Quantitative analysis of allergens in peanut varieties and assessment of effects of food processing on peanut allergens

By

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Peanut, a major allergenic food, has life-threatening potential and is difficult to be totally avoided due to its common use in processed foods. Thermal processing can influence the allergenic properties of peanuts. However, the kinetics of the reactions caused by thermal processing has not been characterized. In our study, kinetics of the commonly used thermal processing methods on a commercial peanut cultivar (Virginia) using five time intervals was conducted. Water-soluble and SDS-sample buffer soluble proteins were extracted sequentially, and analyzed by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western-blot using human plasma containing IgE antibodies. The relationships between thermal processing (time) and log transformed water-soluble/total extractable major allergen content could be explained by a simple linear regression kinetic model for most of the processing methods (except high-pressure steaming). Among all the methods with optimal processing point, frying for 6 min had relatively lower IgE binding (linear epitopes) ratio may be due to the fact that this processing condition causing break down, cross-linking and aggregation of Ara h 2, and relatively lower solubility.
Besides thermal processing, enzymatic processing also is considered to be an effective method in the allergenicity of peanuts. Eleven peanut lines (Coded MS-1~MS-11, MS-9 is the check and a common cultivar namely Valencia) were pre-screened from 122 peanut lines harvested in 2015 for allergen levels. These pre-screened lines were re-planted in 2016 for further analysis. One line, MS-7, was selected for lower Ara h 1 (8.5-9.5% of total protein) and Ara h 2 (4.2-6.6% of total protein) content in 2015 and 2016. Roasted MS-9 (check) peanut powders were used for enzymatic treatment for enzyme selection. A first order kinetic reaction model was conducted to describe the relationship between enzyme concentration (0-400AzU/g) and IgE-binding property reduction. Among eight food-grade enzymes, bromelain, papain and ficin hydrolysates had lower IgE-binding properties in terms of high IgE-binding property reducing rate (K, ≥ 0.4) and were selected for the following study. MS-7 (selected) & MS-9 (at level of 200AzU/g) hydrolyzed by three selected enzymes (200AzU/g) were used for IgE binding property comparison, TGase crosslinking and functional properties study. After hydrolyzed by the selected enzymes (200 AzU/g), the emulsion and foaming stabilities were decreased. Emulsion and foaming stabilities were increased in TGase (5U/g protein) crosslinked hydrolysates, which were even higher than soy protein isolate (SPI). The IgE-binding properties of TGase treated hydrolysates were similar to the hydrolysates without TGase treatment. MS-7 hydrolysates (with/without TGase) possessed less IgE-binding properties and similar functionality as compared with MS-9 hydrolysates.

**Keywords:** Peanut allergen, thermal processing, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), kinetic analysis, water-soluble, IgE binding properties, peanut screening, enzymatic processing, Transglutaminase (TGase), functional properties
DEDICATION

I would like to dedicate this dissertation to my lovely family: my parents, Qinghai Meng, Lijun Zhao; my parents-in-law, Baosheng Tan, Suhua Xie; and my lovely wife, Yuqing Tan.
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Peanut (*Arachis hypogaea*) is an important oil and food crop. It is the third major oilseed of the world. Due to the low cost and high nutrition, it is one of the most popular foods worldwide. However, peanut allergy is one of the most common causes of food-related fatal anaphylaxis (Sáiz, Montealegre, Marina, & García-Ruiz, 2013). The number of people with peanut allergies in the United States appears to be increasing (Commin, Kim, Orgel, & Kulis, 2016).

To date, 17 peanut allergens [Ara h 1(*Arachis hypogaea*) 1 - Ara h 17] have been identified that are listed by the Allergen Nomenclature Sub-Committee (Subcommittee, 2017) of the International Union of Immunological Societies. Of the 17 recognized peanut allergens, Ara h 1, Ara h 2, and Ara h 3 are considered the major allergens because they are abundant and recognized by serum IgE of > 50% of peanut allergic individuals (Mueller, Maleki, & Pedersen, 2014). Among these, Ara h 2 is regarded as the most potent for severe allergic reactions and higher IgE-binding properties (Schocker, Baumert, Kull, Petersen, Becker, & Jappe, 2016). Currently, the only available treatment is complete peanut avoidance. However, avoiding peanuts in food product is difficult because of its ubiquitous use as an ingredient in processed foods.

It has been reported that the major allergen (Ara h 1, Ara h 2, Ara h 3) levels varied among hundreds of peanut cultivars (Kang, Gallo, & Tillman, 2007). Previous
study has been conducted on 99 of the 112 Peanut Mini core accessions for the quantification of major allergen composition. Low Ara h 1, Ara h 2 or Ara h 3 level accessions have been found among the 99 Mini core collections (Kang, Gallo, & Tillman, 2007). However, the planting year and location may influence the distribution of the peanut allergens, it would be necessary to analysis allergen levels of the Mini core accessions with different harvest location (Clovis, NM) and year (2015) to test if the result would be consistent with the previous study (harvested in Tifton, GA) (Kang, Gallo, & Tillman, 2007), and to select less allergen level peanut for processing study.

Thermal and enzymatic processing have been applied to process peanuts for allergen reduction. The effects of thermal processing, including frying, boiling, steaming, roasting, microwaving and autoclaving, on allergenic properties of peanuts have been studied extensively (Cabanillas et al., 2015; Cabanillas et al., 2012; Chung & Reed, 2014; Maleki, Chung, Champagne, & Raufman, 2000; Mondoulet et al., 2005; Rao, Tian, Tao, Tang, Li, & Xue, 2016; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2009; Verhoeckx et al., 2015). Even though thermal processing cannot eliminate all the allergenic potential (Verhoeckx et al., 2015), to understand how the peanut major allergens are changed under thermal processing in a quantitative way with kinetic models is important. Until now, kinetic reaction models have not been reported to assess the changes of soluble allergen content as a function of processing time under various thermal processing methods.

Enzymatic processing also has been reported as a strategy to reduce the allergenicity of peanut proteins by hydrolyzing them into smaller peptides that significantly decrease IgE-binding properties (Cabanillas et al., 2012; Shi et al., 2013;
Yu, Ahmedna, Goktepe, Cheng, & Maleki, 2011). Protein (including peanut) hydrolysates may lose certain functional properties after enzyme hydrolysis; and taste bitter due to the existence of hydrophobic amino acid residues (FitzGerald & O'cuinn, 2006). Transglutaminase (TGase) has been reported to increase the functional properties and sensory characteristics of protein hydrolysates in previous studies (Agyare, Addo, & Xiong, 2009; Fan et al., 2005; Song et al., 2013). Until now, no studies have been conducted on peanut hydrolysates treated with TGase. In this study, TGase was applied on the prescreened low allergen peanut hydrolysates to test the allergenicity and functional properties.

The major objectives of our study were to: (1) develop the kinetic model to characterize how the major water-soluble allergen content changes during thermal processing (2) analyze the allergen IgE-binding properties of the peanut allergens following the different thermal processing methods; (3) screen peanut lines with lower allergen content from hundreds of peanut lines harvested in Clovis, NM (2015); (4) select efficient food grade enzymes for peanut allergen reducing in screened (low allergen) and check (regular) roasted peanut; (5) test if TGase processing would be a suitable treatment to improve functional properties of peanut hydrolysates.
CHAPTER II
LITERATURE REVIEW

2.1 Peanut overview

Peanut, was first grown 5000 years ago in South America, is the seeds of an annual legume. There are many peanut varieties cultivated in USA, with more than 44% of the American peanut crop consumed as peanut butter (USDA-PSP, 2017). Runner, which is the dominant peanut type grown in the U.S. and accounts for the large increase in yield, is a very important source of peanut butter. Virginia has the largest kernel, is mostly roasted and sold in its shell. Spanish peanut, with a smaller kernel and covered with a reddish-brown skin, is used mostly in peanut candy production as well as for salted nuts and peanut butter. Valencia is a small and sweet peanut, which is rarely used in processed foods but usually roasted and sold in the shell (Peanut-Phadia, 2017).

Peanut are mainly consumed as peanut butter, as snacks (roasted, salted, plain or dry roasted), in candy and in baked foods. Peanut is also widely used for producing cooking oils (refined/ crude, aromatic/ no-aromatic). In China, peanut oil ranked second as a source of fat and oil only behind soy oil (Peanut-Phadia, 2017). Peanut oil, if not highly purified, may contain peanut allergens (Teuber, Brown, & Haapanen, 1997). All the peanut food products mentioned above contain allergens and cannot be consumed by peanut-allergic person.
2.2 Structure and theory of protein as allergen

Allergies emerge with regard to certain proteins termed allergens, which could trigger immediate (type I) hypersensitivity reactions (Bredehorst & David, 2001). Food allergy elicited from two major phases called sensitization and reaction (Lee, Wright, & Rachaputi, 2016).

Briefly, in the first stage (sensitization), the ingested food components are swollen by antigen-containing cells such as dendritic cells and macrophages when they pass through the epithelial barrier into the circulatory system. The allergenic proteins are processed into small peptides by dendritic cells and then presented to T-cells, which are then polarized to Th2-cells. The immune response continues with the help of Th2-cells, inducing a large quantity of specific IgE antibodies in B-cells. These specific antibodies travel through the blood in the circulatory system until reaching the mast cells (each has several IgE-antibody receptors on the surface) located in the trachea, intestinal mucosa or basophils in the blood. The sensitization phase is finished after the IgE antibodies attach themselves to the surface of mast cells. (Lee, Wright, & Rachaputi, 2016).

In the second stage (reaction), when the same food protein (termed as allergen this time) is ingested and circulated in the blood, it interacts with the IgE antibodies located on the surface of the mast cells or basophils. The mast cells or basophils are activated after allergen binding to IgE. The activated mast cell or basophils release inflammatory chemicals including histamine, which would cause various symptoms, such as hives, swelling, wheezing, asthma, nausea, itching and anaphylaxis (Lee, Wright, & Rachaputi, 2016). The critical step to a specific allergic reaction is the binding of at least two IgE
antibody molecules to a multivalent allergenic protein (allergen) (Bredehorst & David, 2001).

Allergens have several characteristics in common. First, allergens are foreign proteins with molecular mass usually varying from 5,000 to 70,000 kDa. Furthermore, a particular foreign protein must be present in substantial amounts, and in the patient’s environment or food for a long time in order to become an allergen (Bredehorst & David, 2001). Besides these two characteristics, structural characteristics (such as epitopes in linear and conformational forms) of allergens also appear to play an important role in the inducing of immune response and allergic reactions.

2.2.1 Linear epitopes

The binding sites recognized by IgE antibodies are called IgE epitopes which are normally classified as linear and conformational. A linear epitope contains continuous amino acids, while a conformational epitope consists of discontinuous amino acids distributed over the protein sequence and near to each other only when the protein is folded correctly (Chen et al., 2016). In other words, conformational epitopes depend on the three-dimensional structure of allergens.

Linear epitopes have been identified for varieties of food allergens including those from soybean (Sun, Shan, Yan, Zhang, & Guan, 2013), peanut (Burks et al., 1997), cow’s milk (Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001) and other resources. All analyzed allergens had multiple linear binding sites, which could reflect the polyclonal nature of immune response. Major allergens of peanuts Ara h 1, Ara h 2 and Ara h 3 contain 21, 14 and 4 linear epitopes for IgE binding, respectively (Lee,
Wright, & Rachaputi, 2016). The IgE binding could be decreased or abolished by the mutations of single amino acid within each epitope (Bredehorst & David, 2001).

2.2.2 Conformational epitopes

Compared with linear epitopes, conformational epitopes have been studied less due to being more complicated and difficult to be determined comprehensively. However, the conformational epitopes also play a very important role in IgE binding. Recent study has determined more than 4 conformational epitopes of Ara 2 and Ara h 6 in peanut by using mimotopes (Chen et al., 2016). For several allergens, the IgE binding activity of conformational epitopes could be reduced (lost) by disulfide bond dissolution or thermal denaturation. Conformational epitopes which recognized by antibody could be affected by roasting, leading a decreased binding to Ara h 1 from roasted peanut (Mueller et al., 2013). However, heat-denatured protein could present new allergenic sites by conformational changes (Bredehorst and David, 2001). Some conformational peptides in Ara h 1, Ara h 2, and Ara h 3 were identified, and the length of the sequence were all composed of over 10 amino acids (Muller et al., 2013).

2.3 Peanut allergen

Peanut contains no less than 32 different proteins, of which at least 18 have been identified as able to bind to specific IgE (Dean, 1998; Scurlock & Burks, 2004). The major peanut allergens belong to the seed storage proteins of conglutin, vicilin, and glycinin families (Bohle et al., 2003). To date, 17 peanut allergens [Ara h 1 (Arachis hypogaea 1) – Ara h 17] have been identified and are listed by the WHO/IUIS Allergen Nomenclature Sub-Committee (Subcommittee, 2017). Among the 17 recognized peanut
allergens, Ara h 1, Ara h 2 and Ara h 3 are the major concern because of their abundance (>30% of total protein content of peanut) and strong contribution (recognized by > 50% serum IgE from peanut-allergic individuals) to IgE binding (Chassaigne, Nørgaard, & van Hengel, 2007; Mueller, Maleki, & Pedersen, 2014). The SDS-PAGE of peanut extracts containing Ara h 1, Ara h 2 and Ara h 3 are shown in Figure 2.1. According to the study of Bianchi-Hall, Keys, Stalker, and Murphy (1993), the electrophoresis profile of peanut protein subunits are also divided into five main classes: namely the conarachin region (molecular weight > 50 kDa, including Ara h 1); acidic arachin region (molecular weight 38–49.9 kDa, including Ara h 3); the intermediate molecular weight region (23–37.9 kDa); basic arachin region (molecular weight 18–22.9 kDa, including Ara h 3) and the low molecular weight protein (14–17.9 kDa, including Ara h 2 & Ara h 6).

Figure 2.1 Seed protein profiles of screened peanut (Arachis hypogaea L.) in SDS-PAGE

(Kang, Gallo, & Tillman, 2007)
2.3.2 Ara h 1

Ara h 1 is a 63.5 kDa – 65 kDa protein, which shows one protein band in reducing SDS-PAGE with estimated molecular weights of 63 kDa according to Kang, Gallo, and Tillman (2007) (Figure 2.1). Ara h 1 (vicilin, 7S globulin) shares the same overall fold with Ara h 3 (legumin, 11S globulin) and belongs to the cupin superfamily (Dunwell, Purvis, & Khuri, 2004). These types of proteins are considered as storage proteins and energy sources for plants during germination (Chruszcz et al., 2011). The 7S and 11S globulins are the majority of the proteins in many seeds that belong to the human diet (Chruszcz et al., 2011). Ara h 1 has 12 to 16% of the peanut total protein (Koppelman et al., 2001). Its stable trimeric structure could protect IgE binding epitopes from degradation (Burks, Sampson, & Bannon, 1998; Maleki et al., 2000).

Ara h 1 has been reported to become highly aggregated and insoluble after thermal treatments, including boiling, roasting and frying. The insoluble feature after aggregation made Ara h 1 difficult to detect in processed food products with the methods that rely on allergen solubility, such as ELISA (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2009). It was identified as at least 23 different linear IgE-binding epitopes were located throughout the length of the Ara h 1 (Burks, Shin, Cockrell, Stanley, Helm, & Bannon, 1997). It is reported that Ara h 1 and Ara h 2 are recognized by 70-90% of patients with peanut allergy (Clarke, Kilburn, Ob'Hourihane, Dean, Warner, & Dean, 1998). However, some patients may not react to either Ara h 1 or Ara h 2 (Bernard et al., 2003). Variations occurred among populations and is demonstrated by the studies of populations from different places: Ara h 1 could be recognized by more than 85% of the patients who came from a North America population (Arkansas, USA; 9 individuals)
(Burks, Williams, Helm, Connaughton, Cockrell, & O'Brien, 1991), and by 35% 
(Netherland; 14 individuals) (De Jong, Van Zijverden, Spanhaak, Koppelman, Pellegrom, 
& Penninks, 1998), 65% (Southampton, UK; 34 individuals) (Kleber-Janke, Crameri, 
Appenzeller, Schlaak, & Becker, 1999) and 70% (Southampton, UK; 91 individuals) 
(Clarke, Kilburn, Ob'Houriheane, Dean, Warner, & Dean, 1998) of patients belonging to 
three European populations.

There is a study on comparing the different properties of natural and recombinant 
Ara h 1 (Chruszcz et al., 2011). It is found that natural Ara h 1 formed higher molecular 
weights in solution. While the full-length recombinant Ara h 1 was partially unfolded and 
existed as a monomer. Ara h 1 core (residues 170–586) could formed trimeric assemblies, 
which indicating that core regions of the Ara h 1 residue would be enough for Ara h 1 
trimer and higher oligomer formation. In addition, natural form of Ara h 1 had higher 
anti-digestion activity as compared with recombinant form by means of in vitro gastric 
and duodenal digestion assays.

2.3.3 Ara h 2

Ara h 2 is a 17.5 kDa protein and a major allergen, which shows two protein 
bands in reducing SDS-PAGE with estimated molecular weights of 20 and 17 kDa 
according to Kang, Gallo, and Tillman (2007) (Figure 2.1). It is a conglutin seed storage 
protein and a trypsin inhibitor (Burks, Sampson, & Bannon, 1998). Ara h 2 has four 
disulfide bonds and five helices with each helix linked by at least one disulfide bond to 
another (Mueller et al., 2011). It belongs to the 2S albumin superfamily (Burks, Williams, 
Connaughton, Cockrell, O'Brien, & Helm, 1992) and has 545 amino acids (Burks, 
Cockrell, Stanley, Helm, & Bannon, 1995). Ara h 2 has 5.9 to 9.3% of the peanut total
protein (Koppelman et al., 2001). The 2S albumin can also be found in many other legumes, for examples: soybeans (Shibasaki, Suzuki, Tajima, Nemoto, & Kuroume, 1980), and nuts such as cashew nuts (Teuber, Sathe, Peterson, & Roux, 2002), brazil nuts (Ampe, Damme, Castro, Sampaio, Montagu, & Vandekerckhove, 1986), walnuts (Teuber, Dandekar, Peterson, & Sellers, 1998), and other oil seeds such as sunflower (Kelly & Hefle, 2000) and sesame seeds (Tai, Wu, Chen, & Tzen, 1999).

More than 85% of serum IgE (among 40 patients) (Kleber-Janke, Crameri, Appenzeller, Schlaak, & Becker, 1999) and over 90% of serum IgE from peanut-sensitive individuals, recognize Ara h 2 (Stanley et al., 1997). The protein bands of Ara h 2 are 18 and 21 kDa, which are generally regarded as Ara h 2 doublet (Burks, Williams, Connaughton, Cockrell, O'Brien, & Helm, 1992). Ara h 2, together with Ara h 6, contributes to the majority of the effector activity in crude peanut extract (Zhuang & Dreskin, 2013), which further confirms its potency as a peanut major allergen. For both Ara h 2 and Ara h 6, it is reported that disruption of the disulfide bonds decreases protein stability, trypsin resistance and allergenicity of these proteins (Starkl et al., 2012). In a study by Maleki et al. (2003), it is shown that a partial disruption of disulfide bonds may result in increased trypsin inhibitory activity, which mimics the effects of roasting on Ara h 2. During Maillard reactions in roasted peanuts, higher order structure of Ara h 2 does not appear as the same way of Ara h 1, even the protein has been greatly covalent modified. The IgE binding properties of Maillard Ara h 2 has been observed to increase. For example, mannose or xylose added to the sample while roasting Ara h 2, increased the competition by 4.1-fold compared with no sugar added samples (Maleki, Chung, Champagne, & Raufman, 2000).
2.3.4 **Ara h 3**

Ara h 3 is a 60 kDa protein and a major allergen, which shows five protein bands in reducing SDS-PAGE with estimated molecular weights of 45, 40, 36, 22 and 14 kDa according to Kang, Gallo, and Tillman (2007) (Figure 2.1). It contributes to 50-57% of the total protein content of the peanut (Koppelman et al., 2001). It is recognized by 45% of a group of patient population (Rabjohn, Helm, Stanley, West, Sampson, Burks, et al., 1999). It is a glycinin (11S) seed storage protein (Burks, Sampson, & Bannon, 1998). Ara h 3 has a number of polypeptides, varying from about 14 to 45 kDa, which can be categorized as acidic and basic subunits, similar to the soy glycinin’s subunit classification (Koppelman et al., 2003).

In a study by Kang and coworkers (2007), 60 accessions in the U.S. Peanut collection (all belonging to a mincore collection), along with 88 Florida Peanut breeding program lines were analyzed for major peanut allergen levels. An accession from India had the lowest level of Ara h 1 (7.0%). An accession from Nigeria had the highest level of Ara h 1 (18.5%), but the lowest level of Ara h 2 (6.2%). An accession from Zambia had the highest level of Ara h 2 (13.2%), but the lowest level of Ara h 3 (21.8%). Two accessions, 20 lines, and 2 Peanut cultivars (Florunner and Georgia Red) contained little or no Ara h 3 isoform (36 kDa). Ara h 3 is regarded as less allergenic as compared with Ara h 1 and Ara h 2. However, in a study of a group of Italian children with peanut-allergy, it was found that they were particularly sensitized to the basic subunit of Ara h 3 (Restani et al., 2005).
2.4 Cell and animal methods for detecting allergenicity

2.4.1 Cell methods for detecting allergenicity

Basophil histamine release (BHR) assay and β-hexosaminidase release methods are based on the amount of two mediators named “basophils” and “mast cells”, respectively. These two mediators (“basophils” and “mast cells”) are released by blood cells from allergic patients following the binding of the allergen to the cell receptors, respectively. The quantity of histamine or β-hexosaminidase is corresponding to the amount of the specific allergen (Kirsch et al., 2009).

The “flow-cytometric allergen stimulation test” (BAT), released (e.g. histamine, leukotriene C4) and surface receptors (e.g. CD63, CD203c) appearing on activated basophils coming from allergic patients after exposure to allergens. The quantification is performed under the help of dye-labelled antibodies, which bind to active receptors and are detected by flow cytometry. The quantification of the allergen by using BAT is based on the measured fluorescence (Kirsch et al., 2009).

2.4.2 Animal methods for detecting allergenicity

Mouse is the commonly used animal in food allergy research. Not only for the small size and short breeding cycle, but the immunological sequence involved in the development of sensitization and the elicitation from the allergic reactions is also similar to humans (Eigenmann, Antonella Muraro, Sampson, & Wahn, 2008). Additionally, with the help of the development of various immunological and molecular reagents and transgenic animal researches, which make the mouse model as the powerful method for investigating immunological mechanisms as affected by food allergies (Bøgh et al., 2016). Besides mouse, dog or pig also can be used for allergenicity testing.
The sensitization process of the animal is needed before the allergenic testing. Sensitization is conducted with either purified protein or extracts or feed containing whole food materials. The antigens are provided orally by feeding, dermal application, intraperitoneal (i.p.) injection or intranasal (i.n.) dosing. The methods for measuring the allergenicity in animals are similar to those used to evaluate allergy in humans. For example, antigen-specific IgE is a primary marker of sensitization in humans. In allergen exposed mice, antigen-specific IgE or IgG1 levels are commonly measured as useful markers of a Th2 response and potential allergy (Marsteller, Bøgh, Goodman, & Epstein, 2015). Other tests in animals include protein-specific dermal mast cell degranulation with anaphylaxis, which is similar to the skin prick tests (SPT) with allergenic extracts for diagnosing humans (Dearman, & Kimber, 2009). Allergen-specific production of Th2 cytokines, release of histamine usually could cause the symptoms, including anaphylaxis, hypothermia, hypotension or reduced pulmonary function in various animal models (Berin & Mayer, 2009; Finkelman, 2007).

2.5 Thermal processing

2.5.1 Common thermal processing methods

Raw peanut has toxic components just as other legumes. For example, soybean is not edible unless it is fully cooked. Peanut is commonly cooked by boiling, steaming, frying, roasting, high-pressure cooking or a combination of the separate methods. The effects of thermal processing on protein structure and legumes allergenicity are shown in Figure 2.2 and Figure 2.3 (Verma, Kumar, Das, & Dwivedi, 2012). Thermal processing can alter the allergen’s epitopes conformationally, which can enhance, reduce or maintain
the allergenicity. The processing method, temperature, and time all could affect the allergen structure and IgE binding capacity.

It has been discovered in China, a lessor rate of peanut allergy occurs compared with America owning to the differences in thermal processing methods. In China, peanut is commonly boiled and fried, while in America, peanut is typically dry roasted. A study carried out by Beyer and coworkers (2001) showed the effect of the cooking methods with regard to peanut’s allergenicity of water-soluble fractions. In this study, fried, boiled, and roasted peanuts were compared by their allergenic properties (IgE binding properties to patient’s plasma). The results of SDS-PAGE, ELISA and Immunoblot showed that the roasted peanuts retained the highest allergen content. Meanwhile, the allergen amount of fried and boiled peanuts was reduced at different levels.

In the study of Mondoulet and coworkers (2005), roasted, boiled and raw peanut were compared for allergenicity. It was demonstrated that the decrease in allergenicity of boiled peanuts resulted mainly from a transfer of low-molecular-weight allergens (Ara h 2/6) into the water during cooking. A later study demonstrated that Ara h 1 aggregates formed by boiling were distinct from those formed by roasting morphologically due to a partial loss of Ara h 1 secondary structure and formation of rod-like branched aggregates, and had lower allergenic activity (Blanc et al., 2011).

Maleki, Chung, Champagne, and Raufman (2000) found that the IgE-binding properties of roasted peanut had approximately 90-fold higher levels than the raw peanuts from the same peanut cultivars. It was discovered that the Amadori products by Maillard reaction during roasting boosted the reactivity to IgE antibody. And this is due to the structural changes of the proteins in roasted peanuts. The study also indicated that Ara h 1
has cross-linked to form higher order structures which could be recognized and bounded by serum IgE when subjected to the Maillard reaction.

Figure 2.2 Effect of thermal processing on protein structure.

(Verma, Kumar, Das, & Dwivedi, 2012)
The study of Zhang and coworkers (2016) demonstrated that processing, including boiling, glycation and frying causes alterations of the protein structures and core epitopes of Ara h 2. Allergenicity was possibly reduced by the collapse of its tertiary/secondary structure, and a reduction in the core epitope binding capacity. Roasting showed higher allergenicity, which means stronger core epitope binding capacity.
2.5.2 Autoclaving

Cabanillas et al. (2012) found that by using the processing method of autoclaving (moisture not mentioned), the IgE binding capacity of roasted peanut proteins was reduced. Autoclaving at 2.56 atm (37.6 psi, 129 °C) for 30 min, produced a significant decrease of IgE-binding capacity of peanut allergens. The solubility of proteins from autoclaved roasted peanut were lower than that from roasted peanut (analyzed by SDS-PAGE and western-blot). The result of circular dichroism spectroscopy (which could detect the protein secondary structure of protein) showed that most of the α-helical structure was lost after autoclave treatments. It is known that many of the IgE binding epitopes in the major allergens of peanuts (i.e. Ara h 1, Ara h 2 and Ara h 3) are located on the α-helical regions (including both linear and conformational epitopes) of these proteins. Also, after 10 min of digestion by trypsin of roasted peanut and autoclaved roasted peanut, the autoclaved roasted peanut was more thoroughly digested and lost most of the IgE binding capability from peanut allergic patients. The effect of autoclave treatment resulted in conformational change of the peanut allergen. Their later study (Cabanillas et al., 2015) expanded the forms of peanuts to raw, fried and roasted for analyzing the influence of thermal processing on the IgE binding properties. The results reconfirmed that thermal/pressure treatments at specific conditions has the capacity to decrease IgE binding properties of water-soluble protein extracts from peanut (solubility not mentioned). Another study conducted by Long et al. (2016), in which peanut protein extracts were treated with high pressure processing and/or thermal treatment (25–75 °C) for 2.5–30 min, and the immunoreactivity of water-soluble peanut protein extracts (solubility not mentioned) were analyzed by ELISA using pool sera containing peanut
specific IgE. Compared to the boiling treatment, a significant reduction in IgE binding was observed when the samples were treated at 55 °C, 87k psi for more than 10 min.

2.5.3 **Heat processing effects on food matrix**

Khuda et al. (2012) tested the allergen recovery after the cookies (include peanut powders) were baked. Five commercial ELISA kits showed that decreased (as much as 80%) recovery of water-soluble peanut allergens after baking for 30 min. Analytical coefficients of variation for most kits increased with baking time, but decreased with incurred allergen level.

2.5.4 **Combination of heat and steam pressure processing**

It is reported by Cuadrado et al. (2011) that DIC (instant controlled pressure drop) after high pressure steam treatment showed a general decrease in IgE binding to legume (including peanut) proteins that was correlated to a higher steam pressure and longer treatment, but did not affect the total protein content of legume seeds. DIC treatment at 6 bar (87 psi, 158 °C) for 3 min resulted in a remarkable decrease in the allergenic (especially for soybean) proteins.

2.5.5 **Heat processing effect on extraction properties**

The study of Schmitt and coworkers (2009) found that the thermal processing (boiling, frying and roasting) could alter solubility of peanut proteins. With increased time of all heat processing tested, overall protein water-solubility was reduced; and IgE binding properties in water was increased in insoluble proteins.
2.6 Non-thermal processing

2.6.1 Fermentation

Heat processing can affect the integrity of the allergenic protein by changing its three-dimensional confirmation and then will affect IgE binding to conformational epitopes, whereas food processing methods that cleave the protein (e.g. fermentation) will also affect IgE binding to linear epitopes (van Hengel, 2007). A new product called fermented peanut-soy milk by Probiotic L. acidophilus (combined with P. acidilactici and S. cerevisiaeas), is a novel functional beverage developed by do Amaral Santos and coworkers (2014). However, the allergenicity of the fermented product was not tested in this study. Further study is needed to show the allergen reducing extent for this type of product in order to develop hypoallergenic fermented peanut foods.

2.6.2 Ultrasound assisted enzymatic treatment

The study of Li coworkers (2013) demonstrated that ultrasound treatment (1 h at 50 Hz) can significantly enhance the efficiency of enzyme treatment (α-chymotrypsin to peanut ratio of 0.25%, w/w) to reduce the major allergens (Ara h 1, Ara h 2) by more than 90% in roasted peanuts by molecular destruction of shear force. Compared with the blanching (heat processing) methods, the ultrasonic treatment could preserve more flavoring compounds due to lower processing temperature.

2.6.3 High pressure

Peanuts are easily to be infected by the mold fungus Aspergillus flavus during storage, accumulating highly toxic substance aflatoxin. Treatment with 600 MPa and 800 MPa on peanuts for 10 min could significantly (P <0.05) reduce the immunoreactivity
compared with the control group by 69.2% and 73.3%, respectively. Meanwhile, the
growth of the mold fungus *Aspergillus flavus* during 30 days’ storage was significantly
lower than the control group. The result indicated that high pressure processing could
preserve ground peanut quality by reducing food immunoreactivity and by controlling
fungus *Aspergillus flavus* in peanuts (Huang, Yang, & Wang, 2014).

2.6.4 Irradiation

Irradiation with up to 10 kGy is proven to be safe by the WHO (WHO-FI, 1988),
and with maximum doses of 8 kGy (for seeds) is permitted by the FDA (FDA-CFR,
2017). Luo et al. (2013) conducted a study to test the effect of irradiation at different
doses (1, 3, 5, or 10 kGy) on the peanut allergen reduction (especially on Ara h 6). By
using SDS-PAGE, ELISA and Immunoblot, the peanut whole protein extract and purified
Ara h 6 all showed allergen reduction with the doses of irradiation increased. At the dose
of 10 kGy, 95% of the allergen lost binding activity to the IgG antibody for both whole
peanut protein extract and purified Ara h 6. The CD-spectrum showed that Ara h 6
exhibited decreasing α-helix proportion with increasing irradiation doses. The
conformational change of the allergen could be confirmed and correlated with the
reduction of the allergenicity.

2.6.5 Phenolic-modified

Tannic acid, which has a higher affinity for proteins (especially proline rich
proteins) of the legumes, has been found to form an important stable complex (Chung &
Reed, 2014). The complex formed at the concentration of 1 mg/ml tannic acid and 5
mg/ml peanut butter extract exhibited less-digestive properties (allergens were not
released) at simulated physiological pH (i.e., pH 2 and 8), and caused 75% reduction for the IgE binding or immunoreactivity by the data of ELISA. The ELISA and immunoblot data showed that the IgE binding capacity of the complex was totally lost when the concentration of tannic acid was increased to 2 mg/ml. However, the sensory test of the complexes was not conducted in this study. The author suggests an idea: tannic acid might have potential to be used as a recipe for accidental ingestion of peanut ingredient containing foods.

2.6.6 Chemical modification (nano-encapsulate)

In a study done by Rebouças et al. (2012), poly(methyl vinyl ether-co-maleic anhydride) nanoparticles loaded with peanut proteins (using the solvent displacement method) could be a delivery system to reach the goal of immunotherapy. Comparing with freeze-dried nanoparticles, spray-dried nanoparticles were more stable with smaller mean particle size and lower release rate along with the storage time increased. After intradermal (injection) immunization of mice with peanut proteins encapsulated in poly(anhydride), a strong mixed TH1/TH2-type immune response to peanut allergen was observed. Their later study (Rebouças et al., 2014) showed that oral immunization with peanut protein incorporated poly(anhydride) nanoparticles also had an immune response to peanut allergen. The spray-dried nanoparticles also presented lower IgE binding properties compared with free peanut protein or freeze-dried nanoparticles. The result above proved that spray-dried nanoparticle can be used as a delivery-system for peanut protein encapsulation for immunotherapy. However, human study is needed in the future for application.
2.7 Enzymatic processing

2.7.1 Enzymatic processing on allergenicity of legumes

A study by Cabanillas et al. (2012) described the influence of allergenicity of ground roasted peanut protein extract by two types (endoprotease alcalase and exoprotease flavourzyme) of enzymatic processing. Compared with flavourzyme’s 64% reduction after 300 min, endoprotease alcalase decreased (by 100% after 90 min) the soluble fraction of roasted peanuts’ IgE reactivity better. A later study of Shi et al. (2013) further expanded one more digestive enzyme pepsin to alcalase and flavourzyme, and also included a Basophil activation test to evaluate the enzymatic effect on soluble roasted peanut extract. The result indicated that all the hydrolysis of peanut flour (light roasted with 12% fat) reduced IgE binding capacity, but did not eliminate the total IgE cross-linking capacity.

The effect of hydrolysis with different in vitro enzymatic treatment systems on raw and roasted peanut powders were conducted and compared (Quist, Phillips, & Saalia, 2009). Raw peanut hydrolyzed with alcalase had a slightly higher degree of hydrolysis (DH, 23%) than pepsin-pancreatin (21%) after 24 hr. However, roasted peanut powders hydrolyzed with alcalase had lower DH (21%) than pepsin-pancreatin (25%) after 24 hr (Quist, Phillips, & Saalia, 2009). In a study by Zheng and coworkers (2015), five enzymes (1% w/w) were used for hydrolyzing defatted peanut flour (DPF) and peanut protein isolate (PPI); alcalase and flavorzyme were found to be more efficient in DPF and PPI hydrolyzing than neutrase, papain and proteamex.
2.7.2 Functionality of proteins/hydrolysates

2.7.2.1 Proteins as emulsifiers in oil/water emulsions

Proteins possess amphiphilic nature and could reduce the interfacial tension at the oil-water interface. The amphiphilic nature and film-forming abilities of proteins are of great interest for their emulsifying properties. Different from small molecular weight emulsifiers, proteins diffuse at a slower rate due to larger molecular weight (Figure 2.4A). After arrival at the interface, partial denaturation (or unravelling) is often needed to expose buried hydrophobic amino acids to the surface (Figure 2.4B). The proteins then re-align themselves to locate their surface hydrophobic amino acids within the oil phase and hydrophilic amino acids within the aqueous phase. At the end, viscoelastic film was developed at the interface (Figure 2.4C). The viscoelastic film could resist mechanical stresses, and provide electrostatic (Figure 2.5A) (depending on the conditions of solvent) and steric (depending on protein) stabilization (Figure 2.5B) (Lam & Nickerson, 2013).

The physiochemical properties of proteins are key factors in determining their emulsifying abilities. For example, the surface hydrophobicity of the protein could influence the ability for the protein to absorb to the oil side of the interface, where high hydrophobicity was desired for higher emulsion capacities (Kim, Decker, & McClements, 2005). In contrast, the surface charge of the protein influences the solubility of the proteins within the aqueous face, where high solubility is preferred for greater interface diffusion (Karaca, Low, & Nickerson, 2011). After the viscoelastic film is formed, droplets can possess a negative or positive charge depending on whether pH of emulsion is higher or lower than the isoelectric point of protein, respectively. High electrostatic repulsion between oil droplets tends to result in greater emulsion stability.
However, under pH conditions near to the isoelectric point (or high ionic strength) of the protein, droplet flocculation/aggregation occurs easily and leads to coalescence and instability (McClements, 2015). The ‘loops’ or ‘tails’ of the protein fractions may expend from the interface containing mainly hydrophilic amino acids, which could create steric stabilization to restrict droplets from aggregation (Lam & Nickerson, 2013).

Partial hydrolysis of protein has been studied and exhibited improved emulsion properties (Lam & Nickerson, 2013). The improvement of emulsion properties after partial hydrolysis is accounted for increasing solubility, revealing hidden hydrophobic groups, increasing the surface hydrophobicity and reduce its molecular weight, which allows for better attachment to the interface of oil and water (Govindaraju & Srinivas, 2006). The hydrolysis of protein and associated functionality improvement are dependent upon the degree of hydrolysis which resulted from various factors such as the time, temperature and selected enzyme (Lamsal, Jung, & Johnson, 2007; Jung, Murphy, & Johnson, 2005). If the protein is highly hydrolyzed, the high concentration of hydrolyzed protein would not adhere to the water-oil interface but saturate in the continuous phase. Therefore, only limited hydrolysis of the protein would have potential to improve the functionality (Conde & Patino, 2007).
Figure 2.3  A depiction of globular proteins migrating to the water–oil interface (A) followed by reorientation (B) and viscoelastic film formation (C).

The red dots represent hydrophobic moieties found in proteins (Lam & Nickerson, 2013).
2.7.2.2 **Enzymatic processing on allergenicity of legumes**

Alcalase treated peanut protein hydrolysates at different degree of hydrolysis (10-40%) were tested for functional properties and ACE inhibitory (Jamdar, Rajalakshmi, Pednekar, Juan, Yardi, & Sharma, 2010). Hydrolysis (degree of hydrolysis > 10%) improved the solubility of peanut protein by more than 40% at pH of 5. The ACE inhibitory also increased by more than 20% after hydrolysis. Peanut protein isolate showed better emulsifying and foaming properties (as much as over 100%) than peanut protein hydrolysate.

Besides peanut, soy protein isolates treated with different food-grade proteases (alcalase, colorase, flavourzyme, neutrase, PTN, papain, pepsin, proteomax, protease N-01) were used for allergenicity, functionality, and sensory property analyses (Meinlschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016). Alcalase (0.5% w/w), pepsin (0.5% w/w), and papain (0.5% w/w) were more effective in the degradation of the
major soybean allergens (β-conglycinin and glycinin) of 100%, 100%, and 95.9%, respectively, which were analyzed by means of electrophoresis. Among all the hydrolysates, flavourzyme and papain were considered as more attractive due to less bitter taste (by as much as 70% less) and improved sensory profile (smell, taste, mouthfeeling). The hydrolysates improved oil-binding properties (from initially 0 to above 2 mL/g) and decreased water-binding properties by at least 30%. The foaming activity was increased by more than 100% but with lower foaming stability and density (Meinlschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016). In their later study, the soy protein treated by two-step enzymatic processing (papain followed by flavourzyme) exhibited effective allergen degradation (100%), less bitter taste (by 30%) and higher foaming capacity (by 300%) compared with SPI (Meinlschmidt, Schweiggert-Weisz, Brode, & Eisner, 2016). The modified soy products may have potential to be used as food additives for protein enhancement with high foaming properties, or could be made into high protein bar/beverage with hypoallergenic requirement. However, in vivo study and color testing are needed for further application.

2.7.3 Transglutaminase (TGase) processing

2.7.3.1 Introduction to TGase

Transglutaminase (TGase; protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) catalyzes an acyl transfer reaction between the γ-carboxyamide group of a peptide-bound glutaminyl residue (acyl donors) and a variety of primary amines (acyl acceptors), including the amino group of lysine (Figure 2.4A). When it is short of amine substrates, TGase catalyzes the hydrolysis of the γ-carboxyamide group of the glutaminyl residue, resulting in deamidation (Figure 2.4C). When the ε-amino group of a peptide-bound lysyl
residue is the substrate, peptide chains are connected covalently through a type of
emulsion (Figure 2.4B) (Yokoyama, Nio, & Kikuchi, 2004). The cross-linking reaction
caued by TGase results in the polymerization of protein/peptide molecules with a
significant increase in molecular mass. The modification of proteins by TGase has been
studied extensively (Clare et al., 2008; Hu, Zhao, Sun, Zhao, & Ren, 2011; Agyare,
Addo, & Xiong, 2009; Fan et al., 2005) and has been shown to be an effective method for
improving the functional properties of proteins/hydrolysates.

\[
\text{A} \quad \text{Glu-C-NH}_2 + \text{RNH}_2 \longrightarrow \text{Glu-C-NHR} + \text{NH}_3
\]

\[
\text{B} \quad \text{Glu-C-NH}_2 + \text{H}_2\text{N-Lys} \longrightarrow \text{Glu-C-NH-Lys} + \text{NH}_3
\]

\[
\text{C} \quad \text{Glu-C-NH}_2 + \text{H}_2\text{O} \longrightarrow \text{Glu-C-OH} + \text{NH}_3
\]

Figure 2.5 Reactions catalyzed by transglutaminase (TGase) (A-C).

(A) Acyl transfer. (B) Crosslinking of Gln and Lys residues in proteins or peptides. The
resulting bridge is called an ε-(γ-glutamyl) lysine (GL) bond. (C) Deamidation
(Yokoyama, Nio, & Kikuchi, 2004).
2.7.3.2 TGase treatment on protein/protein hydrolysates

A study of Clare and coworkers (2008) found that peanut flour dispersions could decrease IgE binding capability when treated with TGase by using casein as cosubstrate to form a polymer. In a study of Hu and coworkers (2011), peanut protein isolate (PPI) was treated by high-pressure microfluidization and/or TGase cross-linking. It was found that individual microfluidization or TGase cross-linking changed the physicochemical and functional properties effectively. TGase cross-linking caused the unfolding of PPI structure, resulting in the decrease levels of α-helix and β-turns and the increase levels of β-sheet and random. Compared with individual treatments, microfluidization followed by TGase cross-linking improved the emulsion properties significantly (with mean particle size decreased to 50% of the PPI’s emulsion). In addition, the combined treatments induced looser structure of peanut protein isolate and resulted in more obvious changes in the physicochemical properties (Hu, Zhao, Sun, Zhao, & Ren, 2011).

In a study of Agyare, Addo, and Xiong (2009), TGase-treated wheat gluten hydrolysate (GH, partially hydrolyzed with chymotrypsin) was tested for emulsifying and foaming properties as compared with no TGase-treated hydrolysate. The TGase treatment had significant improvement on foaming capacity by 7% (compared with non-TGase treated GH) at pH 4.0 (measured at pH 4.0), but had no effect at pH 6.5. On the contrary, the foaming stability of TGase treated hydrolysate was not significantly different at pH 4.0 and 6.5 compared with GH. The changes in functional properties of TGase-induced wheat gluten hydrolysates were dependent on the amphiphilic nature of gluten peptides and increased electrostatic repulsion resulting from deamidation (measured by carboxyl groups production) (Agyare, Addo, & Xiong, 2009).
In a study of Fan, et al. (2005), the mixtures of soy protein isolate and soy protein hydrolysates (SPM) were used for a gel-forming study. The soy protein hydrolysates were obtained by using pepsin (pH 3, enzyme/protein = 1/200). The treatment of TGase was conducted by using 2.5% of TGase adding into SPM at pH 7 (55 °C, 60 min). The radical scavenging activity of the mixtures containing soy protein isolate and its hydrolysate increased as the percentage of hydrolysate increased, while the gel-forming ability decreased as the percentage of hydrolysate increased. The addition of TGase (pH 7) improved the gel-forming ability of SPI and hydrolysate mixture. It has been found that a mixture of 70% SPI and 30% hydrolysate by adding TGase resulted in a gel with better gelling property compared with non-TGase treated SPM and higher radical scavenging activity compared with SPI (Fan et al., 2005).

2.8 Peanut varieties with less allergen

A study done by Koppelman et al. (2001) shows that no significant differences in the amounts of Ara h 1 (12-16%) and Ara h 2 (5.9-9.3%) in seeds based on market type (Valencia, Virginia, Spanish, or Runner) or location where the peanut was cultivated. In a later study, 60 accessions in the U.S. Peanut core collection and 88 Florida Peanut breeding program lines were analyzed. The results showed wider range of Ara h 1 levels (7.0-18.5%) and Ara h 2 levels (6.2-15.7%) with more diverse peanut samples. An accession from India (PI 372305) had the lowest level of Ara h 1 (7.0%). An accession from Nigeria (PI 372305) had the highest level of Ara h 1 (18.5%), but the lowest level of Ara h 2 (6.2%). An accession from Zambia (PI 494795) had the highest level of Ara h 2 (13.2%), but the lowest level of Ara h 3 (21.8%). No or little 36 kDa Ara h 3 isoform was contained in two peanut cultivars (Florunner and Georgia Red) (Kang, Gallo, & Tillman,
2007). Since there were no seeds with significant lower contents of all the major allergens, the author concluded that the major allergens, which constitute the most for the seed storage protein, may compensate for each other to balance the total amino acid composition in peanut seed.

2.9 Peanut allergen under simulated in vitro digestion

It is believed from the previous studies that food allergens (even after cooking) possess certain gastric stability in order to reach the intestinal mucosa where absorption and sensitization occur (induce allergy) (Astwood, Leach, & Fuchs, 1996). The stability of the food allergens after processing may be changed but need to be tested. Simulated digestive system (in vitro) mimics human digestive system’s function by using simulated saliva, gastric juice, duodenal juice and bile juice. The digested foods could be analyzed and provide important references for food component (for examples: protein, lipid, starch, and fiber) digestibility. Several studies have been conducted on the digestibility of peanut allergen under simulated digestion systems. There was a study about comparing the digestibility of peanut and hazelnut under gastric digestion and duodenal conditions (Vieths, Reindl, Müller, Hoffmann, & Haustein, 1999). It was found that roasted peanut allergens were more stable during digestion compared with unprocessed hazelnut. Both Ara h 1 and Ara h 2 turned out to be digestion-resistant in some instances with the resistance increased by 3.6-fold after roasting (undergone Maillard reaction) (Maleki, Chung, Champagne, & Raufman, 2000; Maleki et al., 2003). It is believed that the allergenicity of peanut, soybean, milk and egg allergens may be partially due to the resistance to proteolysis in the stomach and intestine. Therefore, stability to digestion would be a prerequisite for sensitization to food these food allergens (Astwood, Leach, &
Fuchs, 1996). In a study of Maleki, Schmitt, Galeano and Hurlburt (2014), the digestibility of peanut allergens from boiled, fried, and roasted peanuts were analyzed and compared. The result showed that boiled and raw peanut proteins had similar digestibility in most cases, except Ara h 1 in boiled peanut was more resistant to digestion. Fried and roasted peanut proteins were more resistant to digestion than raw and boiled peanuts, and had more IgE binding fragments after digestion.
CHAPTER III
QUANTITATIVE AND KINETIC ANALYSES OF PEANUT ALLERGENS AS AFFECTED BY FOOD PROCESSING

3.1 Abstract

Peanut, a major allergenic food, contains four major allergens with differences in allergenic potency. Thermal processing can influence the allergenic properties of peanuts. However, the kinetics of the reactions caused by thermal processing has not been characterized. Our objective is to characterize the reaction kinetics of the thermal processing methods, including wet processing (boiling with/without high-pressure, steaming with/without high-pressure), deep frying and dry processing (microwaving and roasting) using five time intervals. Water-soluble and SDS-sample buffer soluble proteins were extracted sequentially, and analyzed by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western-blot using human plasma containing IgE antibodies. Proximate chemical analyses were conducted on peanut powder to understand the changes in the major constituents of peanut following various processing conditions. The relationships between processing time and extractable major allergen content could be explained by a simple linear regression kinetic model (except high-pressure steaming). The proximate analysis showed that wet processing increased moisture content (wet basis) and decreased lipid content (dry basis). Protein content was not significantly changed by various processing methods. Among all the methods with optimal processing
point, frying for 6 min had a relatively lower IgE binding (linear epitopes) ratio, possibly due to the processing condition causing break down, cross-linking and aggregation of Ara h 2, and a relatively lower solubility.

**Key words:** Peanut allergen, thermal processing, proximate analysis, kinetic analysis, water-soluble, SDS-sample buffer soluble, IgE binding properties

### 3.2 Introduction

Peanut is an important oil and food crop. It is the third major oilseed of the world. Due to the low cost and high nutrition, it is one of the most popular foods worldwide. However, peanut allergy is one of the most common causes of food-related, fatal anaphylaxis (Sáiz, Montealegre, Marina, & García-Ruiz, 2013). The number of people with peanut allergies in the United States appears to be increasing (Pansare & Kamat, 2010). To date, 17 peanut allergens (Ara h 1[Arachis hypogaea 1] - Ara h 17) have been identified that are listed by the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies (Subcommittee, 2014). Of the 17 recognized peanut allergens, Ara h 1, Ara h 2, Ara h 3 and Ara h 6 are considered the major allergens because they are abundant and recognized by serum IgE of >50% of peanut allergic individuals (Mueller, Maleki, & Pedersen, 2014). Among these, Ara h 2 is regarded as the most potent for severe allergic reactions and higher IgE-binding properties (Schocker, Baumert, Kull, Petersen, Becker, & Jappe, 2016). Currently the only available treatment is complete peanut avoidance. However, avoiding peanuts in food product is difficult because of its ubiquitous use as an ingredient in processed foods.

Thermal and non-thermal processing methods are the two major strategies that have been used for allergen reduction in peanuts. The effects of thermal processing,
including frying, boiling, steaming, roasting, microwaving and autoclaving, on allergenic properties of peanuts have been studied extensively (Cabanillas et al., 2015; Cabanillas, Maleki et al., 2012; Chung & Reed, 2014; Maleki, Chung, Champagne, & Raufman, 2000; Rao, Tian, Tao, Tang, Li, & Xue, 2016; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2009; Verhoeckx et al., 2015). Thermal processing may reduce or alter IgE binding due to degradation of allergens into small peptide fragments, and/or formation of aggregates, and interactions with other proteins, carbohydrates and lipids (Chung & Reed, 2014). Ara h 1, Ara h 2 and Ara h 3 have 21, 14 and 4 epitopes for IgE-binding, respectively (Lee, Wright, & Rachaputi, 2016). Thermal processing may also alter the IgE-binding potency by changing the conformation of epitopes. Boiling and frying have been reported to reduce allergic properties of peanuts compared to roasting (Beyer et al., 2001). Roasted peanuts may bind significantly higher IgE than raw peanuts due to the Maillard reaction (Maleki, Chung, Champagne, & Raufman, 2000). High-pressure processing (2.56 atm, for 30 min) has been shown to reduce the allergenic properties of roasted peanuts (Cabanillas et al., 2012). Thermal processing has been found to alter the solubility and structure of major peanut allergens and may reduce allergenic properties under some conditions (Cabanillas et al., 2012; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2009), although thermal processing may not eliminate all of the allergenic potential (Verhoeckx et al., 2015). In order to understand how the major peanut allergens are changed under thermal processing in a quantitative way, developing kinetic models is important. Until now, a kinetic model has not been reported to assess the changes of soluble allergen content as a function of processing time under various thermal processing methods. Also, a comparison of IgE-binding properties of water-
soluble/water-insoluble allergens in peanut processed by commonly used methods (such as high-pressure processing, steaming, boiling, microwaving, roasting, frying) within palatable texture ranges would be meaningful.

In this study, a common market type peanut variety (Virginia Jumbo) was analyzed under various processing conditions that produced palatable peanut texture. Our objective was to develop the kinetic model to characterize how the major water-soluble allergen content changes during processing, which may be useful in understanding and predicting the effects of processing on water-soluble allergen levels. The major constituents of peanut, including moisture, lipid, protein, ash and carbohydrate were analyzed under various processing treatments. The allergen IgE-binding properties of the peanut allergens were analyzed following the different processing methods.

3.3 Materials and methods

3.3.1 Materials

Virginia Jumbo peanut (Waldron Farms, Citra, FL) was purchased from a food supplier. The whole-shell peanuts were sun-dried (on a clean sheet over the concrete ground to the moisture content of kernel below 5%) and shell-removed before processing or protein extraction. The proximate data of raw Virginia peanut was shown in Table 3.1. Human plasma from six individuals with only peanut allergy (IgE levels CAP-FEIA >100 kU/L; contained anti-Ara h 1, Ara h 2 and Ara h 3 antibodies) were purchased from PlasmaLab International (Everett, WA). All the chemicals (analytical grade) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A).
3.3.2 Thermal processing on Virginia Jumbo peanut

Thermal processing methods, including boiling, steaming, frying (170 °C), roasting (177 °C), microwaving (2.45 GHz), high-pressure (1.02 atm, 15 psi) (HP) boiling and high-pressure (1.02 atm) (HP) steaming. Fifty grams of peanut per batch were used for each processing method. Boiling (no presoaking treatment) was conducted in a regular boiler with tap water (peanut-to-water ratio of 1: 30, w/w, to make sure peanuts were immersed in water completely during the cooking process) and boiled at 30, 60, 90 and 120 min, respectively. HP boiling (no presoaking treatment) was conducted in an All American® high-pressure cooker (model 915, Wisconsin Aluminum Foundry, Manitowoc, WI), with peanut-to-water ratio of 1: 30 (w/w) and processed at 10, 20, 30 and 40 min, respectively. In order to conduct steaming/HP steaming easily, peanuts were presoaked in tap water (peanut-to-water ratio 1: 10, w/w) at room temperature for 12 hr. The presoaked peanuts (about 1.5 times bigger) were used for steaming at 30, 60, 90 and 120 min, respectively, with a regular steamer, respectively. HP steaming was conducted in All American® high-pressure cooker (model 915, Wisconsin Aluminum Foundry, Manitowoc, WI) at 5, 10, 15, 20 min, respectively. Deep oil frying was conducted with peanut-to-corn oil ratio of 1:20 (w/w), in a temperature-controlled fryer (Model 35034, Hamilton Beach Co., Washington, NC). The time of frying was 2, 4, 6, and 8 min, respectively. Roasting was conducted in a temperature controlled Oster® toaster oven (model TSSTTVSK01, Jarden Co., NY). The time of roasting was 4, 8, 12 and 16 min, respectively. Microwaving was conducted in a microwave oven (model OGG61403-B, Jarden Co., NY). The time of microwaving was 0.75, 1.5, 2.25 and 3 min, respectively.
3.3.3 **The protein extraction method for raw and processed peanut**

The protein extraction for raw and processed peanut was according to earlier study (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2009) with some modifications. Briefly, approximately 5 g of the pre-frozen peanuts were milled with Magic bullet blender (Model No. MBR-1101, Capbran Holdings, LLC, Los Angeles, CA) into coarse powders. Then approximately 4 g of the peanuts ground with mortar and pestle in liquid nitrogen into fine powder (could pass through 0.5 mm sieve). Five hundred mg of peanut powder was defatted by adding 10 mL of acetone and then shaken for 2 hrs in an orbital shaker. The suspension was centrifuged (2800 g for 15 min) and the pellet was dried under an exhaust hood overnight at room temperature. The defatted flours were stirred in 3 ml of 0.02 M sodium phosphate, pH 8.5, plus 10 mM EGTA at room temperature (RT) for 2 hrs. The aqueous fractions (water-soluble fractions) were collected by centrifugation (2800 g) for 15 min at room temperature. The pelleted parts after centrifugations were mixed with standard electrophoresis SDS-sample buffer (1% SDS + 5 mM dithiothreitol) and boiled for 5 min to extract proteins from the water-insoluble residue. The samples in SDS-samples buffer were then centrifuged at 2795 g for 15 min. The collected supernatants in SDS-sample buffer (SDS-sample buffer soluble fractions) and water-soluble fractions were analyzed for protein content determination by the Bradford’s Method (Bradford, 1976). The sum of water-soluble and SDS-sample buffer soluble protein fraction was taken as the total extractable protein.
3.3.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western-blot Analysis

The SDS-PAGE was slightly modified according to earlier studies (Kang, Gallo, & Tillman, 2007; Laemmli, 1970; Meng, Chang, Gillen, & Zhang, 2016) with a Mini Protein Tetra System (BioRad, Hercules, CA). Water-soluble and SDS-sample buffer soluble extracts (adjusted to 2 mg/mL) was mixed with an equal volume of 2× SDS-PAGE sample buffer containing 5% β-mercaptoethanol. The mixture was boiled for 5 min and then centrifuged for approximately 5 to 10 seconds. Electrophoresis was performed on 13.5 % (non-gradient) acrylamide gels for 1 h at 50 V followed by 1.5 h at 100 V. At the end of electrophoresis, gels were stained with Coomassie Brilliant Blue R-250. For the quantification of peanut major allergens (Ara h 1, Ara h 2 and Ara h 3), gels were scanned and analyzed by a Molecular Imager (Bio-Rad ChemidocTM XRS+, Hercules, CA) equipped with Image LabTM Analysis Software (version5.2). The major allergens and their relative composition were calculated based on the band intensity in the SDS-PAGE analyzed by the Image LabTM Analysis Software. Allergen protein content was calculated based on the ratio of band intensities in the SDS-PAGE and protein content (shown in the following section).

Changes in allergenic properties of existing allergens due to processing were analyzed by immunoassay of western-blot using plasma containing IgE antibodies from peanut allergic individuals as described by an earlier study (Chung & Reed, 2012). Briefly, protein extracts were transferred from SDS-PAGE gel to an Immobilon-P membrane. After blocking with a SuperBlock solution (Cat No. 37515, ThermoFisher, Waltham, MA), the membrane was incubated for 30 min with a pooled plasma diluted 1:20 (v/v) in Superblock/TBS-Tween 20 (1:1, v/v). The membrane was then washed with
TBS/Tween and incubated with a rabbit anti-human IgE-peroxidase (1:250) and then washed 3 times for 10 min each with TBS Tween. After washing, the membrane was then incubated in the SuperSignal™ West Pico Chemiluminescent Substrate (Fisher Scientific, Pittsburgh, PA) for about 1 min. The incubated membrane was scanned and analyzed by a Molecular Imager (Bio-Rad ChemidocTM XRS+, Hercules, CA) equipped with Image LabTM Analysis Software (version5.2).

### 3.3.5 Quantification of allergens and estimate of total allergen IgE binding properties

Quantification of allergens was calculated based on the band intensity of SDS-PAGE, protein content (dry basis) and according to the following formula:

\[
\text{Allergen content (g/100g peanut dry basis)} = \text{Ratio of band intensities} \times \text{protein content [g/100g peanut dry basis, analyzed by the method of Bradford (1976)]}
\]

Quantification of Processed (Raw) peanut Allergen IgE binding properties were based on the allergen content and intensities of western-blot and SDS-PAGE which shows as below:

\[
\text{Processed peanut Allergen IgE binding properties} (\%) = 100 \times \text{Processed peanut Allergen content} \times (\text{western-blot intensities/ corresponded SDS-PAGE Intensities}) / [\text{Raw peanut Allergen content} \times (\text{western-blot intensities/ corresponded SDS-PAGE Intensities})]
\]

The raw peanut Allergen IgE binding properties [Raw peanut Allergen content \times (western-blot intensities/ corresponded SDS-PAGE Intensities)] were considered as 100%
3.3.6 Proximate analysis

The proximate analysis was performed to understand the processing effects on the major seed constituents. Moisture, lipid, protein and ash content were analyzed according to the standard AOAC methods of oven drying, Soxhlet extraction, Kjeldahl and furnace ashing, respectively (Method 934.01; Method 2003.06; Method 955.04; Method 942.05) (Latimer, 2012). Carbohydrate content calculated by subtracting the lipid, protein and ash content (g/100g peanut in dry base) from the total weight (100g/100g peanut on dry basis).

3.3.7 Kinetic analysis

Simple linear regression model was fitted as a first-order reaction, which was similar to our earlier thermal processing study on isoflavone in soybean (Zhang, Chang, & Liu, 2015). This model was conducted by using time (min) as the variate and log transformed water-soluble/total-extractable allergen content (µmol/100g peanut, dry basis) as the response.

\[- \frac{d[\text{allergen content}]}{dt} = K[\text{allergen content}] \]  \hspace{1cm} (1)

Integration from time 0 to t shows

\[- \int \frac{d[\text{allergen content}]}{[\text{allergen content}]} = \int K dt \]  \hspace{1cm} (2)

\[\log[\text{allergen content}]_t/\log[\text{allergen content}]_0 = -Kt \]  \hspace{1cm} (3)

\[\log[\text{allergen content}]_t = -Kt + b \]  \hspace{1cm} (4)

K represents rate constant, which means log[allergen content] reduction per min.

The result was analyzed by using RStudio (Version 1.0.136, Boston, MA) to get the R square, constant rate K (slope) and P value.
3.3.8 Statistical analyses

Experiments were performed in duplicate for processing and triplicate for analysis. Data were analyzed by 2014 SAS (Version 9.4, SAS Inst. Inc., Cary, NC, U.S.A.) and RStudio (Version 1.0.136, Boston, MA). Significant differences among means were determined by the Duncan multiple range test procedure for independent samples at P < 0.05.

3.4 Results and Discussions

3.4.1 Proximate analysis of peanut processed by seven types of thermal processing treatments

Table 3.1 shows the proximate composition of peanut under various thermal processing methods. The moisture content (wet base) of raw peanut (control) was 4.95%. The moisture of different processed peanuts varied widely, with frying for 8 min containing the lowest amount of moisture (1.54%), and boiling for 2 hr containing the highest amount of moisture (28%). Wet processing methods (boiling and steaming with/without HP) contained higher moisture content with approximately 5 times more than the control (dry peanut without processing). For microwaving and roasting, the moisture content of processed peanuts was not significantly different from the control. The result was not consistent with the study of Khalil and Chughtai (1983) who reported roasted peanut possessed with a lower moisture content (about 2%). The raw material used and the roasting time for the reported study (Khalil & Chughtai, 1983) was 30 min while for our study was 4-16 min, and had relatively less heat. Different roasting conditions in processing our peanut were considered in our study for the palatable acceptability was considered in our study, whereas palatability was not mentioned in the
reported literature (Khalil & Chughtai, 1983). The palatability conditions of various processed peanuts were evaluated and the descriptions were listed on Table 3.1. The moisture content changes were represented by the weight gain and loss of the peanut after cooking.

Lipid content (dry basis) varied from about 41% to 49% among all the processing methods. Microwaving and roasting had no significant moisture changes (from the raw state) after processing. All wet processing methods decreased the lipid content (dry basis). The reason may be due to the free lipids leached out of the peanut kernels into the hot water when the processing time increased. Frying peanuts may also cause the loss of the lipid content at high temperatures, however the oil used for frying could be absorbed on the surface of the peanut and compensated for the lipid loss of the fried kernels. This can explain the few oil content changes were observed for the fried peanut. The lipid extraction time for processed peanut samples was doubled (48 hrs) compared to raw peanut. This indicated the lipid was more difficult to extract with the organic solvent after thermal processing, perhaps due to change in the overall peanut matrix (i.e. enhanced lipid protein interactions). The protein may bind to lipids to form aggregates (Chung & Reed, 2014), which would make the extraction more difficult.

The protein content (dry basis) varied from about 24% to 27%, which changed little among different methods. Roasting for 12 min had the highest protein content, while steaming for 1.5 hr had the lowest protein content. Boiled peanuts were found to lose some low molecular weight peptides/proteins during processing based on an earlier study (Mondoulet et al., 2005). Besides boiling, steaming also can result in loss of proteins into the water during the process of presoaking. However, the total protein content of peanut
was not influenced much (less than 2% changes) with the method of boiling/steaming (Table 3.1).

The ash content of peanut following different methods varied from about 1% to 5% (Table 3.1). The roasted and raw peanut ash content was similar to a previous study in which five varieties of peanuts were analyzed (Khalil & Chughtai, 1983). Boiling (with/without HP) resulted in lower amounts of the ash, due possibly to the loss of minerals in the water. The carbohydrate content (dry basis) was calculated by subtracting the sum of analyzed proximate content above (lipid, protein, ash in dry basis) from the total content (100g/100g peanut dry basis). The contents of carbohydrate were varied from 21.96% to 31.43% (Table 3.1). Raw peanuts possessed about 17.22% carbohydrate based on the review by Nwokolo and Smartt (1996). The relative wide ranges of carbohydrate content determined in peanuts following different processing methods in this study may be due to the variations in lipid and ash content. Peanuts from all the wet processing methods had higher ratio of carbohydrate content due to their relatively low ratio of lipid content.

3.4.2 Processing effect on water-soluble major peanut allergen by SDS-PAGE and western-blot analysis

The effect of processing times (within palatable ranges) on the water-soluble allergens was assessed by SDS-PAGE (Figure 3.1) and western-blot (Figure 3.2), respectively. The objective of SDS-PAGE analysis was to visualize and quantitate the allergen content changes following various processing methods. While, the objective of western-blot analysis was to determine the presence, levels and reactivity of the allergen under various treatment conditions. For the SDS-PAGE analysis, all the protein
concentrations were normalized (1 mg/ml in SDS sample buffer) and equal amounts of total protein (8 μg) were loaded for each sample. The relative band intensities of water-soluble proteins (allergens) before and after processing are shown in the Figure 3.1. In microwaved peanuts, all the major allergens (as labeled) tended to show a similar distribution until the last min of processing. In the cases of roasting, HP steaming, frying, boiling and steaming, the intensity of the of Ara h 1 (62 kD) monomer band was reduced when processing time increased.

Meanwhile, at the top of the separating gel, high molecular mass protein aggregate bands were formed and became darker with increased processing time for microwaving, roasting, boiling, HP boiling and deep-frying conditions (Figure 3.1). The aggregated protein bands were more prominent in the SDS-sample buffer soluble fractions of roasted and deep oil fried peanut (Figure 3.3). The result was similar to some earlier studies (Rao, Tian, Tao, Tang, Li, & Xue, 2016; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2009). The correlations between water-soluble Ara h 1 decreases and SDS-sample buffer soluble aggregate formation were significant for the conditions of roasting and deep oil frying (R > 0.97, P < 0.05). This result confirmed that Ara h 1 formed water-insoluble aggregates after heat processing due to covalent cross-linking and hydrophobic interactions (Rahaman, Vasiljevic, & Ramchandran, 2016). The intensities of smeared bands under 10 kD also became darker when frying time increased to 8 min (Figure 3.1), at which peanuts began to burn. This exact reaction occurred in roasting, when peanuts are cooked long enough (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2009). This indicated that thermal processes could cause the water-soluble proteins to become
aggregated via chemical cross-linking or broken down due to free-radicals attack on the protein side chains and peptide bonds.

While under wet processing (boiling with/without HP, steaming with/without HP), the Ara h 1 monomer shows significantly more changes in intensity over the times measured here compared with the dry processing (microwaving, roasting and frying) methods (Figure 3.1). Especially for steaming and HP steaming, the Ara h 1 levels in the water-soluble fractions (Figure 3.1) were more significantly reduced over the time of treatment compared with other methods even at the beginning of processing. Therefore, steaming had a significant impact on the solubility of Ara h 1. Similar to Ara h 1, distinct water-soluble Ara h 2 bands (Figure 3.1) also had relatively lower intensity in wet processing methods. All the SDS-PAGE of wet processed peanuts showed smeared bands at low molecular mass range, which had some similarity with fried processing. Among wet and fried processing methods, HP steaming had a higher ratio of smeared bands, which may be due to the thoroughness of cooking under pressure. The presoaked processing made the peanut soft due to penetration of moisture. Therefore, a more thorough protein hydration could make the proteins more susceptible to be structural alteration, the HP cooking seemed to effect further the protein structures of the presoaked peanut allergens. HP boiling for 40 min and HP steaming for 20 min could decrease the water-soluble Ara h 3 content to very low levels, indicating the HP processing conditions could be arranged to alter the solubility of Ara h 3.

Figure 3.2 showed the effect of corresponded processing methods on the water-soluble fractions by western-blot with pooled human plasma containing peanut-specific IgE antibody. Wet processing methods, which had low SDS-PAGE band intensity in the
three major allergens (in water-soluble fractions), showed similar less IgE reaction in the western-blot analysis. The reason was due to the low protein content in the water-soluble part (Figure 3.1). The Ara h 1 allergen had less intensity comparing with the Ara h 2, which might be due to the sensitivity of pooled patients’ plasma (Figure 3.2). The plasma used in this study had been confirmed for its anti- Ara h 1, Ara h 2 and Ara h 3 reactivity by the company. Although Ara h 1 and Ara h 2 accounted for 70-90% of patients’ recognition (Burks, Sampson, & Bannon, 1998; Clarke, Kilburn, Ob'Hourihane, Dean, Warner, & Dean, 1998), the relative IgE binding to these major allergens were different. Among all the methods, microwaving, roasting, deep frying exhibited higher IgE-binding properties (water-soluble fractions, 2mg/ml) in terms of high intensity bands on western-blot. However, water-soluble protein content need to be taken into consideration for evaluating the water-soluble allergen IgE binding properties and will be discussed in the following sections.

3.4.3 Processing effect on SDS-sample buffer soluble peanut protein by SDS-PAGE and western-blot analysis

The water-insoluble part of the peanut protein extracts still contained a certain amount (as much as about 60g/100g total protein) of proteins, especially when the processing time increased (Table 3.1). This result was consistent with the earlier study (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2009). All thermal processing methods showed higher band intensities in SDS-sample buffer soluble part than in the water-soluble part due to a combination of chemical modification and aggregation. The extent of changes in water-soluble/SDS-sample buffer soluble proteins are characterized in Figure 3.5 and Figure 3.6, and will be discussed in the following section. For
microwaving, roasting and deep frying, along with an increase in processing time, the intensity of the aggregated bands at the top of the separating gel (> 250 kD) and in the stacking gel (those bands did not move into the separating gel) were also increased (Figure 3.3). This was similar to the soluble protein SDS-PAGE (Figure 3.1) but with higher band intensity, indicating that peanut proteins were less water-soluble following aggregation. On the top of the stacking gels and separating gels, there are apparent aggregates formed along with the processing time increased in most of the methods, especially in dried and fried processing methods. The aggregates could partially explain the loss of major allergens such as Ara h 1 and h 3, and it would be meaningful to calculate the amount of aggregates to better understand the protein structure changes after processing. Quantitative analysis of aggregates content will be discussed in the following sections.

The western-blot of the SDS-sample buffer soluble part is shown in Figure 3.4. Steaming, boiling and HP boiling also showed higher intensity compared with the water-soluble part (Figure 3.2), especially the intensity of Ara h 2 (19, 17 kD), which was significantly higher in Figure 3.4. The method applied in this study (steaming, boiling and HP boiling) could alter the solubility of Ara h 2 (Figure 3.1) but could not eliminate the IgE antibody-reactivity in the whole processed peanut since there were still strong IgE-binding properties remaining in the western-blot of the SDS-sample buffer soluble part (Figure 3.2, 3.4). However, HP steaming and frying resulted in less band intensity in the last time points (HP steaming 20 min and frying 6-8 min) (Figure 3.4). The IgE binding to the 17 kD isoform of Ara h 2 following deep frying for 6-8 min was much less than in other processing methods (Figure 3.4), which is most likely due to aggregation of
this Ara h 2 isoform with other intact and degraded proteins and formation of the higher and/or lower molecular weight smears, particularly visible in the higher time points. HP steaming for 20 min also showed certain decreases in band intensity compared with the raw peanuts or lower time points. The band intensity of Ara h 1 monomer was not very dark, but also could not accurately be quantitated due to smearing (which also showed IgE antibody reactivity) as a result of oligomer formation, degradation and reassociation of protein and peptide fragments via chemical cross linking. The study of Schmitt, Nesbit, Hurlburt, Cheng, and Maleki (2009) had similar but stronger bands of Ara h 1 monomer. The difference may be due to the variations in the levels or sensitivity of plasma.

3.4.4 Quantitative and kinetic analysis of peanut water-soluble allergen under various processing methods

The solubility of peanut protein was effected by thermal processing methods and processing times. The water-soluble protein yield of raw peanuts was 72% of total protein. However, the solubility of the protein was decreased along with increased processing time. Frying 8 min resulted in the lowest water-soluble protein yield of 6.4% of total protein (dry basis), which decreased 66.5% of the solubility (Table 3.1). Allergen quantitative determination was based on the band intensities and protein contents. The peanut processing effect on water-soluble protein (allergen) content is shown in Figure 3.5. Water-soluble Ara h 1, Ara h 2 and Ara h 3 content (measured by the method of Bradford17) of raw peanuts are 3.2, 1.9 and 9.5 g/100 g peanut (dry basis), respectively (Figure 3.5). Boiling, HP boiling, Steaming, HP steaming and deep frying all decreased the water-soluble Ara h 1 and Ara h 2 content into nearly zero amount (less than 0.1g/100
g peanut for Ara h 1 and less than 0.2 g /100 g peanut for Ara h 2) by the end of cooking within the palatable ranges (Figures 3-A). These findings were consistent with a recent study of Comstock, Maleki, and Teuber (2016), in which they found boiling and frying could decrease the solubility of Ara h 1 and Ara h 2 due to the conformation changes.

The allergen contents decreased more dramatically at the beginning of the processing than at the later time points (Figure 3.5). The curves for all the patterns were similar, the general relationship between protein (SDS-PAGE band) content and processing time appeared to fit the first-order reaction kinetics model (Zhang, Chang, & Liu, 2015). Therefore, a simple linear regression model was fitted and conducted by using time (min) as variate (X) and log transformed water-soluble allergen content (g/100g peanut, dry basis) as response (Y). The rate constant K (slope of the linear regression), R square and P value were all listed in the Table 3.2. Boiling, Steaming, HP boiling, frying, roasting and microwaving suited first-order reaction model well with all R squares higher than 0.85 (P < 0.05) and most of them higher than 0.95 (P < 0.01). Meanwhile, HP steaming had relative lower R squares for Ara h 1, Ara h 2 and Ara h 3, indicating the reduced fitness in the first-order reaction models. The reason may be due to the overnight presoaking process, which provided more hydration (water activity) combined with HP processing that, in turn, contributed more significant-conformational changes than other processing methods. Frying resulted in the highest R square on water-soluble Ara h 1, Ara h 2 and Ara h 3 (0.99 for all of them) among all the processing methods, indicating the highest fitness of the applications to the first-order reaction model.
The rate constant $K$, which represented the unit time (min) of log [water-soluble allergen] decreased. The rate constants were the highest in the frying with 0.24, 0.13 and 0.14 for Ara h 1, Ara h 2 and Ara h 3 (Table 3.2). In contrast, the rate constants were relative low in boiling and steaming (about 10 - 20 folds lower than frying) among all the methods. The temperature used for boiling and steaming were the lowest (100 °C) among all the processing methods. The relatively lower temperature maybe the reason for the low constant rate. The temperature used for frying was 170 °C, which was not the highest compared with roasting (177 °C) and microwaving. However, deep frying was conducted by immersing the peanuts into the high temperate oil which would heat up the peanut efficiently and evenly. While roasting and microwaving were conducted by heating the surrounding by air, which might not heat homogeneously when compared with deep frying. Microwaving is well known to produce less uniform heating due to the positional effect. However, microwaving caused significant water-soluble allergen reduction compared to roasting in this study, probably due to the higher heat penetration. HP boiling appeared to reduce the water-soluble allergen in a manner similar to roasting.

3.4.5 Quantitative analysis of SDS-sample buffer soluble allergen and kinetic analysis of total extractable peanut allergen under various processing methods

SDS-sample buffer soluble protein of the raw peanuts was 26% of the total protein (Table 3.1). The sum of water-soluble and SDS-sample buffer soluble protein was 98%, which was equivalent to the total protein from the raw peanuts. Quantitative analysis of SDS-sample buffer soluble peanut allergen is presented in Figure 3.6. Compared with Figure 3.5, the SDS-sample buffer soluble peanut allergen did not appear to increase at the same level as that of the decreases in the water-soluble peanut allergen.
with the processing time. This may be due to the chemical modifications or conformational changes in the allergens which can result in aggregate formation and smears, which were difficult to quantify. The quantification of smears is difficult due to the large area occupied and the lack of distinct bands. The aggregates at the top of stacking gels and separating gels were quantified and shown in Figure 3.7. All the methods resulted in higher amount of aggregates at the end points of processing. Frying-8 min resulted in the highest amount of aggregates (about 1.72g/100 g peanut). However, the end points of wet processing (with/without HP) methods resulted in lowest amount of aggregates (about 0.63g/100g peanut).

There were still a certain amount (as much as 55% of total protein, Table 3.1) of highly insoluble protein residues in the peanut powders after a two-step extraction (sodium phosphate buffer extraction followed by standard electrophoresis SDS-sample buffer extraction). The highly insoluble protein residues may bind tightly with carbohydrates or lipids after the heat processing (Chung & Reed, 2014). Total extractable peanut allergen content was calculated by taking the sum of water-soluble (extracted by sodium phosphate buffer) (Figure 3.5) and water-insoluble (but extracted by standard electrophoresis SDS-sample buffer) (Figure 3.6) allergen content. The plotted graph of the processing effect on total extractable allergen content is shown in Figure 3.8. The total extractable allergen (percentage of total peanut protein, sum of Ara h 1, Ara h 2 and Ara h 3) content (data are not shown) was lower in processed peanuts (12-46%) than in raw peanuts (65%). The total extractable allergen content (percentage of total protein content) at the end of processing point for various processed peanuts exhibited a descending order: microwaving (46%), steaming (34%), boiling (29%), HP steaming
(25%), HP boiling (23%), roasting (21%) and frying (15%). Burnt peanut (Table 3.1) processed by roasting (16 min) and frying (8 min) had lower total allergen content. The result could partially explain the processing effect on the protein structure changes.

The shape of each plotted graph under various processing methods (Figure 3.8) was similar to Figure 3.5, which was considered to fit first-order reactions. The simple linear regression model was fitted and conducted by using time (min) as variate and log transformed total extractable allergen content (g/100g peanut, dry basis) as response. Roasting, microwaving, frying and HP boiling fitted the model well on all three major allergens with adjusted R square higher than 0.85 (P<0.05) (Table 3.3). Boiling fitted the model well on Ara h 1 and Ara h 3. Steaming fitted the model well on Ara h 2 and Ara h 3. HP steaming did not fit well on all the major allergens, which was similar to the previously part (water-soluble allergen). Frying showed the highest R square (0.99) in both (water-soluble and total extractable allergen) kinetic analyses, showing the high predictable possibility using the first-order reaction model.

3.4.6 Water-soluble and SDS-sample buffer soluble allergen IgE binding properties under various processing methods

From the western-blot figures (Figure 3.2 and 3.4), the bands of Ara h 2 (both isoforms at 19 kD and 17 kD) were clearer than the other two allergens (Ara h 1 and Ara h 3), which were difficult to quantify due to smears and less binding by the IgE from human plasma. Therefore, only the IgE binding to Ara h 2 was quantified here (Figure 3.9, 3.10). It is necessary to note the IgE binding property evaluated in this study were mostly based on the binding property of linear epitopes, due to the SDS-sample buffer soluble allergen was already denatured after extraction. In addition, the protein after
mixing with sample-buffer for reducing SDS-PAGE also had denatured. The reducing reagent (2-Mercaptoethanol) used for extraction and SDS-PAGE may have altered the conformation of the protein, which made it difficult to evaluate the IgE-bindings of the conformational epitopes. IgE binding to the water-soluble Ara h 2 under the best selected conditions of each processing method is shown in Figure 3.9. Compared to untreated peanut, after roasting for 12 min, the IgE binding to Ara h 2 (19 kD), Ara h 2 (17 kD) was retained 71% and 59%, respectively, which were significantly higher than the other methods except microwaving. Microwaving for 3 min also retained relatively higher degree of IgE binding to Ara h 2 with about 50% of the raw peanut for both isoforms. The IgE binding to the Ara h 2 in the SDS-sample buffer soluble fractions, under various processing methods is shown in Figure 3.10. The Ara h 2 in almost all of the processing methods tested had greater IgE binding properties compared with the control, except for frying and HP steaming. Frying for 6 min reduced the IgE binding to distinct (unsmearred) Ara h 2 (19 kD) and Ara h 2 (17 kD) to 70% and 38%, respectively. HP steaming for 15 min reduced the IgE binding properties to distinct (unsmearred) Ara h 2 (19 kD) to 75%. The results are consistent with previous observations that the proteins aggregate and fall out of solution gradually becoming less visible on SDS-PAGE gel in the water-soluble fractions. Also frying peanuts for 6 min caused break down, cross-linking and aggregation of Ara h 2 (similar to Ara h 1 and Ara h 3) in the SDS-sample buffer soluble fractions and therefore diffusion of the IgE binding to the distinct bands over time. High level of aggregation can be seen in 6 and 8 minute lanes of the fried peanuts (Figure 3.3), all of which is due, in part, to the Maillard reaction. It is reported that the structure of Ara h 1 after roasting (Maillard reaction) is retained and with better
IgE-binding properties, which may be due to alterations such as chemical modifications to single amino acids or increased epitope exposure (Nesbit et al., 2012). The Ara h 2 also had higher IgE-binding properties after the Maillard reaction (Maleki, Chung, Champagne, & Khalifah, 2001), and with higher anti-trypsin digestibility (Maleki et al., 2003). While the IgE binding to the Ara h 2 isoforms is diffusing, for the purposes of our assessments we showed this as a decreased in IgE binding. However, roasted peanut possess higher IgE binding properties of Ara h 2 in both water-soluble and SDS-sample buffer soluble extracts than peanut treated by most of the processing methods, which may be due to the Mallard reaction (Beyer et al., 2001; Maleki, Chung, Champagne, & Raufman, 2000) that had been reported to enhance IgE binding. If the peanuts were roasted for longer times, the Ara h 2 bands would mimic what is seen in the peanuts fried for 8 min. Another recent study (Rao, Tian, Tao, Tang, Li, & Xue, 2016), showed that proteins in the roasted peanuts (130 °C, 20 min) were collectively less structured when compared with lower roasting temperatures and boiled peanuts. However, no conclusions had been given about the structure of individual allergens based on that study. Meanwhile in the same study, the antibody-reactivity of high temperature roasted peanut (>130 °C) was stronger than others (peanut roasted at ≤130 °C) (Rao, Tian, Tao, Tang, Li, & Xue, 2016).

HP boiling (Figure 3.10) resulted in higher IgE binding to the allergens in the SDS-sample buffer soluble fractions than most of the processing methods tested in our study. However, HP processing [at 2.56 atm (37.6 psi) for 30 min] had previously been shown to reduce the IgE-binding capacity significantly (Cabanillas et al., 2012). In our study, the processing conditions were much milder (1.02 atm, 40 min), which may not
alter the allergen structure enough to alter IgE binding. Similar to this earlier study (Cabanillas et al., 2012) with mild HP processing conditions [1.18 atm (17.3 psi), 30 min] the SDS-sample buffer soluble allergen IgE binding capacity was not decreased significantly. In contrast, HP steaming for 20 min resulted in lower IgE binding properties than HP boiling for 40 min and steaming for 180 min. The reason may be due to the combination of pre-soaking and high pressure that led to the alterations of the proteins to reduce IgE bindings. The objective of this study was to analyze the peanut allergen changes under various processing conditions that would maintain peanuts in palatable condition. Peanut after HP boiling (40 min) and HP steaming (20 min) are very soft and started to be overcooked. Higher processing temperature (pressure) and longer processing time may decrease the water-soluble peanut allergen content and overall binding properties more thoroughly but may damage the food quality of peanuts. Thermal processing could alter the conformational epitopes of allergens, which could enhance, reduce or maintain the allergenic potential (Chung & Reed, 2014).

Besides peanut, it is had also been reported that many milk allergenic proteins could be structurally changed after thermal processing and are heat sensitive in the order of immunoglobulins (Ig) < bovine serum < albumin (BSA) < β-LG and α-LA (Bu, Luo, Chen, Liu, & Zhu, 2013). Caseins are heat-stable due to lack of secondary, tertiary and quaternary structures that can be disrupted by heating, indicating that heating of milk can only partly reduce its allergenicity (Verhoeckx et al., 2015). Ehn et al. (2004) found that the IgE binding ability of β-LG after heating β-LG solution or milk at 74 °C was only decreased slightly, whereas the IgE binding ability of β-LG was decreased significantly after heating at 90 °C by means of enzyme-linked immunosorbent assay (ELISA)
Inhibition study. In the study of Bu et al. (2009), the effects of heat treatment on the antigenicity of α-LA and β-LG in whey protein isolate (WPI) by means of in vitro competitive ELISA inhibition tests was evaluated and the WPI was dissolved in water. It is found that the antigenicity of α-LA and β-LG increased along with temperature increased from 50 to 90 °C. However, antigenicity of both proteins decreased significantly, when the heating temperature was above 90 °C. When treated at 120 °C for 20 min, the antigenicity of α-LA decreased by 25% compared with the untreated sample (Bu et al., 2009). The increase in whey protein antigenicity from 50 to 90 °C may be accounted for the exposure of allergenic epitopes buried inside the native molecule resulted from unfolding of conformational structure during heat denaturation (Kleber & Hinrichs 2007). The decrease of antigenicity at above 90 °C may be due to the destruction or masking of conformational epitopes exposed to the molecule surface by the aggregation of sulfhydryl/disulfide exchange and subsequent (Kleber & Hinrichs 2007). Under more severe thermal processing conditions, the Maillard reaction may lead to loss of linear epitopes, which resulted in reduced antigenic response (Davis and Williams 1998; Fritsche 2003). All factors of the processing method, the temperature, and the time can affect the allergen structure and IgE binding capacity.

In our study, the IgE-binding property majorly represent the linear epitopes. Since during the extraction procedure, SDS-sample buffer (containing DTT) was used so that structure of the processed protein was altered, which made it difficult to analyze the conformational epitopes IgE-binding properties. In the future study, we could apply the purification method (Nesbit et al., 2012) to obtain purified allergen without using reducing buffer for conformational epitopes IgE-binding analysis. In the study of Nesbit
and coworkers (2012), the purified Ara h 1 after thermal processing become denatured and unfolded, and lost significant IgE binding compared to folded Ara h 1. Compared with the purified protein system, the whole food approach may be different since the water content is low and there are many other constituents present in the peanuts, and that conformational changes may not occur exactly as it is in a purified protein system. Therefore, more studies with whole food systems using novel methods are needed in the future to understand the mechanism of the allergenicity changes in this more complicated scenario after thermal processing.

3.5 Conclusions

We believe that food industries would like to develop hypoallergenic food. Our results showed that normal thermal processing would not significantly reduce or eliminate the IgE binding properties of peanuts. Thermal processing can alter the structure of allergen (protein) which may be reflected by the decreased solubility and changes in the appearance of the protein bands (formation of protein aggregates and smears) on SDS-PAGE. The relationship between thermal processing and major allergen content may, to some extent, be explained by a kinetic model, which would help us to predict and compare the quantity and efficiency of water-soluble/total extractable allergens and proteins. The proximate analysis of the processed peanut showed that wet processing increased moisture content and decreased lipid content. The protein content was minimally changed among all the processing methods. Among all methods with optimal processing point, frying for 6 min was the only process which greatly reduced both water-soluble and SDS-sample buffer soluble Ara h 2 IgE binding properties among the seven processing methods studied, although this observation may be due to diffusion...
of the distinct bands from a combination of degradation, modification and oligomerization.

In summary, our results showed the processing effect on water-soluble/SDS-sample buffer soluble and total extractable allergens, and demonstrated the potentially predictive, kinetic models can be developed for most of the cases to characterize alteration of allergen in peanuts during processing. The proximate analysis provided very useful references to understand the proximate mass changes under different thermal processing methods. HP steaming (with presoaking) resulted in lower IgE binding properties compared with regular steaming and HP boiling. This study may provide valuable information for the peanut processing industry to further improve processing strategies, such as using the combinations of selected methods to reduce the allergenicity of peanut allergens. However, the allergenic properties of the residue after SDS-buffer extraction was not analyzed in the current study. Further study is needed to analyze the total allergenic properties of the whole peanut.
Table 3.1 The proximate composition and soluble/insoluble fractions (protein) of peanut (Virginia) under various thermal processing methods.

<table>
<thead>
<tr>
<th>Processing method/ time (min)</th>
<th>Palatability condition</th>
<th>Moisture*</th>
<th>Lipid**</th>
<th>Protein**</th>
<th>Ash**</th>
<th>Carbohydrate**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Raw</td>
<td>4.95±0.09 h</td>
<td>47.82±0.11 abc</td>
<td>26.63±0.12 abc</td>
<td>2.92±0.01 de</td>
<td>22.63±0.61 jk</td>
</tr>
<tr>
<td>M0.75</td>
<td>Not fully cooked</td>
<td>4.79±0.50 h</td>
<td>47.52±3.09 abc</td>
<td>25.55±0.06 ghi</td>
<td>2.82±0.63 def</td>
<td>24.11±1.95 ghijk</td>
</tr>
<tr>
<td>M1.5</td>
<td>Not fully cooked</td>
<td>4.63±0.34 h</td>
<td>47.12±0.39 abcde</td>
<td>24.92±0.03 klmn</td>
<td>2.80±0.01 def</td>
<td>25.16±2.67 fghij</td>
</tr>
<tr>
<td>M2.25</td>
<td>Well cooked</td>
<td>4.67±0.45 h</td>
<td>48.12±0.39 ab</td>
<td>26.05±0.19 defg</td>
<td>3.03±0.27 cde</td>
<td>22.80±2.19 ijk</td>
</tr>
<tr>
<td>M3</td>
<td>Well cooked</td>
<td>4.5±0.08 h</td>
<td>48.17±1.50 ab</td>
<td>25.12±0.30 jkl</td>
<td>2.90±0.10 def</td>
<td>23.81±1.09 hijk</td>
</tr>
<tr>
<td>R4</td>
<td>Not fully cooked</td>
<td>5.20±0.09 h</td>
<td>47.39±1.20 abcd</td>
<td>25.01±0.39 klm</td>
<td>1.98±0.09 ghi</td>
<td>25.61±0.67 efgh</td>
</tr>
<tr>
<td>R8</td>
<td>Not fully cooked</td>
<td>5.10±0.29 h</td>
<td>47.53±1.44 abc</td>
<td>24.68±0.24 lmn</td>
<td>2.04±0.01 ghi</td>
<td>25.76±0.78 efgh</td>
</tr>
<tr>
<td>R12</td>
<td>Well cooked</td>
<td>4.73±0.03 h</td>
<td>48.38±1.36 ab</td>
<td>26.95±0.10 a</td>
<td>1.99±0.05 gh</td>
<td>22.68±0.86 jk</td>
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<tr>
<td>R16</td>
<td>Little burnt</td>
<td>5.00±0.36 h</td>
<td>48.76±0.06 a</td>
<td>26.84±0.04 ab</td>
<td>2.44±0.39 fg</td>
<td>21.96±1.68 k</td>
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<tr>
<td>HPB10</td>
<td>Not fully cooked</td>
<td>21.14±1.92 f</td>
<td>45.34±0.96 cdefgh</td>
<td>25.09±0.37 jkl</td>
<td>3.02±0.02 cde</td>
<td>26.55±1.90 defg</td>
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<td>HPB20</td>
<td>Not fully cooked</td>
<td>23.40±0.14 cd</td>
<td>44.39±0.13 fgh</td>
<td>25.53±0.27 hij</td>
<td>2.04±0.29 gh</td>
<td>28.04±1.56 cde</td>
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<tr>
<td>HPB30</td>
<td>Well cooked</td>
<td>25.70±0.27 b</td>
<td>43.12±0.96 hij</td>
<td>25.30±0.04 ijk</td>
<td>1.35±0.01 ij</td>
<td>30.23±0.45 abc</td>
</tr>
<tr>
<td>HPB40</td>
<td>Well cooked</td>
<td>27.95±0.08 a</td>
<td>43.01±4.80 hij</td>
<td>26.13±0.16 cdef</td>
<td>1.09±0.18 ij</td>
<td>29.76±0.28 abc</td>
</tr>
<tr>
<td>HPS5</td>
<td>Not fully cooked</td>
<td>21.91±0.17 ef</td>
<td>45.34±0.26 cdefgh</td>
<td>26.28±0.58 cde</td>
<td>3.73±0.36 b</td>
<td>24.65±1.30 fghij</td>
</tr>
<tr>
<td>HPS10</td>
<td>Not fully cooked</td>
<td>24.25±0.27 c</td>
<td>45.97±0.43 bcdefg</td>
<td>26.83±0.19 ab</td>
<td>1.60±0.25 hi</td>
<td>25.60±2.02 efgh</td>
</tr>
<tr>
<td>HPS15</td>
<td>Not fully cooked</td>
<td>24.30±0.50 c</td>
<td>43.93±0.10 ghij</td>
<td>25.90±0.76 defgh</td>
<td>2.04±0.29 gh</td>
<td>28.12±1.76 cde</td>
</tr>
<tr>
<td>HPS 20</td>
<td>Well cooked</td>
<td>24.07±1.15 cd</td>
<td>41.14±0.54 j</td>
<td>25.89±0.42 defgh</td>
<td>2.11±0.06 g</td>
<td>30.86±0.77 ab</td>
</tr>
</tbody>
</table>

The results are the means of 3 determinations ± SD within a column followed by different letters are significantly different (P < 0.05). *Expressed on wet basis (g/100g peanut). **Expressed on dry basis (g/100g peanut). ***Expressed on dry basis (g/100g total protein). Defatted peanut powder firstly extracted by sodium phosphate buffer to obtain water-soluble fraction; the pellet was then extracted by SDS-sample buffer to obtain SDS-sample buffer soluble fraction. M: Microwaving; R: Roasting; HPB: High-pressure boiling; HPS: High-pressure steaming; DF: Deep frying; B: Boiling; S: Steaming.
Table 3.1 (continued)

<table>
<thead>
<tr>
<th>Processing method/time (min)</th>
<th>Palatability condition</th>
<th>Moisture*</th>
<th>Lipid**</th>
<th>Protein**</th>
<th>Ash**</th>
<th>Carbohydrate**</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF2</td>
<td>Not fully cooked</td>
<td>2.98±0.11 i</td>
<td>47.41±2.15 abc</td>
<td>24.90±0.23 klmn</td>
<td>3.42±0.11 bc</td>
<td>24.27±2.34 fghijk</td>
</tr>
<tr>
<td>DF4</td>
<td>Well cooked</td>
<td>2.31±0.31 ij</td>
<td>48.04±2.14 abc</td>
<td>24.03±0.10 op</td>
<td>3.40±0.21 bc</td>
<td>24.53±0.78 fghijk</td>
</tr>
<tr>
<td>DF6</td>
<td>Well cooked</td>
<td>1.60±0.06 j</td>
<td>48.09±1.83 ab</td>
<td>25.09±0.23 jkl</td>
<td>3.26±0.70 bcd</td>
<td>23.56±1.34 hijk</td>
</tr>
<tr>
<td>DF8</td>
<td>Start to burn</td>
<td>1.54±0.10 j</td>
<td>48.26±0.73 ab</td>
<td>24.53±0.25 mno</td>
<td>2.99±0.02 cde</td>
<td>22.42±2.56 ghijk</td>
</tr>
<tr>
<td>B30</td>
<td>Not fully cooked</td>
<td>19.47±0.37 g</td>
<td>46.65±0.82 abcdefg</td>
<td>26.32±0.14 cde</td>
<td>3.65±0.61 b</td>
<td>23.38±0.89 hijk</td>
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<td>B60</td>
<td>Well cooked</td>
<td>21.27±0.30 f</td>
<td>46.34±4.08 abcdefg</td>
<td>26.36±0.38 bcd</td>
<td>2.05±0.07 gh</td>
<td>25.25±0.69 fghij</td>
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<td>B90</td>
<td>Well cooked</td>
<td>24.14±1.88 c</td>
<td>46.97±0.23 abcdef</td>
<td>26.19±0.02 cdef</td>
<td>1.44±0.01 ij</td>
<td>25.39±2.21 fghi</td>
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<tr>
<td>B120</td>
<td>Over cooked</td>
<td>25.88±1.99 b</td>
<td>44.47±0.60 efgh</td>
<td>25.70±0.13 fghi</td>
<td>1.36±0.27 ij</td>
<td>28.46±1.34 bcd</td>
</tr>
<tr>
<td>S30</td>
<td>Not fully cooked</td>
<td>22.93±0.79 de</td>
<td>44.78±0.66 defgh</td>
<td>24.90±0.19 klmn</td>
<td>4.57±0.57 a</td>
<td>25.76±2.09 efgh</td>
</tr>
<tr>
<td>S60</td>
<td>Well cooked</td>
<td>23.49±0.59 cd</td>
<td>44.19±1.35 gh</td>
<td>25.82±0.53 efgh</td>
<td>3.10±0.05 cde</td>
<td>26.90±2.89 def</td>
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<tr>
<td>S90</td>
<td>Well cooked</td>
<td>24.24±0.34 c</td>
<td>42.96±2.03 hij</td>
<td>23.71±0.29 p</td>
<td>2.90±0.31 def</td>
<td>30.44±1.40 abc</td>
</tr>
<tr>
<td>S120</td>
<td>Soft</td>
<td>24.30±0.28 c</td>
<td>41.30±0.87 ij</td>
<td>24.50±0.53 no</td>
<td>2.77±0.20 ef</td>
<td>31.43±0.99 a</td>
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</table>

The results are the means of 3 determinations ± SD within a column followed by different letters are significantly different (P < 0.05). *Expressed on wet basis (g/100g peanut). **Expressed on dry basis (g/100g peanut). ***Expressed on dry basis (g/100g total protein). Defatted peanut powder firstly extracted by sodium phosphate buffer to obtain water-soluble fraction; the pellet was then extracted by SDS-sample buffer to obtain SDS-sample buffer soluble fraction. M: Microwaving; R: Roasting; HPB: High-pressure boiling; HPS: High-pressure steaming; DF: Deep frying; B: Boiling; S: Steaming.
<table>
<thead>
<tr>
<th>Processing method/ time (min)</th>
<th>Water-soluble fractions</th>
<th>SDS-sample buffer soluble fractions</th>
<th>Processing method/ time (min)</th>
<th>Water-soluble fractions</th>
<th>SDS-sample buffer soluble fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>72.66±6.21 a</td>
<td>27.22±2.08 i</td>
<td>HPS10</td>
<td>9.95±0.67 nopq</td>
<td>38.53±3.1 fgh</td>
</tr>
<tr>
<td>M0.75</td>
<td>46.98±3.45 c</td>
<td>44.54±3.2 de</td>
<td>HPS15</td>
<td>9.28±0.62 opq</td>
<td>48.48±3.43 bcd</td>
</tr>
<tr>
<td>M1.5</td>
<td>46.38±3.65 cd</td>
<td>44.91±3.34 cde</td>
<td>HPS 20</td>
<td>8.75±0.45 pq</td>
<td>50.13±4.03 bc</td>
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<tr>
<td>M2.25</td>
<td>40.89±3.28 e</td>
<td>44.72±3.52 cde</td>
<td>DF2</td>
<td>38.42±3.02 ef</td>
<td>41.12±3.12 efg</td>
</tr>
<tr>
<td>M3</td>
<td>33.72±2.64 g</td>
<td>48.52±3.73 bcd</td>
<td>DF4</td>
<td>20.99±1.37 jk</td>
<td>34.25±2.45 h</td>
</tr>
<tr>
<td>R4</td>
<td>52.35±3.79 b</td>
<td>28.51±1.89 i</td>
<td>DF6</td>
<td>11.45±0.78 mnop</td>
<td>37.21±2.88 gh</td>
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<tr>
<td>R8</td>
<td>51.41±3.98 b</td>
<td>29.65±1.97 i</td>
<td>DF8</td>
<td>6.38±0.46 q</td>
<td>34.55±2.65 h</td>
</tr>
<tr>
<td>R12</td>
<td>36.65±2.89 g</td>
<td>31.36±2.76 hi</td>
<td>B30</td>
<td>46.53±3.66 cd</td>
<td>37.21±2.69 gh</td>
</tr>
<tr>
<td>R16</td>
<td>23.85±1.96 ij</td>
<td>33.39±2.65 h</td>
<td>B60</td>
<td>24.67±1.84 ij</td>
<td>43.45±3.44 def</td>
</tr>
<tr>
<td>HPB10</td>
<td>25.54±2.01 hi</td>
<td>46.90±3.73 bcd</td>
<td>B90</td>
<td>18.89±1.49 kl</td>
<td>50.51±4.01 b</td>
</tr>
<tr>
<td>HPB20</td>
<td>19.64±1.55 kl</td>
<td>47.88±3.76 bcd</td>
<td>B120</td>
<td>14.01±1.01 mn</td>
<td>48.85±3.77 bcd</td>
</tr>
<tr>
<td>HPB30</td>
<td>18.25±1.43 kl</td>
<td>48.14±3.75 bcd</td>
<td>S30</td>
<td>42.51±3.29 de</td>
<td>51.97±3.99 b</td>
</tr>
<tr>
<td>HPB40</td>
<td>15.55±1.21 lm</td>
<td>59.44±3.99 a</td>
<td>S60</td>
<td>29.10±2.01 h</td>
<td>58.69±4.59 a</td>
</tr>
<tr>
<td>HPS5</td>
<td>13.18±1.01 m</td>
<td>50.58±3.54 b</td>
<td>S90</td>
<td>9.80±0.68 opq</td>
<td>60.05±4.55 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S120</td>
<td>9.05±0.71 pq</td>
<td>60.42±4.73 a</td>
</tr>
</tbody>
</table>

The results are the means of 3 determinations ± SD within a column followed by different letters are significantly different (P < 0.05). *Expressed on wet basis (g/100g peanut). **Expressed on dry basis (g/100g peanut). ***Expressed on dry basis (g/100g total protein). Defatted peanut powder firstly extracted by sodium phosphate buffer to obtain water-soluble fraction; the pellet was then extracted by SDS-sample buffer to obtain SDS-sample buffer soluble fraction. M: Microwaving; R: Roasting; HPB: High-pressure boiling; HPS: High-pressure steaming; DF: Deep frying; B: Boiling; S: Steaming.
Table 3.2  Rate constant, adjusted R square and P value of water-soluble Ara h 1, Ara h 2 and Ara h 3 content under different processing methods based on the first order assumption (calculated from the data of Figure 3.5).

<table>
<thead>
<tr>
<th>Processing method</th>
<th>Allergen</th>
<th>Rate constant(K)</th>
<th>R square</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microwaving</td>
<td>Ara h 1</td>
<td>0.18</td>
<td>0.96</td>
<td>0.009**</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.12</td>
<td>0.98</td>
<td>0.04*</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.1</td>
<td>0.98</td>
<td>0.003**</td>
</tr>
<tr>
<td>Roasting</td>
<td>Ara h 1</td>
<td>0.05</td>
<td>0.93</td>
<td>0.024*</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.02</td>
<td>0.98</td>
<td>0.005**</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.03</td>
<td>0.95</td>
<td>0.012*</td>
</tr>
<tr>
<td>HP boiling</td>
<td>Ara h 1</td>
<td>0.05</td>
<td>0.96</td>
<td>0.008**</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.03</td>
<td>0.85</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.05</td>
<td>0.98</td>
<td>0.003**</td>
</tr>
<tr>
<td>HP steaming</td>
<td>Ara h 1</td>
<td>0.13</td>
<td>0.8</td>
<td>0.104</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.05</td>
<td>0.8</td>
<td>0.102</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.26</td>
<td>0.72</td>
<td>0.17</td>
</tr>
<tr>
<td>Frying</td>
<td>Ara h 1</td>
<td>0.24</td>
<td>0.99</td>
<td>0**</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.13</td>
<td>0.99</td>
<td>0.001**</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.14</td>
<td>0.99</td>
<td>0.001**</td>
</tr>
<tr>
<td>Boiling</td>
<td>Ara h 1</td>
<td>0.01</td>
<td>0.96</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.01</td>
<td>0.87</td>
<td>0.013*</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.01</td>
<td>0.99</td>
<td>0.001**</td>
</tr>
<tr>
<td>Steaming</td>
<td>Ara h 1</td>
<td>0.14</td>
<td>0.88</td>
<td>0.027**</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.01</td>
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<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.01</td>
<td>0.96</td>
<td>0.008**</td>
</tr>
</tbody>
</table>

* P value less than 0.05. ** P value less than 0.01.

Log[allergen content] = -Kt + b. K represents constant rate.
Table 3.3  Rate constant (K), R square and P value of total extractable Ara h 1, Ara h 2 and Ara h 3 subunit content under different processing methods based on the first order assumption (calculated from the data of Figure 3.8).

<table>
<thead>
<tr>
<th>Processing method</th>
<th>Allergen</th>
<th>rate constant(K)</th>
<th>R square</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microwaving</td>
<td>Ara h 1</td>
<td>0.18</td>
<td>0.96</td>
<td>0.009**</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.07</td>
<td>0.99</td>
<td>0**</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.03</td>
<td>0.94</td>
<td>0.017*</td>
</tr>
<tr>
<td>Roasting</td>
<td>Ara h 1</td>
<td>0.05</td>
<td>0.94</td>
<td>0.017*</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.02</td>
<td>0.97</td>
<td>0.008**</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.03</td>
<td>0.95</td>
<td>0.006**</td>
</tr>
<tr>
<td>HP boiling</td>
<td>Ara h 1</td>
<td>0.02</td>
<td>0.94</td>
<td>0.018*</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.03</td>
<td>0.88</td>
<td>0.044*</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.01</td>
<td>0.89</td>
<td>0.05*</td>
</tr>
<tr>
<td>HP steaming</td>
<td>Ara h 1</td>
<td>0.02</td>
<td>0.65</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.02</td>
<td>0.66</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.01</td>
<td>0.60</td>
<td>0.28</td>
</tr>
<tr>
<td>Frying</td>
<td>Ara h 1</td>
<td>0.16</td>
<td>0.99</td>
<td>0.001**</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.10</td>
<td>0.99</td>
<td>0**</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.06</td>
<td>0.99</td>
<td>0.001**</td>
</tr>
<tr>
<td>Boiling</td>
<td>Ara h 1</td>
<td>0.002</td>
<td>0.87</td>
<td>0.05*</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.002</td>
<td>0.75</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.002</td>
<td>0.97</td>
<td>0.006**</td>
</tr>
<tr>
<td>Steaming</td>
<td>Ara h 1</td>
<td>0.002</td>
<td>0.81</td>
<td>0.094</td>
</tr>
<tr>
<td></td>
<td>Ara h 2</td>
<td>0.003</td>
<td>0.97</td>
<td>0.008**</td>
</tr>
<tr>
<td></td>
<td>Ara h 3</td>
<td>0.001</td>
<td>0.90</td>
<td>0.04*</td>
</tr>
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</table>

* P value less than 0.05. ** P value less than 0.01.

Log[allergen content] = -Kt + b. K represents constant rate.
Figure 3.1  SDS-PAGE analysis of the various thermal processing effects on water-soluble peanut (Virginia) protein.

Seven processing methods were conducted at 5 time intervals, respectively. The processing methods and time (min) are shown below corresponding gel, respectively. M represents the molecular mass marker (molecular masses are shown beside the markers). Major allergen of Ara h 1 (63 kD), Ara h 2 (17, 19kD), Ara h 3 (22, 38, 40 kD) and Ara h 6 (14 kD) are indicated on the right.
Figure 3.2 Western-blot analysis of the various thermal processing effects on water-soluble peanut (Virginia) protein.

Seven processing methods were conducted at 5 time intervals, respectively. The processing methods and time (min) were labeled below the corresponded membranes, respectively. Molecular masses are shown on the left. Major allergens of Ara h 1 (63 kD), Ara h 2 (17, 19 kD), and Ara h 6 (14 kD) are indicated on the right. Pooled human plasma containing IgE antibody against peanut allergens were used for analysis.
Figure 3.3  SDS-PAGE analysis of the various thermal processing effects on SDS-sample buffer peanut (Virginia) protein.

Seven processing methods were conducted at 5 time intervals, respectively. The processing methods and time (min) are shown below corresponding gel, respectively. M represents the molecular mass marker (molecular masses are shown beside the markers). Major allergen of Ara h 1 (63 kD), Ara h 2 (17, 19kD), Ara h 3 (22, 38, 40 kD) and Ara h 6 (14 kD) are indicated on the right.
Figure 3.4  Western-blot analysis of the various thermal processing effects on SDS-sample buffer soluble peanut (Virginia) protein.

Seven processing methods were conducted at 5 time intervals, respectively. The processing methods and time (min) were labeled below the corresponded membranes, respectively. Molecular masses are shown on the left. Major allergen of Ara h 1 (63 kD), Ara h 2 (17, 19kD), and Ara h 6 (14 kD) are indicated on the right. Pooled human plasma containing IgE antibody against peanut allergens were used for analysis.
Figure 3.5  Processing effect on peanut water-soluble allergen content (g/100g peanut dry basis) based on SDS-PAGE.

Quantification analysis of allergen content was based on the band intensities and protein content.
Figure 3.6  Processing effect on peanut SDS-sample buffer soluble allergen content (g/100g peanut dry basis) based on SDS-PAGE.

Quantification analysis of allergen content was based on the band intensities and protein content.
Figure 3.7  Processing effect on peanut water-insoluble (but SDS-sample buffer soluble) aggregate allergen content (g/100g peanut dry basis) based on SDS-PAGE.

Quantification analysis of aggregate content was based on the band intensities and protein content.
Figure 3.8  Processing effect on peanut total extractable allergen content (g/100g peanut dry basis) based on SDS-PAGE.

Total allergen content was the sum of water-soluble and SDS-sample buffer soluble allergen content.
Figure 3.9  Processing effect on water-soluble allergens (Ara h 2) IgE binding properties.

Control represents raw peanuts, all the processing methods’ time were selected with the optimal processing point (Table 3.1) in this study, respectively. Microwaving: 3 min; Roasting: 12 min; High-pressure boiling: 40 min; High-pressure steaming: 20 min; Deep frying: 6 min; Boiling: 90 min; Steaming: 90 min. The control (raw) peanuts’ total IgE binding properties for each of the Ara h 2 isoforms were set as 100 percent.
Figure 3.10  Processing effect on SDS-sample buffer soluble allergens (Ara h 2) IgE binding properties.

Control represents raw peanuts, all the processing methods’ time were selected with the optimal processing point (Table 3.1) in this study, respectively. Microwaving: 3 min; Roasting: 12 min; High-pressure boiling: 40 min; High-pressure steaming: 20 min; Deep frying: 6 min; Boiling: 90 min; Steaming: 90 min. The control (raw) peanuts’ total IgE binding properties for each of the Ara h 2 isoforms were set as 100 percent.
CHAPTER IV
PEANUT ALLERGEN REDUCING BY MEANS OF PEANUT SCREENING, ENZYMATIC HYDROLYSIS AND TRANSGLUTAMINASE

4.1 Abstract

Eleven peanut lines [Coded MS-1~MS-11, MS-9 (check) is a common cultivar namely Valencia] were pre-screened from 122 peanut lines harvested in 2015 for allergen levels. These pre-screened lines were re-planted in 2016 for further analysis. One line named MS-7 was selected for lower Ara h 1 (8.5-9.5% of total protein) and Ara h 2 (4.2-6.6% of total protein) content in 2015 and 2016. Roasted MS-9 (check) peanut powders were used for enzymatic treatment for enzyme selection. A first order kinetic reaction model was conducted to describe the relationship between enzyme concentration (0-400AzU/g) and IgE-binding property reduction. Among eight food-grade enzymes, bromelain, papain and ficin hydrolysates had lower IgE-binding properties in terms of high IgE-binding property reducing rate (K) and were selected for the following study. MS-7 (selected) & MS-9 (at level of 200AzU/g) hydrolyzed by three selected enzymes (200AzU/g) were used for IgE binding property comparison, TGase crosslinking and functional properties study. After hydrolyzed by the selected enzymes (200 AzU/g), the emulsion and foaming stabilities were decreased. Emulsion and foaming stabilities were increased in TGase (5U/g protein) crosslinked hydrolysates. The IgE-binding properties
of TGase treated hydrolysates were similar to the hydrolysates without TGase treatment. MS-7 hydrolysates (with/without TGase) possessed less IgE-binding properties and similar functionality as compared with MS-9 hydrolysates.

**Keywords:** Peanut allergen, peanut screening, enzymatic processing, IgE-binding properties, papain, ficin, bromelain, transglutaminase (TGase), functional properties.

### 4.2 Introduction

Peanut (*Arachis hypogaea* L.), a seed crop legume with high protein and sensory attributes is widely used for human food purpose (Koppelman et al., 2016). However, peanut allergy is considered to be one of the most severe food allergies owing to the persistency and the life-threatening potential (Cabanillas et al., 2012). In the Western countries, including USA and UK, about 1% of the population has been diagnosed with peanut allergy (Verhoeckx et al., 2015). Currently, the only treatment for allergy is total avoidance. However, totally avoiding peanuts is difficult due to its widespread use as a common food ingredient. Consequently, it would be meaningful to develop a possible strategy which can decrease the peanut allergen to a significantly lower level to ensure the safety of all customers.

It has been reported that the major allergen (*Ara h 1, Ara h 2, Ara h 3*) levels varied among hundreds of peanut cultivars (Kang, Gallo, & Tillman, 2007; Koppelman et al., 2016). The “core of core collection” (Mini core) including 112 accessions, which developed by Holbrook and Dong (2005), contains the genetic diversity of the entire 831 core accessions of U.S. germplasm collection. Previous study has been conducted on 99 of the 112 accessions for the quantification of allergen composition. Low *Ara h 1, Ara h 2* or *Ara h 3* level accessions have been found among the 99 Mini core collections (Kang,
In our study, 97 of the Mini core accessions and 25 accessions from other resources (harvested in Clovis, NM; 2015) were collected and analyzed to select a less allergen level peanut for this processing study.

Several methods, including thermal and nonthermal processing methods have been used for allergen reduction studies. Few of them have been proven to reduce the allergen content significantly to a lower level. Enzymatic processing treated with exogenous or digestive enzymes has been reported as a strategy to reduce the allergenicity of peanut proteins by hydrolyzing them into smaller peptides, which would significantly decrease IgE-binding properties (Cabanillas et al., 2012; Shi et al., 2013; Yu, Ahmedna, Goktepe, Cheng, & Maleki, 2011). Compared with digestive enzymes, food grade exogenous enzyme processing would be a more suitable choice due to more diversities and the lower cost of the enzymes. In order to make high quality hypoallergenic protein additives, comparing the allergen reduction extent by using food-grade enzyme on peanuts followed by functional properties testing is necessary. Currently, a comprehensive study dealing with both allergenicity and functional properties of peanut hydrolysates made from a wide array of enzymes is not available.

Protein hydrolysates may lose functional properties, depending on the type of enzyme and degree of hydrolysis. For example, if the protein is highly hydrolyzed, the high concentration of hydrolyzed protein would not adhere to the water-oil interface but saturated in the continuous phase, which resulted in decreased emulsion properties (Conde & Patino, 2007). The taste can become more bitter than intact protein extracts due to existing of hydrophobic amino acid residues after hydrolyzed (FitzGerald & O'cuinn, 2006). Transglutaminase (TGase; protein-glutamine γ-glutamyltransferase, EC 2.3.2.13)
catalyzes an acyl transfer reaction between the γ-carboxyamide group of a peptide-bound glutaminyl residue (acyl donors) and a variety of primary amines (acyl acceptors). The cross-linking reaction caused by TGase results in the polymerization of protein/peptide molecules with a significant increase in molecular mass. The modification of proteins by TGase has been studied extensively (Clare et al., 2008; Hu, Zhao, Sun, Zhao, & Ren, 2011; Agyare, Addo, & Xiong, 2009; Fan et al., 2005) and has been shown to be an effective method for improving the functional properties of proteins/hydrolysates. Until now, no study has been conducted on peanut hydrolysates that are crosslinked with TGase. In this study, TGase was applied on the peanut hydrolysates to test their allergenicity and functional properties.

The objectives of this study were to: 1) screen for lower allergen content peanut lines from 122 peanut lines harvested in Clovis, NM (2015); 2) select efficient food grade-enzymes for hydrolyzing peanut protein to further reduce allergen potency in roasted peanut powders; 3) test functional properties (emulsifying, foaming capacity and stability) after hydrolysis; and 4) test TGase for processing as a suitable treatment to improve functional properties of peanut hydrolysates.

4.3 Materials and methods

4.3.1 Materials

Peanuts (shell-removed) with 122 lines harvested in Clovis, NM (2015) were provided by New Mexico State University. Ninety-seven lines of these 122 germplasms belonged to the US Mini Core USDA- Griffin collection (with PI number). Twenty-five lines of them belong to other resources, including experimental lines, a check line (Valencia, code MS-9 in this study) and private company lines. Peanut lines with low
major allergen (Ara h 1, Ara h 2, or Ara h 3) levels were selected based on the analysis of SDS-PAGE. The screened peanuts (coded MS-1 ~ MS-11, including check line MS-9) were replanted and harvested in Clovis, NM (2016) to increase the quantity for enzymatic processing study.

Food-grade exogenous enzymes alcalase, neutrase, Novo-proD, and protamex were provided by Novozyme (Franklinton, NC). Papain, ficin, bromelain, and thermolysin were purchased from Sigma-Aldrich (St. Louis, MO). Human plasma from six individuals with only peanut allergy (IgE levels CAP-FEIA >100 kU/l; contained anti-Ara h 1, Ara h 2 and Ara h 3 antibodies) were purchased from PlasmaLab International (Everett, WA). All the other chemicals of the analytical grade were purchased from Sigma Company.

4.3.2 The protein extraction method for raw peanut

The protein extraction for raw peanut powder was according to the earlier study (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2009) with some modifications. Briefly, approximately 5 g of the pre-frozen peanuts were milled with Magic bullet blender (Model No. MBR-1101, Capbran Holdings, LLC, Los Angeles, CA) into coarse powders then about 4 g ground with mortar and pestle in liquid nitrogen into fine powder (could pass through 0.5 mm sieve). Five hundred milligrams of peanut powders in a 15 mL centrifuge tube were defatted by adding 10 mL acetone and shaken for 2 hr in an orbital shaker. The suspension was centrifuged (2795 g for 15 min) and the pellet was dried under an exhaust hood overnight at room temperature. The defatted flours were shaken in a 15-mL centrifuge tube with 3 mL of 0.02 M sodium phosphate, pH 8.5, plus 10 mM EGTA for 20 min at room temperature for 2 hr. The supernatant after 30-time dilution
was collected after centrifugation (2800 g) for 15 min at room temperature. The protein content of the obtained supernatant was determined by the Bradford’s method (Bradford, 1976).

### 4.3.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE method was modified according to earlier studies (Kang, Gallo, & Tillman, 2007) with a Mini Protein Tetra System (BioRad, Hercules, CA). Soluble protein extracts (adjusted to 2 mg/mL) were mixed with an equal volume of sample buffer containing 5% β-mercaptoethanol. The mixture was boiled for 5 min for denaturation. Electrophoresis was performed on 13.5 % (non-gradient) acrylamide gels for 1 hr at 50 V followed by 1.5 hr at 100 V. Protein Standard (ranges from 10-250 kDa, Product No.1610373, Bio-Rad, Hercules, CA) was used to identify and estimate the major allergens and their molecular weights. For the quantification of peanut major allergens (Ara h 1, Ara h 2 and Ara h 3), gels were scanned and analyzed by a Molecular Imager (Bio-Rad Chemidoc™ XRS+, Hercules, CA) equipped with Image Lab™ Analysis Software (version 5.2). The major allergens and their relative composition were calculated based on the band intensity and total area of their subunits. Allergen protein levels were determined and expressed as the percentage of total detectable protein.

### 4.3.4 Two-dimensional (2D) electrophoresis of screened peanut protein extracts

Two-dimensional electrophoresis was performed according to a previous study (Chassaigne, Trégoat, Nørgaard, Maleki, & van Hengel, 2009) with modifications. Peanut powder (0.5g, without defatting procedure) was extracted by 3mL 20mM TBS buffer, pH 7.4, plus 150 mM NaCl for 2 hr. The protein extractions (aqueous fractions)
were collected by centrifugation (2800 g) for 15 min at room temperature. Cleanup Kit (BioRad, Hercules, CA) was used to purify and precipitate the protein extractions for isoelectric focusing (IEF). Prepared proteins (150 microgram) were loaded onto each precast immobilized pH gradient (IPG) strip (pH 3–10, BioRad, Hercules, CA) and focused using a Protean IEF System (BioRad) overnight. After focusing, the proteins were reduced by immersing the IPG strips in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% dithiothreitol) for 15 min, and alkylated with 2.5% w/v iodoacetamide in equilibration buffer for 15 min. The IPG strips were then placed directly onto 13.5% polyacrylamide-SDS mini gels (8.6 x 6.8 cm) for the second-dimensional electrophoresis. After electrophoresis, the proteins in the gels were stained with Coomassie blue R-250 and then destained with a destaining solution (40% methanol and 10% acetic acid). After destaining, the gels were scanned and analyzed by a Molecular Imager (Bio-Rad Chemidoc™ XRS+, Hercules, CA) equipped with Image Lab™ Analysis Software (version 5.2). Each identical spot (based on the result of western-blot and the previous literature) was quantified with Image Lab™ Analysis Software and expressed as percentage of the total allergen content.

4.3.5 Pre-treatment (Roasting) on screened and check peanuts

Roasting (177 °C, 10 min) was conducted on the selected peanut (MS-7) and control peanut (MS-9) in a temperature controlled Oster® toaster oven (model TSSTTVSK01, Jarden Co., NY). The roasted peanuts were ground into fine powders (without defatting procedure) according to the same method mentioned above.
Food grade enzymes were applied on the roasted peanut powder for hydrolyses. The protein yield of roasted peanut was considered as 100 g; the protein yield of the hydrolysates/residues was expressed in red color. Water-soluble hydrolysates were used for TGase treatments.
4.3.6  Enzymatic processing methods and kinetic analysis

The method was according to Quist, Phillips, Saalia. (2012) with some modifications (Figure 4.1). Azocoll was used as standard substrate to measure the proteolytic activities of enzymes. The activity-measured enzymes were applied to enzymatic processing with five concentration levels (0, 100, 200, 300, 400 AzU/g) on roasted MS-9 peanut to understand the relationship between enzyme concentration and IgE-binding properties (tested by ELISA and described in the following sections) and conducting a kinetic reaction model (first order) to explain this relationship. At the level of 400 AzU/g, the weight percentage of enzymes (% of peanut powder’s protein) were listed as follows (E/Protein): 2.66% (papain); 9% (ficin); 10.8% (bromelain); 3.8% (neutrase); 0.06% (alcalase); 0.8% (protemax); 5.12*10^{-4}% (thermolysin); 5.2*10^{-2}% (Novo-ProD).

This model was conducted by using the concentration (AzU/g) as the variate and log transformed IgE binding properties (% of untreated roasted MS-9 peanut) as the response.

\[-d[IgE \text{ binding properties}] / dc = K[IgE \text{ binding properties}] \] \hspace{1cm} (1)

Integration from time 0 to c shows

\[-\int d[IgE \text{ binding properties}] / [IgE \text{ binding properties}] = \int K dc \] \hspace{1cm} (2)

Log[IgE binding properties]_{c} / Log[IgE binding properties]_{0} = -Kc \] \hspace{1cm} (3)

Log[IgE binding properties]_{c} = -Kc + b \] \hspace{1cm} (4)

K represents rate constant, which means log[IgE binding properties] reducing rate by adding 100 AzU/g of enzyme. C represents concentration (AzU/g).
The result was analyzed by using RStudio (Version 1.0.136, Boston, MA) to get the R square, constant rate K (slope) and P value.

From the five concentrations mentioned above, 200 AzU/g [median concentration] of papain, ficin and bromelain were selected for further analysis with both MS-7 and MS-9 peanut hydrolysis with/without TGase crosslinking. Three grams of roasted peanut powder were mixed with 27 mL of 0.01 M potassium phosphate buffer (pH 7.5, this neutral-skewed pH would be suitable for most of the enzymes) for pre-incubation. The mixed samples were pre-incubated at 50 °C for 30 min. The reaction was initiated by adding 3 mL of phosphate buffer with/without enzymes. The reaction was terminated by heating the mixture at 85 °C for 15 min after reacted for 2 hr. The mixture was centrifuged at 2780 g by the centrifuge (SorvallTM LegendTM X1, Thermo, Marietta, OH) for 15 min to collect supernatant (soluble fraction). The pelleted parts after centrifugation were mixed with standard electrophoresis SDS-sample buffer (1% SDS + 5 mM dithiothreitol) and boiled for 5 min to extract proteins from the insoluble residue. The samples in SDS-samples buffer were then centrifuged at 2795 g for 15 min. The collected supernatants in SDS-sample buffer (insoluble fractions) and soluble fractions were analyzed for allergenicity by western-blot. The soluble fractions were freeze-dried and used for functional properties analyses and TGase crosslinking.

4.3.7 Transglutaminase (TGase) processing on peanut hydrolysates and kinetic analysis

Three peanut hydrolysates (hydrolyzed by papain, ficin, bromelain at 200 AzU/g) were used for the TGase treatment. The processing method was based on a previous study (Hu, Zhao, Sun, Zhao, & Ren, 2011). Protein hydrolysates were added with TGase
at a protein to substrates ratio (E/S) of 0.5 U/g (which was selected from 0, 1, 5, 10 U/g in our preliminary study for having clear high molecular mass band at the top of the SDS-PAGE gel) and incubated at 37 °C for 2 hr. The reactions were stopped by immediately heating up to 85 °C for 15 min and then freeze-dried for functional properties analyses.

The functional properties of TGase (0, 2.5, 5, 7.5, 10 U/g) treated hydrolysates and peanut protein were analyzed and the relationship between TGase concentration (c) and functional properties (at pH 8) were described by a kinetic reaction model (Peleg, 1988).

\[ F(c) = F_0 + \frac{c}{(K_1 + K_2c)} \]  (1)

\[ F(c): \text{the functional properties when TGase’s concentration at } c \text{ Unit/g. } F_0 \text{ is the functional properties at TGase concentration } = 0, K_1 \text{ is Peleg’s rate constant and } K_2 \text{ is Peleg’s capacity constant. The equilibration value } F_{c \rightarrow \infty} \text{ could be shown as follow:} \]

\[ F_{c \rightarrow \infty} = F_0 + \frac{1}{K_2} \]  (2)

The Equation (1) could be transformed into a linear form as follow:

\[ \frac{c}{[F(c) - F_0]} = K_1 + K_2c \]  (3)

The result was analyzed by using RStudio (Version 1.0.136, Boston, MA) to get the R square and P value.

4.3.8 Western-blot Analysis

A two-dimensional gel of two peanut lines (MS-7 and MS-9, raw peanut) and a one-dimensional gel obtained from processed peanuts (MS-7 and MS-9) were analyzed by western-blot analysis using plasma containing IgE antibodies from peanut allergic individuals as described by an earlier study (Chung & Reed, 2012). In brief, protein extracts were transferred from SDS–PAGE gel to an Immobilon-P membrane.
membrane then was blocked with a SuperBlock solution (contains a proprietary protein in pH 7.4 PBS, Cat No. 37515, ThermoFisher, Waltham, MA) to provide low background interference. After blocking, the membrane was incubated for 1 hrs with a pooled plasma diluted 1:20 (v/v) in Superblock/TBS-Tween 20 (1:1, v/v). The membrane was washed with TBS-Tween 20 and incubated with a rabbit anti-human IgE-peroxidase (1:250) for 1 hr, and then washed with TBS-Tween 20 for 3 times (10 min each time). After washing, the membrane was then incubated in the SuperSignal™ West Pico Chemiluminescent Substrate (Fisher Scientific, Pittsburgh, PA) for 1 min to develop reaction for detection of horseradish peroxidase (HRP) activity from secondary antibodies. The incubated membrane was scanned and analyzed by a Molecular Imager (Bio-Rad Chemidoc™ XRS+, Hercules, CA) equipped with Image Lab™ Analysis Software (version 5.2).

4.3.9 **Indirect enzyme-linked immunoabsorbent assay (ELISA) Analysis**

The quantification of IgE binding properties was determined by ELISA with a slight modification of the method of Chung and Reed (2012). Briefly, a 96-well micro plate was coated by coating buffer (100mM sodium bicarbonate) at 37 °C for 2 hr with protein extracts/hydrolysates (20 μg/mL; 100 μL each). After incubation and washing with TBS/Tween 20 (0.05%), the plate was blocked with a Superblock buffer solution (200 μL per well) overnight at room temperature. After blocking and washing, a diluted pooled plasma containing IgE antibodies (1:20) (100 μL) from the peanut-allergic individuals was added to the plate and incubated for 30 min at room temperature. The plate was washed again with TBS-Tween 20 (0.05%), followed by the addition and incubation of a rabbit anti-human IgE peroxidase conjugate (1:500) for 30 min at room temperature. After incubation and wash, a substrate of o-phenylenediamine (OPD) (0.5
mg/mL), containing 0.03% hydrogen peroxide in 0.1 M citrate buffer, pH 5.5 was added. After 10 min, the color reaction was stopped by adding 4 N H$_2$SO$_4$ (50 μL). The absorbance was read at 490 nm. All values are mean of triplicate. The IgE binding properties of MS-9 roasted peanut extract (without enzyme treatments) was set as 100%.

4.3.10 Emulsifying properties

Emulsifying properties were determined based on the reported methods (Jamdar, Rajalakshmi, Pednekar, Juan, Yardi, & Sharma, 2010; Klompong, Benjakul, Kantachote, & Shahidi, 2007). The ability of a protein works as an emulsifier for emulsion formation is related to its ability of adsorbing to and stabilizing the oil-water interface. The emulsion capacity of a protein should be related to the interfacial area which can be coated by the available protein (Ivey et al., 1970). In a similar way, the emulsion stability should be related to the constancy of the interfacial area. The interfacial area could be calculated by the turbidity, which can be measured by spectrophotometer (Pearce & Kinsella, 1978). In brief, the corn oil (10 mL) and 30 mL of 1% protein solution dispersion (protein concentration was measured by Bradford’s method) were mixed with pH adjusted to 2, 4, 6, 8 and 10. The mixture was homogenized by a homogenizer (Omni International, Kennesaw, GA, USA) at 20,000 rpm for 1 minute. Fifty microliters of emulsion was pipetted from the bottom of the tube at 0 and 10 min after homogenization and followed by mixing with 5 mL of 0.1% SDS solution. The absorbance of the diluted solution was measured with a spectrophotometer at wavelength of 500 nm. The absorbance measured at 0 min ($A_0$) and 10 min ($A_{10}$) after emulsion forming were used to calculate the emulsifying activity index (EAI) and emulsion stability (ESI) as follows:

$$EAI (m^2/g) = (2 \times 2.303 \times A_{500 \text{ nm}}) / F \times \text{protein weight (g)},$$
\( F \) is the oil volume fraction of 0.25.

\[
ESI (\%) = (A_0 - A_{10}/A_0) \times 100.
\]

### 4.3.11 Foaming properties

Foaming capacity (FC) and foaming stability (FS) of hydrolysates were determined based on previous methods (Jamdar, Rajalakshmi, Pednekar, Juan, Yardi, & Sharma, 2010; Klompong, Benjakul, Kantachote, & Shahidi, 2007) with some modifications. Twenty milliliters of 0.5% protein solution was adjusted to pH 2, 4, 6, 8, and 10, followed by homogenization at 16,000 rpm for 2 min at room temperature. The foamed sample was transferred into a 60-mL cylinder, the total volume was recorded after 30 sec. The FC was calculated based on the following equation:

\[
FC (\%) = (A_0 - B) \times 100 / B,
\]

Where \( A_0 \) is the volume after foaming (mL), \( B \) is the volume before foaming (mL).

The whipped sample was allowed to stand at room temperature for 10 min and the volume of whipped sample was then recorded. FS was calculated as follows:

\[
FS (\%) = A_t \times 100 / A_0,
\]

Where \( A_0 \) is the volume after foaming (mL), \( A_t \) is the volume after standing (mL).

### 4.3.12 Hydrophobicity, viscosity and net charge

The surface hydrophilicities of the roasted peanut protein/hydrolysates were measured according to the method described by Xu, Mukherjee and Chang (2018). The protein/hydrolysates were diluted by 0.01 M phosphate buffer (pH 7.0), and the protein mass fraction of the solution determined to be 0.02%. After that, 7 µl of ANS (8 mmol/L)
was added into 500 µL of protein solution in the absence of light, and left in the dark for one minute. The relative fluorescence intensity (RFI) of the mixture (100 µL) was determined at excitation and emission wavelengths of 365 and 484 nm, respectively, using a FlexStation 3 Microplate Reader equipped with the SoftMax Pro Microplate Data Acquisition & Analysis Software (Molecular Devices, Sunnyvale, CA, USA).

The apparent viscosity of the roasted peanut protein/hydrolysates (with/without TGase treatment) was measured by a Brookfield cylindrical viscometer (Model LVT, Brookfield Engineering Laboratories, Inc. Stoughton, MA, USA) at 22°C, and a cylinder speed of 60 rpm (with #61 spindle) using a Brookfield UL adapter.

The surface charge of the emulsions (according to the method described in 4.3.10) was investigated by a Zeta-potential Instrument (Zetasizer Nano-ZS290, Malvern, UK) at 25 °C and a current of 0.07 mA. 1 mL aliquot of each emulsion, prepared as above, was serially diluted into 10,000 times with type 2 water. The zeta potential was measured in triplicate and the mean of the zeta potential was calculated for the intact protein and the hydrolysate emulsions (Xu, Mukherjee, & Chang, 2018).

4.3.13 Statistical analyses

Experiments were performed in duplicate for enzymatic processing and triplicate for analysis. Data were analyzed by 2014 SAS (Version 9.4, SAS Inst. Inc., Cary, NC, U.S.A.). Significant differences among means were determined by the Duncan multiple range test procedure for independent samples at P < 0.05.
4.4 Results and Discussions

4.4.1 Peanut screening

4.4.1.1 Peanut lines harvested in 2015

The allergen content (% of total protein) was calculated based on the band intensities of the SDS-PAGE, which was the same calculating method as a previous study (Kang, Gallo, and Tillman, 2007). Among 97 lines of peanuts from mini core collections (harvested in 2015), Ara h 1 varied from 6.33 to 17.02%; Ara h 2 varied from 3.66 to 9.29%; Ara h 3 varied from 46.62 to 61.97% (Table A. 1). In the study of Kang, Gallo, and Tillman (2007), 99 accessions of peanuts from mini core collections were used for protein extraction before allergen determination, 60 relatively higher protein yield accessions were used for SDS-PAGE analysis. Fifty accessions of peanuts in our study were the same as the previous study (Kang, Gallo, & Tillman, 2007). A correlation of major peanut allergens (Ara h 1, Ara h 2) between our study and Kang’s study was analyzed to test if the relative allergen level would be changes by the different harvest years and locations. The correlation was not significant ($R^2 < 0.2$, $P > 0.05$), indicating that the year and location might affect the level of allergen. Therefore, it is necessary to screen the peanuts for each location/year to determine the protein composition for the peanut crop. Among 25 lines from other resources (Table A. 2), Ara h 1 varied from 11.60% to 18.10%; Ara h 2 varied from 6.14 to 9.75%, Ara h 3 varied from 43.98% to 53.08% (Table A. 2). Among 122 peanut lines in total, two lines (MS- 1 and MS-8) were selected with lower Ara h 1; two lines (MS-5 and MS-6) were selected with lower Ara h 2; five lines (MS-2, MS-3, MS-4, MS-10, MS-11) were selected with lower Ara h 3; one line (MS-7) was selected with lower Ara h 1 & Ara h 2 (Table 4.1).
4.4.1.2 Prescreened peanut lines harvested in 2016

Eleven prescreened peanut lines were replanted and harvested in 2016. Ara h 1 varied from 7.84 to 15.52%; Ara h 2 varied from 6.65 to 8.95%; Ara h 3 varied from 46.05 to 57.85% (Table 4.1). MS-1 and MS-8 had relatively lower Ara h 1 (6-10%) in 2015 and 2016 (Table 4.1). MS- 5 and MS-6 had relatively lower Ara h 2 (3.6-7.5%) in both years (Table 4.1). MS-2 and MS-3 had relatively lower Ara h 3 (46-48%) in both years (Table 4.1). MS-7 had relatively lower Ara h 1 (8.4-9.5%) & Ara h 2 (4.2-6.7%) in both years (Table 4.1). However, the total content of these three major allergens were not significantly different among all the accessions within the same year (Table 4.1). In the study of Kang and coworkers (2007), 60 accessions in the U.S. Peanut collection (all belonged to mincore collection), along with the 88 Florida Peanut breeding program lines were analyzed for major peanut allergen levels. An accession from India had the lowest level of Ara h 1 (7.0%). An accession from Nigeria had the highest level of Ara h 1 (18.5%), but the lowest level of Ara h 2 (6.2%). An accession from Zambia had the highest level of Ara h 2 (13.2%), but the lowest level of Ara h 3 (21.8%). Our result was consistent with this report, which cited major allergen distribution varied among all the lines but with similar total content.

Since among the major allergens of Ara h 1, Ara h 2 and Ara h 3, the allergenicity ranks from high to low is Ara h 2, Ara h 1 and Ara h 3 (Lee, Wright, & Rachaputi, 2016), the peanut cultivar with lower content of Ara h 1 & Ara h 2 is preferred. Therefore, MS-7 was chosen for enzymatic processing for relatively stable allergen level in both years with lower Ara h 1 & Ara h 2 levels. MS-9, Valencia was also chosen for the next stage study as the control.
4.4.1.3 Two-dimensional (2D) SDS-PAGE and western-blot analyses of MS-7 and MS-9 protein extracts

The major protein (allergen) distribution of MS-7 and MS-9 were compared based on the result of two-dimensional SDS-PAGE and western-blot (Figure 4.2 & 4.3).

According to the result of western-blot by using human plasma containing (anti-peanut) IgE (Figure 4.2B & 4.3B) and previous literatures (Chassaigne, Trégoat, Nørgaard, Maleki, & van Hengel, 2009; Schmidt et al., 2009), 19 distinct IgE-binding spots were labeled in the corresponded SDS-PAGE (Figure 4.2A & 4.3A) with number 1 to 19. Spot 1 belongs to ten isoallergens of Ara h 1; spot 2 to 5 belong to isoallergens of Ara h 2; spot 6 to 19 belong to isoallergens of Ara h 3 (Chassaigne, Trégoat, Nørgaard, Maleki, & van Hengel, 2009; Schmidt et al., 2009). The quantification of each spot was conducted and shown in Table 4.2, which was expressed as the percentage of the total allergen content.

The contents of Ara h 1 isoallergens (spot 1) and Total Ara h 2 isoallergens (spot 2 to 5) were significantly higher in MS-9 (Table 4.2). On the contrary, the contents of Total Ara h 3 isoallergens (Spot 6 to 19) were significantly higher in MS-7 (Table 4.2). The result was mutual supported with one-dimensional SDS-PAGE (Figure 4.1, Table 4.1), and consistant with the study of Kang, Gallo, and Tillman (2007) with the major allergens in peanut seed would compensate for each other with the similar total allergen content. In the study of Schmidt and coworkers (2009), an Indonesia peanut line (Kacang Asin) was compared with a common peanut variety Virginia. There were virtually nearly no Ara h 1 band/spot in the 1D/2D SDS-PAGE of Kacang Asin, the Ara h 2 allergen level was also apparently lower in this Indonesia peanut line. Meanwhile, the Ara h 3 level was higher in this Indonesia variety. Comparing with this previous study (Schmidt et al., 2009), the Ara h 1 level (< 10%) of MS-7 in the current study was relatively higher. However, both
of these two varieties (Kacang Asin and MS-7) had relatively lower Ara h 1 & 2, which would be preferred for food industry. The Ara h 2 spots (spots 2-5) had less intensities as compared to Ara h 1 isoallergen spots (spot 1), the same situation had also been found in an earlier study of Chassaigne and coworkers (2009) by using the same protein extraction method (TBS buffer, pH 7.4). The relatively mild extraction method may be result in a less extraction rate, which is reflected by lower band intensities. The relative mild treatment (neutral pH extraction) would have less influence on the protein denaturation during extraction and result in a better resolution of 2D-gel, but 1D SDS-PAGE (protein extracted with higher pH of 8.5) may reflect the extract ratio of the peanut allergen better. However, the relative peanut isoallergen content between two varieties would be considered as the same.

4.4.2 Quantification and kinetic study of enzymatic processing effect on IgE-binding properties of roasted MS-9 peanut hydrolysates

IgE-binding properties of peanut hydrolysates hydrolyzed by 8 enzymes with different concentrations (0-400 AzU/g) were shown in Table 4.3. The IgE binding property (tested by ELISA) of roasted MS-9 peanut protein extract was set as 100%. Enzyme treatments with increased concentration resulted in decreased IgE binding properties. At 400 AzU/g level, the hydrolysate treated by ficin had no IgE-binding properties, followed by those treated by bromelain and papain with 1.2% and 2.31% remaining, respectively, which was significantly less than other enzymes (12-92% remaining). The SDS-PAGE of the enzymatic processing effect on the peanut protein pattern changes are shown in Figure B.1 (Appendix B) for reference. In Figure B.1, it is
apparently found that papain, ficin and bromelain had lower band intensity at Ara h 1 – Ara h 3 as compared with other enzymes at ranges of 0-400 AzU/g.

The relationship between enzyme concentration and IgE-binding properties could be explained by a first order reaction model, in which enzyme concentration is the variate and log transformed IgE-binding properties is the response (Table 4.4). In this reaction model, K is the constant rate, and represents the log [IgE binding properties] reducing rate by adding 100 AzU/g of enzyme. The K value of ficin was the highest (0.9) among all the enzymes, followed by bromelain (0.47) and papain (0.4). This result was mutually supported with the SDS-PAGE (Figure B.1), indicating that the peanut allergenicity and the visible allergen peptide band were decreased simultaneously. All the other five enzymes had K values of no more than 0.21, thermolysin had the lowest K values at 0.01. Even though all the enzyme used were endopeptidases, which are less specific and hydrolyze more completely than trypsin or α-chymotrypsin (Li, Yu, Goktepe, & Ahmedna, 2016), the ability of hydrolysis is varied widely in terms of IgE binding property reduction. The result above could support the conclusion that food allergens are sometimes characterized by their high stability under certain (including digestive) enzymes, with their epitope structures remaining unchanged (Li, Yu, Goktepe, & Ahmedna, 2016). However, they can be hydrolyzed by some food grade proteases, which can decrease or eliminate the IgE bindings depending on type and concentration of the enzyme.

In an earlier study, alcalase was considered as a more efficient protease to hydrolyze defatted peanut flour and peanut protein isolate (dispersion) than neutrase, papain and protamex with the same enzyme concentration of 1% (Zheng, Zhao, Xiao,
Sun-Waterhouse, Zhao, & Su, 2015). In our study, the enzyme concentrations of alcalase (0.06%) was lower than other enzymes (≥ 0.08%). The IgE binding property of thermolysin treated hydrolysates in our study was decreased the least (6% reduction by 400 AzU/g enzyme). However, in an earlier study (Pownall, Udenigwe, & Aluko, 2010), thermolysin (0.5% w/w, on the basis of protein weight) was successfully applied on pea protein hydrolysis to produce low molecular weight peptides. However, in our study, the concentration of thermolysin at 400 AzU/g was approximately 5.12*10^-4% (w/w, on the basis of protein weight), which was much lower than the literature. The enzymes with the same unit of proteolytic activity may have various concentration by weight. Since no study has been conducted under the same proteolytic activity measured by a standard substrate (Azocoll), most of researchers used the enzymes based on weight percentage (E/protien) or enzyme unit provided by manufacturer. Our method is easier to repeat and provide meaningful references for comparing the efficiency of hydrolysis. Our objective was to differentiate the enzymatic hydrolyzing efficiency based on the same unit.

At the level of 200AzU/g, papain, ficin and bromelain resulted in relatively lower IgE-binding properties with 15.4%, 5.2% and 9.9% among the eight enzymes (Table 4.3), respectively. Papain, ficin and bromelain all belong to cysteine proteinases and endopeptidase (Turk, Turk, & Turk, 1997). The relatively similar attributes made these three enzymes exhibit the similar extent of hydrolysis compared with the other five enzymes. In a recent study, papain was found to also have better gliadins (major allergen in wheat flour) reduction (≥ 90% reduction of control) than the other four enzymes (flavoenzyme, pepsin, trypsin, chymotrypsin) at the level of 254U/g (enzyme Unit provided by manufacturer) (Li, Yu, Goktepe, & Ahmedna, 2016).
Besides MS-9, MS-7 (low allergen peanut) was also used for hydrolysis in the following study. Therefore, 400AzU level of ficin (100% reduction in IgE binding properties) may eliminate the IgE-bindings for both variety, which made it difficult to compare. Moreover, protein under extensively hydrolyzed may have functional property (Jamdar et al., 2010) and may be too small to be crosslinked by TGase. Hence, 200 AzU/g (median concentration among all the conditions tested above) of papain, ficin and bromelain were chosen for the following hydrolysis study.

4.4.3 Comparration of the allergenicity of hydrolysates produced from MS-7 & MS-9 peanuts with selected enzymes at 200AzU/g

SDS-PAGE of (MS-7 & MS-9) hydrolysates and allergen remaining percentage are shown in Figure 4.5A and Table 4.6, respectively. The MS-9 protein extract (without hydrolyzing) contains 0.4 mg/mL, 0.6 mg/mL and 4.9 mg/mL of Ara h 1, Ara h 2 and Ara h 3, respectively. The allergen contents of Ara h 1, Ara h 2 and Ara h 3 in MS-9 protein extract (without hydrolyzing) were set as 100% for calculating the allergen remaining level in percentage. Ficin and bromelain reduced the more distinct major allergen band of Ara h 1 and Ara h 2 than papain in MS-7 & MS-9 hydrolysates. However, the remaining percentage of Ara h 3 in the hydrolysates showed in descending order: bromelain, papain and ficin. Therefore, ficin resulted in relatively less remaining percentage of Ara h 1 – Ara h 3. Ara h 3 band at 22 kDa was the only visible major allergen band in the SDS-PAGE of ficin and bromelain hydrolysates (Figure 4.5A). The reason might be that Ara h 3 (22 kD) content had 4-6 times higher than Ara h 2 and Ara h 1 in protein extracts before hydrolysis. In an earlier study (Zhao, Liu, Zhao, Ren, & Yang, 2011), it has also been found that the subunits of acid arachin (Ara h 3 of 36-40
kD) were the most susceptible to alcalase hydrolysis, followed by conarachin (Ara h 1) and the basic subunits of arachin (Ara h 3 of 22 kD).

According to Figure 4.5 and Table 4.6, the major allergen remaining percentages of water-soluble Ara h 1 and Ara h 2 were all significantly lower in MS-7 (selected peanut). However, the remaining percentages of water-soluble Ara h 3 were significantly higher in MS-7. The result was consistent with the major allergen content in raw peanut (Table 4.1), in which the major allergens had similar total amounts with discrepancies only in single allergen contents, and this situation remained after enzymatic hydrolysis.

The IgE binding properties of water-soluble hydrolysates for both varieties were shown in Figure 4.5B. Papain hydrolysate has the highest IgE binding properties (11-15%) followed by bromelain (7-10%) and ficin (3-5%). MS-7 hydrolysates had lower IgE-binding properties compared with the MS-9 (check peanut). The reason may be that MS-7 had lower content of Ara h 2 (Tables 4.1 & 4.2), which are reported to be the most potent peanut allergen (Schocker, Baumert, Kull, Petersen, Becker, & Jappe, 2016). The western-blot figure (Figure 4.6B) could support our assumption that the band intensities of Ara h 2 were darker than others, and may contribute more to the overall allergenicity.

Soybean protein isolate (SPI) was hydrolyzed by papain in earlier studies (Meinlschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016; Panda, Tetteh, Pramod, & Goodman, 2015) and the results showed that most of the major allergens (β-conglycinin and glycinin) and IgE binding properties were reduced (Panda, Tetteh, Pramod, & Goodman, 2015). However, in the same study mentioned above, the major allergens of soybean changed slightly after hydrolyzing by bromelain (about 0.08%), and the IgE binding properties were increased (Panda, Tetteh, Pramod, & Goodman, 2015). The discrepancy
of the current and previous studies could attribute to: 1) the concentration was relatively low (about 0.08%) in earlier studies, and the mild hydrolysis may result in an increase in allergenicity because of exposure of new antigenic epitopes due to protein breakdown; 2) the different allergen structure and composition of peanut and soybean could also influence the extent of hydrolysis.

Proteins in the residues of hydrolysates extracted by SDS-sample buffer (1% SDS + 5 mM dithiothreitol) and analyzed by SDS-PAGE and western-blot (Figure 4.6). The major allergen (Figure 4.6A) and IgE-binding properties (Figure 4.6B) were higher in MS-9 comparing with MS-7, especially for the residues of ficin and bromelain hydrolysates. The result was consistent with the study of Li and coworkers (2016), in which the enzyme also had an impact on the allergen band intensity of SDS-PAGE on both soluble and insoluble parts. The results indicated that hydrolysate residue of MS-7 would be more suitable to replace peanut powder in the food industry, however, in vivo study and sensory evaluations are needed for further applications.

4.4.4 Transglutaminase (TGase) processing effect on allergenicity of peanut hydrolysates

The roasted peanut protein (PP) and hydrolysates were crosslinked by TGase. The SDS-PAGE of TGase (5 Unit/g protein) crosslinked water-soluble roasted peanut protein (TPP) and hydrolysates are shown in Figure 4.5A. The major allergens remaining percentages of TGase crosslinked peanut protein (TPP) and hydrolysates are shown in Table 4.6 and Table 4.7, respectively. TPP possessed a significantly lower percentage of major allergen bands (about 50% lower than roasted peanut protein) (Table 4.5). However, the allergen remaining percentage of TGase crosslinked hydrolysates in terms
of Ara h 1 and Ara h 2 changed slightly (decreased 1-3%) (Figure 4.5A, Table 4.6). The distinct band remaining percentage of Ara h 3 in TGase crosslinked hydrolysates changed greatly, with about 5-10% decrease than untreated hydrolysates (Figure 4.5A, Table 4.6). The reason for the difference may be that Ara h 1 and Ara h 2 had lower content in hydrolysates than Ara h 3, and made it much more difficult to alter the Ara h 1 and Ara h 2 amount after TGase cross-linking. As shown in Table 4.6, the allergen remaining percentage of Ara h 1 and Ara h 2 were lower in MS-7, while the Ara h 3 content was lower in MS-9. Before TGase treatment, no bands were found at the top of the separating and stacking gels in the roasted peanut protein and the hydrolysates’ SDS-PAGE (Figure 4.5A). After TGase treatment, the larger polymers were formed at the top of separating and stacking gels for TGase crosslinked hydrolysates and TPP (Figure 4.5A). This finding was consistent with an earlier study of Feng and coworkers (2014) in which peanut major allergen of conarachin (Ara h 1) and arachin (Ara h 3) were able to form crosslinking with TGase-catalyzing.

The IgE binding properties of TGase treated roasted peanut protein (TPP) were not significantly different from no-TGase treated roasted peanut protein (PP) (Figure 4.6B, Table 4.5). The IgE-binding properties of TPP was not reduced despite of the decreasing of allergen bands. The result was consistent with earlier studies that TGase treated peanut protein (Clare, Gharst, & Sanders, 2007), dairy whey protein (Wroblewska, Jedrychowski, Hajos, & Szabo, 2008) and Ovalbumin in egg white (Ma, Lozano-Ojalvo, Chen, Lopez-Fandiño, & Molina, 2015) exhibited similar allergenicity to untreated protein. The IgE binding properties of Ara h 2 in TPP were similar to the PP as showed in western-blot (Figure 4.6B), indicating that the majority of Ara h 2 was not
cross-linked into new polymer. Meanwhile, Ara h 1 and Ara h 3 of TPP formed into larger polymer as shown in the top of separating and stacking gels and reduced their distinct band intensity comparing with PP (Figure 4.6A). The new formed polymer of TPP possessed IgE-binding properties as shown in Figure 4.6B, indicating that the epitopes of the major allergen of TPP were not masked or altered after TGase cross-linking. The TGase treated hydrolysates also had similar IgE-binding properties to non-treated hydrolysates (Figure 4.5B, Table 4.6). While in an earlier study conducted by Wroblewska and coworkers (2008), TGase treated dairy whey protein hydrolysates (hydrolyzed by alcalase) had lower allergenicity (about 80% lower) as compared with untreated hydrolysates. It could be explained by cross-linking, acyl-transfer, and deamidation of hydrolysates resulting in the structural changes of proteins, which might influence the IgE-binding reaction (Wroblewska, Jedrychowski, Hajos, & Szabo, 2008). In our study, the allergenicity of the TGase crosslinked hydrolysates were not significantly changed, the reason may be due to the different type of proteins between peanut and whey. Even though no significant IgE-binding property reduction was found, at least one thing could be confirmed that no neo-allergen was formed after TGase treatment. In a recent study (Yuan et al., 2017), reduced allergenicity was found in tropomyosin (major allergen of shrimp) treated by TGase-catalyzed glycosylation (with Glucosamine) which correlate with the loss of secondary structure. In our study, the IgE-binding properties of PP and hydrolysates after TGase treatment was not significantly changed, which was evaluated by ELISA (Table 4.7). The reason may be due to the relatively higher number of the epitopes in peanut allergen’s Ara h 1 – Ara h 3 (21, 14
and 4, respectively) for IgE-binding (Lee, Wright, & Rachaputi, 2016) as compared with tropomyosin (2) (Rao, Rajagopal, & Ganesh, 1998).

### 4.4.5 Functional properties of peanut hydrolysates without/with TGase treatments.

#### 4.4.5.1 Functional properties (at pH 2-10) of peanut hydrolysates without/with TGase (5 Unit/g) treatments.

The effect of pH on the emulsifying activity index (EAI) and emulsion stability index (ESI) of MS-7 (selected) roasted peanut powder hydrolysates (fat removed by filtration with No. 4 filter paper) are shown in Figure 4.7 (A&B), Roasted peanut protein (PP) was used as the control and soy protein isolate (SPI) was used as a reference. The functionality of MS-9 (roasted peanut) hydrolysates were also conducted and the result was similar with MS-7. Therefore, in this entire section, MS-7 hydrolysates were chosen for discussion. The emulsion activity index (EAI) of all the samples including hydrolysates and PP were relatively lower at pH 4 than those at other pH levels (Figure 4.7A). The isoelectric point (lowest solubility) of soy and peanut protein isolate are all around pH 4.5 (Jamdar, Rajalakshmi, Pednekar, Juan, Yardi, & Sharma, 2010; Wolf, 1970), at which the protein would precipitate and had lowest solubility; this explaining the EAI at pH 4 were lower with other conditions. The EAI of SPI were the highest with all the pH environments. The hydrolysates treated by bromelain and papain had relatively higher EAI than PP; the hydrolysates treated by ficin were the lowest among all the non-TGase treated samples. From the SDS-PAGE of the hydrolysates (Figure 4.5A), it could be found that ficin resulted in the lowest (about 7% retained as compared with PP) intensities of larger molecular weight (> 20 kDa) bands, in other words, the portion of larger peptides were broken down into small pieces more thoroughly than the other two
enzymes. The result was similar to the previous study (Jamdar, Rajalakshmi, Pednekar, Juan, Yardi, & Sharma, 2010), in which at neutral pH condition, the rank of EAI from high to low were exactly opposite to the degree of hydrolysis. The relatively higher degree of hydrolysis perhaps contributed to lower EAI (Jamdar, Rajalakshmi, Pednekar, Juan, Yardi, & Sharma, 2010).

The emulsion stability index (ESI) of all the hydrolysates (without TGase treatment) were significantly lower than that of SPI (less than about 15-20%) and PP (less than about 5-10%) for all the pH ranges (Figure 4.7B). In general, ESI should decrease by extensive hydrolysis (with high degree of hydrolysis) which tends to produce more smaller peptides. In our study, the viscosity of roasted peanut protein/hydrolysates were analyzed. The hydrolysates appeared to have lower viscosity (17.2-23.5 cP) compared with untreated protein (28.3 cP) (Table A.3). Among the three hydrolysates, ficin resulted in the lowest viscosity of hydrolysates which may be due to lowest content (about 7% of roasted protein extract) of larger molecular weight (>20 kDa) bands in SDS-PAGE (Figure 4.5A). The small peptides in hydrolysates would result in reduced viscosity of the continuous phase and to reduce emulsion stability (Surówka, Żmudziński, Fik, Macura, & Łasocha, 2004). It is widely believed that emulsifier must have an amphiphilic nature. Therefore, hydrophobicity was one of the major factors to influence the emulsion/foaming capacity/stability. In our study, the hydrophobicity of the no-TGase treated hydrolysates were lower than that of the peanut protein (Table A.3). The reason may be due to that some hydrophobic amino acids (such as alanine, valine, leucine and isoleucine) tend toward the interior of the protein molecule on folding as they are smaller in size and are more flexible (Mahmoud, Malone, & Cordle, 1992).
After treated with 5 Unit/g of TGase, the EAI and ESI of hydrolysates are shown in Figure 4.7 (C&D). The EAI of TGase treated hydrolysates (Figure 4.7C) were significantly higher than untreated hydrolysates (Figure 4.7A) at most of the pH conditions, especially at higher pH values. The EAI of TGase treated bromelain and papain hydrolysates were shown to be no less than SPI and higher than TPP. Protein cross-linking reactions were promoted by TGase through an acyl transferase mechanism including protein-bound glutaminyl residues and primary amines such as the ε-amino group of lysine residues (Clare, Gharst, & Sanders, 2007). The result was consistent with the earlier study that the digested (with chymotrypsin) soy protein hydrolysates treated with TGase had higher emulsion properties than soy protein polymer (soy protein treated with TGase) and the untreated digests of soy protein polymer (El Fadil, Khan, Matsudomi, & Kato, 1996).

It is assumed by Babiker (2000) that the improvement of emulsifying properties in TGase-crosslinked soy protein hydrolysate maybe due to an increase in the negative charges resulting from the hydrolysis of the amide groups in glutamine and asparagine. In our study, the negative charge of the emulsion with no-TGase treated hydrolysates was higher than that treated with peanut proteins. More carboxylic and amino groups would become liberated along with the treatment of proteolytic enzymes on peptide bonds. Since the hydrolysis was maintained at pH 7.5 (≥ isoelectric point of most amino acids and peptides), the carboxylic groups would dissociate to carboxylate ions (COO-) resulting in an increase in net negative charge (Mahmoud, Malone, & Cordle, 1992). After TGase treatment, the negative charges were not significantly changed, indicating the carbonate ions remained after TGase modification by means of amine incorporation,
crosslinking and deamination (Ma, Lozano-Ojalvo, Chen, Lopez-Fandiño, & Molina, 2015).

In an earlier study (El Fadil, Khan, Matsudomi, & Kato, 1996), TGase treated soy protein hydrolysates had decreased hydrophobicity but increased emulsion and foaming property. They assumed that the loss of hydrophobicity after TGase crosslinking may be due to that the exposed hydrophobic residues of the polymerized peptides were buried inside the polymerized molecules (El Fadil, Khan, Matsudomi, & Kato, 1996). However, in our study, the TGase-treated peanut protein/hydrolysates had significantly higher hydrophobicity, which indicating unfolding of the protein (arachin and conarachin)/peptides by means of TGase treatment (Qiu et al., 2017). In addition, the viscosity after TGase treatment was significantly increased due to large polymer formation (Table A.3), which may contribute to the improvement of emulsion properties by increasing the viscosity of the continuous phase and emulsion stability.

Therefore, in our study, the improvement in the emulsifying properties maybe partially due to the formations of polymerization, increased hydrophobicity and viscosity (Table A.3). Like EAI, the ESI of TGase crosslinked hydrolysates also increased significantly by about 5 to 10% compared with the untreated hydrolysates.

The foaming capacity (FC) and stability (FS) of MS-7 roasted peanut powder hydrolysates (without TGase treatment) are shown in Figure 4.8 (A&B). At pH 2-6, the FC of all the hydrolysates were higher than that of SPI. At pH 6-10, the FC of the bromelain and ficin hydrolysates were higher than PP. However, at pH 2-10, the FS of all the hydrolysates were lower than PP and SPI. The results confirmed the earlier study that hydrolysis of peanut protein improves the foaming capacity but with poor stability
The reason may be that even if smaller peptides can absorb more air into the solution than the larger peptides, they cannot maintain the stable form with enough strength (Kristinsson & Rasco, 2000). For the grapeseed protein and the peanut protein Isolates, hydrolysates with a degree of hydrolysis as little as 5% and 10% would decrease their foaming stability, respectively (Vioque et al., 2000; Jamdar et al., 2010). Besides the length of peptide chain, the structure of the peptide chain also played an important role for determining the emulsifying and foaming stability. The roasted peanut protein (PP) in our study had less foaming stability than SPI, the reason may be that PP is denatured after roasting. After thermal processing such roasting, major peanut allergen Ara h 1 became aggregated and resulted in reduced solubility (Maleki & Hurlburt, 2004). It is well known that a good emulsifier has an amphiphilic nature, which meaning it has high solubility in both water and oil. The protein with reduced solubility may be caused by folded and hindered hydrophilic peptide chains after denaturation and aggregation.

The foaming capacity (FC) and foaming stability (FS) of TGase crosslinked PP (TPP) and hydrolysates are shown in Figure 4.8 (C&D). The FC of all the hydrolysates were higher than TPP and SPI. The FC of TGase treated bromelain hydrolysates was more than 100% at pH 2-10. The FC of the TGase crosslinked hydrolysates was higher than untreated hydrolysates (50-80% more, depending on enzymes and pH). The FC of TGase crosslinked PP (Figure 4.9C) higher than PP (Figure 4.9A) with less extent by 5-20% depended on the differenting pH. This result was consistent with an earlier study, indicating the foaming properties of the TGase treated hydrolysates were greatly improved compared to the TGase treated protein (Babiker, 2000). The improvement of
the foaming properties after TGase treatment revealed the protein association or polymerization was considered a structural (secondary structure) factor influencing the foaming property (Kato, Takahashi, Matsudomi, & Kobayashi, 1983; Yuan et al, 2017). The FS of TGase treated hydrolysates and TPP all increased considerably (from 24-245%) than non-TGase treated hydrolysates and PP, respectively. The TGase treated bromelain hydrolysates had similar FS with SPI and TPP. The result indicated that TGase treatment could increase the emulsion and foaming properties of hydrolysates by forming larger aggregates. By comparing, the three enzymes treated by TGase, bromelain and papain hydrolysates gave higher EAI, ESI, FC and FS than ficin. Ficin hydrolysates had the lowest content (about 7% of roasted protein extract) of the larger molecular weight (>20 kDa) bands in SDS-PAGE (Figure 4.5A), which made the small peptides more difficult to cross-link the form of larger polymers, and further enhance the functionality.

4.4.5.2 Kinetic analysis (pH 8) of functional properties as affected by different concentrations of TGase (0-10 Unit/g)

The kinetic analysis was conducted to describe the relationship between TGase concentrations and functional properties (EAI, ESI, FC, FS) at pH 8 (the highest value among the pH range of 2-10). Various concentrations (0, 2.5, 5, 7.5 and 10 Unit/g) of TGase were applied to roasted peanut protein/hydrolysates. As shown in Figure 4.9, along with the increasing of TGase concentration, the functional properties were also increased. The pattern of the correlation could be explained by the Peleg’s model (Peleg, 1988). By using the Peleg’s model, the functional property can be described by initial value (F₀), rate constant rate K₁, capacity constant K₂ and concentration (c) (Table 4.7). The equilibration value could also be predicted when the TGase concentration increased.
to very high. When the TGase concentration become high enough, the product of $K_2$ and $c$ would be much bigger than $K_1$, so that $K_1$ might be neglected (Table 4.7). Therefore, capacity constant $K_2$ and Initial value $F_0$ determine the equilibration value. Among three types of hydrolysates, bromelain hydrolysates added with TGase (0-10 Unit/g) had the highest functional properties (EAI, ESI, FC and FS), followed by papain and ficin. The equilibration value was comparable to the actual value, indicating that initial value was a major factor for determining the equilibration value. It is important to note that the equilibration value here is more like the optimal value, with the concentration of TGase a little higher than 10 Unit/g but not infinity high. Furthermore, the value of the functional property at TGase concentration of 10 Unit/g is already very close to (about 98% of equilibration value) estimated equilibration value. The curves of the functional property show and confirm this trend (Figure 4.9), in which from 0-5 Unit/g, the functional property increased more rapidly, and become flat at the relatively higher concentration (5-10 Unit/g). The functional property of TGase treated PP/hydrolysates may increase along with the formation of polymer by TGase crosslinked within certain ranges. The formation of the larger polymer could easily and more effectively distribute itself on the oil/water interface. However, if the polymer become extremely large, it may act as the “bridge” among all the oil droplets, and consequently disrupt the stability of the emulsion (Tang, Yang, Chen, Wu, & Peng, 2005).

4.5 Conclusions

Peanut (MS-7) was selected with a lower allergen of Ara h (1+2) from 122 lines based on the analyses of SDS-PAGE. The roasted peanut hydrolysates treated by papain, bromelain and ficin exhibited lower IgE-binding properties comparing with neutrase,
alcalase, protamex, Novo-ProD and thermolysin. TGase treated hydrolysates possessed significantly higher functional properties comparing with untreated hydrolysates. The IgE-binding properties of TGase-treated hydrolysates were not increased. The differences between two peanut lines (MS-7 and MS-9) on functional properties were not significant. The results above indicated that the combination of peanut screening, enzymatic hydrolysis and TGase treatment could obtain hydrolysates with lower allergenic properties and improved functional properties (such as emulsifying and foaming properties), which would be further studied for food ingredient application.

4.6 Limitation of the study

(1) In the first study (Chapter 3), only linear epitopes of the allergenic properties were conducted.

(2) In the second study (Chapter 4), sensory testing is not conducted to test for the bitterness (or any other unpleasant sensations) and acceptability of the hydrolysates.

(3) In the second study (Chapter 4), kinetics analyses of the reactions under various conditions beyond concentration (substrate concentration, temperature, time) were not studied.

(4) In the second study (Chapter 4), competitive ELISA was not conducted, the current ELISA may not reflect the exact IgE binding of the hydrolysates, since hydrolysates may have hydrophobic nature and not easily bind to the bottom of micro-plate.

(5) In the first and second study (Chapter 3&4), six patient’s plasmas were used in the ELISA and western-blot analyses, while more than ten patients would be more representable to the population with peanut allergy.
(6) In the first and second study (Chapter 3&4), allergenicity testing methods beyond western-blot and ELISA were not conducted.

4.7 Future research recommendation

(1) In vivo methods of allergenicity testing such as cell; in vivo methods of allergenicity testing such as mouse and human studies are necessary in future study to prove the specific allergen reducing effects.

(2) Food matrixes (products), such as cookies, high protein bars or high protein drinks can be made with the thermal/ enzymatic processed peanut powders.

(3) More varieties of enzymes or a combination of several enzymes must be tested to improve allergen reducing efficiency, functionality and sensory quality. Kinetics analyses of the reactions under various conditions (enzyme concentration, substrate concentration, temperature, time) should be studied.

(4) Similar research strategies may be applied to other allergenic foods, such as soybeans.
Table 4.1 The major allergen content of 11 selected peanuts harvested in year 2015 and 2016.

<table>
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<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Ara h1*</td>
<td>MS-1</td>
<td>6.33±0.22 g</td>
<td>10.33±0.52 cd</td>
<td>6.55±0.58 d</td>
<td>8.95±0.90 a</td>
<td>57.17±2.95 a</td>
<td>51.70±3.65 abc</td>
<td>70.05±3.75 a</td>
<td>70.98±5.07 a</td>
</tr>
<tr>
<td>Ara h3*</td>
<td>MS-2</td>
<td>14.36±0.80 cd</td>
<td>12.90±0.77 b</td>
<td>7.27±0.71 c</td>
<td>7.11±0.59 bc</td>
<td>47.27±2.72 c</td>
<td>52.06±3.29 abc</td>
<td>68.90±3.93 a</td>
<td>72.07±4.66 a</td>
</tr>
<tr>
<td>Ara h3*</td>
<td>MS-3</td>
<td>12.53±1.02 e</td>
<td>15.52±1.05 a</td>
<td>6.85±0.52 d</td>
<td>7.22±0.48 bc</td>
<td>48.67±2.40 bc</td>
<td>46.05±4.10 c</td>
<td>68.05±3.94 a</td>
<td>68.78±5.62 a</td>
</tr>
<tr>
<td>Ara h3*</td>
<td>MS-4</td>
<td>17.02±0.16  e</td>
<td>15.26±1.69 a</td>
<td>6.95±0.65 d</td>
<td>8.07±0.59 ab</td>
<td>46.62±1.25 c</td>
<td>46.82±2.38 c</td>
<td>70.59±3.76 a</td>
<td>70.15±4.66 a</td>
</tr>
<tr>
<td>Ara h2*</td>
<td>MS-5</td>
<td>14.60±0.59  c</td>
<td>13.57±1.32 ab</td>
<td>3.66±0.27 f</td>
<td>7.11±0.67 bc</td>
<td>52.88±3.46 b</td>
<td>50.83±4.05 bc</td>
<td>69.55±4.35 a</td>
<td>71.51±6.04 a</td>
</tr>
<tr>
<td>Ara h2*</td>
<td>MS-6</td>
<td>13.58±0.90  de</td>
<td>12.19±1.32 bc</td>
<td>3.66±0.27 f</td>
<td>7.47±0.90 bc</td>
<td>59.33±5.43 ab</td>
<td>53.78±3.33 ab</td>
<td>76.58±6.00 a</td>
<td>73.44±5.55 a</td>
</tr>
<tr>
<td>Ara h1/Ara h2*</td>
<td>MS-7</td>
<td>8.41±0.10 f</td>
<td>9.49±1.31 de</td>
<td>4.16±0.45 ef</td>
<td>6.65±0.51 c</td>
<td>61.97±2.32 a</td>
<td>56.45±5.05 ab</td>
<td>74.54±3.87 a</td>
<td>72.59±6.88 a</td>
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<tr>
<td>Ara h1*</td>
<td>MS-8</td>
<td>8.62±0.11 f</td>
<td>7.84±1.22 e</td>
<td>4.26±0.32 e</td>
<td>8.43±1.02 ab</td>
<td>60.14±3.85 a</td>
<td>57.85±4.91 a</td>
<td>73.03±3.28 a</td>
<td>74.12±7.15 a</td>
</tr>
<tr>
<td>Check*</td>
<td>MS-9</td>
<td>13.11±0.13 d</td>
<td>12.29±1.12 bc</td>
<td>6.46±0.21 d</td>
<td>7.93±1.00 abc</td>
<td>51.13±2.40 b</td>
<td>52.23±3.38 abc</td>
<td>70.70±3.74 a</td>
<td>72.45±5.51 a</td>
</tr>
<tr>
<td>Ara h3*</td>
<td>MS-10</td>
<td>16.03±0.33 b</td>
<td>12.59±1.47 b</td>
<td>8.65±0.04 b</td>
<td>8.04±0.93 ab</td>
<td>46.70±1.29 c</td>
<td>51.78±4.12 abc</td>
<td>71.38±4.67 a</td>
<td>72.42±6.52 a</td>
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<tr>
<td>Ara h3*</td>
<td>MS-11</td>
<td>18.10±2.22 a</td>
<td>12.83±1.12 b</td>
<td>9.75±0.38 a</td>
<td>7.29±0.99 bc</td>
<td>43.98±4.66 c</td>
<td>55.05±5.61 ab</td>
<td>71.83±3.26 a</td>
<td>75.18±7.73 a</td>
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<td>Range</td>
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<td>6.33-18.10</td>
<td>7.84-15.52</td>
<td>3.66-9.75</td>
<td>6.65-8.95</td>
<td>43.98-61.97</td>
<td>46.05-57.85</td>
<td>68.05-76.58</td>
<td>68.78-75.18</td>
</tr>
</tbody>
</table>

The allergen content was expressed by the % of total soluble protein. Data in bold represents the content were lower in both 2015 and 2016. Check cultivar is Valencia.
Table 4.2  Quantification of major allergen spots between two peanut cultivars based on two-dimensional gels.

<table>
<thead>
<tr>
<th>No.</th>
<th>Allergen</th>
<th>Percentage of total allergen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MS-7</td>
</tr>
<tr>
<td>1</td>
<td>Ara h 1</td>
<td>28.02±2.22 b</td>
</tr>
<tr>
<td>2</td>
<td>Ara h 2</td>
<td>0.44±0.05 b</td>
</tr>
<tr>
<td>3</td>
<td>Ara h 2</td>
<td>0.86±0.06 b</td>
</tr>
<tr>
<td>4</td>
<td>Ara h 2</td>
<td>1.43±0.13 b</td>
</tr>
<tr>
<td>5</td>
<td>Ara h 2</td>
<td>1.68±0.14 b</td>
</tr>
<tr>
<td>2-5</td>
<td>Total Ara h 2</td>
<td>4.41±0.31 b</td>
</tr>
<tr>
<td>6</td>
<td>Ara h 3</td>
<td>8.08±0.57 a</td>
</tr>
<tr>
<td>7</td>
<td>Ara h 3</td>
<td>1.91±0.13 a</td>
</tr>
<tr>
<td>8</td>
<td>Ara h 3</td>
<td>1.00±0.07 a</td>
</tr>
<tr>
<td>9</td>
<td>Ara h 3</td>
<td>1.77±0.12 a</td>
</tr>
<tr>
<td>10</td>
<td>Ara h 3</td>
<td>0.91±0.06 a</td>
</tr>
<tr>
<td>11</td>
<td>Ara h 3</td>
<td>2.23±0.16 a</td>
</tr>
<tr>
<td>12</td>
<td>Ara h 3</td>
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<td>Ara h 3</td>
<td>3.48±0.24 a</td>
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<tr>
<td>14</td>
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<td>Ara h 3</td>
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<td>Ara h 3</td>
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<tr>
<td>19</td>
<td>Ara h 3</td>
<td>2.34±0.16 a</td>
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<td>6-19</td>
<td>Total Ara h 3</td>
<td>35.57±2.49 a</td>
</tr>
<tr>
<td>1-19</td>
<td>Total allergens</td>
<td>67.00±4.69 a</td>
</tr>
</tbody>
</table>

The results are the means of 3 determinations ± SD within a row followed by different letters are significantly different (P < 0.05). All spots are demonstrated with IgE-binding properties by western-blot and previous literature. *Spot 1: isoallergens of Ara h 1; spot 2-5: isoallergens of Ara h 2; spot 6-19: isoallergens of Ara h 3 (Chassaigne, Trégoat, Nørgaard, Maleki, & van Hengel, 2009; Schmidt et al., 2009).
Table 4.3  IgE binding properties of peanut hydrolysates under eight enzyme hydrolysis with different concentrations.

<table>
<thead>
<tr>
<th>Conc. (AzU/g)</th>
<th>Ficin</th>
<th>Bromelain</th>
<th>Papain</th>
<th>Neutrase</th>
<th>Alcalase</th>
<th>Protemax</th>
<th>Thermolysin</th>
<th>Novo-ProD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>15.4±1.13</td>
<td>27.31±2.11</td>
<td>34.12±3.09</td>
<td>73.65±6.23</td>
<td>66.28±5.12</td>
<td>50.12±3.98</td>
<td>98.24±3.23</td>
<td>72.32±5.55</td>
</tr>
<tr>
<td>200</td>
<td>5.2±0.36</td>
<td>9.90±0.89</td>
<td>15.40±1.39</td>
<td>46.00±3.22</td>
<td>34.06±2.38</td>
<td>29.83±2.68</td>
<td>96.23±2.44</td>
<td>45.28±4.08</td>
</tr>
<tr>
<td>300</td>
<td>1.40±0.04</td>
<td>4.10±0.18</td>
<td>6.23±0.26</td>
<td>34.53±2.41</td>
<td>25.23±1.96</td>
<td>21.11±2.05</td>
<td>94.12±2.45</td>
<td>31.98±1.96</td>
</tr>
<tr>
<td>400</td>
<td>0.00±0.00</td>
<td>1.20±0.02</td>
<td>2.31±0.11</td>
<td>25.10±1.84</td>
<td>16.56±1.11</td>
<td>13.75±1.09</td>
<td>92.11±3.55</td>
<td>23.12±2.01</td>
</tr>
</tbody>
</table>

The reaction was conducted with 0-400 AzU/g of enzymes and reacted for 2 hr at 50 °C (pH 7.5). *IgE binding properties were tested by ELISA with pooled human plasma from allergic person.

Table 4.4  Rate constant, adjusted R square and P value IgE binding properties under different enzyme hydrolysis based on the first order assumption (calculated from the data of Table 4.3).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Rate constant (K)</th>
<th>R²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficin</td>
<td>0.9</td>
<td>0.91</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Bromelain</td>
<td>0.47</td>
<td>0.94</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Papain</td>
<td>0.4</td>
<td>0.93</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Neutrase</td>
<td>0.15</td>
<td>0.95</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Alcalase</td>
<td>0.20</td>
<td>0.92</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Protemax</td>
<td>0.21</td>
<td>0.90</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>0.01</td>
<td>0.97</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Novo-ProD</td>
<td>0.16</td>
<td>0.96</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Log[IgE binding properties]=−K*C. K: constant rate, represents log [IgE binding properties] reducing rate by adding 100 AzU/g of enzyme. C: concentration of enzyme (AzU/g protein).
Table 4.5  Allergen remaining and IgE-binding percentages of roasted MS-9 & MS-7 water-soluble protein extracts treated without (-)/with (+) TGase (5 U/g).

<table>
<thead>
<tr>
<th>TGase*</th>
<th>Peanut</th>
<th>Ara h 1 (%)</th>
<th>Ara h 2 (%)</th>
<th>Ara h 3 (%)</th>
<th>IgE binding properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>MS-9</td>
<td>100±3.45 a</td>
<td>100±4.23 a</td>
<td>100±2.78 b</td>
<td>100±4.11 a</td>
</tr>
<tr>
<td>-</td>
<td>MS-7</td>
<td>81.23±6.67 b</td>
<td>79.65±6.12 b</td>
<td>108.73±6.98 a</td>
<td>91.36±7.34 b</td>
</tr>
<tr>
<td>+</td>
<td>MS-9</td>
<td>41.00±3.28 c</td>
<td>59.00±4.72 c</td>
<td>43.00±3.44 de</td>
<td>98.00±6.82 a</td>
</tr>
<tr>
<td>+</td>
<td>MS-7</td>
<td>32.00±2.24 d</td>
<td>52.00±4.68 d</td>
<td>41.00±2.46 e</td>
<td>90.30±5.48 b</td>
</tr>
</tbody>
</table>

*Protein extracts treated without TGase was expressed as ‘-’; with TGase was expressed as “+”. The results are the means of 3 determinations ± SD within a column followed by different letters are significantly different (P < 0.05). The allergen remaining percentage (based on SDS-PAGE) and IgE binding properties (Based on ELISA with pooled human plasma containing IgE antibody against peanut allergens) of MS-9 roasted peanut proteins were set as 100%.
Table 4.6 Allergen remaining and IgE-binding percentages of roasted MS-9 & MS-7 water-soluble hydrolysates treated without (-)/with (+) TGase (5 U/g).

<table>
<thead>
<tr>
<th>TGase*</th>
<th>Peanut</th>
<th>Enzyme</th>
<th>Ara h 1 (%)</th>
<th>Ara h 2 (%)</th>
<th>Ara h 3 (%)</th>
<th>IgE binding properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>MS-9</td>
<td>Papain</td>
<td>1.52±0.15 a</td>
<td>25.28±2.53 a</td>
<td>17.74±1.77 ef</td>
<td>15.40±1.39 a</td>
</tr>
<tr>
<td>-</td>
<td>MS-9</td>
<td>Ficin</td>
<td>0.37±0.04 de</td>
<td>15.58±1.56 cd</td>
<td>13.34±1.33 gh</td>
<td>5.20±0.36 e</td>
</tr>
<tr>
<td>-</td>
<td>MS-9</td>
<td>Bromelain</td>
<td>0.40±0.04 d</td>
<td>16.19±1.62 cd</td>
<td>28.60±2.86 b</td>
<td>9.90±0.89 bc</td>
</tr>
<tr>
<td>-</td>
<td>MS-7</td>
<td>Papain</td>
<td>1.29±0.11 b</td>
<td>19.72±1.18 b</td>
<td>21.28±1.70 cd</td>
<td>11.20±1.39 b</td>
</tr>
<tr>
<td>-</td>
<td>MS-7</td>
<td>Ficin</td>
<td>0.32±0.02 de</td>
<td>10.75±0.86 fgh</td>
<td>15.34±1.23 fg</td>
<td>3.40±0.36 f</td>
</tr>
<tr>
<td>-</td>
<td>MS-7</td>
<td>Bromelain</td>
<td>0.34±0.02 de</td>
<td>11.66±0.93 fg</td>
<td>32.03±2.56 a</td>
<td>7.10±0.89 d</td>
</tr>
<tr>
<td>+</td>
<td>MS-9</td>
<td>Papain</td>
<td>1.30±0.10 b</td>
<td>24.00±1.92 a</td>
<td>11.00±0.88 hji</td>
<td>14.40±1.04 a</td>
</tr>
<tr>
<td>+</td>
<td>MS-9</td>
<td>Ficin</td>
<td>0.08±0.01 f</td>
<td>13.00±1.04 ef</td>
<td>9.65±0.77 j</td>
<td>4.80±0.58 e</td>
</tr>
<tr>
<td>+</td>
<td>MS-9</td>
<td>Bromelain</td>
<td>0.35±0.03 de</td>
<td>13.30±0.94 ef</td>
<td>20.00±1.60 de</td>
<td>9.67±0.44 c</td>
</tr>
<tr>
<td>+</td>
<td>MS-7</td>
<td>Papain</td>
<td>1.00±0.01 c</td>
<td>17.00±0.90 c</td>
<td>13.00±0.91 hgi</td>
<td>10.60±0.68 bc</td>
</tr>
<tr>
<td>+</td>
<td>MS-7</td>
<td>Ficin</td>
<td>0.06±0.01 f</td>
<td>10.20±0.82 gh</td>
<td>10.21±0.82 ij</td>
<td>3.00±0.27 f</td>
</tr>
<tr>
<td>+</td>
<td>MS-7</td>
<td>Bromelain</td>
<td>0.28±0.02 e</td>
<td>8.90±0.71 h</td>
<td>23.30±1.86 c</td>
<td>6.92±0.10 d</td>
</tr>
</tbody>
</table>

*Hydrolysates (hydrolyzed by 200 AzU/g enzyme) treated without TGase was expressed as ‘-’; with TGase (5U/g) was expressed as ‘+’. The results are the means of 3 determinations ± SD within a column followed by different letters are significantly different (P < 0.05). The allergen remaining percentage (based on SDS-PAGE) and IgE binding properties (Based on ELISA with pooled human plasma containing IgE antibody against peanut allergens) of MS-9 roasted peanut proteins were set as 100%.
The initial value, Peleg’s rate constant ($K_1$), Peleg’s capacity constant ($K_2$), estimated equilibration value and $R^2$ square of functional properties (EAI, ESI, FC, FS) at pH 8 based on Peleg’s model.

<table>
<thead>
<tr>
<th>Functional Properties</th>
<th>Type of Hydrolysates</th>
<th>Initial value ($F_0$)</th>
<th>$K_1$</th>
<th>$K_2$</th>
<th>Estimated Equilibration value ($F_{c \rightarrow \infty}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bromelain</td>
<td>588</td>
<td>0.0057</td>
<td>0.0076</td>
<td>720</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Ficin</td>
<td>410</td>
<td>0.0060</td>
<td>0.0057</td>
<td>585</td>
<td>0.94</td>
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<tr>
<td></td>
<td>Papain</td>
<td>495</td>
<td>0.0067</td>
<td>0.0049</td>
<td>700</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>PP</td>
<td>448</td>
<td>0.0034</td>
<td>0.0045</td>
<td>670</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>Bromelain</td>
<td>25</td>
<td>0.1451</td>
<td>0.1488</td>
<td>32</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>Ficin</td>
<td>20</td>
<td>0.2512</td>
<td>0.1568</td>
<td>26</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>Papain</td>
<td>19</td>
<td>0.0702</td>
<td>0.0895</td>
<td>30</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>PP</td>
<td>28</td>
<td>0.8481</td>
<td>0.6499</td>
<td>30</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>Bromelain</td>
<td>48</td>
<td>0.0112</td>
<td>0.0100</td>
<td>148</td>
<td>0.95</td>
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<tr>
<td></td>
<td>Ficin</td>
<td>30</td>
<td>0.0141</td>
<td>0.0144</td>
<td>100</td>
<td>0.96</td>
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<tr>
<td></td>
<td>Papain</td>
<td>45</td>
<td>0.0159</td>
<td>0.0106</td>
<td>139</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>PP</td>
<td>35</td>
<td>0.0615</td>
<td>0.0444</td>
<td>58</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>Bromelain</td>
<td>39</td>
<td>0.0152</td>
<td>0.0172</td>
<td>97</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Ficin</td>
<td>13</td>
<td>0.0225</td>
<td>0.0166</td>
<td>73</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>Papain</td>
<td>38</td>
<td>0.0327</td>
<td>0.0206</td>
<td>87</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>PP</td>
<td>62</td>
<td>0.0239</td>
<td>0.0276</td>
<td>98</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Peleg’s model: $F(c) = F_0 + c/(K_1 + K_2c)$. $F(c)$: the functional properties when TGase’s concentration at $c$ Unit/g. PP: roasted peanut protein. The hydrolysates were obtained by adding 200 AzU/g of enzymes into peanut powders and reacted for 2 hr at 50 °C, pH 7.5. TGase treatment was conducted by adding 0.5 U/g TGase into hydrolysates and reacted for 2 hr at 37 °C, pH 7.5. PP: Roasted peanut protein without hydrolysis.
Figure 4.2  SDS-PAGE analysis of the eleven selected peanuts with lower allergens (harvested in 2016).

M represents the molecular mass marker (molecular masses are shown beside the markers). Ara h 1 (63 kD), Ara h 2 (17, 19 kD), Ara h 3 (22, 36, 38 & 40 kD) and Ara h 6 (14 kD) represents the major allergen of the peanuts. Lane 1-11 represents screened peanuts (MS-1– MS-11) polypeptides. MS-9 (Valencia) represents the check.
Figure 4.3  2D SDS-PAGE (A) and Western-blot (B) of MS-7 (selected) peanut (harvested in 2016) protein.

Human plasma (containing anti-peanut IgE antibody) was used for western-blot analysis. Major allergenic spots (1-8) were labeled in the correlated location in the SDS-PAGE. Spot 1: Ara h 1; Spot 2-5: Ara h 2; spot 6-19: Ara h 3 (Chassaigne, Trégoat, Nørgaard, Maleki, & van Hengel, 2009; Schmidt et al., 2009)
Figure 4.3 (continued)
Figure 4.4  2D SDS-PAGE (A) and Western-blot (B) of MS-9 (check) peanut (harvested in 2016) protein.

Human plasma (containing anti-peanut IgE antibody) was used for western-blot analysis. Major allergenic spots (1-8) were labeled in the correlated location in the SDS-PAGE. Spot 1: Ara h 1; Spot 2-5: Ara h 2; spot 6-19: Ara h 3 (Chassaigne, Trégoat, Nørgaard, Maleki, & van Hengel, 2009; Schmidt et al., 2009)
The TGase treatment was conducted on hydrolysates added with TGase (0.5 U/g protein) and reacted for 2 hr at 37 °C (pH 7.5). PP: roasted peanut powder extract; (T)PA, (T)F and (T)B represent (TGase treated) papain, ficin and bromelain hydrolysates, respectively. TPP: TGase treated PP. Ara h 1 (63 kD), Ara h 2 (17, 19kD), Ara h 3 (22, 36, 38 & 40 kD) and Ara h 6 (14 kD) represents the major allergen of the peanuts. MS-9 is the check peanut. MS-7 is the selected line with lower Ara h 1 and Ara h 2 content. MS-9 is the check peanut. MS-7 is the selected line with lower Ara h 1 and Ara h 2 content. Pooled human plasma containing IgE antibody against peanut allergens were used for western-blot analysis.
<table>
<thead>
<tr>
<th>PP</th>
<th>PA</th>
<th>F</th>
<th>B</th>
<th>PP</th>
<th>PA</th>
<th>F</th>
<th>B</th>
<th>TPP</th>
<th>TPA</th>
<th>TF</th>
<th>TB</th>
<th>TPP</th>
<th>TPA</th>
<th>TF</th>
<th>TB</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-7</td>
<td>MS-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGase (-)</td>
<td>TGase (+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Figure 4.5 (continued)
Figure 4.6  SDS-PAGE (A) western-blot (B) of roasted MS-7 residues (SDS sample-buffer soluble) after hydrolyzed with selected food grade enzymes.

The reaction was conducted with 200 AzU/g of enzymes and reacted for 2 hr at 50 °C (pH 7.5). PP: roasted peanut protein; PA: hydrolyzed by papain; F: hydrolyzed by ficin; B: hydrolyzed by bromelain. Ara h 1 (63 kD), Ara h 2 (17, 19 kD), Ara h 3 (22, 36, 38 & 40 kD) and Ara h 6 (14 kD) represents the major allergen of the peanuts. Pooled human plasma containing IgE antibody against peanut allergens were used for western-blot. MS-9 is the check peanut. MS-7 is the selected line with lower Ara h 1 and Ara h 2 content.
Figure 4.7  Emulsion activity index (EAI) and emulsion stability index (ESI) of MS-7 (selected) peanut hydrolysates treated without (A&B) and with (C&D) TGase.

PP: roasted peanut powder extract; SPI: soy protein isolate. (T)PA, (T)F and (T)B represent (TGase treated) papain, ficin and bromelain hydrolysates, respectively. TPP: TGase treated PP.
Figure 4.7 (continued)

PP: roasted peanut powder extract; SPI: soy protein isolate (no TGase treatment). (T)PA, (T)F and (T)B represent (TGase treated) papain, ficin and bromelain hydrolysates, respectively. TPP: TGase treated PP.
Figure 4.8  Foaming capacity (FC) and foaming stability (FS) of MS-7 (selected) peanut hydrolysates treated without (A&B) and with (C&D) TGase.

PP: roasted peanut powder extract; SPI: soy protein isolate. (T)PA, (T)F and (T)B represent (TGase treated) papain, ficin and bromelain hydrolysates, respectively. TPP: TGase treated PP.
Figure 4.8 (continued)

PP: roasted peanut powder extract; SPI: soy protein isolate (no TGase treatment). (T)PA, (T)F and (T)B represent (TGase treated) papain, ficin and bromelain hydrolysates, respectively. TPP: TGase treated PP.
Figure 4.9  Emulsion activity Index (EAI, at pH 8), emulsion stability index (ESI, at pH 8), foaming capacity (FC, at pH 8) and foaming stability (FS, at pH 8) of three enzyme hydrolysates (MS-7) treated by different concentration (0-10 AzU/g protein) of TGase.

EAI, ESI, FC and FS are shown in Figure A, B, C and D, respectively. B, F, PA, PP represent bromelain, ficin, papain hydrolysates and roasted MS-7 peanut protein, respectively.
EAI, ESI, FC and FS are shown in Figure A, B, C and D, respectively. B, F, PA, PP represent bromelain, ficin, papain hydrolysates and roasted MS-7 peanut protein, respectively.
REFERENCES


Burks, A. W., Williams, L. W., Connaughton, C., Cockrell, G., O'Brien, T. J., & Helm, R. M. (1992). Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *Journal of Allergy and Clinical Immunology, 90*, 962-969.


APPENDIX A

MAJOR ALLERGEN CONTENT OF 122 PEANUT LINES HARVESTED IN 2015
Table A.1  The major allergen content of 97 USDA-minicore peanut cultivars harvested in year 2015 (Clovis, NM).

<table>
<thead>
<tr>
<th>No.</th>
<th>PI number</th>
<th>Ara h 1</th>
<th>Ara h 2</th>
<th>Ara h 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>152146</td>
<td>13.93±1.14</td>
<td>8.31±0.24</td>
<td>53.69±2.32</td>
<td>75.92±3.30</td>
</tr>
<tr>
<td>2</td>
<td>155107</td>
<td>13.19±1.10</td>
<td>8.29±0.66</td>
<td>54.89±2.76</td>
<td>76.36±3.72</td>
</tr>
<tr>
<td>3</td>
<td>157542</td>
<td>14.74±1.19</td>
<td>7.36±0.51</td>
<td>50.82±2.30</td>
<td>72.91±3.21</td>
</tr>
<tr>
<td>4</td>
<td>158854</td>
<td>13.84±0.82</td>
<td>8.65±0.71</td>
<td>50.81±1.93</td>
<td>73.30±2.66</td>
</tr>
<tr>
<td>5</td>
<td>159786</td>
<td>14.27±1.05</td>
<td>6.91±0.12</td>
<td>50.28±2.30</td>
<td>71.46±3.47</td>
</tr>
<tr>
<td>6</td>
<td>162655</td>
<td>14.24±1.14</td>
<td>8.24±0.76</td>
<td>54.09±1.78</td>
<td>76.56±2.28</td>
</tr>
<tr>
<td>7</td>
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<td>13.83±1.27</td>
<td>7.25±0.24</td>
<td>54.89±2.76</td>
<td>76.36±3.72</td>
</tr>
<tr>
<td>8</td>
<td>196622</td>
<td>14.75±0.21</td>
<td>7.34±0.10</td>
<td>49.34±0.29</td>
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</tr>
<tr>
<td>9</td>
<td>196635</td>
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<td>7.43±0.19</td>
<td>49.56±2.77</td>
<td>71.43±3.06</td>
</tr>
<tr>
<td>10</td>
<td>200441</td>
<td>14.10±0.20</td>
<td>8.36±0.48</td>
<td>52.19±1.32</td>
<td>74.64±2.00</td>
</tr>
<tr>
<td>11</td>
<td>240560</td>
<td>13.90±0.24</td>
<td>8.89±0.18</td>
<td>52.40±1.46</td>
<td>75.19±1.89</td>
</tr>
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The allergen content was expressed by the % of total soluble protein.
The allergen content was expressed by the % of total soluble protein.
Table A.1 (continued)

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The allergen content was expressed by the % of total soluble protein.
Table A.2  The major allergen content of 25 peanut cultivars from other resources harvested in year 2015 (Clovis, NM).

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<th>No.</th>
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</table>

The allergen content was expressed by the % of total soluble protein.
APPENDIX B

SDS-PAGE OF ROASTED PEANUT HYDROLYZED BY EIGHT FOOD GRADE ENZYMES WITH DIFFERENT CONCENTRATIONS
Figure B.1  SDS-PAGE of roasted peanut (MS-9) hydrolyzed by eight types of food grade enzymes with different concentrations (0-400 AzU/g protein).

The reaction was conducted with 0-400 AzU/g of enzymes and reacted for 2 hr at 50 °C (pH 7.5).
Figure B.1 (continued)

The reaction was conducted with 0–400 AzU/g of enzymes and reacted for 2 hr at 50 °C (pH 7.5).
APPENDIX C

HYDROPHOBICITY, VISCOSITY AND NET CHARGE OF THE ROASTED ONE PEANUT PROTEIN/HYDROLYSATES
Table A.3  Hydrophobicity, viscosity of roasted MS-7 peanut protein/hydrolysates and net charge of the emulsion prepared by roasted peanut protein/hydrolysates.

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<th>Viscosity (cP)</th>
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<td>PP</td>
<td>246.3±13.2 a</td>
<td>-29.9±2.5 a</td>
<td>32.2±2.4 a</td>
</tr>
<tr>
<td>+</td>
<td>Papain</td>
<td>199.6±10.6 bc</td>
<td>-35.6±2.4 b</td>
<td>26.7±2.1 bc</td>
</tr>
<tr>
<td>+</td>
<td>Ficin</td>
<td>160.5±9.7 ef</td>
<td>-38.8±2.3 b</td>
<td>19.4±1.8 ef</td>
</tr>
<tr>
<td>+</td>
<td>Bromelain</td>
<td>197.5±13.2 bcd</td>
<td>-37.2±2.1 b</td>
<td>25.2±1.9 cd</td>
</tr>
</tbody>
</table>

*Hydrolysates (hydrolyzed by 200 AzU/g enzyme) treated without TGase was expressed as ‘-’; with TGase (5U/g) was expressed as “+”. The results are the means of 3 determinations ± SD within a column followed by different letters are significantly different (P < 0.05).