

## ABSTRACT

SHI, XIAOLEI. Improving the Value of Peanut Flour using Enzymatic Hydrolysis.  
(Under the direction of Dr. Jack P. Davis.)

Peanut flour is an established food ingredient; however, the potential exists to improve, through processing techniques, the functional and/or bioactive properties of this ingredient. Peanut flour is also the active ingredient in peanut oral immunotherapy (OIT) trials; however, during OIT the potential exists for an adverse immune response. The objectives of this research were to understand the potential of enzymatic hydrolysis: 1) to enhance functional and nutritional/bioactive properties of peanut flour as a food ingredient and 2) to generate peptides from peanut flour with improved OIT properties.

Soluble fractions of 10 % (w/v) light roasted peanut flour dispersions were hydrolyzed with the following proteases: Alcalase (pH 8.0, 60 °C), pepsin (pH 2.0, 37 °C), Flavourzyme (pH 7.0, 50 °C), or sequentially with Alcalase and Flavourzyme. The trinitrobenzenesulfonic acid (TNBS) method was used to determine degree of hydrolysis (DH). Protein solubility was determined by the BCA protein assay and soluble nitrogen measurements. Angiotensin-converting enzyme (ACE) inhibition was determined according to standard procedures. SDS-PAGE was used to visualize generated peptide profiles. Size exclusion chromatography (SEC) was used to fractionate hydrolysates and to measure the mass frequency of resulting fractions. Immunoreactivities of hydrolysates and respective unhydrolyzed controls were evaluated by: 1) Western blotting against immunoglobulin type E (IgE) to determine IgE binding potential, and 2) functional assay – basophil activation test to understand IgE cross-linking capacity.

Protein contents of soluble fractions varied significantly ( $p < 0.05$ ) with pH, which was adjusted according to optimal conditions for different proteases. DH varied significantly ( $p < 0.05$ ) across all proteases and generally increased ( $p < 0.05$ ) with increasing hydrolysis times, ultimately ranging from 14.9 to 37.4%. Alcalase hydrolysis showed the greatest capacity to release ACE inhibitory peptides from peanut flour, indicating the potential use of Alcalase hydrolysates as the starting material for further purification of ACE inhibitory peptides.

SDS-PAGE was used to understand protein degradation by the various proteases. Hydrolysis by all tested proteases resulted in substantial degradation of the major peanut allergens (Ara h 1, Ara h 2, and Ara h 3/4) and the accumulation of novel bands in the 0-28 kDa molecular weight range. Western blotting experiments revealed these novel peptides retained some residual IgE binding reactivity, with the relative capacity to degrade known peanut allergens being: Alcalase > Flavourzyme > pepsin. Additional Western blotting experiments using specific anti-Ara h 1 and anti-Ara h 2 rabbit sera confirmed that these novel IgE reactive peptides were primarily Ara h 2 fragments regardless of protease tested.

Despite extensive reduction in IgE *binding* as revealed by initial Western blotting experiments, a more robust and biologically relevant basophil activation test ( $n = 4$ ) revealed that all individual hydrolysates were comparable ( $p > 0.05$ ) to unhydrolyzed controls in IgE *cross-linking* capacity. Sequential Alcalase-Flavourzyme hydrolysates were generated with the goal to further degrade peanut allergens. The sequential hydrolysates had higher ( $p < 0.05$ ) DH values and retained substantially less IgE binding capacity than any of individual hydrolysates, indicating a more complete degradation. Furthermore, mass frequency profiles of the sequential hydrolysates as determined by SEC suggested extensive hydrolysis, with

only 6.1 % peptides having a mass > 2.0 kDa. The basophil activation tests of these sequential hydrolysates and respective fractions are ongoing.

These results indicate that hydrolysis of peanut flour can be successfully used as a processing strategy to generate hydrolysates with enhanced ACE inhibitory potential and reduced IgE binding capacity. However, the unchanged IgE cross-linking capacity indicated these hydrolysates may not be superior to intact proteins in peanut flour for OIT. Work is ongoing to better understand these phenomena as described in the ‘Conclusions and Future Work’ section of this document.

Enzymatic Hydrolysis for Improving the Value of Peanut Flour Protein

by  
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## **DEDICATION**

I dedicate this work to my parents. Thank you for your continuous caring and love throughout my lifetime and always showing me support to achieve my goals.

## **BIOGRAPHY**

Xiaolei Shi was born on August 11, 1987 in Rizhao, Shandong, P. R. China. She is the daughter of Jifu Shi and Jinying Xia of Shandong, China. Xiaolei graduated from Rizhao Yizhong high school, where she dedicated as the deputy secretary of the student union. She continued her education at the Huazhong Agricultural University, Wuhan, China. She majored in Plant Protection for the first year and then transferred to Food Quality and Safety. There, Xiaolei obtained scholarship each year from 2007 to 2010 and was an active show hostess. Xiaolei earned a Bachelor of Science in May 2010 and then continued her education as a Masters student in Food Science at North Carolina State University, Raleigh, NC, USA. She is an active member of the Food Science Club. In 2012, she presented in the Food Chemistry division poster section at the IFT Annual Meeting. Upon graduation, she will continue her education as a PhD student in Food Science, under the direction of Dr. Jack P. Davis.

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**CHAPTER 1:**  
**Literature Review**

## **The Peanut**

The peanut (*Arachis hypogaea* L.), is an annual legume grown in the tropics and temperate regions in nearly 100 different countries. With a farm value of more than one billion U.S. dollars, the peanut ranks the 12th most valuable cash crop grown in the United States. Unlike other countries where the end product is mainly peanut oil, most peanuts grown in the U.S. are used for whole seed consumption and the major value-added products of peanut are peanut butter, confections, and snack products [1].

The U.S. ranks as the world's third largest peanut producer, after China and India. Since most of the peanuts in India are consumed domestically, the U.S. is one of the world's leading peanut exporters. Other significant exporters are Argentina and China [2].

There are four basic market types of U.S. peanuts: Runner, Virginia, Spanish and Valencia. Each type differs in size and flavor. Runner peanuts are medium-size varieties of the Virginia type and typically account for 80 % of U.S. peanut production in recent decades due to their high yields, and the vast majority of Runners are used in peanut butter production. Virginia peanuts account for 15 % of U.S. production and are characterized by the largest kernel size. The main product of this type is in-shell roasted or shelled-salted peanuts. Spanish-type peanuts account for 4 % of U.S. production and feature a reddish-brown skin. This type is usually produced into peanut candy. Valencia peanuts account for less than 1 % of U.S. production, typically with three or more small kernels in one pod. Since the seeds of this type are quite sweet, they are usually sold as in-shell roasted as well as boiled peanuts [3].

## **Peanut Components**

Peanuts are typically characterized by approximately 44-56 % lipid, 22-30 % protein and a low percentage of carbohydrates as well as ash [4]. The peanut oil and protein are discussed in detail below. As for the carbohydrates, the peanut kernel contains about 4 % starch, 2 % cellulose and 4 % pectin [5]. The most abundant sugar is sucrose, which varies from 2.86 to 6.35 % among various peanut cultivars, followed by stachyose and raffinose [6]. Peanuts are also good sources of some minerals and vitamins. Peanuts contain much more potassium than sodium, and they are also good sources of phosphorous and magnesium. Also, peanuts contain abundant levels of vitamin B-complex and choline [7].

## **Peanut Oil**

Peanuts are good source of lipids, proteins and fatty acids. Peanuts are naturally high in oil content, around 44-56 % [7]. Peanut oil consists of 96.1 to 96.4 % triglycerides, 2.4 to 2.9 % phospholipids, 0.69 to 0.80 % sterols, 0.1 to 0.4 % free fatty acids, and 0.10 to 0.14 % glycolipids [8]. One molecule of triglyceride is composed of one glycerol and three fatty acid moieties. The estimated fatty acid composition for peanut is summarized in Table 1 [4]. Since palmitic (C 16:0), oleic (C 18:1), and linoleic acids (C 18:2) constitute about 90 % of peanut oil, proportions of these fatty acids are most important from both human nutrition perspectives and oil stability [7]. Oleic acid, the main monounsaturated fatty acid in peanut, is reported to have health benefits in reducing the risk of cardiovascular disease, preventing cancer, increasing insulin sensitivity and ameliorating some inflammatory diseases [8]. Linoleic acid, the main polyunsaturated fatty acid in peanut, is considered to increase the

chances of oxidation and rancidity of peanut oil, thus limiting the shelf life [9]. High oleic, low linoleic peanut lines with approximately 80% oleic acid and 3% linoleic acid contents have been discovered and disseminated using peanut breeding [10]. High oleic peanuts and derived peanut oils are less prone to oxidation and hence have substantially increased shelf lives compared to traditional peanut seed [11].

**Table 1.** Typical fatty acid composition of traditional (non High Oleic) peanut oil. Table was adapted from [4].

Fatty acid	Percentage (%)
Palmitic (C16:0)	10 %
Stearic (C 18:0)	3 %
Oleic (C 18:1)	45 %
Linoleic (C 18:2)	35 %
Behenic (C 22:0)	2 %
Others	5 %

Peanut oil also contains other minor components, including phospholipids, sterols, tocopherols, pigments and phenolic compounds, of which tocopherols and phenolic compounds are considered health beneficial due to their antioxidant and bioactive capacity [7]. Four forms of tocopherol have been identified in peanut as  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ , with  $\gamma$ -tocopherol showing the highest antioxidant capacity and  $\delta$ -tocopherol having the lowest [12].

### **Peanut Protein**

The protein content of whole peanut seeds ranges from approximately 22 to 30 % and has been traditionally grouped into two categories: globulins (salt soluble) and albumins (water soluble) [7, 13]. Globulins consist of arachin, conarachin and non-arachin. Arachin and conarachin are the major storage proteins and together these proteins account for more than

85 % of the total peanut protein content. Arachin differs from conarachin mainly by the amount of sulfur content: 0.4 % for arachin and 1.9 % for conarachin [14]. Peanut albumins consist of agglutinins, lectin-reactive glycoproteins, protease inhibitors, alpha-amylase inhibitors, and phospholipases [15].

The solubility of peanut proteins is very sensitive to pH. The isoelectric point of most peanut proteins falls between pH 4.0 and 5.0, but this range may shift in different media [7]. Near their isoelectric point, peanut proteins have net neutral electrostatic charges which minimize protein solubility and this lack of solubility can negatively impact the proteins' emulsifying properties [16, 17]. Above or below the isoelectric point, peanut proteins show sharp rises in solubility [18]. Roasting significantly decreases the extractability of peanut proteins at pH 5.0-10.0, while minimally impacting protein solubility from pH 2.0 to 5.0 [18]. Starting with 5 % flour/water dispersions, the most significant difference was observed at pH 7.0 in which nearly 30.0 % soluble protein was extracted from raw peanut flour compared to about 10% from roasted flours [18].

The solubility of completely defatted peanut protein varies with different extraction media [19]. In this study, relatively pure isolates of arachin and non-arachin proteins were extracted from ground, defatted peanut seeds using different extraction media, which were deionized water, sodium phosphate buffer (I = 0.03; pH 7.9), 10 % NaCl and 1 M NaCl + 20 mM sodium phosphate buffer (pH 7.0). These extracts were dialyzed against water to lower the level of salts and were adjusted to pH 1.0 to 12.0 with 1 N NaOH or 1 N HCl. Peanut proteins extracted from 10 % NaCl and 1 M NaCl + 20 mM sodium phosphate buffer (pH 7.0) media showed isoelectric points between pH 3.0 and 3.5, while those extracted from

sodium phosphate buffer and deionized water were approximate at pH 4.5 and 5.0, respectively. Also, protein solubility in extraction media containing sodium chloride increased from 1.3 to 7.5 mg/ml from pH 3.5 to 6.5, while the highest protein solubility for peanut protein dispersed in deionized water was 2.6 mg/ml at pH 9.0, and the highest protein solubility for peanut protein dispersed in phosphate buffer was 4.0 mg/ml at pH 10.0.

Peanut flour, peanut meal, peanut concentrates and peanut isolates are four primary protein-enriched peanut materials obtained from peanut seed. Except for peanut meal, all are food grade ingredients [2]. Peanut concentrates and isolates can be prepared by simultaneous separation of peanut protein and oil from raw peanut seeds using an aqueous media [20]. The main difference between concentrates and isolates is that isolates are further purified to remove non-protein components and have protein contents > 90 %. In the US, the term peanut meal is used to describe the primary by-product of commercial peanut oil production. This material is high in protein (~55%) and low in oil (< 3 %); however, the potential contamination of peanut meal with aflatoxin currently limits the applications of this material to animal feed [21].

### **Peanut Flour**

Peanut flour is a commercially available, high protein, low fat food ingredient prepared from roasted peanut seed. The raw peanut seeds are cleaned, blanched and electronically color sorted to select high quality peanuts for subsequent roasting, pressing and grinding to obtain defatted peanut flour [2]. Peanut flour can be light, medium or dark roasted, peanut flour contains 47 to 55 % high quality protein with high essential amino acid content and usually

contains 12 or 28 % fat. Current products containing peanut flour include confectioneries, seasoning blends, bakery mixes, frostings, fillings, and nutritional bars [2]. Since peanut flour is partially defatted, it works well as a fat binder in applications such as confection centers [22].

### **Enzymatic Hydrolysis**

Although peanut flours, concentrates and isolates are all edible peanut products and have good potential as nutritional ingredients, they still show limited functional properties compared with soy protein in many aspects, such as emulsification, foamability, solubility and nutritional properties [23]. Enzymatic hydrolysis, chemical hydrolysis or microbial fermentation can be used to breakdown the protein molecules to obtain well defined peptide profiles. Enzymatic hydrolysis is most widely used for the purpose of enhancing the functional, nutritional/bioactive and immunological properties of peanut protein because it is efficient and controllable [24]. Sequential treatment using endoproteases and exoproteases coupled with post-hydrolysis procedures is considered as an effective way to obtain protein hydrolysates with improved characteristics [25]. Endoproteases cleave peptide bonds of non-terminal amino acids, whereas exoproteases break peptide bonds from their end amino acids. Enzymatic hydrolysis usually results in three distinct effects: 1) a decrease in molecular weight; 2) an increase in the number of ionisable groups; 3) exposure of hydrophobic groups previously concealed [26].

Enzymatic hydrolysis is commonly carried out under strictly controlled conditions, as all enzymes have optimal pH and temperature conditions for maximum activity [27]. The extent

of proteolysis is commonly expressed as the degree of hydrolysis (DH), which refers to the percentage of peptide bonds cleaved within the parent protein [28]. Termination of enzymatic hydrolysis at the desired DH is important, as extensive hydrolysis may result in undesirable bitterness and loss of bioactivity. Common means to inactivate enzymatic proteolysis include heat or pH adjustment, which denature the enzymes rendering them inactive [29].

The three approaches most widely used for the quantification of DH during food protein hydrolysis are the pH stat, o-phthalaldehyde (OPA) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) methods [28]. The pH stat technique can be used when hydrolysis is carried out at neutral or alkaline conditions. The release of protons during hydrolysis leads to a reduction in the pH of the reaction medium, thus DH can be estimated from the amount of base required to maintain a constant pH during the reaction [30]. The OPA method is based on the specific reaction between OPA and primary amino groups, in the presence of a thiol to form 1-alkylthio-2-alkyl-substituted isoindoles, which can be detected spectrophotometrically at 340 nm or fluorometrically at 455 nm [31]. TNBS reacts with primary amino groups specifically to form a chromophore with a maximum absorbance at 340 nm, and this response can in turn be used to calculate a DH [27].

DH was shown to have a direct relationship with conformational and functional properties of peanut protein isolate (PPI) [32]. In this study, a 5 % (w/v) PPI dispersion was hydrolyzed with Alcalase, which is a serine protease, at pH 7.0 and 50 °C. At the desired DH, enzymatic hydrolysis was terminated by incubation at 85 °C for 10 min. The DH was determined by the OPA method. After the thermal treatment, the obtained hydrolysates were freeze-dried and then stored at -18 °C prior to further use. This study showed improved protein solubility and

gel-forming ability of PPI within the DH range of 2.1–5.4 %. Compared with unhydrolyzed PPI, hydrolysates had less compact tertiary conformation and better thermal stability.

Roasted peanut flours (Golden Peanut Company, Alpharetta, GA) and raw peanut flours prepared from blanched peanuts (Tara Foods, Albany, GA) were used as starting materials for an enzymatic hydrolysis study [33]. Pepsin–pancreatin was used to mimic human digestion, while Alcalase was used for an industrial perspective. Peanut flours were hydrolyzed at protein/extraction ratios of 1:80 (w/v) with either enzyme system in a water bath maintained at 37 °C for the sequential pepsin–pancreatin hydrolysis (weight ratio = 1: 250 for pepsin, 1: 25 for pancreatin), or 60 °C for the Alcalase hydrolysis (E/S = 0.3 AU/g protein). DH was measured using TNBS method. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to detect the peptide mass distributions. Results revealed that the Alcalase mediated peanut flour hydrolysis showed more rapid band disintegration than pepsin-pancreatin while both enzyme systems reached 20-25 % DH after 24 hours hydrolysis under the reported conditions. Also, the peptides with molecular weight (MW) < 20 kDa seemed more resistant to pepsin-pancreatin hydrolysis. These peptides were suspected to be Ara h 2 fragments, which are known to be very persistent to enzymatic hydrolysis and have a MW less than 17 kDa.

### **Selected Proteases**

Alcalase, pepsin and Flavourzyme are all water soluble, commercially available proteases. Alcalase and Flavourzyme are extensively used in industry while pepsin is the primary digestive protease in the stomach of humans. These proteases could be used individually, or

sequentially, to prepare degraded proteins with improved functionality and immunological properties for both commercial and research purposes. Alcalase, pepsin and Flavourzyme were the focus of current experiments. All three enzymes are characterized by their own specificity and optimal conditions (Table 2) [34-36]. Alcalase is a serine protease and works primarily as an endoprotease. The activity units for Alcalase are the Anson units (AU), which is defined as the amount of enzyme which, under specified conditions, digests urea-denatured hemoglobin at an initial rate such that there is liberated an amount of TCA-soluble product per minute which gives the same color with Folin-Ciocalteu Phenol reagent as one milliequivalent of tyrosine at 25 °C/pH = 7.50. Flavourzyme has both endoprotease and exoprotease activities. The most important commercial application of Flavourzyme is for debittering of hydrolysates in which it is used as an exoprotease. The activity units for Flavourzyme are LAPU. One LAPU is the amount of enzyme which hydrolyzes 1  $\mu\text{mol}$  of L-leucine-p-nitroanilide per minute. Flavourzyme can be used for extensive hydrolysis at E/S ratio > 10 LAPU. One unit (U) of pepsin is defined as the enzyme activity that increases the absorbance at 280nm of 0.001/min at pH 2.0 and 37 °C with hemoglobin as substrate.

**Table 2.** Proteases selected for peanut flour hydrolysis. This table was adapted from [34-36].

	<b>Alcalase</b>	<b>Flavourzyme</b>	<b>Pepsin</b>
<b>Type</b>	endoprotease	exoprotease; endoprotease	endoprotease (exoprotease)
<b>Source</b>	<i>Bacillus licheniformis</i>	<i>Aspergillus oryzae</i>	porcine stomach mucosa
<b>Specificity/ Application</b>	large uncharged; hydrophobic amino acid, especially serine type	protein-debittering (< 10 LAPU/g); extensive hydrolysis (10-50 LAPU/g)	low specificity; hydrophobic, aromatic amino acid
<b>Optimal conditions</b>	pH 8.0; 60 °C	pH 7.0; 50 °C	pH 2.0; 37 °C

## Bioactive Peptides

Bioactive peptides are breakdown fragments of proteins generated by proteases present in the gastro-intestinal tract, or by enzymes present during food processing. These peptides have bioactivity only after release from the parent protein source [37]. A general bioactive mechanism across these peptides is the capacity to modulate the metabolic enzymes that participate in the pathogenesis of chronic diseases. Examples of reported bioactivities include antihypertensive, antioxidant, anticancer, anti-amnesic and immunomodulatory properties as well as cholesterol-lowering functions [38]. Bioactive peptides usually contain 3–20 amino acid residues, but in some cases this range may be extended [39]. Enzymatic hydrolysis, chemical hydrolysis and microbial fermentation can be used to generate bioactive peptides, with enzymatic hydrolysis being most common as this approach is efficient, controllable and economical [40]. Various traditional protein sources, including milk, egg, fish, meat, soy,

rice and potato have been used for the preparation of peptides with improved bioactive properties [24].

One focus within research involving bioactive peptides is Angiotensin I converting enzyme (ACE; kinases II peptidyl dipeptide hydrolase) inhibitory peptides. ACE is important for blood pressure regulation by converting inactive prohormone angiotensin I (decapeptide) to angiotensin II (octapeptide), thus causing increased blood pressure through vasoconstriction, by increased systemic resistance and stimulated secretion of aldosterone [37]. Increased blood pressure is known as hypertension, and can cause blood vessel changes in the retina, abnormal thickening of the heart muscle, stroke, heart attack and arterial aneurysm [41]. ACE inhibitory peptides have to be released from the original protein for physiological benefits through either *in vivo* gastrointestinal digestion or during protein processing. From previous studies, ACE inhibitory peptides have been isolated from multiple plant proteins such as soybean [42], corn [43], rapeseed [44], canola [45] and peanut [46]. ACE inhibitory peptides derived from food proteins are used as milder and safer therapeutic agents to treat hypertension compared to synthetic drugs [47].

Peanut protein is considered a natural source of ACE inhibitory peptides [46]. Alcalase hydrolysis of peanut proteins resulted in increased ACE inhibition and antioxidant capacities with increasing of DH [48]. In this study, 10 % (w/v) PPI dispersion was adjusted to pH 8.0 and incubated at 50 °C for 30 min and then Alcalase was added at E/S ratio of 1:10 (w/v) at various times to achieve DH values of 10, 20, 30 and 40 %. As DH increased, ferrous ion chelating activity, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity and ACE inhibitory activity of peanut protein hydrolysates (PPH) increased, indicating that DH

affected functional properties, antioxidant and ACE inhibitory activities of peanut protein. Recently, purified ACE inhibitory peptides from Alcalase digests of peanut flour were obtained after membrane filtration and RP-HPLC [46]. The most potent fraction resulted in up to an 8-fold increase in ACE inhibitory activity over the crude hydrolysate, which indicates the effectiveness of this isolation procedure.

### **Peanut Allergy**

Peanut allergy is defined as the adverse immune response to peanut proteins and is considered as one of the most severe food allergies due to its potential life-threatening complications. About 1 % of the westernized population are suffering from peanut allergy and this prevalence has been listed even as high as 2-6 % for children [49, 50]. The allergenic reactions range from mild to severe. Among all the food-induced allergies, peanut allergy is the most common cause of the fatal anaphylaxis in emergency departments [51].

Anaphylaxis is a life-threatening type of allergic reaction involving two or more symptoms which can occur in many combinations (Table 3), such as hives, swelling of tongue, and even heart attack. Some symptoms are not necessarily life-threatening, but the most severe ones restrict breathing and blood circulation and may cause shock and even death [52].

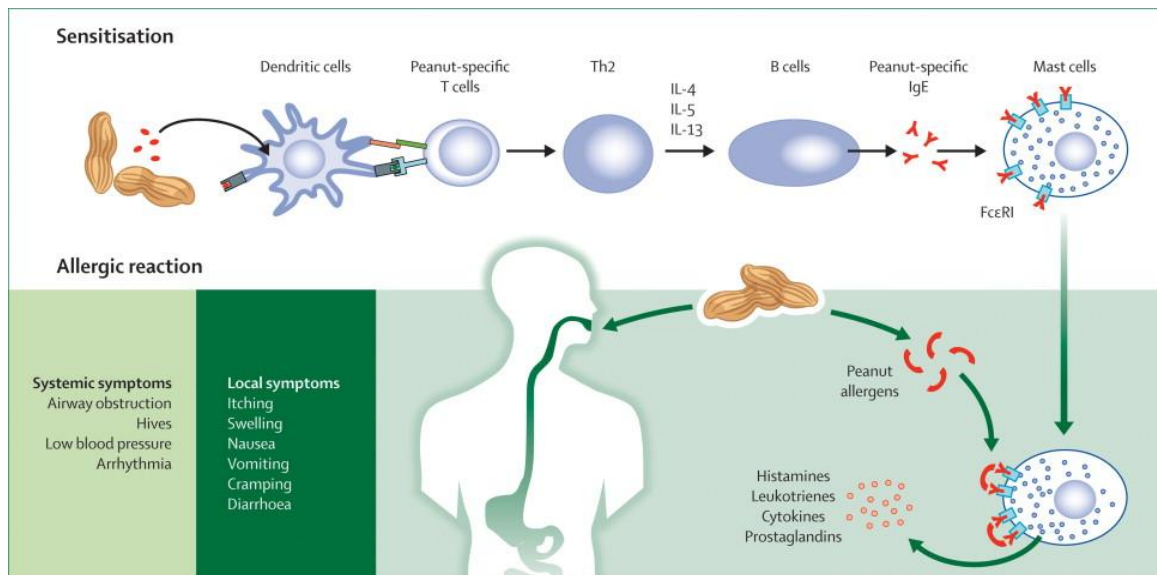
**Table 3.** Sites and symptoms of anaphylaxis. Table is adapted from [52].

Site	Symptoms
Skin	Itching, hives, redness, swelling
Nose	Sneezing, stuffy nose, runny nose
Mouth	Itching, swelling of lips or tongue
Throat	Itching, tightness, difficulty swallowing, hoarseness
Chest	Shortness of breath, cough, wheeze, chest pain, tightness
Heart	Weak pulse, passing out, shock
GI tract	Vomiting, diarrhea, cramps
Nervous system	Dizziness or fainting

Peanut allergy is immunoglobulin E (IgE) mediated and classified as a Type I allergy. The mucosal surface of the gastrointestinal tract is the site for introduction of peanut allergens. There are two stages of the peanut induced allergenicity: sensitization and allergic reaction. The process causing peanut allergy is described in Figure 1. When the first exposure to peanut allergens occurs, the peanut proteins are processed and presented by dendritic cells as peptide fragments to specific naive CD4+ T cells, stimulating the T cells to differentiate into T helper type 2 (Th2) cells. Th2 cells stimulate B cells to secrete peanut specific IgE, which is a type of antibody that is only found in mammals and is thought a key mediator for most food allergies. During this process, Th2 cells release several cytokines, of which IL-4 is considered the major one that enhances IgE secretion. Under various stimulations, naive CD4+ T cells can also differentiate into T regulatory cells, T helper type 1 (Th1) cells or T helper type 17 (Th17) cells. IgE deposition on the surface of mast cells marks the end of the sensitization period. The second exposure is also called the allergic reaction. This process begins when peanut allergens cross-link IgE deposited on the surface of basophil cells,

which is a type of white blood cell, resulting in degranulation, or the release of various inflammatory mediators, such as cytokines, chemokines, lipid mediators and histamines [53]. These chemicals will cause local symptoms, such as itching and tongue swelling, and even systemic symptoms, like airway obstruction and low blood pressure.

The priming process during which the proteins induce IgE secretion and subsequent attachment to mast cells/basophils takes longer time, and involves more stages (B cell priming, T cell activation and differentiation, IgE secretion, and IgE attachment) than the allergic reaction process in which proteins/peptide trigger IgE cross-linking. Because of this, allergic symptoms may not occur until the second exposure to peanut allergens. The peptides that are required to priming the immune system may contain different epitopes that are required for IgE cross-linking [54, 55].



**Figure 1.** Sensitization and allergic reaction to peanut. Cited from [53].

## **Peanut Allergens**

Recently, 11 peanut allergens, namely Ara h 1 to Ara h 11 have been recognized by the International Union of Immunological Societies (IUIS) Nomenclature Subcommittee and they are listed in Table 4 according to relative molecular weight [56]. Ara h 1 and Ara h 2 are considered the major peanut allergens as together they are recognized by > 90 % of peanut allergic patients, with Ara h 2 being recognized most frequently in the peanut allergic subjects [57, 58]. Ara h 3 is only been recognized by a subpopulation of patients with a history of peanut sensitivity [59]. A recent study suggested an important role for Ara h 6 [60]. Ara h 2 and Ara h 6 have a sequence homology of approximately 60 %, and both are resistant to heat and gastrointestinal digestion due to a core structure reinforced by disulfide bonds [60]. Ara h 3 was originally identified as a 14 kDa protein, but a full coding gene was expressed as a 60 kDa protein containing both acidic and basic chains [61, 62]. In peanut seed, Ara h 3 is present in post-translational form consisting of acidic subunit at approximate 42-45 kDa and basic subunit at approximate 25 kDa [62, 63]. Ara h 4 was described independently, however, sequence comparisons between Ara h 3 and Ara h 4 cDNAs show a high sequence homology of 91.3 % [61].

**Table 4.** The nomenclature of peanut allergens, including plant family, biochemical name, Svedberg units and relative MW. Table is adapted from [56].

Allergenic protein	Plant family (Biochemical family, Svedberg units)	MW (kDa)
Ara h 1	vicilins (globulins, 7S)	64
Ara h 2	conglutin (albumins, 2S)	17
Ara h 3	legumins (globulins, 11S)	60
Ara h 4	legumins (globulins, 11S)	37
Ara h 5	Profilin	15
Ara h 6	conglutin (albumins, 2S)	15
Ara h 7	conglutin (albumins, 2S)	15
Ara h 8	Pathogenesis-related protein	17
Ara h 9	Nonspecific lipid-transfer protein	9.8
Ara h 10	Oleosin	16
Ara h 11	Oleosin	14

Recently, a study investigated the effect of enzymatic hydrolysis to reduce peanut allergens in peanut kernels [64]. Individual or mixed  $\alpha$ -chymotrypsin and trypsin were used to treat peanut protein already extracted from raw and light roasted peanut kernels (Good Earth Peanut Company, Skippers, VA) for 5 hours. The concentrations used of the enzyme solutions were 0, 0.01, 0.05, 0.10 or 0.15 % (w/w) of  $\alpha$ -chymotrypsin or trypsin or 1: 1 mixture of the two. ELISA results confirmed that Ara h 1 and Ara h 2 in peanut kernel extracts were reduced up to 100 % and 98 % at 0.12 % enzyme concentration for 3 h, respectively.

Food processing affects peanut allergenicity [65-68]. Generally, roasting is considered to increase the allergenic properties of peanut, whereas boiling and frying tend are thought to reduce allergenicity. On the view of peanut allergens, the mechanism is explained that roasting induced chemical modifications by Maillard reaction. Modifications of Ara h 1 and Ara h 2 may result in new IgE binding epitopes, hence increasing the allergenicity [69].

Also, roasted Ara h 1 aggregates to form dimers, trimers and large complexes, which are resistant to digestion [65]; however, boiling may produce Ara h 1 aggregates distinct from those formed by roasting. These aggregates may have reduced peanut allergenicity [66].

Another recently published paper investigated the effects of enzymatic hydrolysis on roasted peanut protein allergenicity [63]. Individual and sequential hydrolysis with Alcalase and Flavourzyme were used to treat roasted peanut protein extract. Decreased IgE binding capacity, and decreased Ara h 1, Ara h 2 and Ara h 3 levels were observed from protein hydrolysates using Western blotting and ELISA experiments.

### **Treatment for Peanut Allergy**

Currently, no FDA-approved treatment strategy is being widely used other than strict avoidance and ready access to epinephrine injection [53]. However, strict avoidance shows limited benefits for many reasons: 1) accidental exposure may occur due to the difficulty in interpreting labels and potential adulteration of peanut allergens in commercial food products [70]; 2) strict avoidance is likely to increase the severity of peanut allergy symptoms when accidental exposure occurs [71]; 3) compared with other food allergens, peanut particles are more easily air-borne. The situation is even worsened by the fact that a small dose of peanut allergens can trigger the allergic symptoms due to the fact that thresholds of peanut allergenic reactivity can vary dramatically among individuals. A previous study suggested that threshold doses for peanut allergic reactions ranged from a dose as low as 100 µg up to 1 g of peanut protein [72]. A diagnosis of peanut allergy negatively impacts the affected patients and their families, resulting in anxiety and reduced quality of life [73].

An epinephrine injection is the first drug administered for acute anaphylactic symptoms in emergencies. At recommended dosage and route of administration, epinephrine introduces vasoconstriction effects, which restricts the dilation of the vessels and alleviates hypertension. However, epinephrine injection does not guarantee the effectiveness of treatment and the patients may still die of delayed administration, inadequate doses, and underlying disease, such as cardiovascular disease, asthma and perhaps other serious systemic disorders [74].

### **Oral Immunotherapy and Sublingual Immunotherapy**

In 1997, Nelson and Lahr Ten delivered immune protection to peanut allergic patients by subcutaneous injections of aqueous peanut extract; however, this research stalled because of repeated severe adverse reactions [75]. Since this time, multiple immunotherapy options for food allergy have been proposed, including those for peanut allergies. From safety and efficacy perspectives, the most promising strategies are oral immunotherapy (OIT) and sublingual immunotherapy (SLIT). OIT and SLIT can induce clinical desensitization and even tolerance. Desensitization refers to increased threshold of ingested food antigen required to cause allergic symptoms, whereas tolerance refers to the long lasting non-reactivity to food allergen [76].

Recently, oral immunotherapy (OIT) has been proposed to provide long term protection to peanut allergic patients using a long-term buildup protocol [77]. OIT starts with regular oral ingestion of gradually increased dose of an allergen to desensitize an individual with the hope that over time, tolerance can then be induced [33]. The immunological mechanism for OIT

has not been clearly established, but there is evidence that the introduction of T regulatory cells ( $T_{reg}$ ) and a shift from Th2 response to Th1 response contribute to desensitization and tolerance [53, 78]. Importantly, the mast cell and basophil responses were also shown to decrease during OIT, indicating that desensitization has occurred. In a recent double-blind, placebo-controlled trials, 28 children aged 1 to 16 years were enrolled in the OIT with peanut flour (n = 19) or placebo (n = 9). Sixteen/Nineteen subjects who received peanut flour OIT reached the highest food challenge dose of 5000 mg [79].

SLIT is slightly different from OIT in delivery method. In SLIT, an allergen extract is delivered in small drops under the tongue and kept in the mouth for a few minutes and then swallowed in “sublingual-swallow” protocol instead of directly ingested [80]. SLIT is also administered in “sublingual-spit” protocol, which involves spitting out the allergen after having kept it under the tongue.

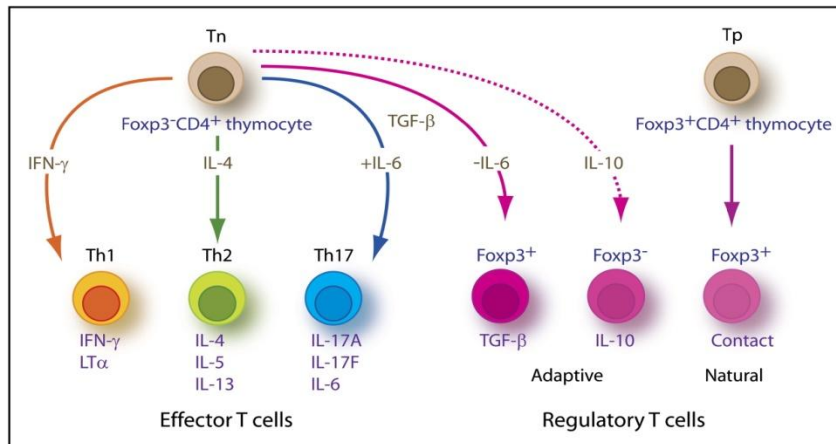
It is difficult to make a direct comparison of OIT and SLIT for peanut allergy treatment. Recently, Chin and Kim reported that OIT induced more extensive immunologic changes associated with desensitization than SLIT using double-blind, placebo-controlled studies with maintenance doses of 2 mg for SLIT and 4000 mg for OIT, respectively [81]. From their results, OIT induced more extensive immunologic changes associated with desensitization. However, the unestablished models for SLIT and OIT limit the credibility of the comparison. Also, long term immunological changes and clinical efficacy were not involved in this study. OIT is accompanied by the potential for allergic symptoms. From research evaluating the safety of peanut OIT protocol in children, symptoms throughout various phases of OIT were

described. According to the result, there is high risk for allergic side effect in the initial escalation phase, and the symptoms are mainly upper respiratory tract (79 %) and abdominal symptoms (68 %). The risk for allergic side effects decreased in buildup phase (46 %) and further decreased in home dose phase (3.5 %) [82].

### **Immune Response in OIT**

The immunological mechanism for OIT has not been clearly established, but the introduction of  $T_{reg}$  and a shift from Th2 to Th1 response is considered important [78]. Also, one kind of immunoglobulin – IgG4 is reported to show a close association with OIT. Previous studies suggested IgG4 levels may be boosted during OIT and act as a blocking agent against the actions of IgE, thereby reducing the IgE mediated food allergy [83, 84]. In previous work, peanut allergic patients showed a progressive decrease in activated basophils during the first 6 months after OIT [76].

Table 2 shows the subsets of CD4<sup>+</sup> T cells that can differentiate from naive CD4<sup>+</sup> T cells. Both naive and differentiated CD4<sup>+</sup> T cells are featured in expressing surface protein cluster of differentiation 4 (CD4), which is the co-receptor for the T cells to interact with antigen presenting cells [85]. Th1, Th2 and Th17 are all effector T cells differentiated from naive CD4<sup>+</sup> T cells, while  $T_{reg}$  act on suppressing the function of the effector T cells. Th1 usually deals with cellular immune response, whereas Th2 is a response to IgE mediated allergic response. Regulatory T cells come in many forms with naturally being CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells, which express surface proteins CD4 and CD25 as well as transcription factor FoxP3 [86].



**Figure 2.** CD4+ T cells proliferation. Cited from [85].

Multiple tests are being used to monitor the immunological effects and clinical efficacy of OIT. CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells can be investigated by flow cytometry of peripheral blood mononuclear cells [76]. IgE and IgG<sub>4</sub> can be measured in serum samples using the ImmunoCAP 100 instrument (Phadia AB) [76]. CD63 on basophils is a good marker for basophil activation test (BAT), as it can be stained by CD63 fluorescein isothiocyanate and be tracked by cytometry. [53].

Immunological effects and clinical efficacy of peanut OIT were examined over the course of 36 months [76]. In this experiment, 27/29 subjects passed the oral food challenge up to a total of 3.9 g peanut protein, indicating introduction of clinical desensitization. The desensitization was accompanied by a significant decrease in serum peanut specific IgE and increase in IgG<sub>4</sub>. CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells increased 1.5-fold in 6-12 months but returned to baseline levels thereafter. Another study showed that pepsin hydrolyzed peanut extract (E/S = 13U/g protein) were deplete IgE binding according to Western blotting using peanut

allergic patients sera ( $n = 5$ ), but retained T cell stimulating activity according to lymphocyte stimulation studies [87]. Basophil degranulation levels decreased significantly for the first 6 months but increased less significantly after 6 months. These results revealed the co-relationship of desensitization of peanut allergy and immunological response.

### **The Potential of Hydrolyzed Peanut Flour in OIT**

Peanut flour is currently used as the active ingredient for peanut OIT as it is high in peanut protein content and it has the handling ease of flour. However, there is risk for extensive allergic adverse effects induced by native proteins in peanut flour. Peptides have been studied as a safer alternative for peanut OIT, namely peptide immunotherapy (PIT) using either synthetic peptides or peptides derived from peanut protein. Peptides are being used to develop vaccines for cat allergies, but have not been used in human immunotherapy [88]. Immunotherapy with peptides could offer substantial advantages over OIT with whole peanut proteins because of the reduced potential for IgE cross-linking [89]. The peptides selected for PIT should ideally contain the epitopes targeting CD4<sup>+</sup> T cells to induce T<sub>reg</sub>, but not have the capacity to trigger IgE cross-linking.

The potential for Ara h 2 peptides to be used in OIT was investigated using model systems [90]. Ara h 2 T cell epitopes were identified by the combination of generation of Ara h 2 specific CD4<sup>+</sup> T cell lines, T cell lines proliferation assays and cytokine detection [90, 91]. These researchers identified short peptides containing T cell epitopes that target allergen specific CD4<sup>+</sup> T cells but lack the capacity to bind IgE [90]. These peptides are thought potentially good candidates for immunotherapy due to these immunological properties.

Immunomodulatory effects were reported using egg white enzymatic hydrolysates to manage IgE mediated immune response [92]. In this study, increased levels of T<sub>reg</sub> cells and a shift from Th2 to Th1 response were observed in mice fed egg white hydrolysates. This study suggested that food derived peptides obtained by food processing methods have the potential to induce immune suppression.

For OIT applications, it appears that enzymatically hydrolyzed peptides should maintain the capacity to prime T<sub>reg</sub> while minimizing IgE cross-linking capacity. Further research is needed in this area to confirm this hypothesis and to generally advance this field of study.

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**CHAPTER 2:**

**Enzymatic Hydrolysis of Peanut Flour to Improve  
Functionality and Bioactivity**

## **Abstract**

The potential of enzymatic hydrolysis to improve the functional and bioactive properties of peanut flour was investigated. Soluble fractions prepared from 10 % (w/v) light roasted peanut flour (50 % protein, 12 % fat) dispersions were hydrolyzed with Alcalase (pH 8.0, 60 °C), pepsin (pH 2.0, 37 °C), Flavourzyme (pH 7.0, 50 °C), or sequentially with Alcalase and Flavourzyme under controlled conditions for 60 min.

The trinitrobenzenesulfonic acid (TNBS) method was used to determine the degree of hydrolysis (DH) after various enzyme treatments. The bicinchoninic acid (BCA) assay and total nitrogen measurements were used to determine protein solubility. Angiotensin-converting enzyme (ACE) inhibition of various hydrolysate fractions was determined.

DH after 60 min reached approximately 22, 15, 27 and 38 % for Alcalase, pepsin, Flavourzyme and sequential Alcalase-Flavourzyme hydrolysates, respectively. This data reflected the different ( $p < 0.05$ ) specificities of the various enzymes utilized. Soluble protein was pH dependent as determined by BCA and soluble nitrogen measurements. Across the pH range tested from 2.0 to 8.0, the minimum protein solubility was observed at pH 4.0-6.0, which is near the isoelectric point of many peanut proteins, and the solubility increased sharply above and below this range. Hydrolysis did not significantly ( $p > 0.05$ ) affect protein solubility based on soluble nitrogen measurement, as soluble protein fractions were used as substrates. Solution turbidity was minimally ( $p > 0.05$ ) impacted by enzyme treatments, except Flavourzyme hydrolysis, which increased ( $p < 0.05$ ) the turbidity of soluble fractions. Alcalase hydrolysates showed the greatest capacity to release ACE inhibitory peptides from

peanut flour protein, indicating the potential use of Alcalase hydrolysates as the starting material for further purification of ACE inhibitory peptides. These results suggest that the nutritional/bioactive properties of peanut flour could be improved via enzymatic hydrolysis due to the production of small peptides.

## **Introduction**

Peanut flour is a commercially available high protein, low fat food ingredient prepared from roasted peanut seed. After roasting to the desired conditions, peanut seeds are partially defatted, typically to either 12 or 28 % fat, and ground to produce peanut flours. Peanut flour typically contains 40 to 55 % protein, 10 to 30 % fat and 20 to 30 % carbohydrate/fiber [1, 2]. Current or potential products that may contain peanut flours include confections, seasoning blends, bakery mixes, frostings, fillings, and nutritional bars [3]. Because peanut flour is partially defatted, it works well as a fat binder in applications such as confectionary products [4]. Although peanut flour is a good source of high quality protein, the functional and nutritional/bioactive potentials of this ingredient class have not been fully explored.

Proteins can serve various functional and nutritional/bioactive purposes in foods. Functional properties include the capacity to form and stabilize the characteristic structure of individual foods, and some functional properties important for these purposes include emulsion capacity, foamability, gelation capacity, water absorption capacity and solubility, among others [5]. Biological activities of proteins/peptides are health benefits that extend beyond amino acid incorporation as necessary for adequate human health [5]. Multiple bioactivities have been reported for peptides released from proteins and examples include antihypertensive, antioxidant, anticancer, anti-amnesic and immunomodulatory properties as well as cholesterol-lowering functions [6]. Enzymatic hydrolysis, chemical hydrolysis, and microbial fermentation have all been investigated as potential processing strategies for peptide production, with enzymatic hydrolysis being most widely practiced due to efficiency and food safety advantages [7]. Various traditional protein sources, including milk, egg, fish,

meat, soy, rice and potato, have been used for the preparation of protein hydrolysates [8]. Hydrolysis has also been widely used as a processing strategy to improve gastrointestinal absorption efficiency of plant proteins [9]. Compared with individual enzymatic hydrolysis treatments of protein sources, sequential treatment using an endoprotease and an exoprotease coupled with post hydrolysis procedures has been proposed as an effective way to obtain peptides with improved functional and bioactive properties [10].

Microbiologically fermented peanut flour had improved protein solubility compared to unmodified control flour, which is important as solubility influences multiple other functional properties, such as emulsification, foaming, water absorption, etc. [11]. Previous work demonstrated enzymatic hydrolysis using pepsin, bromelain, and trypsin generally increased protein solubility of peanut flour at the isoelectric pH range (4.0-5.0), whereas hydrolysis fully destroyed the emulsion capacities in model systems [12, 13].

One especially active area within bioactive peptide research is the documentation and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides derived from food proteins. These peptides have the potential to be used as milder and safer therapeutic agents to treat hypertension compared with synthetic drugs [7]. A previous study revealed that Alcalase treated peanut protein isolates resulted in increased ACE inhibition and antioxidant capacities with increased DH from 10 to 40 % [14]. Pepsin, trypsin, chymotrypsin and pancreatin were used to release ACE inhibitory peptides from arachin, the major storage globulin of peanut, and maximum ACE inhibition was observed with pepsin hydrolysis [15]. These results potentiated the use of peanut protein hydrolysates as therapeutic diets toward blood pressure regulation.

Recently, ACE inhibitory peptides from protein hydrolysates have been further purified by high performance liquid chromatography (HPLC) fractionation. In a study pertaining to canola meal hydrolysates, separation on a Sephadex G-15 gel permeation column (GPC) yielded a fraction with an superior  $IC_{50}$  (concentration of an inhibitor required for 50 % inhibition) value of 2.3  $\mu\text{g}$  protein/ml compared to 27.1  $\mu\text{g}$  protein/ml for bulk canola meal hydrolysates [16]. ACE inhibitory peptides were purified from Alcalase digests of peanut flour by membrane filtration and reverse phase HPLC [17]. The most potent fraction resulted in up to an 8-fold increase in ACE inhibitory activity over the crude hydrolysate, which indicated the effectiveness of this isolation procedure to obtain a substrate with higher ACE inhibition.

In the current study, the primary objective was to compare the capacity of different enzymes to release the ACE inhibitory peptides, thus verify the starting material for further purification. The secondary objective involves purification of the most active ACE inhibitory peptides from the primary hydrolysates and determination of the specific peptides sequence. This part is our future work.

## **Materials and Methods**

**Materials.** Light roasted 12 % fat peanut flour was provided by Golden Peanut Company (Alpharetta, GA). Alcalase from *Bacillus licheniformis* (2.4 AU/g, Batch 056K1213, EC 232-752-2), Flavourzyme from *Aspergillus oryzae* (500 LAPU/g, Batch 084K0543, EC 232-752-2), pepsin from porcine stomach mucosa (460 units/mg solid, Sigma-Aldrich, Batch 086K1319, EC 232-629-3) and trinitrobenzenesulfonic acid (TNBS) were purchased from

Sigma-Aldrich Chemical Co. (St. Louis, MO). L-Leucine and Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) were obtained from Fluka BioChemika (Buchs, Switzerland). HCl and NaOH were purchased from Fisher Scientific (Fair Lawn, NJ).

**Peanut Flour Hydrolysates Preparation.** 10 % (w/v) dispersions of peanut flour were prepared using deionized water. The 10 % dispersions were equilibrated at room temperature for 10 min. After the incubation period, the pH was adjusted by 2N NaOH or 2N HCl to 8.0, 2.0, or 7.0, the respective optimum pH values for Alcalase, pepsin and Flavourzyme hydrolysis. After another 20 min incubation period, the dispersions were centrifuged at  $9150 \times g$ ,  $10-15\text{ }^{\circ}\text{C}$  for 20 min. After centrifugation, 15 ml supernatant samples were collected as controls. Enzymatic hydrolysis was carried out in a water bath under constant stirring using an overhead stirrer at 60, 37, and  $50\text{ }^{\circ}\text{C}$ , the optimum temperatures for Alcalase, pepsin and Flavourzyme hydrolysis, respectively. Alcalase was added at an enzyme/substrate (E/S) ratio of 0.048 Anson Units/g peanut proteins. Pepsin was added at an E/S ratio of 18400 Unit/g proteins. Flavourzyme was added at an E/S ratio of 10 Leucine amino peptidase units (LAPU)/g peanut protein. The E/S ratio selected for Alcalase is within normal ranges commonly reported for this enzyme in food applications. The E/S ratio for Flavourzyme is the maximum dosage for endoprotease activity. E/S ratio for pepsin was 10 times of the upper range of the pepsin concentration in human stomach. Ten ml samples were taken at 0, 5, 30 and 60 min of hydrolysis and collected in 15 ml conical tubes. Enzymes were inactivated at  $90\text{ }^{\circ}\text{C}$  for 15 min. Unhydrolyzed controls were heated equivalently. After cooling to room temperature, dispersions were microfuged at  $14000 \times g$  for 10 min to

separate insoluble material. Supernatants (hydrolysates) were collected in 1.5 ml plastic vials and frozen at -80 °C for further analyses.

**Degree of Hydrolysis (DH).** DH was determined using the TNBS acid method [18].

Hydrolysates (0.05 mL) were added to 0.95 mL of 1 % SDS and vortexed. Each sample (0.25 ml) was then transferred into 20 ml amber vials containing 2.0 mL of 0.2125 M sodium phosphate buffer (pH 8.2) and 2 mL of 0.1 % TNBS were added. Then, test tubes were vortexed and equilibrated at 50 °C for 60 min in the dark. The TNBS reaction was quenched immediately after the incubation by adding 4 mL of 0.1 N HCl. All test tubes were cooled to room temperature and absorbencies measured at 340 nm using a Monochromator Microplate Reader (Tecan., Austria). Concentrations of 0-3 mM L-Leucine were prepared equivalently for the standard curve. DH values were calculated using the following formula:

$$DH = h/h_{tot} \times 100 \%$$

Where h, hydrolysis equivalents, is the number of peptide bonds cleaved during hydrolysis and  $h_{tot}$  is the total number of peptide bonds in a given protein. The total number of peptide bonds of the peanut protein substrate was determined by fully hydrolyzing soluble fractions from peanut flour dispersions with 12 N HCl for 24 h at a ratio of 1: 1 at 90 °C. Hydrolysis equivalents, h, were determined by reference to the L-Leucine standard curve.

**BCA (bicinchoninic acid) Assay.** A BCA™ Protein Assay Kit (Pierce, Rockford, IL) was used to determine protein concentration of the peanut flour hydrolysates and unhydrolyzed controls. In an alkaline environment, the peptide bonds have reducing capacity to reduce Cu<sup>2+</sup> ions from the cupric sulfate to Cu<sup>+</sup>. The amount of Cu<sup>2+</sup> reduced is proportional to the

amount of peptide bonds. A purple-colored complex with a strong absorbance at 562 nm is formed when two molecules of BCA chelate with one  $\text{Cu}^+$  ion.  $\text{Abs}_{562}$  is nearly linear with increasing protein concentrations across 20 to 2,000  $\mu\text{g}/\text{ml}$ . A standard curve was prepared using bovine serum albumin solutions.

Hydrolysates and the unhydrolyzed controls were diluted at a ratio of 1:20 with deionized water. Then 25 $\mu\text{L}$  of all diluted samples and BSA standards were loaded into 96 well micro-plate mixed in with 200  $\mu\text{L}$  working reagent (1: 50 mixture of reagent A: 4 % cupric sulfate: sodium carbonate, sodium bicarbonate, and reagent B: BCA and sodium tartrate in 0.1 M sodium hydroxide.). The test micro-plate was incubated for 30 min at 37 °C in Monochromator Microplate Reader (Tecan., Austria) and immediately followed by  $\text{Abs}_{562}$  determination.

**Total Nitrogen Assay.** One hundred ml 10 % w/v peanut flour dispersions were prepared and the pH was adjusted to 7.0 and 8.0 using 2N NaOH or to 2.0, 3.0, 4.0, 5.0 and 6.0 using 2N HCl, respectively. The volumes of 2N HCl or 2N NaOH used are reported in Table 1. Then, these dispersions were centrifuged at  $9,150 \times g$ , 10-15 °C for 10 min. The supernatants at pH 2.0, 7.0 and 8.0 were hydrolyzed with pepsin, Flavourzyme and Alcalase, respectively. The supernatants at each pH as well as the hydrolysates were incubated at 90 °C for 15min and stored frozen. Soluble nitrogen was determined using a 2400 CHN Elemental Analyzer (Perkin Elmer, Norwalk, CT).

For total nitrogen assay, soluble protein (mg/ml) values were calculated from the following formula using conversion factor 5.46 for peanut. This conversion factor is based on the typical 18.31 % nitrogen content in peanut protein.

$$\text{Soluble Protein (mg/ml)} = \text{Soluble Nitrogen (N mg/mL)} \times 5.46$$

**Table 1.** The volume of 2N HCl or 2N NaOH added to adjust the pH of 100 ml 10 % peanut flour dispersions to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 from original pH = 6.83. Volumes were calculated using conversion factor of 20 drops/ml.

pH values	2.0	3.0	4.0	5.0	6.0	7.0	8.0
2N HCl added (ml)	6.5	3.35	2.3	1.2	0.3		
2N NaOH added (ml)						0.25	0.6

**Turbidity.** Turbidity is defined as the cloudiness of a solution/fluid due to suspended particles, which scatter, reflect and attenuate the incident light rather than transmits the light in straight lines [19]. Turbidity was measured by 2100AN Laboratory Turbidimeter (Hach, Ames, IA) in three replications and expressed in nephelometric turbidity units (NTU). NTU is defined as the intensity of light at a specified wavelength scattered or attenuated by suspended particles or absorbed at a method-specific angle (usually 90 degrees), from the path of the incident light compared to synthetic chemically prepared standards [19]. Dark color suspended particles can affect turbidimetric measurements [20].

**Microplate Kinetic Assay of Angiotensin I Converting Enzyme (ACE) Inhibition.** To determine ACE inhibitory activity, all samples were normalized to 5 mg/ml protein amount based on BCA protein assay. ACE was reconstituted fresh daily with 1 mL of deionized water. Ten  $\mu\text{L}$  ACE solutions (2.5 units/mg) along with 10  $\mu\text{L}$  of deionized water (control) or

samples were placed separately to prevent reaction in wells of a 96 well micro-plate. The substrate, 0.88 mM N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) in 50 mM Tris-HCl buffer (pH= 7.5), containing 0.3 M NaCl, was preheated to 37 °C for 15 min prior to use. One-hundred fifty µL preheated FAPGG buffer was added to each well to start the reaction, and the plate was placed into a 96 well Monochromator Microplate Reader (Tecan, Austria) pre-heated to 37 °C. Absorbance at 340 nm (Abs<sub>340</sub>) was measured in 30 second intervals for 30 min. The mean slope/sec over a linear interval from 10 to 25 min of the Abs<sub>340</sub> vs. time (s) curve was determined to calculate % ACE inhibition as follows:

$$\begin{aligned} \% \text{ ACE Inhibition} &= [1 - (\rho A_{\text{inhibitor}}/\rho A_{\text{control}})] \times 100 \\ \rho A_{\text{inhibitor}} &= \text{slope of curve containing inhibitor} \\ \rho A_{\text{control}} &= \text{slope of curve without inhibitor (DI water control)} \end{aligned}$$

**Statistical Analyses.** Statistics were performed as effects for enzyme and time to analyze the DH, BCA protein, soluble nitrogen and turbidity data from repeated measures design. All statistics were performed using JMP 9.0 (Cary, NC). P-values less than 0.05 were considered statistically significant.

## **Results and Discussion.**

**Degree of Hydrolysis.** DH measures the percent of cleaved peptide bonds during hydrolysis. Alcalase, pepsin, Flavourzyme and sequential hydrolysis were used to achieve various DH values. DH was monitored over the course of 60 min (Figure 1). DH increased rapidly with hydrolysis time for all treatments and had not clearly plateaued after 60 min. Statistical analysis indicated that enzyme type and hydrolysis time affected DH significantly ( $p < 0.05$ ). At 60 min hydrolysis time, DH ranged from 14.9 to 37.4 %. The highest DH observed was

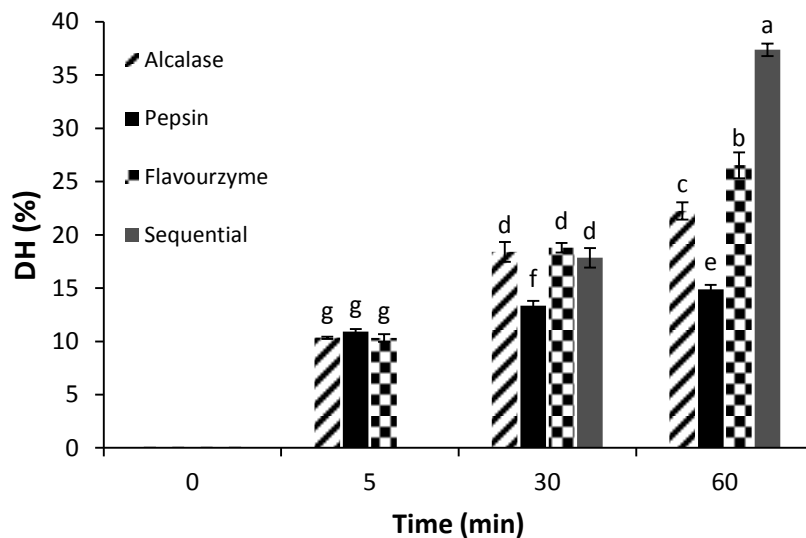
for the sequential Alcalase-Flavourzyme hydrolysate, followed by Flavourzyme hydrolysate and then the Alcalase digest.

Differences in DH data reflect the preferences and specificities for each enzyme to protein digestion under these specified conditions. Alcalase is an endoprotease of the serine type and has broad substrate specificity [21], thus reached a much higher DH than pepsin, which is also endoprotease but has a more strict specificity for hydrophobic amino acids, especially aromatic residues [21]. After 5 min, the rate of Alcalase hydrolysis surpassed pepsin hydrolysis and showed a significant increase from 18.4 to 22.2 % between 30 and 60 min, while pepsin showed a steady increase from 13.4 to 14.9 %. At the E/S ratio of 10 LAPU/g protein, which is the maximum dosage for endoprotease activity used (10 LAPU/g protein), Flavourzyme was likely to produce more free amino acids. DH of Flavourzyme hydrolysates increased steadily with hydrolysis time, surpassing Alcalase and pepsin at 60 min. The sequential Alcalase-Flavourzyme hydrolysis activity accelerated the DH dramatically due to extensive cleaving activity.

A previous study supported our results that sequential hydrolysis surpassed individual hydrolysis in terms of DH. In a recently released paper, 2 % roasted peanut protein extract solutions were hydrolyzed by the sequential and individual action of Alcalase (E/S ratio = 0.2 AU/g protein) and Flavourzyme (E/S ratio = 100 LAPU/g protein) [22]. At 60 min, DH reached about 14 % for Alcalase, 17 % for Flavourzyme and 20 % for sequential Alcalase-Flavourzyme. Compared with current study, lower DH values were observed during the first 60 min hydrolysis for each treatment, but the same relative orders of hydrolysis capacity for the enzyme systems were consistent between the current study and [22]. The discrepancies

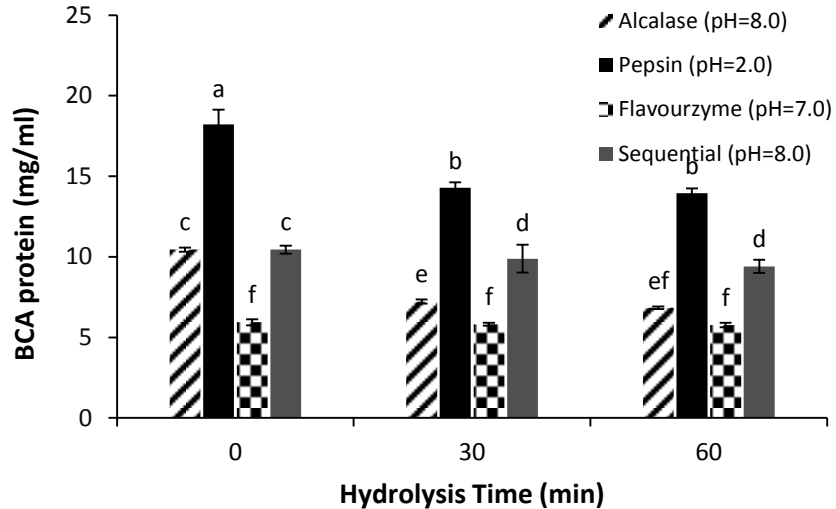
may be caused by the differing method of protein extraction and different enzyme levels. For the current study, peanut protein was extracted by deionized water for soluble protein; however, in the previous study, peanut protein was extracted by 0.05 M Tris-HCl and 0.5 N NaCl and dialyzed by a membrane with a cutoff of 3.5 kDa [22].

Another study compared the effect of industrial used enzyme and human digestive enzymes on hydrolysis of peanut flour protein by using Alcalase hydrolysis and *in vitro* pepsin-pancreatin digestion to hydrolyze both defatted peanut flours (Golden Peanut Company, Alpharetta, GA) and raw peanut flours prepared from blanched peanuts (Tara Foods, Albany, GA) [9]. DH was monitored over the course of 24 hours. The final DH reached 21 to 25 % for both Alcalase and pepsin-pancreatin hydrolysis regardless of raw or roasted peanut flour.



**Figure 1.** Degree of hydrolysis for the Alcalase, pepsin, Flavourzyme and sequential (Alcalase and Flavourzyme) hydrolysates as a function of time. Data bars are means  $\pm$  standard deviation. Data labels above the bar indicate the statistical significance.

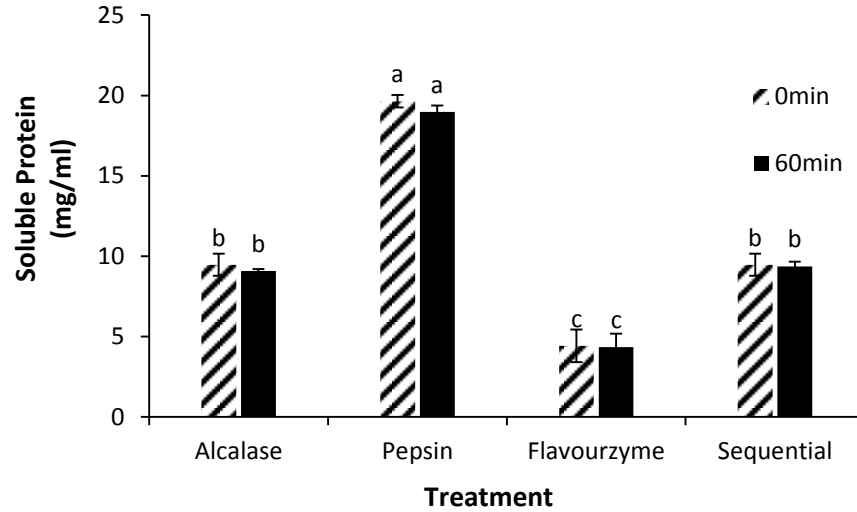
**Protein Solubility.** For the current study, protein content was determined by: (1) the BCA assay or (2) determining soluble nitrogen content and converting this measurement to total protein. BCA protein solubility data is summarized in Figure 2. Prior to enzyme addition (0 min), protein contents were only pH dependent. For the unhydrolyzed controls, soluble fractions at pH 2.0 (pepsin) had the highest level of protein of 18.2 mg/ml, followed by soluble fractions at pH 8.0 (Alcalase and sequential) of 10.4 mg/ml and pH 7.0 (Flavourzyme) of 5.9 mg/ml. Across 60 min hydrolysis time, there are significant ( $p < 0.05$ ) decreases in BCA protein in Alcalase, pepsin and sequential Alcalase-Flavourzyme hydrolysates, whereas there is no significant ( $p > 0.05$ ) change of BCA protein for Flavourzyme hydrolysates. In theory, the protein solubility is only pH dependent because there is no decrease of protein content during the hydrolysis as this is a 'closed system', i.e. proteases were added to pre-solubilized protein extracts. In practice, there are two possible explanations for the negative discrepancies: 1) BCA assay is generally less sensitive to small MW peptides and does not detect di-peptides or free amino acids; 2) During hydrolysis, pH changed over time, leading to the changes of solubility. Over the course of 60 min, pH ranged from 8.0 to 6.58 for Alcalase, 2.0 to 2.52 for pepsin and 8.0 to 6.77 for sequential hydrolysis, respectively. The final pH values of the hydrolysates are closer to the isoelectric point than the initial pH, perhaps partially explaining the decrease of BCA protein contents over hydrolysis time. Flavourzyme showed minimized changes in pH, ranging from 7.0 to 6.88, which is consistent with the constant levels of BCA protein for Flavourzyme hydrolysis.



**Figure 2.** BCA protein (mg/ml) within soluble fractions as a function of hydrolysis time with different proteases. Data bars are means  $\pm$  standard deviation.

Generally, it is assumed that the peanut proteins arachin and conarachin contain approximately 18.31 % nitrogen. Thus, the protein content of a sample is calculated from the determined nitrogen content by multiplying by a nitrogen-to-protein conversion factor, 5.46 (i.e., 100/18.31) [23]. However, the inherent limitations of this indirect determination are the results can be affected by nonprotein nitrogen [24].

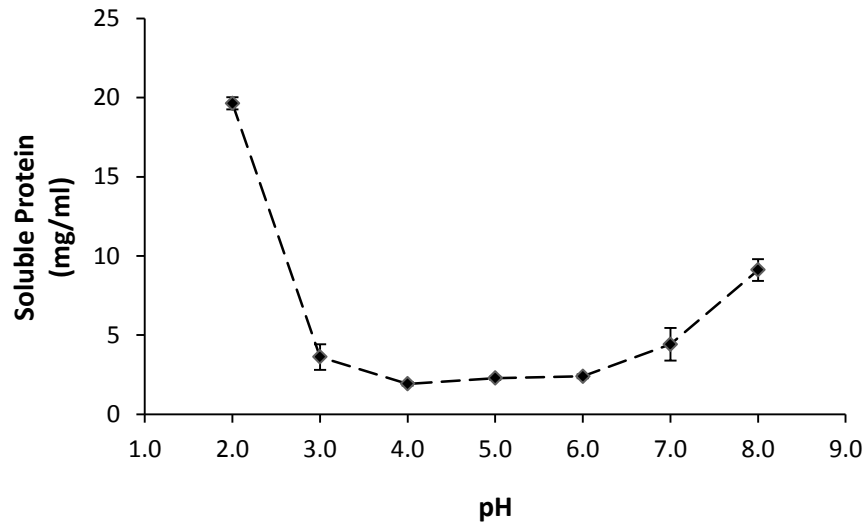
In addition to BCA protein measurement, the effect of hydrolysis on the protein content was also examined using nitrogen solubility assay (Figure 3). These results supported our hypothesis that the soluble protein remained almost the same levels during hydrolysis for all treatment ( $p > 0.05$ ), and the protein solubility is only pH dependent. The slight discrepancies of BCA protein and soluble protein converted from soluble nitrogen are likely due to their inherent limitations mentioned above.



**Figure 3.** Soluble Nitrogen (mg/ml) within soluble fractions as a function of treatment and hydrolysis time. Data bars are means  $\pm$  standard deviation.

To better understand the effect of pH on the peanut protein solubility, peanut flour dispersions were prepared and pH values were adjusted to a range from 2.0 to 8.0, with an interval of 1.0 pH value. Solubility of peanut proteins is known to be highly pH dependent with a minimum around pH 5.0 which is close to the isoelectric point for many of the predominant seed storage proteins [25]. For the current study, the pH effects on the solubility of peanut flour soluble dispersions were determined using nitrogen solubility (Figure 4). Across the pH range tested (pH 2.0–8.0), the minimum protein solubility was observed at pH 4.0–6.0, which is around the isoelectric point, and the maximum solubility was at pH 2.0 or higher than pH 8.0. Compared with the soluble fractions at pH 2.0 and 8.0, which are the optimal pH values for pepsin and Alcalase respectively, the fractions at Flavourzyme optimal pH (pH = 7.0) showed much lower solubility. This indicates that the proteins at pH 7.0 are more inclined to aggregate, but it is unclear whether this aggregation is reversible or not.

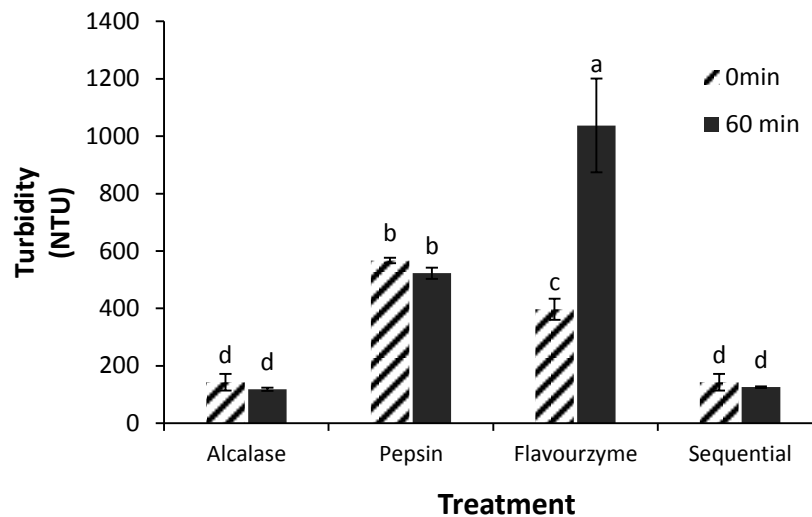
In a previous study, solubility profiles of roasted peanut flour protein in water showed sharp rises in solubility with pH changes both above and below pH 5.0, with significantly higher peanut protein solubility at pH 2.0 as compared to pH 7.0 and 8.0 [11], and these findings are consistent with our results. Another study confirmed the isoelectric point of peanut protein is around pH 5.0, but showed equivalent protein solubility at pH 2.0, 7.0 and 8.0 [24]. Such discrepancies with the current study could be attributable to 1) the slightly different conditions were used in preparing aqueous flour extracts for the current study as compared to earlier referenced studies; 2) the thermal processing we used in our research to prepare samples.



**Figure 4.** Protein (mg/ml) within soluble fractions with the 90 °C/15min treatment as a function of pH. Data bars are means  $\pm$  standard deviation.

**Turbidity.** Solution turbidity is another important consideration for these treatments and this data is summarized in Figure 5. For unhydrolyzed controls, turbidity was greatest at pH 2.0. This could be due to protein aggregation induced by the acidic environment. Additionally,

color of the dispersed phase can affect turbidity measurements [20], and as seen in Figure 2 of the appendix, solutions at pH 2.0 had a notable dark color which could be affecting current turbidity measurements. Further data is needed to better understand these phenomena. For Alcalase, pepsin, and sequential hydrolysis, the enzyme effects showed limited impact on turbidity ( $p > 0.05$ ) while flavourzyme increased the turbidity significantly ( $p < 0.05$ ).

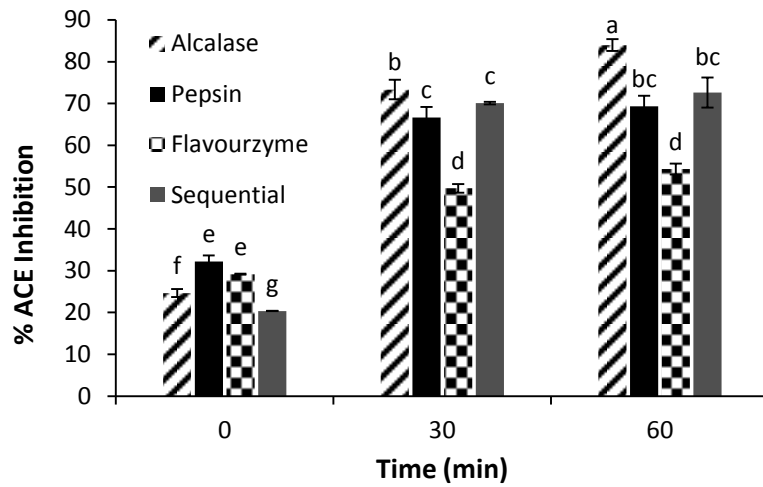


**Figure 5.** Turbidity within soluble fractions as a function of pH. Data bars are means  $\pm$  standard deviation. Data labels indicate the statistical significance.

**ACE Inhibition.** Peanut flour protein is considered as natural source of ACE inhibitory peptides. For the current study, the ability of proteases to release ACE inhibitory peptides was studied. ACE inhibition activity is presented as the function of enzyme type and hydrolysis time (Figure 6). ACE inhibition increased ( $p < 0.05$ ) with hydrolysis time for all hydrolysates. ACE inhibition started from a small range from 20.0 to 33.1 %, but the final ACE inhibition at 60 min hydrolysis time ranged from 54.3 % for Flavourzyme to 83.9 % for Alcalase. The highest ACE inhibition was observed for 60 min Alcalase hydrolysate with DH

of 25.1 %. This result is consistent with a previous study, which suggested Alcalase hydrolyzed peanut protein showed significantly increased ACE inhibition [14]. As for sequential hydrolysates, very limited changes of ACE inhibition were observed after Flavourzyme addition, suggesting this exoprotease activity is not as effective to release ACE inhibitory peptides compared with Alcalase, which is primarily an endoprotease. This assumption can be further confirmed by the minimum increase of ACE inhibition with Flavourzyme hydrolysis. Pepsin, though not that effective in achieving a high DH, is quite effective to improve the ACE inhibitory activity. Two main points could be concluded from the ACE inhibition changes with pepsin treatment: 1) ACE inhibition activity is nearly plateaued after 30 min, indicating these peptides are resistant to digestive enzyme thus they have the potential to survive from physiological digestion and be absorbed in the gastrointestinal tract [17]; 2) the specificity and preference of pepsin cleaving type is likely to make it very effective and efficient to release ACE inhibitory peptides. In a previous work, the pepsin hydrolyzed arachin showed > 90 % ACE inhibition and three peptides were purified with sequences of NLAG, NAQRP and IETWNPNNQ, respectively, according to amino acid analyses and N-terminal sequencing [15].

It is not clear whether the ACE inhibitory peptides derived for Alcalase and pepsin hydrolysates are structurally similar. Previous work revealed that the ACE inhibitory potential increased when the peptides < 6 amino acids with more hydrophobic side chains at the carboxy-terminus and decreased side chain length of the penultimate amino acid [26, 27]. Future work may involve purification of the ACE inhibitory peptides and determination of the amino acid sequence of major ACE inhibitory peptides.



**Figure 6.** ACE inhibitory activity of Alcalase, pepsin, Flavourzyme and sequential (Alcalase and Flavourzyme) hydrolysates of peanut flour. % ACE inhibition was determined at a concentration of 5 mg/ml based on BCA protein content.

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## **Chapter 3.**

### **Some Allergenic Properties of Peanut Flour Hydrolysates**

## **Abstract**

Peanut flour is currently being used as the active ingredient in oral immunotherapy (OIT) applications designed to desensitize peanut allergic patients. This strategy for treating peanut allergy is proving quite promising; however, there is a risk for allergic adverse reactions. In the current study, enzymatic hydrolysis of peanut flour was investigated as a processing strategy to minimize such allergic reactions and hence improve OIT applications of peanut flour. To investigate this potential, soluble fractions of 10 % (w/v) light roasted peanut flour dispersions were hydrolyzed with Alcalase (pH 8.0, 60 °C), pepsin (pH 2.0, 37 °C), Flavourzyme (pH 7.0, 50 °C), or sequentially with Alcalase and Flavourzyme. SDS-PAGE was used to visualize peptide distribution. Immunoreactivity was evaluated by Western blotting and basophil activation test (BAT). Generally, the SDS-PAGE banding intensity of major peanut allergens, Ara h 1, Ara h 2 and Ara h 3/4 decreased and a series of smaller molecular weight (MW) (< 22 kDa) peptide fragments were produced after hydrolysis. Western blotting using pooled human serum from peanut allergic patients (n = 6) revealed the relative capacity ranking of the enzymes as to degrade known peanut allergens: Alcalase > Flavourzyme > pepsin. Also, a series of smaller MW peptides were produced in the region of 0-22 kDa. These peptide fragments were derived primarily from Ara h 2 as demonstrated by Western blotting experiments using anti-Ara h 1 and anti-Ara h 2 rabbit sera. Despite extensive reduction in IgE binding as revealed by Western blotting, BAT assay (n = 4) data indicated that individual hydrolysates were comparable ( $p > 0.05$ ) to unhydrolyzed controls in IgE cross-linking capacity. Sequential hydrolysates were generated, with a higher DH and less IgE binding compared with any individual hydrolysate. The BAT of bulk sequential

hydrolysates and fractions from size exclusion chromatography is ongoing. Mass frequency profiles of sequential Alcalase-Flavourzyme hydrolysate showed that peanut flour protein was extensively degraded, resulting in only 6.1 % peptides > 2.0 kDa.

These results indicate that enzymatic hydrolysis significantly decreased the IgE binding but retained IgE cross-linking capacity, suggesting that these hydrolysates may not be superior to peanut flour for OIT at this stage to decrease the potential for allergic side effects.

## **Introduction**

Peanut allergy is the adverse immune response to peanut proteins and is considered as one of the most severe food allergies due to its life-threatening nature and persistency. Peanut allergy is immunoglobulin E (IgE) mediated. About 1 % of the western populations are suffering from peanut allergy and this prevalence has even been reported as high as 2-6 % for children [1, 2]. Unlike egg and milk food allergy, peanut allergy usually persists through adulthood, negatively impacting the life quality of affected patients and their families [3].

Currently, no treatment strategy is being widely used other than strict avoidance and ready access to epinephrine injection [2]. However, strict avoidance shows limited benefits because accidental exposure may occur without knowledge and peanut allergic patients may have severe reactions to minute doses of peanut allergens [4]. Also, epinephrine injection does not guarantee the efficacy to alleviate the life threatening symptoms due to many factors, including delayed administration and inadequate doses. Some diseases may also restrict the efficacy of epinephrine injection, such as cardiovascular disease, asthma and perhaps other serious systemic disorders [5].

Recently, oral immunotherapy (OIT) has been proposed to provide long term protection to peanut allergic patients. OIT starts with regular oral ingestion of gradually increasing doses of allergens, from 0.1 mg to 300 or 4000 mg depending on the study, to desensitize an individual with the hope that over time, tolerance can be induced [6]. The immunological mechanism for OIT has not been clearly established, but the introduction of T regulatory cells ( $T_{reg}$ ) and a shift from Type 2 helper T cells (Th2) to Type 1 helper T cells (Th1)

response was considered an important immune response [7]. Of further importance, was that the mast cell and basophil responses were shown to decrease as well during OIT, indicating that desensitization had occurred [8].  $T_{reg}$ , Th1 and Th2 are all T cells differentiated from native CD 4<sup>+</sup> T cells.  $T_{reg}$  acts to inhibit other T cells. Th1 deals with cellular immune responses, whereas Th2 plays important role in IgE mediated allergic response. Also, recent studies suggest during the treatment of OIT, IgG4, which is another type of immunoglobulin, may be boosted in the serum of peanut-allergic patients and act as a blocking agent against the actions of IgE, thus alleviating the IgE mediated food allergy [9, 10].

Peanut flour is a commercially available high protein, low fat food ingredient prepared from roasted peanut seed. Due to its high content of peanut protein in a roasted format, and due to its handling ease which allows it to be readily weighed/measured, peanut flour is currently being used as the active ingredient for OIT. Despite the general success of using peanut flour in OIT up to date, there are risks that patients can have extensive negative immunological responses during OIT to even minute doses of the flour [6]. One strategy being investigated that could minimize such negative side effects is the use of peptides derived from peanut proteins, or synthesized to mimic peanut peptides. For example, in one recent study, peptides were selected with minimal IgE reactivity, but containing T-cell epitopes of the major peanut allergens, namely Ara h 2, for their potential as safer alternatives in immunotherapy applications [3]. Such peptides are expected to drive T regulatory cell responses while minimally affecting IgE cross-linking, which are conditions thought ideal for desensitization. A potential problem with selecting peptides only derived from Ara h 2, is that such peptides will not reflect the composition of other peanut allergens and hence may cause incomplete

desensitization for subpopulations of peanut allergic patients that are sensitive to non-major peanut allergens. The major peanut allergens as sorted by molecular weight are described in Table 1 [11]. Among them, Ara h 1 and Ara h 2 are considered as major allergens as they are recognized by greater than 90% of peanut allergic patients, with Ara h 2 being most frequently recognized in peanut allergic subjects [12, 13]. Ara h 3, however, has only been recognized by serum IgE from a subpopulation of patients with a history of peanut sensitivity [14]. Ara h 4 has about 91.3 % sequence homology to Ara h 3 [15]. Ara h 1 is sensitive to digestive enzymes but some IgE binding epitopes were reported to survive model gastrointestinal enzyme digestion [16]. Ara h 2 (and the homologous Ara h 6) is considered resistant to digestive enzymes because it has a stable core structure due to disulfide bonding in 2S albumin proteins [17]. Ara h 3 is reported to be more liable to gastric enzymes than Ara h 1 [16]. Ara h 2 and Ara 6 have shown to account for 80 to 90 % of the effector activity, which is the total potency of the substrate for activation of IgE sensitized mast cells/basophils, as determined by a study investigating the effector activity of crude peanut extract after immune-depletion of both Ara h 2 and Ara h 6 [18].

**Table 1.** The nomenclature, biochemical name, plant family, Svedberg units and relative MW of major peanut allergens. Table is adapted from [11].

<b>Allergenic protein</b>	<b>Biochemical name</b>	<b>Plant family</b>	<b>Svedberg units</b>	<b>MW (kDa)</b>
Ara h 1	vicilins	globulins	7S	64
Ara h 2	conglutin	albumins	2S	17
Ara h 3	legumins	globulins	11S	60
Ara h 4	legumins	globulins	11S	37
Ara h 6	conglutin	albumins	2S	15

Western blotting is a method to visualize peanut allergen-serum IgE binding to peanut proteins and is often used as a first screen to understand the allergenic potential of a given preparation. During the sensitization phase of the allergic response, IgE attaches to the surface of mast cells, basophils and other cells. Basophil degranulation is caused by the crosslinking of surface attached IgE by peanut allergens. BAT is used to reveal the IgE cross-linking capacity indirectly by tracking CD63, which is one kind of cell surface protein that can be used as a specific basophil marker for flow cytometric quantification of *in vitro* activated basophils [19]. In the current study, three food grade enzymes were used individually or sequentially to degrade the peanut allergens. Our general hypothesis was that generated peptides would have decreased IgE binding and cross-linking capacity and that there would be enzyme specific effects due to differing specificities of tested enzymes and final DH values tested.

Fractionation was reported to show the potential to further decrease the sensitizing capacity of Ara h 1 digests [20]. These authors hypothesized that Ara h 1 peptides in Ara h 1 digests may aggregate to cause the allergenicity, and the fractionation may eliminate aggregation and thus decrease the allergic response in model systems [20]. However, the effect of fractionation on peanut hydrolysates, which contain multiple peanut allergen fragments, has not been determined.

## **Materials and Methods**

**Materials.** Light roasted 12 % fat peanut flour was provided by Golden Peanut Company (Alpharetta, GA). Alcalase was from *Bacillus licheniformis* (2.4 AU/g, Batch 056K1213, EC

232-752-2); Flavourzyme was from *Aspergillus oryzae* (500 LAPU/g, Batch 084K0543, EC 232-752-2); pepsin was from porcine stomach mucosa (460 units/mg solid, Sigma-Aldrich, Batch 086K1319, EC 232-629-3). HCl and NaOH were provided by Fisher Scientific (Fair Lawn, NJ). NuPAGE® 1.0 mm x 10 well 4-12% Bis-Tris Gels were provided by Invitrogen (Carlsbad, CA).

**Peanut Flour Hydrolysates Preparation.** The procedure was identical to that described in Chapter 2 and aliquots of the same samples were used for this study.

**BCA (bicinchoninic acid) Assay.** The procedure was identical to that described in Chapter 2.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).**

Hydrolysates were normalized to 1µg/µL based on BCA protein data using NuPAGE® LDS Sample Buffer (4X), NuPAGE® Sample reducing Agent (10X), and deionized water. After incubation at 70 °C for 10 min, 10 µg proteins were loaded per well based on BCA protein content in NuPAGE® 1.0 mm x 10 well 4-12 % Bis-Tris Gels (Invitrogen, Carlsbad, CA).

SeeBlue® Plus2 Prestained Standard molecular weight marker was used as a reference.

Electrophoresis conditions were 200 V for 35 min in MES SDS Running Buffer (1×).

SimplyBlue™ SafeStain was used to stain the gels for 60 min. The gels were destained in deionized water overnight and then dried using Gel-Dry™ drying solution.

**Western Blotting.** Hydrolysates were normalized to 1 µg/ µL protein amount using phosphate buffered saline (PBS), NuPAGE® LDS Sample Buffer (4X), and 0.5M dithiothreitol (DTT). Protein contents of 20µg were loaded per lane based on BCA protein

content in NuPAGE® 1mm x 10 well 4-12 % Bis-Tris Gel and separated by electrophoresis using the same conditions as described for SDS-PAGE. MagicMark™XP Western Standard was used as a marker. Gels were transferred onto the Immobilon®Transfer Membranes at 25 V for 90 min using an XCell II™ Blot Module. Ponceau S Solution was used to stain the gel for 5 min. Pooled human serum (Table 2), anti-Ara h 1 rabbit and anti-Ara h 2 rabbit sera were used to bind with the serum IgE. The membranes were incubated in the serum overnight and then immersed in the diluted Biotin-Labeled Affinity Purified Antibody to human IgE produced in goat (1:4000) and SouthernBiotec® Goat Anti-Rabbit IgG-HRP. After incubating in the diluted NeutrAvidin™ Horseradish Peroxidase Conjugate for 30 min, the blots were submerged in SuperSignal® West Pico Chemiluminescent Substrate for 3 min. A Chemi Doc® Imaging System was used to visualize the digital signal of the blots.

**Table 2.** Pooled human serum formulation. Sera were provided by Department of Pediatrics, UNC (Chapel Hill, NC). The peanut-specific IgE of the mixed serum equals 336.6 kU/L.

Serum ID	Peanut-specific IgE (kU/L)	Amount (mL)
PN28	90.3	0.5
PN19	840	2.1
PN29	27	1.3
PN18	11.3	2.9
PN23	42.9	1.1
PN26	159	0.6

**Basophil Activation Test.** This assay was conducted by the Department of Pediatrics, UNC (Chapel Hill, NC). Basophil Activation Test was performed by incubated 0.1 µg/ml hydrolysates or unhydrolyzed controls in peanut allergic patients' whole blood (n=4). After

incubation, red blood cells were lysed and leukocytes were fixed. CD63 was assessed by flow cytometric analysis as the specific protein marker for *in vitro* activated basophil [21].

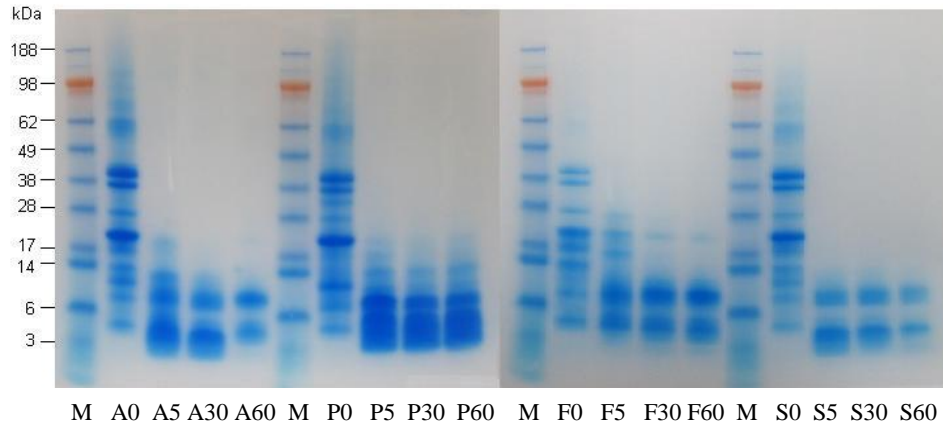
**FPLC Fractionation of Sequential Peanut Hydrolysate.** To fractionate sequentially hydrolyzed peanut flour and obtain the approximate MW distribution of the fractions, AKTA Fast Protein Liquid Chromatography (FPLC) System (AKTA, GE Healthcare) was performed. Approximately 10 mg of total protein for sequentially hydrolysates was loaded onto a Superdex Peptide 10/300 column, pre-equilibrated and then eluted with 30 % aqueous acetonitrile containing 0.1 % trifluoroacetic acid at a flow rate of 0.5 mL/min. Spectra were detected at 280 nm. Fractions resulting from multiple runs were pooled, and solvent was evaporated in a SpeedVac® concentrator (ThermoSavant). The size exclusion column was calibrated for determining the approximate MW distribution by external standards, namely Cytochrome C (12,327 Da), Aprotinin (6,511 Da), Chain B insulin (3,495 Da), Chain A insulin (2,531 Da), Substance P (1,347 Da), Substance P fragment 1-7 (900 Da), and Phenylalanine (165 Da). Elution volumes were used to calculate MW based on the standard curve ( $y = 207086e^{-0.338x}$ ,  $R^2 = 0.9279$ ).

**Statistical Analyses.** Statistics were performed with effects for enzyme and time to analyze the BCA protein data and basophil activation test from repeated measures design. All statistics were performed using JMP 9.0 (Cary, NC).

## **Results and Discussion**

**SDS-PAGE.** SDS-PAGE was used to visualize peptide profiles with increasing hydrolysis time for various proteases (Figure 1). Figure 1 shows the SDS-PAGE banding patterns of

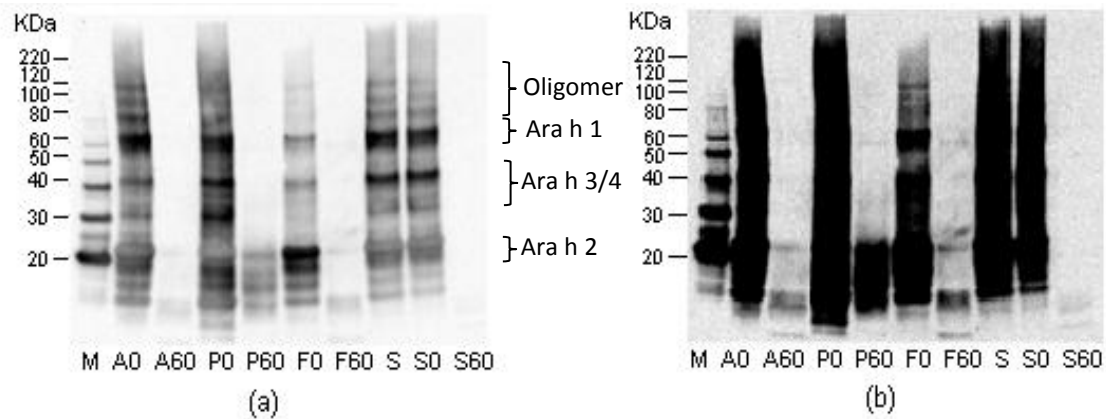
peanut protein before and after hydrolysis with Alcalase, Flavourzyme and pepsin and sequential Alcalase-Flavourzyme. The results indicated that the unhydrolyzed controls had similar banding patterns for the Alcalase, Pepsin and sequential gels, but the intensity of the bands for the Flavourzyme gel was much weaker. This observation appears to be pH dependent. The different profile for Flavourzyme control (pH = 7.0) could be relative to its lower protein solubility than Alcalase control (pH = 8.0) and pepsin control (pH = 2.0) (Chapter 2, Figure 2). Considering the molecular weights of established peanut protein allergens, it could be speculated that Ara h 1(64 kDa), Ara h 3 (42-45 kDa; 25 kDa) and Ara 4 (37 kDa) were degraded most rapidly and a series of smaller MW subunits were produced after enzymatic hydrolysis. Ara h 1, acidic subunits of Ara h 3 (42-45 kDa) and Ara h 4 bands disappeared quickly upon digestion with all treatments, while basic subunits of Ara h 3 (25 kDa) showed some persistency to Flavourzyme. Ara h 2 (17-19 kDa) is even more resistant to enzymatic hydrolysis, as evidenced by the weak but visible bands for all the 60 min individually hydrolyzed samples. Among all 60 min hydrolysates, the darkest Ara h 2 banding was observed from Flavourzyme and pepsin hydrolysates, followed by Alcalase, and the minimal Ara h 2 banding was observed for sequential hydrolysates. A series of smaller MW peptides were produced during hydrolysis with all enzymes, resulting in the bands and smears in the < 22 kDa region. These smaller MW peptides are breakdown fragments of the seed storage proteins and some of them may retain allergenic epitopes. Specifically, pepsin hydrolysates showed distinguished banding patterns with darker and broader bands in the < 22 kDa region while sequential hydrolysates showed the weakest and more confined banding patterns.



**Figure 1.** SDS-PAGE of Alcalase, pepsin, Flavourzyme and sequential Alcalase-Flavourzyme hydrolysates. “M” is the molecular weight ladder. Subsequent lanes are marked 0-60 min of hydrolysis of Alcalase (A), pepsin (P), Flavourzyme (F) and sequential Alcalase-Flavourzyme (S). 10 µg proteins were loaded for each lane.

**Western Blotting.** Western blotting was used to visualize the peptides that could bind with the specific serum IgE. Pooled human serum from peanut allergic patients (n = 6) was used to visualize multiple peanut allergenic proteins and peptides, while anti-Ara h 1 and anti-Ara h 2 rabbit sera were used to visualize Ara h 1 or Ara h 2 specific binding, respectively. Figure 2 shows multiple peanut allergens that bound to specific serum IgE. In general, the peanut allergens were extensively degraded; resulting in significantly decreased IgE binding for all treatments at 60 min. Specifically, sequential hydrolysates displayed minimal IgE binding, reflecting the relatively extensive digestion of peanut seed storage proteins sequentially with an endoprotease (Alcalase) and an exoprotease (Flavourzyme), which is in accordance to previous studies showing that sequential treatment using an endoprotease and exoprotease can be used to effectively obtain extensively hydrolyzed peptides [22]. Pepsin, as a human digestive enzyme, retained most IgE active peptides in 0-22 kDa region.

Individual Alcalase and Flavourzyme hydrolysis resulted in accumulation of three bands of IgE-bindings in < 22 kDa region, while Alcalase showed less IgE reactivity than Flavourzyme. Compared with individual proteolysis, the sequential treatment further degraded the protein, revealing depleted IgE-binding at 22 kDa and significantly decreased the intensity of the other two bands at even lower MW. This is in agreement with recently published work that sequential Alcalase-Flavourzyme hydrolysis was more effective than the individual proteolysis to reduce the IgE binding capacity [23].

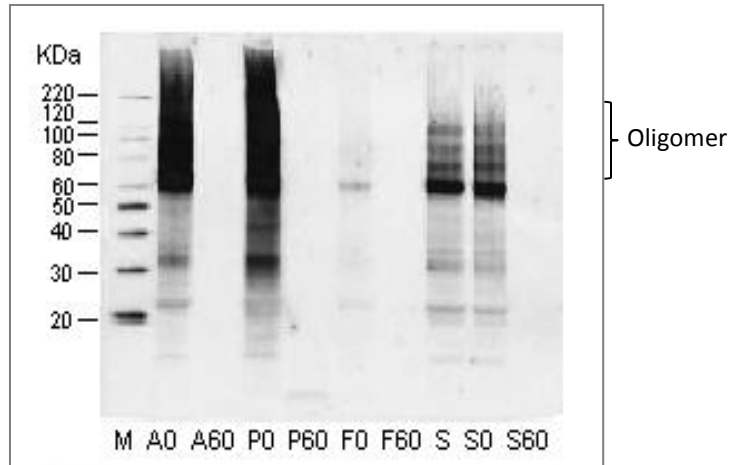


**Figure 2.** Western blots of soluble peanut flour extracts after various protease treatments. Proteases evaluated after 60 min hydrolysis time included Alcalase (A60), Pepsin (P60), Flavourzyme (F60) and sequential hydrolysis with Alcalase and Flavourzyme (S60). 0 and 60 indicate time in minutes. 20 µg of protein were loaded per lane. Blots were reacted against a human sera mixture derived from confirmed peanut allergic individuals (Table 1) in panels (a) and (b). “M” is the molecular weight marker. “S” is the unhydrolyzed control of the sequential hydrolysate without heating at 90 °C, which is the enzyme inactivation temperature. Exposure times for panels (a) and (b) were 208s. The exposure times for panel (a) and (b) were identical; however, the contrast was increased for panel (b) as compared to panel (a) using the image software. 20 µg proteins were loaded for each lane.

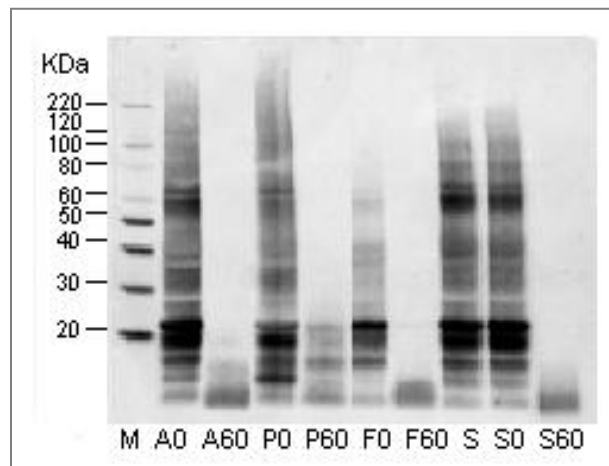
To better characterize peptide profiles and determine the capacity of these peptides to bind specific IgE, Western blots with anti-Ara h 1 and anti-Ara h 2 rabbit sera were used to detect specific Ara h 1 and Ara h 2 IgE binding, respectively. Figure 3 showed that for Alcalase (pH 8.0) and pepsin (pH 2.0) controls, there are obvious IgE binding bands of Ara h1 oligomers, but the Flavourzyme (pH 7.0) control had a lack of Ara h1 oligomers, which might be caused by precipitation of these oligomers at a pH value closer to the pI than other unhydrolyzed treatments.

Figure 3 shows that Alcalase, Flavourzyme and sequential 60 min hydrolysates did not show detectable Ara h 1 IgE binding, but pepsin hydrolysates showed a weak Ara h 1 IgE binding band. The smears and bands in the > 65 kDa region were considered to be Ara h 1 oligomers. In contrast to Ara h 1, Ara h 2 (Figure 4) showed persistency in all hydrolyzed treatments, resulting in accumulated IgE binding in the 0 to 22 kDa region. The IgE bands in the < 22 kDa region likely represent breakdown subunits of Ara h 2 with retained IgE binding capacity. For all unhydrolyzed controls, there are also some Ara h 2 induced IgE banding patterns at higher MW. These high MW bands can be caused by either aggregates of Ara h 2 or cross-reactivity of the IgE to bind with other allergens other than Ara h 2 [24].

In summary, significantly decreased IgE binding was observed after enzymatic hydrolysis. Sequential hydrolysis resulted in minimal IgE binding. Ara h 2 is more resistant to hydrolysis than Ara h 1. Even sequential hydrolysates retained some minimal Ara h 2 IgE binding capacity.

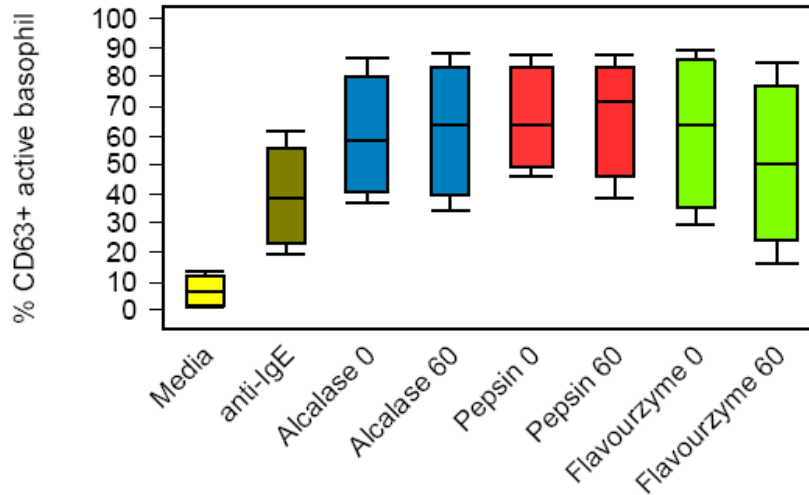


**Figure 3.** Western blotting was reacted against rabbit anti-Ara h 1 at a 1:40000 dilution. Exposure time is 26s. M is the marker. A, P, F and S stand for Alcalase, pepsin, Flavourzyme and sequential Alcalase-Flavourzyme. 0 and 60 indicate time in minutes. 20  $\mu$ g proteins were loaded for each lane. “S” is the unhydrolyzed control of the sequential hydrolysate without heating at 90 °C, which is the enzyme inactivation temperature.



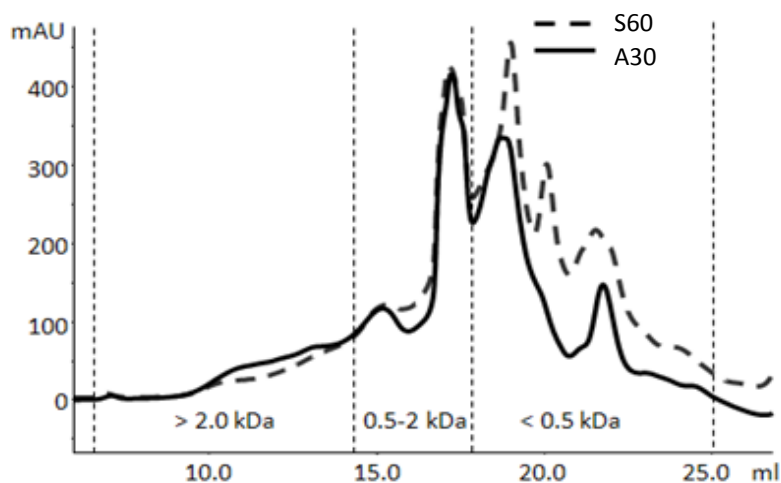
**Figure 4.** Western blotting was reacted against rabbit anti-Ara h 2 at a 1:20,000 dilution. Exposure time is 51s. M is the marker. A, P, F and S stand for Alcalase, pepsin, Flavourzyme and sequential Alcalase-Flavourzyme. 0 and 60 indicate time in minutes. 20  $\mu$ g proteins were loaded for each lane. “S” is the unhydrolyzed control of the sequential hydrolysate without heating at 90 °C, which is the enzyme inactivation temperature.

**Basophil Activation Test.** To analyze the IgE cross-linking capacities of peanut flour hydrolysates and unhydrolyzed controls, patient basophils were stimulated with the 0.1 µg/ml of hydrolysates or unhydrolyzed controls. CD63, which is the specific protein marker for *in vitro* activated basophils, was assessed by flow cytometry (Figure 5). Activated basophil levels of controls and 60 min hydrolysates were comparable for all enzymes ( $p > 0.05$ ), indicating that these samples retained IgE cross-linking capacity after enzymatic hydrolysis. The results from the IgE *cross-linking* capacity assay did not show the same trend as IgE *binding* after hydrolysis. Differences could be partially explained by the different format of the IgE, i.e. free IgE in the Western Blots versus the IgE being attached to mast cell/basophils in the BAT. The latter assay is a functional test and more robust and physiologically relevant. When these IgE molecules are attached to the surface of mast cells/basophils, their conformation may change, thus increasing their affinity for binding with the peanut allergens or derived allergenic fragments. Also, small peptides with reduced IgE reactivity may be forming larger aggregates in human blood, and these aggregates may exhibit cross-linking capacity.



**Figure 5.** Summary of BAT conducted on peanut allergic subjects (n=4). Data shown are from stimulation with 0.1  $\mu\text{g}/\text{ml}$  of indicated protein preparation. 0 and 60 indicate time in minutes. P-value equals 0.875 for pepsin, 0.375 for Alcalase and 0.250 for Flavourzyme. Data provided by collaborators in UNC, Chapel Hill, NC.

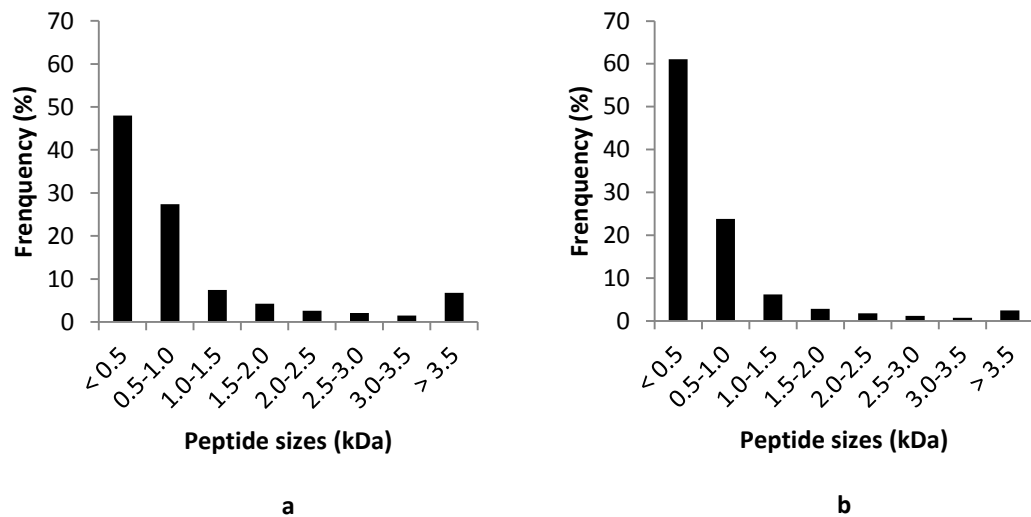
**FPLC Fractionation.** Size exclusion chromatography was used to fractionate samples. The starting substrate was the sequential Alcalase-Flavourzyme hydrolysate (S60) due to its minimal IgE binding observed from the Western blotting. An Alcalase hydrolysate at 30 min (A30) was also fractionated. The objective of this process was to separate the peptides in hydrolysate by size and determine the mass frequency of the peptides in certain MW ranges. It is generally accepted that peptides need to maintain a certain MW (3.5 kDa) to retain allergic properties [25]. Recently, some research revealed an even lower cutoff of 2.0 kDa [20]. When comparing the FPLC fractionation UV trace of sequential hydrolysate and its control at 280 nm (Figure 6), there was a larger portion of small MW peptides or amino acids in  $< 0.5$  kDa for the sequential hydrolysate. For both hydrolysates, the peptides  $> 2.0$  kDa comprised a small portion of the extract, which is in accordance with the peptide profiles shown on SDS-PAGE (Figure 1).



**Figure 6.** UV trace at 280 nm of FPLC fractionation of sequential hydrolysate (S60) and Alcalase hydrolysate at 30min (A30). Molecular weights were calculated from standard curve prepared using external standards.

External standards with known MW were used to obtain the standard curve of elution volume vs. peptide sizes of and the corresponding elution volumes were calculated. Mass frequency was determined using the percent of area within certain MW ranges. Figure 7 shows the peptide mass distribution profiles of the pools of sequential Alcalase-Flavourzyme hydrolysate (S60) and Alcalase digest at 30min (A30). For both S60 and A30 hydrolysates, more than 85 % peptide fragments had MW less than 2.0 kDa, which is generally considered as the cutoff for peptides to maintain the allergenicity [20]. Sequential hydrolysis decreased the frequency of peptides > 2.0 kDa from 11.5 to 6.1% due to the Flavourzyme hydrolysis effects on pre-digested peanut protein. However, the significant decrease of the peptides < 2.0 kDa did not result in depletion of IgE cross-linking capacity according to the BAT result (Figure 5). This part of the research is still ongoing. Our future work includes Western blotting and BAT of the fractions derived from SEC.

A previous study suggested that the retained sensitizing capacity of pepsin hydrolysates of Ara h 1 is caused by peptide aggregation [20]. Purified Ara h 1 was hydrolyzed by pepsin (E/S = 170 U/mg protein) for 2 h and the hydrolysates were fractionated into large complexes and small complexes. Gel permeation chromatography was used to analyze the aggregation profiles in hydrolysate. The results showed that the peptide size of the large complexes was < 4.0 kDa but these peptides formed aggregates with size up to 56 kDa, while the peptides of the small complexes were < 3.0 kDa but they formed aggregates up to 9.0 kDa. Both large and small complexes derived from fractionation lost the sensitizing capacity according to IgE and IgG1 response of rat immunization.



**Figure 7.** Mass frequency profiles of (a) Alcalase hydrolysate at 30min (A30) and (b) sequential Alcalase-Flavourzyme hydrolysate (S60), shown in a histogram, where each bar corresponds to a peptide size interval of 0.5 kDa.

The current study showed enzymatic hydrolysis is effective in degrading peptides from peanut flour protein, resulting in significantly reduced IgE binding as determined by Western blotting experiments. However, at this stage, the enzyme digests did not reduce IgE cross-linking capacity as determined by the more physiologically relevant basophil activation tests. Ara h 2 resistant peptides were detected in all hydrolysates and these peptides are hypothesized to be retaining IgE cross linking capacity; however, more work is necessary to confirm this hypothesis. Sequential hydrolysates and SEC fractions derived from these peptides are currently being evaluated for their potential to degranulate basophils. The potential of generated peptides to form aggregates which could result in basophil degranulation is another hypothesis that could be investigated.

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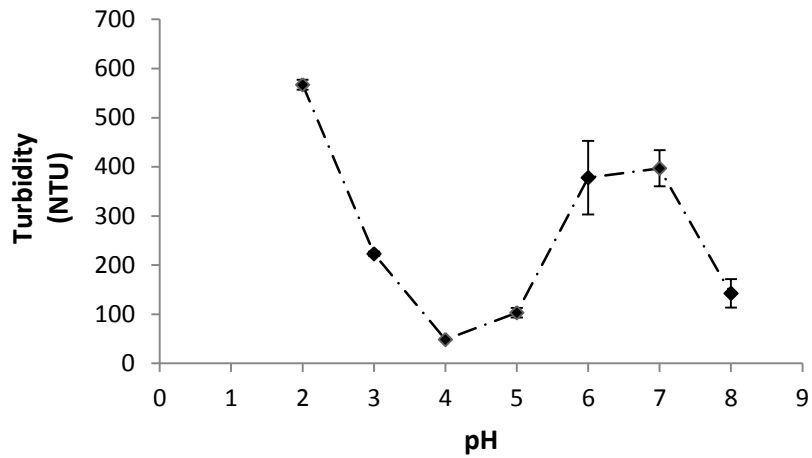
**CHAPTER 4:**  
**Conclusions and Future Work**

Enzymatic hydrolysis of peanut flour extracts resulted in hydrolysates with enhanced ACE inhibitory properties and decreased IgE binding capacity compared to unhydrolyzed controls. Among all the treatments, Alcalase hydrolysis resulted in hydrolysates with the greatest ACE inhibitory capacity. All protease treatments decreased IgE binding capacity with the relative order being: sequential Alcalase-Flavourzyme > Alcalase > Flavourzyme > pepsin. For all hydrolysates, Ara h 2 peptides maintaining IgE reactivity were observed. Surprisingly, all individual hydrolysates had IgE *cross-linking* capacity equivalent to unhydrolyzed controls in a basophil degranulation assay. The formation of peptide aggregates with enhanced IgE reactivity could explain this observation and further work is needed to confirm this hypothesis.

Future work will include testing sequential hydrolysates (highly degraded peptides) and FPLC derived fractions of these hydrolysates in the basophil activation assay to further understand IgE *cross-linking* potential. Furthermore, we are working towards implementing LC-MS/MS methodologies to better characterize hydrolysate structure as related to bioactive and/or allergenic potential.

## **APPENDICES**

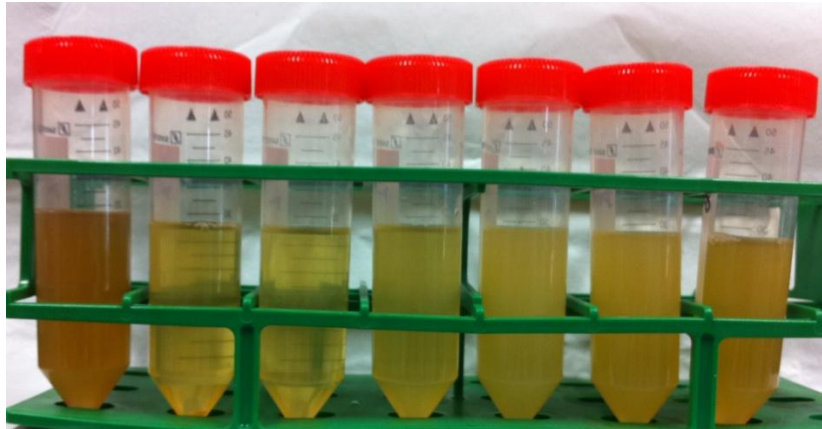
**Appendix A.** Turbidity of peanut flour soluble fractions from pH 2.0-8.0.



Turbidity of supernatants was measured by 2100AN Laboratory Turbidimeter (Hach, Ames, IA) in three replications and expressed in nephelometric turbidity units (NTU).

Data bars are means  $\pm$  standard deviation. Samples were prepared as described in Chapter 2 (pgs 43-44). The decrease in turbidity at pH 4.0-5.0 is due to isoelectric point precipitation of the proteins at this pH and subsequent removal of these proteins by centrifugation. The especially high turbidity at pH 2.0 is thought to perhaps be interference from the notable dark color of these samples as seen in Appendix B and as discussed in Chapter 2.

**Appendix B.** Visual observations of peanut flour soluble fractions from pH 2.0-8.0.



pH =2   pH =3   pH =4   pH =5   pH =6   pH =7   pH =8

Visual observation for peanut flour soluble fractions as a function of pH. Samples were prepared as described in Chapter 2 (pgs 43-44).

## **Appendix C. Acronyms**

ACE angiotensin I converting enzyme

AU Anson Units

AUC area under the curve

BSA basophil activation assay

BCA bicinchoninic acid

BHA butylated hydroxyanisol

BHT butylated hydroxytoluene

BSA bovine serum albumin

DH degree of hydrolysis

DPF defatted peanut flour

DPPH 1,1-diphenyl-2-picrylhydrazyl

DTT dithiothreitol

E/S enzyme/substrate

FAPGG N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine

FDA Food and Drug Administration

FPLC fluid protein liquid chromatography

GPC gel permeation column

GRAS generally recognized as safe

H-ORAC hydrophilic-oxygen radical absorbance capacity

HCl hydrochloric acid

HHPH highly hydrolyzed protein hydrolysates

HPLC high performance liquid chromatography

hr hour

HRP horseradish peroxidase

IgE immunoglobulin E

kDa kilo Dalton

LAPU Leucine Amino Peptidase Units

min minute

MW molecular weight

NaCl sodium chloride

NaOH sodium hydroxide

NTU nephelometric turbidity units

OIT oral immunotherapy

OPA o-phthaldialdehyde

O/L oleic to linoleic acid ratio

PBS phosphate buffered saline

PPH peanut protein hydrolysates

PPI peanut protein isolate

RP-HPLC reverse phase high performance liquid chromatography

SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SLIT sublingual immunotherapy

Th1 T helper cells type 1

Th2 T helper cells type 2

TNBS trinitrobenzenesulfonic acid

T<sub>reg</sub> T regulatory cells

w/v weight per volume