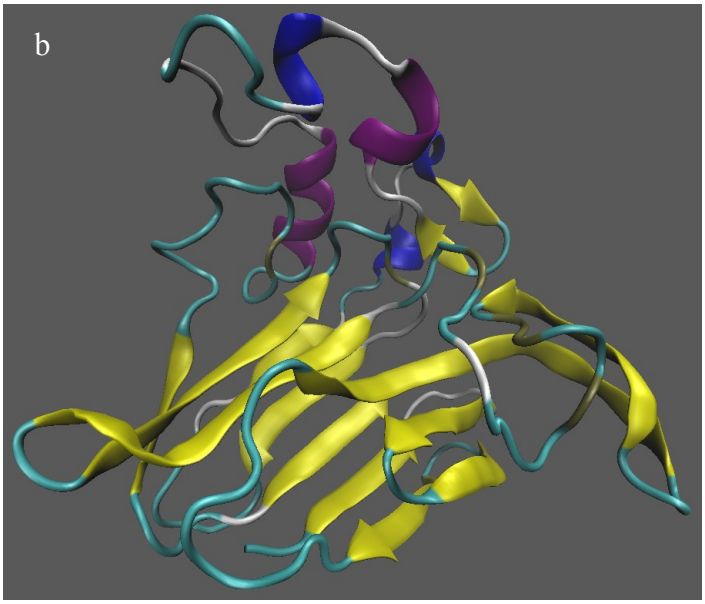


Figure 7.2. (a) FASTA sequence of kiwifruit allergen (Act d 2, PDB Accession Code: 4BCT); (b) Secondary structure of kiwifruit allergen Act d 2 (Source: protein data bank, α-helix: Purple, 3/10 helix: Blue, η-helix: Red, β-sheets: Yellow, Turns: Cyan, coils: White).

7.4. RESULTS AND DISCUSSION

7.4.1. Secondary structure of Act d 2

Act d 2 is one of the members from thaumatin-like proteins (TPLs) family with a 20-26 kDa molecular weight [180]. In the plant kingdom, TPLs are very common proteins that can be highly expressed when plants undergo stresses (e.g., drought) or pathogen infections to activate the defense system [181-183]. From the Protein Data Bank, it can be seen that the secondary structure of act d 2 contains 38% of β-sheet (18 strands; 77 residues) and 13% of helical (6 helices; 28 residues) with 201 amino acid residues (**Figure 7.2**). Six helices consist of four α-helices and two 3/10 helices. As shown in **Figure 7.2a**, four α-helixes are presented between the residues of 150-153 (Iso-Glu), 167-171(Pro-Phe), 174-177 (Asp-Cys), and 187-195 (Asp-Arg). The two 3/10 helices are formed between the residues of 156-158 (Asp-Leu) and 206-208 (Asp-Thr). β-sheet can be found between the residues of 26-31(Thr-Asn), 36-42 (Thr-Val), 44-49 (Gly-Leu), 53-57 (Gln-Ile), 66-75 (Ala-Asn), 82-86 (Gly-Thr), 104-113 (Thr-Phe), 116-122 (Leu-Ser), 130-135 (Ala-Pro), 144-146 (Ile-Cys), 210-214 (Thr-Pro), and 218-223 (Asn-Phe).

Visual molecular dynamics (VMD) STRIDE algorithm has been applied on the peanut protein (Ara h 6) and soybean protein (trypsin inhibitor) to visualize their secondary structure changes under different external stresses such as thermal or electric field [134,342,343]. In the present study, VMD STRID technique was used to evaluate the structural responses of kiwifruit allergen Act d 2 to the external thermal and electric field stresses. The values of Y and X-axis represent the residues of protein and the time/frames, respectively (**Figure 7.3**). Each band with distinct colors matches with the different residues present in the secondary structure of Act d 2 under various simulation conditions. The results showed that Act d 2 is quite stable under different external stresses, which might be attributed to the formation of disulphide bonds between the cysteine residues of 33-224, 74-84, 89-95, 140-213, and 146-1961 [343]. As presented in **Figure 7.3a-b**, some obvious disruption was observed between residues of 91-103 (frame 55-176), 175-181 (frame 67-112), and 199-211 (frame 144-200) when the temperature increased from 300 K to 325 K. A higher amount of loss in turn structure was observed in the protein when the temperature was raised further.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c
d
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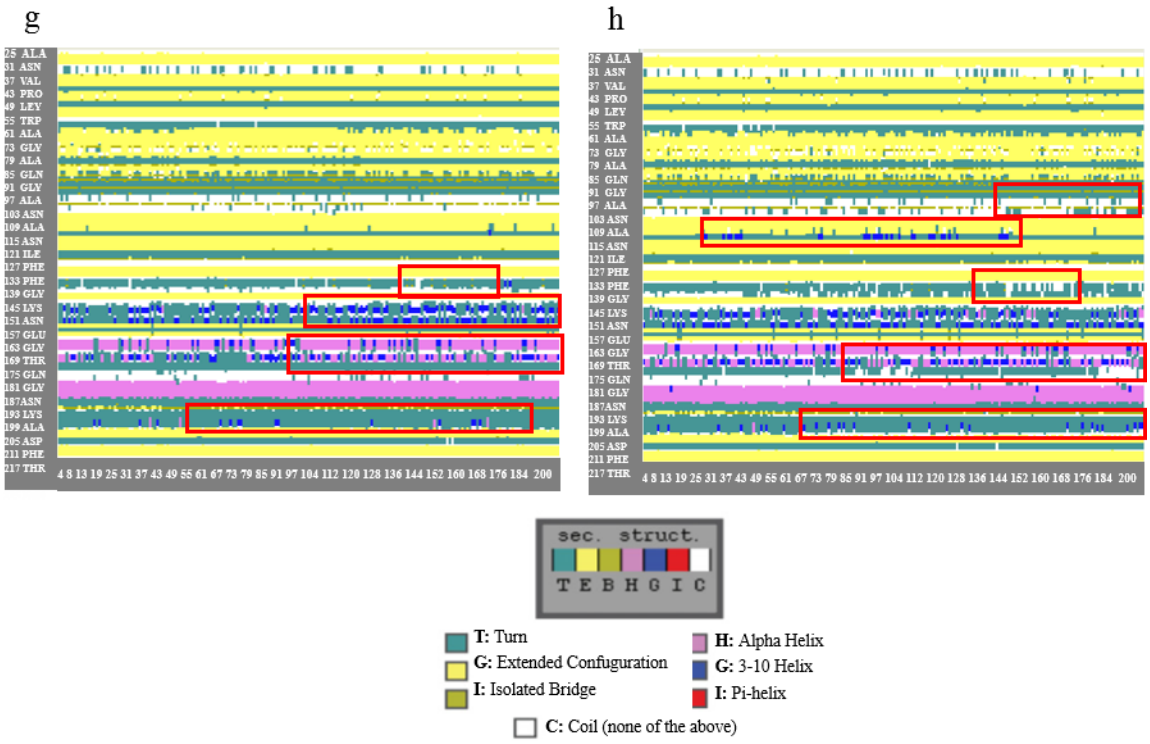

Figure 7.3. Secondary structure changes of Act d 2 after thermal treatment (a) 300 K, (b) 325 K, (c) 350 K, (d) 375 K, and thermal + oscillating electric field treatment at 2450 MHz (e) 300 K, 0.05 V/nm; (f) 325 K, 0.05 V/nm; (g) 350 K, 0.05 V/nm; (h) 375 K, 0.05 V/nm through STRIDE analysis. Note: Color code: Magenta denotes α (alpha)-helix, red denotes π (pi)-helix, cyan denotes turn, blue denotes 3/10 helix.

Specifically, the turn structure between residues of 121-127 was replaced with a high amount of 3/10 helix under 375 K thermal stress (**Figure 7.3d**). At the same temperature, more 3/10 helix structure was produced between residues of 175-187 compared to that of 300 K. It indicates that turns present in Act d 2 were sensitive to heat, once the temperature reached to the required level, it would be replaced with 3/10 helix structure. Similar results in the secondary structure were obtained in the electric field treated soybean allergen molecule [343]. Moreover, as shown in **Figure 7.3e-f**, a significant disruption of turn structures between residues of 55-67 was observed under electric field treatment (0.05 V/nm, 2.45 GHz) at 325 K compared to that of 300 K. Furthermore, the turn structure transformed into beta-strand between residues of 55-67, and 3/10 helix between resides of 151-157 (**Figure 7.3f**).

The α-helix was transferred into turn and 3/10 helix structures between resides of 169-175 (frame 112-184) and 205-211 (frame 91-136) when stimulated with electric field at 325 K. The higher transformation of turn (Asn (residue:151)-Glu (residue: 157)) and alpha-helix (residues:

169-175) into 3/10 helix was observed when temperature increased from 300 K to 350 K and 375 K under electric field (**Figure 7.3g-h**). Similar results were also obtained in Ara h 6 (peanut allergen), and trypsin inhibitor (soybean allergen), when simulated under thermal combined with electric field stresses [134,342]. Therefore, it is clear that the combination of thermal and electric field treatment showed a potential impact on the secondary structure of Act d 2 through changing the turn and helix structures in the protein.

7.4.2. Root mean square deviation (RMSD)

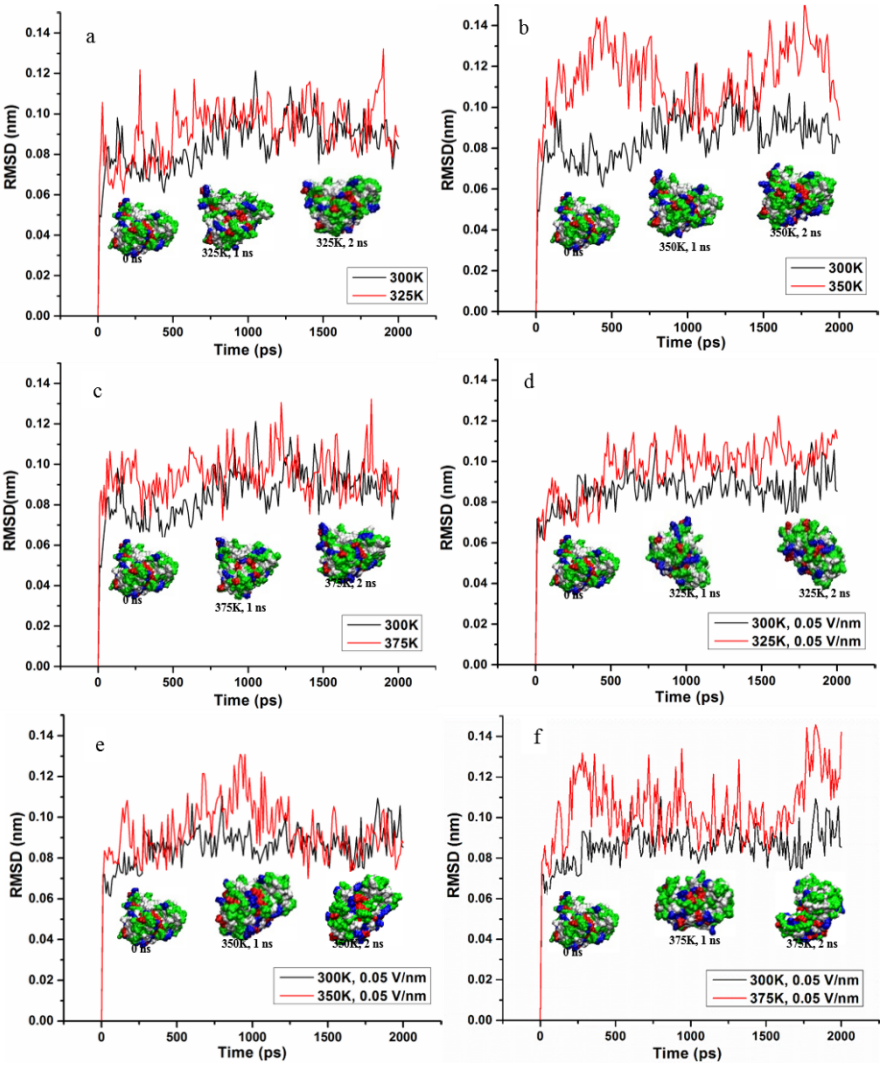

Figure 7.4. RMSD variations observed in kiwifruit allergen, Act d 2 under (a-c) thermal treatment, and (d-f) thermal + oscillating electric field treatment at 2450 MHz.

As shown in **Figure 7.4**, no obvious differences were observed in the RMSD of Act d 2 when simulated under thermal stress at 325 K and 375 K compared to that of 300 K, while the RMSD values obtained from 350 K were obviously higher than that of 300 K and other treatments (Fig.

3a-c). This significant enhancement could be due to the increase of thermal sensitivity of Act d 2 during the thermal simulation at the range of 300-350 K. No further increase of RMSD was observed even at its processing temperature increase to 375 K, which might be attributed to the effects obtained from external thermal stress on the RMSD of Act d 2 reaching its maximum level at 350 K [134]. Similar results have been reported on Ara h 6 peanut allergen [134] and soybean trypsin inhibitor protein [342] when they were simulated under thermal stress at 300-425 K. In comparison to thermal, the combination of the electric field at 0.05 V/nm, 2.45 GHz with thermal stress significantly increased the RMSD values with the rise of treated temperatures (Fig. 3d-f). Specifically, the RMSD of Act d 2 showed a significant jump when the temperature increased further to 375 K (10.41 nm) from 300 K (9.65 nm) (Fig. 3f). These continual increases in RMSD of Act d 2 within a specific temperature range might be due to the cumulative effects from thermal and electric field stresses [342].

7.4.3. Root mean square fluctuations (RMSF)

The fluctuation of atoms present in Act d 2 increased with the rise of applied temperatures from 300 K to 375 K during the thermal simulation (**Figure 7.5a**). Specifically, two sharp peaks were observed around the atom of 1750, and 2250 under 325 K thermal treatment when compared to the control (300 K). Several sharp peaks were found under 350 K thermal simulation at the atom of 300, 600, 800, 1100 and 1800. As the temperature further increased, more sharp peaks formed during the 375 K thermal simulation compared to the others. These sharp peaks were also obtained on milk allergen (bovine β−lactoglobulin) when treated with electric field combined with high temperature [36]. The formation of these sharp peaks might be attributed to secondary structure changes such as turns and helix (**Figure 7.5a-h**). As shown in **Figure 7.5b**, similar results were obtained during the simulation of the oscillating electric field at 2.45 GHz combined with thermal stress. The fluctuation of atoms in the protein molecule showed the highest frequency at 375 K, followed by 350 K, 325 K, and 300 K. It indicates that the mobility of atoms in a protein molecule could be effectively improved with the rise of applied temperatures under electric field stress, which may provide a possibility to modify the structure of protein under certain temperatures.

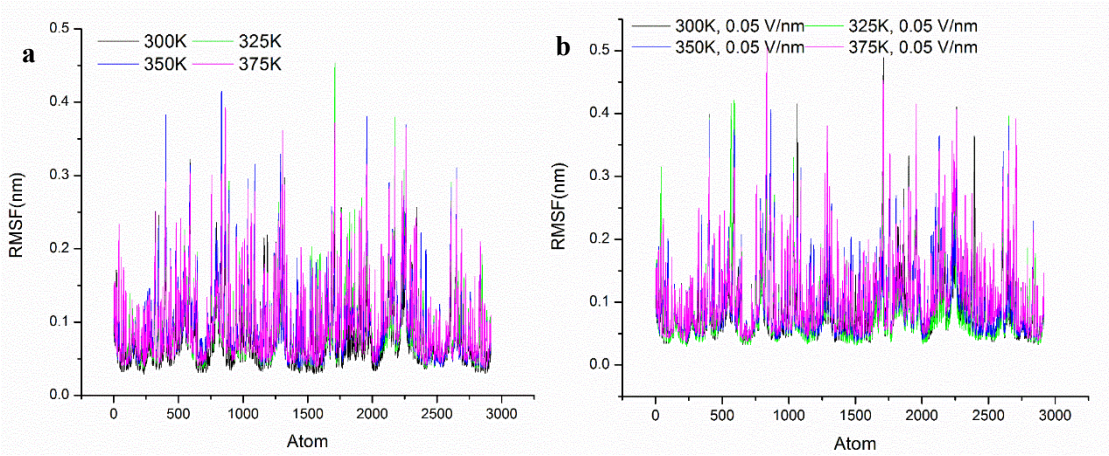

Figure 7.5. Root mean square fluctuations (RMSF) observed in Act d 2, allergen under (a) thermal treatment, and (b) thermal + oscillating electric field treatment at 2450 MHz.

7.4.4. Solvent accessible surface area (SASA) measurement of Act d 2

The SASA of Act d 2 showed a fluctuating trend with the rise of applied temperatures (**Figure 7.6**). Specifically, a significant increase in the surface area of Act d 2 was observed under the thermal simulation at 325 K compared to that at 300 K. This enhancement could be attributed to the new binding sites (**Figure 7.7b**) which were available on the surface of the molecule of Act d 2 under an appropriate thermal condition (325 K) [342]. The surface area of Act d 2 returned to the initial level with the rise of temperature from 300 K to 350 K, and then its value showed a decreasing trend during thermal treatment at 375 K (**Figure 7.6a**). In comparison to the 300 K thermal treatment, the surface area of Act d 2 showed a significant increase during the simulation of the electric field at 0.05 V/nm, 2.45 GHz, 300 K (**Figure 7.6b**). This increase in SASA of Act d 2 resulted from the additional stresses obtained from the electric field, causing a volatile property in protein structures. However, there was a decreasing trend in the SASA of Act d 2 when the applied temperature increased from 300 K to 350 K (**Figure 7.6b**). Moreover, the surface area values of Act d 2 remained almost constant at 375 K. Similar results have been observed on the soybean protein under thermal and electric field simulation [342]. These fluctuations might be due to the application of an electric field which can force the amino acid residues of Act d 2 to turn depending on the direction of the applied electric field [342].

The surface properties have been used to measure the functional characteristics changes of the protein in peanut [134] and soybean [348].

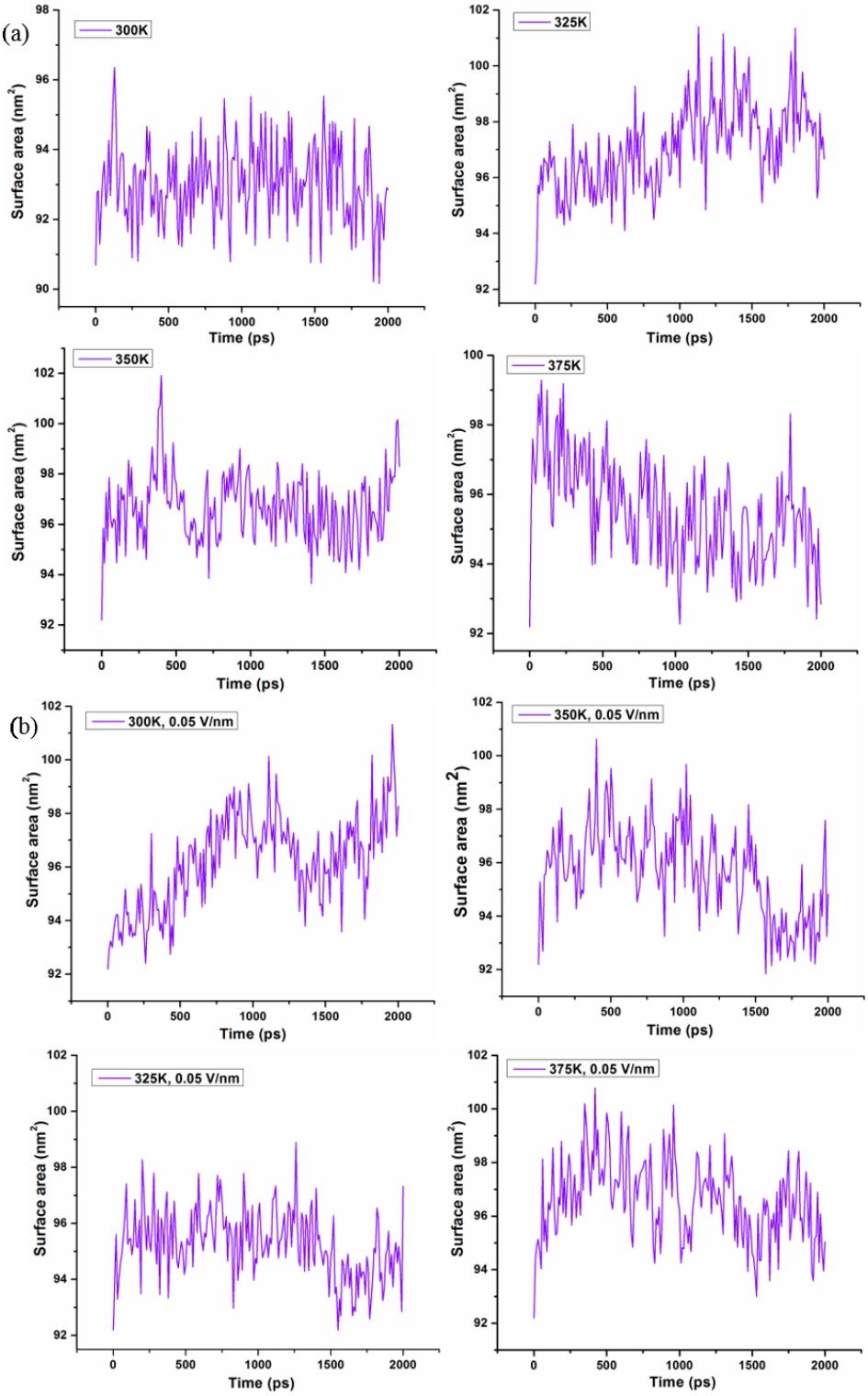

Figure 7.6. Solvent accessible surface area changes of Act d 2, kiwifruit allergen under (a) thermal treatment, (b) thermal + oscillating electric field treatment at 2450 MHz.

In the present study, we obtained the snapshot of the protein molecular structure (Act d 2) treated with two external stresses, including thermal and electric field using VMD. As presented in **Figure 7.7a-d**, there was a slight change in the molecular structure of Act d 2

under thermal stimulation from 300 K to 375 K. It indicates that single thermal stress effectively changed the surface properties of the protein molecule due to its heat-stable properties. However, a significant change in the molecular structure of Act d 2 allergen was observed after an oscillating electric field (0.05 V/nm, 2.45 GHz) was applied at 300 K (**Figure 7.7e**) compared to standalone thermal stress at 300 K (**Figure 7.7a**). This change of molecular structure increased the surface area of Act d 2 (**Figure 7.6**). The secondary structure of Act d 2 was completely modified by the end of the simulation at 0.05 V/nm, 2.45 GHz, and 375 K (**Figure 7.7h**), which led to a significant reduction in the surface area of Act d 2 compared to the initial level (**Figure 7.6b**). These significant changes in the molecular structure of Act d 2 allergen could be attributed to the application of electric field combined with thermal stress. Many studies have reported that there is a strong relationship between the surface properties and the molecular structure [134,348]. The surface properties of the proteins highly rely on the molecular structures of the proteins. Once the structure changed under external stresses, it could cause changes in surface properties leading to significant changes in the functional properties of related proteins or allergens.

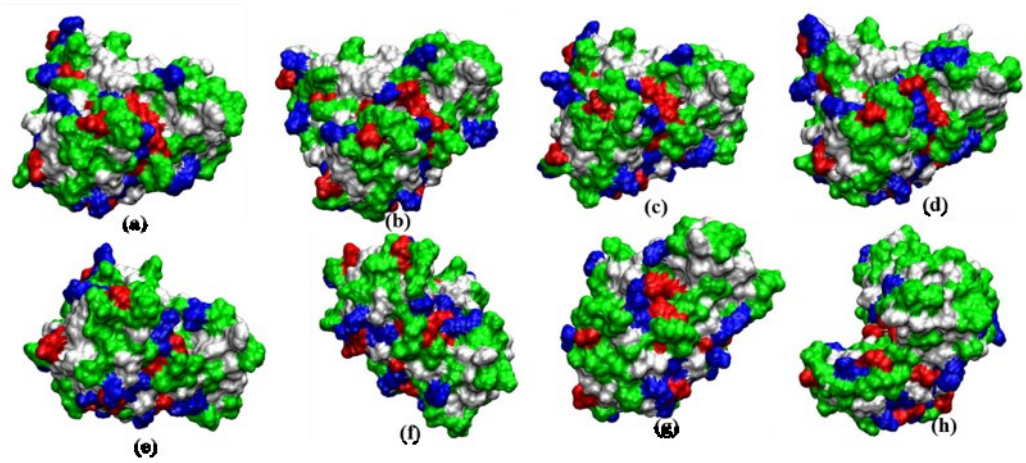

Figure 7.7. Snapshot of surface properties of Act d 2 after thermal simulation (a) molecular structure at 300 K; (b) 325 K; (c) 350 K; (d) 375 K; and electric field simulation at 2450 MHz (e) molecule structure at 300 K; (f) 325 K; (g) 350 K; (h) 375 K. Note: non-polar residues (white), basic (blue), acidic (red) and polar residues (green).

7.5. CONCLUSION

MD simulations have been applied by others to observe the structural changes in food allergens under different external stresses on peanut, soybean, and milk allergens. In this study, we simulated the structural changes of kiwifruit allergen, Act d 2 under thermal processing and electric field treatments. The results showed no significant changes in the molecular structure of Act d 2 when the protein was solely treated with thermal processing from 300 K to 375 K. However, the combination of thermal and electric field (0.05 V/nm, 2450 MHz) treatment significantly changed the molecular structure of Act d 2 kiwifruit allergen, especially when the processing temperature reached 375 K. Further, the surface area, RMSD, and RMSF showed a significant change under this combination treatment. Therefore, the combination of thermal and electric field treatments might be considered as a novel processing method in the reduction of food allergens in the future food industries. In addition, the current study could provide a new path where MD simulations can be used to verify the experimental predictions regarding the stability of secondary structures in proteins.

CHAPTER VIII

GENERAL SUMMARY & CONCLUSION, CONTRIBUTION TO KNOWLEDGE AND RECOMMENDATIONS

8.1. GENERAL SUMMARY AND CONCLUSIONS

The primary emphasis of this thesis was to evaluate the influences of variety, fruit components, maturity and storage conditions on the allergenic potential of kiwifruit and its relationship with antioxidant activity. Furthermore, we also analyzed the influences of thermal processing-microwave treatment and non-thermal ultrasound processing on the nutritional properties and allergenicity of kiwifruit samples. In this thesis, the relationship between these structural changes in the proteins, digestibility, and allergenicity was also studied. The literature review that was presented showed that fruit allergenicity varies in different varieties, maturity stages, fruit components (peel, pulp, and seed), and storages conditions of fruits (Chapter II), while no scientific studies were reported on the kiwifruit. In the second part of the literature review (Chapter III), we found that kiwifruit is considered as the most common fruit allergy, based on the clinical database obtained from the global analyses. Kiwifruit allergy is becoming a major health-related issue because it can trigger a range of allergic symptoms from mild OAS to the severe, life-threatening anaphylaxis. In the literature, we found that the kiwifruit allergenicity showed a strong relationship with the structural properties of proteins, especially their secondary structures (alpha-helix and beta-sheet). Many studies have shown that some food processing methods such as high temperature, could reduce the allergenicity of processed food matrixes might be due to the modification of related protein structures. Although several processing methods such as steam cooking, enzyme, and ethylene treatment can be beneficial to reduce the allergenicity of kiwifruit, the efficacy of these processing methods needs to be improved and well-studied. Novel processing techniques also need to be developed.

8.1.2. Variety, maturity, fruit components, storage and allergenicity

The effects of variety, fruit components, maturity and storage conditions on the allergenic potential of kiwifruit have been evaluated (Chapter IV). Major kiwifruit allergen, Act d 2, was mainly detected in the green kiwifruit, while no detectable level of Act d 2 was observed in golden kiwifruit. The Act d 2 allergen present in seeds is 2-fold and 4-fold higher when compared with pulp and peel, respectively. Thus, peeling and seeds removing from the fruit may reduce the allergenic potential of kiwifruit. Further, the highest Act d 2 allergen content was determined in ripe kiwifruit, followed by overripe fruit and unripe fruit. Thus, green ripe kiwifruit was selected as the subject in the following studies.

Moreover, after 10-day storage, a 50% enhancement in Act d 2 was observed when stored at 20 °C compared with the initial level, while no significant increases in the allergen concentration were observed in fruit stored at -20 °C. Further, the Act d 2 content showed a negative correlation with the ascorbic acid content and total antioxidant activity at various conditions. Therefore, antioxidants are considered as important factors which may involve the regulation of Act d 2 allergen of kiwifruit. Further studies regarding the mechanism of synthesis and regulation in Act d 2 allergen are to be explored.

8.1.3. Microwave treatment

In Chapter V, the influences of microwave treatment (75 °C, 0-5 min) on the nutritional properties and allergenicity of kiwifruit were studied. The results found that microwave processing significantly improved the total antioxidant activity of kiwifruit samples. The digestibility and peptide content of kiwifruit protein was improved due to the changes of secondary structures under high temperatures and electromagnetic field. Further, in comparison with the untreated samples, an 80% reduction in Act d 2 allergen content was observed after 5-min microwave by decreasing the alpha-helix structures and increasing the beta-sheet structures. Thus, microwave processing showed a potential application in reducing kiwifruit allergenicity. Whereas, a negative effect on the color attributes and sugars content of kiwifruit juice was obtained after microwave processing. To maintain the activity of nutrients present in the fruit samples, novel non-thermal processing methods are needed.

8.1.4. Ultrasound processing

In this study, we found that ultrasound processing significantly increased phenolics and flavonoids content and total antioxidant capacity compared to the untreated kiwifruit juice, which is probably due to the disruption of cell structures in kiwifruit tissues under ultrasonication. Also, high-intensity ultrasound processing significantly improved the color attributes (a*, b*, and YI), digestibility, peptide content, and rheological characteristics (flow and viscoelastic behavior) of kiwifruit juice. Further, the major kiwifruit allergen, Act d 2 content was significantly decreased after ultrasound processing compared with the untreated samples. Thus, high-intensity ultrasound processing shows potential applications in improving healthy bioactive compounds and decreasing the allergenicity of kiwifruit samples. However, the responses of patients with a kiwifruit allergy to these modified proteins are still unknown. Therefore, further researches regarding clinical and immunology are in need.

8.1.5. Molecular dynamics simulation

Molecular dynamics (MD) simulations have been studied by others to observe the structural changes in food allergens under different external stresses on peanut, soybean, and milk allergens. In this study, we simulated the structural changes of kiwifruit allergen, Act d 2 under thermal processing and electric field treatments. The results showed no significant changes in the molecular structure of Act d 2 when the protein was solely treated with thermal processing from 300 K to 375 K. However, the combination of thermal and electric field (0.05 V/nm, 2450 MHz) treatment significantly changed the molecular structure of Act d 2 kiwifruit allergen, especially when the processing temperature reached 375 K. Further, the surface area, RMSD, and RMSF showed a significant change under this combination treatment. Therefore, the combination of thermal and electric field treatments might be considered as a novel processing method in the reduction of food allergens in the future food industries. In addition, the current study could provide a new path where MD simulations can be used to verify the experimental predictions regarding the stability of secondary structures in proteins.

8.2. CONTRIBUTION TO KNOWLEDGE

The following are important contributions to knowledge:

1. The study indicated that Act d 2 is present in the green kiwifruit and mainly presented in seeds and pulp of fruit, while was not detected in the gold kiwifruits.
2. Although microwave processing showed a negative impact on the nutritional properties of kiwifruit samples, a significant reduction (85%) in the kiwifruit allergen, Act d 2 was observed, which provides a database for the future studies in allergenicity in food industries.
3. As a non-thermal processing, ultrasound treatment has potential not only in improving bioactive compounds but also in reducing the allergenicity of kiwifruit.
4. Molecular modeling studies were performed for the first time on the kiwifruit allergen, to understand the effect of external stresses on the conformational structure of Act d 2, which provides a visual tool to predict the structural changes of proteins.

8.3. FUTURE WORK RECOMMENDATIONS

Further studies are still in need regarding the following area:

1. Further emphasis is needed on investigating the mechanism of synthesis and regulation in the Act d 2 allergen, during the growth of kiwifruit.
2. Although the allergenic potential of kiwifruit allergen (Act d 2) was decreased to a certain level after microwave or ultrasound processing, the responses of patients with a kiwifruit allergy to these modified proteins are still unknown. Therefore, further researches in relation to the clinical and immunological responses are needed.
3. The effects of the combination processing of microwave and ultrasound on the reduction of kiwifruit allergenicity need to be evaluated in the future.
4. Molecular dynamics simulation provides a visual tool to understand the protein structures changes under various external stresses, while the current processing duration is too short due to the high requirements for the computing time. Thus, in the following research, the simulation duration needs to be increased to 50-100 ns to further analyze the protein structure changes under external conditions.
5. Togethering with the MD simulation, real experiments based on the same parameters should be performed in the future to conform the protein structural changes.

CHAPTER IX

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