

ABSTRACT

SEIFERT, LAUREN ELAINE. Processing Techniques for the Improvement of Peanut Meal. (Under the direction of Dr. Jack P. Davis.)

Peanut meal is characterized as the non-food grade material that remains after the extraction of oil from peanuts (*Arachis hypogaea* L.). Oil is extracted from peanuts that are considered not suitable for human consumption due to discolored, broken, or aflatoxin contaminated seed. Peanut meal is a rich source of protein (45-60%) and can be used in food products if the aflatoxin, a natural contaminant in peanuts, was eliminated. Recent trends have shown that plant proteins are increasingly being used as a less expensive alternative to animal proteins for fulfilling basic nutritional needs. Technologies are needed to expand the applications for this commercially available material, which is currently sold at a low economic value as either animal feed or fertilizer, depending upon the aflatoxin concentration. The objectives of this research were to improve the value of peanut meal: 1) through enzymatic hydrolysis to enhance protein functional and nutritional properties, and 2) by sequestering the aflatoxin from contaminated meal using a non-nutritive adsorbent.

Defatted peanut meal dispersions (10% w/w) were hydrolyzed with commercial proteases (Alcalase, pepsin and Flavourzyme) and soluble fractions (hydrolysates) were collected for subsequent testing. Degree of hydrolysis ranged from approximately 20-60% for Alcalase, 10-20% for pepsin and 10-70% for Flavourzyme from 3-240 min. Low molecular weight peptides (<14 kDa) were observed in all hydrolysates as determined by SDS-PAGE. Results indicated that total soluble material increased a minimum of 30% regardless of protease at 240 min and antioxidant capacity of all hydrolysates was greater than unhydrolyzed controls. Alcalase hydrolysates had the greatest antioxidant capacity and

total soluble material. These results suggest that peanut meal could be made more valuable via enzymatic hydrolysis to create small peptides with improved functional and nutritional properties.

A method of reducing aflatoxin within peanut meal was also investigated, as it is imperative to have <20 ppb for peanut products intended for human applications. The *in vitro* efficacy of sodium bentonite clay, Astra-Ben 20™ (AB20), to sequester aflatoxin from contaminated meal was studied. Aqueous peanut meal dispersions (10% w/w) were adjusted to pH 2 and 8 and randomly assigned to one of three treatments: control (no clay), 0.2% AB20 (w/w), or 2% AB20 (w/w). Results revealed that the addition of clay significantly lowered the aflatoxin concentration in the soluble and insoluble fractions to a level that would be permissible by the FDA for use in food products. The pH of the soluble samples did not significantly affect the ability of clay to bind aflatoxin. Soluble fractions subjected to 2% AB20 treatment had significantly lower protein solubility and total soluble material than their respective pH 2 and 8 controls. This novel research not only provides an avenue for high protein peanut meal soluble fractions to be used safely in human food applications, but also for the insoluble fractions to be sold as animal feed at a higher price due to the decreased level of detectable aflatoxin.

Processing Techniques for the Improvement of Peanut Meal

by
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DEDICATION

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

BIOGRAPHY

Lauren Elaine Seifert was born on December 25, 1982 in Baltimore, Maryland. She is the daughter of Ken and Laura Seifert of Stewartstown, Pennsylvania. Lauren spent the majority of her grade school life as a competitive dancer and graduated from Kennard-Dale High School in June 2000. She continued her education at the Pennsylvania State University in University Park, Pennsylvania. There, Lauren was an active member of the Food Science Club, Food Product Development Team, and Tapestry: The Penn State Dance Company. During her undergraduate studies, she held a summer internship in quality control at Tyson Foods, Inc. Lauren earned a Bachelor of Science in Food Science in May 2004 and began her career as a Quality and Food Safety Manager at Frito-Lay, Inc. at the York, Pennsylvania manufacturing plant. In November 2004, she purchased her first home in York.

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CHAPTER 1:
Literature Review

The Peanut

The peanut, *Arachis hypogaea* Linn seed, is an annual legume that originates from South America and was first cultivated in the 16th century (1). China, India and the United States (U.S.) are the leading peanut growing countries, producing approximately 29 million metric tons annually (2). In the U.S., 99% of the peanuts are grown in seven states (see Table 1).

Table 1. U.S. peanut crop growing regions (2).

State	Percent of U.S. Crop (%)
Georgia	41
Texas	24
Alabama	10
North Carolina	9
Florida	6
Virginia	5
Oklahoma	5

Peanuts are classified into four basic types: Runner, Virginia, Spanish and Valencia (2). Each type has distinctive characteristics. Runner peanuts have become increasingly popular in recent decades due to their high yields and now account for approximately 80% of U.S. production. Runner-type peanuts have a range in seed size and are primarily used in peanut butter production. Virginia peanuts have the largest seed size and are usually sold as roasted in-shell or shelled-salted peanuts. Virginia-type peanuts account for 15% of U.S. production. Spanish-type peanuts have smaller seeds, account for only 4% of U.S. production and are used predominantly in peanut candies. Valencia peanuts, accounting for less than 1% of U.S. production, typically have three or more small seeds in each pod. They are very sweet peanuts and are mainly sold as roasted in-shell or boiled peanuts (2).

Worldwide, peanuts are primarily grown for the seed oil which is commonly used in cooking applications, or as a salad oil (3, 4). However, in the U.S., peanuts are predominantly used for peanut butter, snack nuts and confections (5). Peanut milk, an aqueous extract of peanut kernels, is used in developing countries because it is less expensive than bovine milk and is rich in protein, minerals and essential fatty acids (6).

Peanut Nutritional Composition

The peanut seed contains two cotyledons and the embryo, called the heart, all of which is surrounded by the seed coat, or testa (7). Peanut seed contain approximately 36-54% lipid, 21-36% protein and a low percentage of carbohydrates and ash (7). Nutritional composition is dependent upon a number of factors, as discussed below.

The fatty acid composition of peanut oil is influenced by cultivar, maturity stage and yearly variations in environmental conditions (8, 9). Oleic (18:1), linoleic (18:2), palmitic (16:0) and stearic (18:0) acids are present in the greatest quantities, accounting for approximately 90% of the total fatty acids (8, 9). Peanut oil contains 0.025% and 0.020% of the antioxidants α - and γ -tocopherol, respectively, which aid in peanut oil resistance to oxidation. (10). It has been reported that α -tocopherol is the most bioactive component in some legumes (11).

The carbohydrate content of peanuts is comprised of 2-5% sugar, 4% starch, 4% pectic material and 2% cellulose (7). Sugar content varies with growing conditions, seed maturity, location and cultivar (12). Sucrose is the most prevalent sugar in peanuts, accounting for 2-5% total weight (7), followed by stachyose and raffinose (5). Sugar content increases with maturity and reaches maximum at full maturity (5).

Peanuts contain a significant amount of some vitamins and minerals. Aside from providing elevated levels of tocopherols (vitamin E), peanuts are also a good source of vitamin B, potassium, phosphorous and magnesium (5, 7). Peanuts contain a small amount of vitamin C and trivial amounts of Vitamins A, D, and K (7).

Proteins, Peptides, and Amino Acids

Proteins are biopolymers composed of amino acids (13). There are 20 commonly occurring amino acids, each of which are characterized by an amino group on a carbon atom adjacent to a carboxylic acid group with varying side chains (13). Amino acids are linked together by peptide bonds in proteins. Peptide bonds are formed by a condensation reaction between a carboxyl group and an amino group on two amino acids (13).

Most amino acids are synthesized in the body, however, there are nine essential amino acids that cannot be synthesized by humans, and therefore, must be provided by the diet. These amino acids are: histadine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine and tryptophan. Ingested proteins are degraded by exposure to acids and enzymes during digestion, and the hydrolyzed proteins are then absorbed and utilized by the body.

Nutritional quality and chemical reactivity are a function of protein composition and structure (14). Protein structures are defined as primary, secondary, tertiary and quaternary. Protein primary structure is defined by the linear sequence of amino acid residues. Secondary structure is the orientation of the amino acids leading to the formation of α -helices or β -sheets which are stabilized by hydrogen bonds (13, 14). The overall shape of the protein, or tertiary structure, is determined by the three dimensional orientation of the

rod-like α -helices and flat β -sheets. Proteins typically take on a globular or fibrous shape (14). Protein globules, tightly packed spheres, typically have a hydrophobic core.

Quaternary structure is formed when multiple protein molecules link together to form a larger unit (14).

Peanut Protein

Peanut seed contains between 21-36% protein which can be broadly separated into globulins (salt soluble) and albumins (water soluble) (15, 16). Of the total globulins, approximately one-fourth is conarachin and three-fourths is arachin (15). Both arachin and conarachin are composed of large molecular weight globulins (17, 18). The main distinction between the two globulins is the sulfur content; conarachin contains nearly three times the sulfur content as arachin (15).

Table 2. Molecular weight regions of the 5 main classes of peanut protein subunits, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19).

Peanut Protein Region	Molecular Weight (kDa)
Conarachin	>50
Acidic arachin	38 - 49.9
Intermediate	23 - 37.9
Basic arachin	18 - 22.9
Low molecular weight protein	14 - 17.9

Amino Acids

The amino acid content of the peanuts varies with the type of peanut, cultivar, growing location, year and maturity (5). Peanuts are rich in glutamic acid, aspartic acid and arginine and deficient in the sulfur containing acids, methionine and cysteine (12). Peanuts are a poor source of the essential amino acids lysine, threonine, and methionine (12).

Table 3. Amino acid composition of peanuts (20).

Amino Acid	Quantity (g/100 g protein)
glutamic acid	23.01
aspartic acid	12.91
arginine	12.43
leucine	6.25
phenylalanine	5.41
serine	5.22
glycine	5.15
proline	5.01
tyrosine	4.12
alanine	3.74
valine	3.65
lysine	3.12
isoleucine	2.83
threonine	2.50
histadine	2.22
tryptophan	1.59
methionine	0.52
cysteine	0.33

Peanut Meal

The primary protein-enriched peanut based ingredients include peanut meal, flour, concentrates and isolates. Peanut meal (45-60% protein concentration) is characterized as the non-food grade material that remains after the extraction of oil from peanut seed (3). Oil processing conditions are chosen to optimize the maximum amount of oil extraction with little regard for protein. In the meal, 97% of the total protein is contained in the two globulins, arachin and conarachin (21). Defatted peanut meal protein content is highly dependent on the type of oil extraction technique used (22). Defatted peanut meal can be prepared by hydraulic pressing, screw pressing, solvent (hexane) extraction or pre-pressing followed by solvent extraction (3, 4).

Organic solvents, most commonly hexane (23), are used to further extract oil from peanut seed after pressing; however, these organic solvents may have an adverse effect on protein properties. Neucere and Ory (24) found that carbon tetrachloride, heptane and acetone extraction of peanut meal made the proteins more insoluble than non-solvent treated peanuts. The albumin and conarachin fractions had the largest decrease in solubility suggesting that the isoelectric point shifted closer to neutrality after organic solvent extraction. Chromatography indicated that acetone extraction caused the arachin protein to be partially dissociated into two components. The changes in protein solubility and ionic properties indicate that the protein structures have been modified (24). The effects of arachin exposed to hexane, acetone, hexane-acetone-water, and hexane-acetic acid were also studied (23). This study found that exposure of arachin to acidic hexane caused irreversible dissociation. Hexane, acetone and hexane-acetone-water had no effect on the conformational properties of arachin; this contradicts the data from Neucere and Ory (24).

In the early 1900's, the non-food grade peanut meal by-product of oil pressing was sold as cattle feed at thirty-five dollars per ton (15). Today, aflatoxin contaminated peanut meal is sold as animal feed at approximately one hundred seventy-five dollars per ton if the aflatoxin contamination is between 20 to 300 parts per billion (ppb). If the peanut meal has less than 20 ppb, it can be sold as dairy cattle feed at a premium price of two-hundred ten dollars per ton. Highly contaminated peanut meal, greater than 300 ppb, can be sold as fertilizer or mushroom compost at approximately ninety-five dollars per ton.

Peanut Flour, Protein Concentrate and Isolates

Edible (less than 20 ppb aflatoxin) defatted peanuts can be converted into a number of viable food ingredients, including food grade peanut flours (typically <69% protein concentration), concentrates (70-85% protein concentration) and isolates (86-100% protein concentration) (3, 4). Peanut flours have an established market in the food industry as an ingredient; however, concerns over allergenicity have resulted in a limited utilization of these materials in otherwise non-allergenic foods. Currently, there is not a demand for peanut protein concentrates and isolates.

The amount of protein extracted in water from peanut solids depends on a number of factors. One study examined the effects of pH, temperature, time, solids-to-water ratio, speed of agitation, hydrolysis and soaking on the extraction of protein from peanut solids (25). Extraction time (10-40 min) and speed of agitation (5000-10000 rev/min) did not significantly affect the amount of protein extracted. Papain hydrolysis and soaking the seeds in deionized water at a 1:5 (w/v) ratio for one hour at 20°C significantly increased the amount of protein extracted. A solids-to-water ratio of 1:5 (w/v) yielded higher protein content than a 1:9 (w/v) ratio. Adjusting the pH from 6.95 to 8.00 also resulted in significantly more protein.

Production of protein isolates involves extracting off-flavored compounds, trypsin inhibitors, aflatoxins, flatulence sugars, water insoluble polysaccharides, water soluble sugars and minerals (3, 26). A typical method for peanut protein isolate production begins by extracting the protein from defatted peanut seed flour with an aqueous alkaline solution. Insoluble materials are then removed by centrifugation or filtration. Proteins are then

precipitated at the isoelectric pH and the precipitated protein curd is collected by centrifugation or filtration. The protein isolate is then dried by spray-drying or lyophilization procedures (3).

Basha (21) studied protein isolates from eleven peanut cultivars using two-dimensional polyacrylamide gel electrophoresis and found wide variability among the major polypeptide composition. At least 74 major and between 100 and 125 minor polypeptides were distinguished by isoelectric point and molecular weight (21). The isoelectric points of the major and minor components ranged from pH 4.4 to 8.0 and the molecular weights ranged from 16,000 to 75,000.

A study was conducted on the functional properties of peanut protein isolates from nine different cultivars harvested at the same maturation stage (27). No significant differences were observed among foaming properties or water-holding capacity. However, there were significant differences in protein solubility and emulsification activity which may be due to the genetic variability among the cultivars (27).

Roasting

Commercial products such as peanut butter, salted peanuts and peanut flour are derived from roasted peanuts. Roasting peanuts affects seed color, flavor, nutritive value and physicochemical properties. During roasting, peanuts are heated to an internal temperature of 130° to 150°C, and this is accompanied by a decrease in internal moisture from approximately 5% to 1% as well as a loss of volatile compounds (7). It is important to consider the effects of roasting conditions because although the peanut meal used in this research is not roasted, the processing temperature does reach 115°C.

The color of roasted peanuts is primarily a result of Maillard browning reactions between the sugar and amino acids (28). The brown color of the peanuts increases with increasing roasting time or temperature (5). Newell et al. (29) found that total carbohydrate and amino acid concentration of peanut seed decreased during roasting. The characteristic flavor of roasted peanuts can be attributed to a reaction between sucrose, which undergoes hydrolysis to fructose and glucose, and amino acids to form volatile compounds (29). Specifically, it has been reported that the amino acids: aspartic acid, glutamic acid, phenylalanine and histadine, are the precursors to roasted peanut flavor (29).

Numerous studies have suggested that roasting peanuts causes a decrease in protein solubility (30-32). One study reported that dry roasting whole peanuts at 145°C for one hour reduced the protein solubility to less than half of the unroasted seed protein solubility. Although the antigenic structure of α -arachin was maintained post-heating, other proteins had altered physicochemical properties (30). Neucere et al. (17) suggested that the decrease in protein solubility may be a result of irreversible denaturation or a change in the protein isoelectric point.

The functional properties of peanut protein concentrate prepared from defatted roasted peanut flour (47-55% protein) were evaluated (33). Roasting reduced protein solubility, water holding capacity, oil binding capacity and foaming capacity (33). The maximum protein solubility was at pH 10. The decrease in protein solubility and water holding capacity was attributed to increased surface hydrophobicity of the denatured protein in the roasted samples. Roasting did not significantly affect the emulsifying capacity. Although roasting lowered the foaming capacity, it was concluded that all tested defatted

peanut flours were not good foaming agents and hence not suitable for use in foam food systems. Upon heating to 90°C and cooling to room temperature, peanut protein concentrate derived from roasted peanut flour formed a soft, shear thinning gel at 10% and a firm, shear thinning gel at 12.5% (33).

The effects of roasting temperature and moisture level on peanut protein availability were determined using a rat feeding study. Protein availability decreased as roasting temperatures increased from 110° to 155°C (31). However, wet or dry roasting the seeds had no significant effect on protein availability or the final mean body weight of the rats (31).

Kopper et al. (32) compared the effects of roasting whole peanut seed versus roasting raw peanut flour. Protein solubility in defatted peanut flour decreased more rapidly than in whole peanuts upon roasting at 179°C. The rapid decrease in protein solubility of flour compared to whole seeds was due to the increase in surface area of the peanut flour (32).

Enzymatic Hydrolysis

Proteins play a vital role in food functionality and quality, in addition to fulfilling basic nutritional needs. Protein functionality within a food system is highly dependent on solubility and degree of denaturation. Enzymatic hydrolysis of proteins is an established method of generating peptides that have been shown to enhance functional properties such as foaming, emulsification, and solubility, as well as improving nutritional quality (34).

To control hydrolysis, enzymes must be inactivated after the desired reaction is complete. Common methods of terminating hydrolysis include pH adjustment or heat inactivation. If enzymatic activity is not fully terminated, hydrolysis will continue until no enzyme specific substrate remains. Studies have shown that extensive hydrolysis may result

in bitterness, a typically undesirable food attribute, which is characteristically associated with neutral amino acids with large alkyl or aromatic side chains (35, 36).

The degree of hydrolysis (DH) measures how extensively an enzyme digests a given protein substrate. The amount of peptide bonds that are cleaved, or the degree to which a protein has degraded to smaller peptides or individual amino acids, is indicative of the catalytic action of proteases (36). Numerous methods including pH stat, o-phthaldialdehyde (OPA), and trinitrobenzenesulfonic acid (TNBS) have been established to track DH. The TNBS method, a spectrophotometric assay that measures the reaction of TNBS with primary amines, will be used for this research because it is well-established, accurate and reproducible (34). Measuring DH is critical for comparing and assessing the effectiveness of multiple enzymes within a food system.

A variety of enzymes have been studied for peanut protein hydrolysis (35, 37). Beuchat, et al. (37) studied the effects of pepsin, bromelain and trypsin enzymatic hydrolysis on defatted peanut flour prepared via hexane extraction. A 15% peanut flour slurry was adjusted to pH 2, 4.5 or 7.6, used for pepsin, bromelain and trypsin hydrolysis respectively. Hydrolysis was carried out using a concentration ratio of 1:75 enzyme-protein (w/w) for 10, 30 and 50 min at 50°C followed by heating for 10 min at 90°C to inactivate the enzymes. After enzyme inactivation, samples were cooled and pH was adjusted to 6.9, the original pH of the peanut flour slurry (37). After 10 min of enzymatic hydrolysis, nitrogen solubility of the flours at pH 4-5 was improved, with the greatest solubility occurring after pepsin hydrolysis (37). Polyacrylamide gel electrophoretic banding patterns indicated that the large molecular weight globulin, arachin, was substantially decomposed after undergoing a pH

adjustment along with pepsin and bromelain hydrolysis. All three enzyme treatments caused an increase in water adsorption and completely destroyed the emulsifying capacity of the peanut flour (37).

Similarly, Sekul et al. (35) studied the effects of papain hydrolysis on defatted peanut flour. Hydrolysis was conducted using a 1:10 (w/v) dispersion of non-roasted defatted peanut flour in deionized water and treating it with papain (0.5% total volume) at 45°C for 15 minutes. Results indicated that papain hydrolysis improved the protein solubility from pH 1 to 9, except at pH 2, where the control had slightly higher nitrogen solubility (35). Hydrolysis decreased the emulsifying capacity, increased foaming properties and did not yield any bitter peptides (35). Papain hydrolysis has an advantage over trypsin which completely destroyed the emulsifying capacity. The papain hydrolysis reaction was allowed to naturally terminate until proteolytic activity reached equilibrium (35). Papain hydrolyzed peanut proteins have been characterized as being bland in taste, odorless and having increased solubility (38).

Hydrolysis can also be conducted on protein fractions. Conarachin is comprised of 2 fractions, conarachin I and II, based on differing sedimentation velocities (39). Three major protein fractions, arachin, conarachin I and conarachin II were isolated from defatted peanut meal via preferential ammonium sulfate and gel filtration. Enzymatic hydrolysis was carried out at 37°C on the three protein fractions using pepsin, trypsin and chymotrypsin (20). Pepsin hydrolysis was carried out at pH 2 at an enzyme to substrate ratio of 1:200 for 120 min. Trypsin hydrolysis was carried out at pH 8 at an enzyme to substrate ratio of 1:40 for 60 min. Chymotrypsin hydrolysis was carried out at pH 8.1 at an enzyme to substrate

ratio of 1:50 for 60 min. DH varied among the different enzymes, with pepsin having the slowest rate of hydrolysis. An analysis of the total protein, arachin, conarachin I and conarachin II composition indicated that aspartic acid, glutamic acid and arginine were the predominant free amino acids (20).

The affect of polysaccharides that are commonly used in food manufacturing on the digestion of peanut protein isolate was studied (40). Peanut protein isolate (91% total protein) and polysaccharide solutions were mixed in a sodium phosphate buffer at 1.7% (w/v) then blended together for a final polysaccharide concentration of 0, 1%, 10%, 20%, 30% and 50% (w/w). Gum arabic, low methoxy pectin and xylan were the polysaccharides being studied. The protein-polysaccharide mixtures underwent *in vitro* hydrolysis at 37°C, first by pepsin, then by a mixture of trypsin and chymotrypsin. Pepsin hydrolysis was conducted in a beaker for 2 hours. To mimic *in vivo* digestion, the pH of the pepsin hydrolysis was progressively reduced from 7 to 2 by adding 0.02 N HCl. The pepsin was inactivated by raising the pH to 8 using 2 N NaOH and the sample was transferred to dialysis bags to undergo hydrolysis by a mixture of trypsin and chymotrypsin for 6 hours at pH 8. Gel electrophoresis of the samples revealed that polysaccharides modify the protein retentates (40). Gum arabic and xylan reduced peanut protein isolate digestibility of some proteins and high molecular weight peptides. Low methoxy pectin did not reduce peanut protein isolate hydrolysis, but did decrease the release of nitrogen which was probably due to interactions between low methoxy pectin and low molecular weight peptides and amino acids from the peanut protein isolate hydrolysis (40).

Selected Proteases

Alcalase, pepsin and Flavourzyme are all water soluble, food-grade, commercially available enzymes. These proteases have been well studied and are used to enhance protein functionality in both commercial food and research applications.

Alcalase

Alcalase is a proteinase prepared from the fermentation of *Bacillus licheniformis* (41). This enzyme is an endoproteinase with broad specificity and a preference for large uncharged residues and terminal hydrophobic amino acids. Alcalase hydrolyzes native and denatured proteins and is most active at optimal conditions between 55-65°C and pH 6.0-8.5.

Pepsin

Pepsin, a component of the gastric juice in mammals and the predominant digestive enzyme in humans, is an acidic peptidase (42). The average human stomach secretes approximately 400 mL of gastric juice per meal; of that, 0.2%, or 0.08 g is pepsin (43). Pepsin is a single enzyme with low specificity and preferentially cleaves hydrophobic, aromatic amino acids. Optimal conditions for this enzyme are between pH 1.5-4.0 and 37°C.

Flavourzyme

Flavourzyme is a fungal complex produced from the fermentation of *Aspergillus oryzae* and possesses both endoprotease and exopeptidase activity (44). This enzyme is most active at optimal conditions between 45-55°C and between pH 5.0-7.0. However, Flavourzyme is primarily used for its debittering properties which are most active at pH 7.0. The optimal conditions for exopeptidase activity are also pH 7.0 and 50°C. Low dosages of Flavourzyme (5-10 LAPU/g [One LAPU is the amount of enzyme which hydrolyzes 1 µmol

of L-leucine-p-nitroanilide per minute]) may be used for debittering bitter hydrolysates or extensive hydrolysis can be obtained through higher dosages (10-50 LAPU/g protein) (45).

Bioactive Peptides

Bioactive peptides are dietary protein fragments which exhibit specific biological effects upon consumption. These peptides, usually 3 to 20 amino acid residues, are inactive in their native parent protein but can be released during gastrointestinal digestion or food processing (46). The biological activity of a specific peptide is affected by the protein source, enzyme used and processing conditions (47). Specific amino acid sequences can exhibit positive health benefits on the cardiovascular, endocrine, immune and nervous systems including antihypertensive, antioxidant, antimicrobial, immunomodulatory, hypocholesterolemic, opioid and mineral sequestering activities (46, 48). These short-chain peptides are able to exhibit their biological effects on the body by crossing the digestive epithelial barrier, reaching blood vessels and altering organ function (49). Biological peptide activity typically decreases with increasing chain length (49).

Bioactive peptides can be produced by enzymatic hydrolysis with digestive enzymes, chemical hydrolysis or by microbial fermentation; however, enzymatic hydrolysis is the most commonly used method because it is the most controllable process (46). Pancreatic enzymes such as trypsin, along with other proteinases including Alcalase, chymotrypsin, and pepsin have been utilized to generate bioactive peptides from foods including eggs, soybeans and dairy products just to name a few (46). Bioactive peptides have also been identified in cereal, fish, and plant sources including wheat, corn, rice, barley, buckwheat and sunflower

(47, 48). Bioactive peptides found in peanut proteins could potentially be used in functional foods or nutraceutical products.

Various techniques can be used to identify the specific amino acids in a bioactive peptide. Normal and reverse phase high performance liquid chromatography, ion-exchange chromatography, capillary electrophoresis, capillary isoelectric focusing and size-exclusion chromatography can all be used to separate the peptides from their parent protein (47). SDS-PAGE can then be used to determine the molecular weight and purity of large peptides.

Chen et al. (50) indicated that peanut protein hydrolysates exhibit antioxidant activities by measuring the inhibition of linoleic acid autoxidation, scavenging effect on free radicals, reducing power and inhibition of liver lipid autoxidation. An 8% (w/v) peanut protein solution was preheated at 90°C for 20 min. Following pretreatment, enzymatic hydrolysis was carried out using the protease Alcalase at pH 7.5 and 55°C for 6 hours at an enzyme-protein ratio of 3:100. The hydrolysate was submerged in boiling water for 3 minutes to inactivate Alcalase. The peanut protein hydrolysate had more antioxidant activity than unhydrolyzed peanut protein, but slightly less antioxidant activity than butylated hydroxytoluene (BHT), a synthetic antioxidant (w/v basis). Peanut protein hydrolysate antioxidant activity in a linoleic acid emulsion increased with increasing concentration from 2-40 mg/mL (50). The researchers did not identify the specific amino acid sequences of the bioactive peptides in the peanut protein hydrolysate that were responsible for the antioxidant activity.

Hwang et al. (51) examined the effect of roasting time and enzymatic hydrolysis of roasted defatted peanut seeds on antioxidant activity. Antioxidant activity increased with

increasing roasting time from 0 to 60 min at 180°C (51). Furthermore, the antioxidant activity of the 60 min roasted samples also increased when hydrolyzed with either Esperase or Neutrase. Increasing the concentration of Esperase or Neutrase hydrolysates increased the scavenging activity on DPPH radicals. Esperase hydrolysates, at a concentration of 0.5 mg/mL, had a 90% scavenging effect on DPPH radicals which was similar to the effect of 0.01 mg butylated hydroxyanisol (BHA)/mL, a synthetic antioxidant (51).

Soybean, another legume seed, is rich in proteins, amino acids and carbohydrates (52). Unlike peanuts, soy products are already recognized as functional foods with specific bioactive peptides. Gibbs et al. (52) evaluated soy hydrolysate and the soy-fermented foods, natto and tempeh, for biological activity. Natto is fermented with *Bacillus subtilis* and tempeh is fermented with *Rhizopus oligosporus*. High performance liquid chromatography indicated that the soy hydrolyzed by plasma proteases produced fewer peptides than either of the fermented soy products (52). Most of the isolated bioactive peptides were derived from glycinin, the major soy protein. Various peptides that were isolated from the natto and tempeh have varying degrees of biological activities including angiotensin converting enzyme inhibitory, anti-thrombotic and antioxidant properties (52).

Sunflower protein hydrolysate, derived from defatted sunflower meal, has been useful in treating patients with liver failure (53). The globulin fraction of the sunflower protein was hydrolyzed in a series of steps, resulting in a product comprised of tri- and dipeptides and free amino acids called highly hydrolyzed protein hydrolysate (HHPH). Bitterness of the HHPH was eliminated by Flavozyme[®] hydrolysis (5% substrate, pH 7.0, 55°C). The loss of bitterness was attributed to the reduction in aromatic amino acid content and a relatively low

concentration of arginine and proline. The final product was non-allergenic and primarily composed of tetra-, tri-, and dipeptides, and free amino acids (53).

Nutraceutical peptides have also been identified in flaxseed protein hydrolysate (54). Flaxseed protein isolate was hydrolyzed by Alcalase, then passed through a 1000 Dalton molecular weight cut-off membrane and finally, the permeate was loaded onto a cation-exchange column to derive a cationic protein hydrolysate. The positively charged amino acids act as inhibitors by binding to calmodulin and inhibiting the activity of calmodulin-dependent neuronal nitric oxide synthase, an enzymatic activity which has been reported in stroke, Alzheimer's and Parkinson's disease (54).

Aflatoxin

Aflatoxins are toxic, carcinogenic compounds, which are produced by the fungi *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare (20). There are four major naturally occurring aflatoxins, aflatoxin B₁, B₂, G₁ and G₂ (55). These four compounds are distinguished by their fluorescence color (B=blue; G=green) and their relative chromatographic mobility (56). *Aspergillus flavus* only produces aflatoxin B₁ and B₂ (56). Aflatoxin M₁, found in milk as a metabolite of aflatoxin in cattle feed, is a hydroxylated form of aflatoxin B₁.

Aflatoxins have been identified in peanuts, treenuts, corn, cottonseed and milk (57). Aflatoxin is a natural contaminant in peanuts, but can be minimized by irrigating the field, harvesting at optimum maturity and storing the product in cool and dry conditions (58). The level of aflatoxin is also affected by the growing season, as drought conditions tend to increase aflatoxin levels.

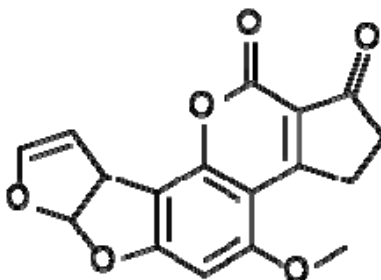


Figure 1. The chemical structure of aflatoxin B₁ (56).

Ingestion of aflatoxins from contaminated food or animal feed can lead to acute liver damage, edema, hemorrhage, alteration in digestion and possibly death (57). Aflatoxin B₁ is the most predominant naturally occurring aflatoxin and has been identified as a potent carcinogen (57). Aflatoxin B₁ is not carcinogenic until it is metabolized to form aflatoxin B₁-8,9-epoxide (56). Aflatoxicosis, or the poisoning that results from ingesting aflatoxins, must be considered when determining if peanut meal can be used as a protein supplement for undernourished populations. The U.S. Food and Drug Administration (FDA) has issued regulatory guidelines limiting the contamination level of aflatoxin to 20 ppb or less in peanut products intended for human consumption (59).

Most peanut oil is a product of aflatoxin-contaminated peanuts. After the oil is extracted, the aflatoxin remains in the by product, peanut meal. The aflatoxin level in the peanut meal must be quantified before it can be sold as animal feed, see Table 4. The susceptibility of animals to aflatoxicosis depends upon 1) their ability to activate aflatoxin B₁ to aflatoxin B₁-8,9-epoxide and 2) their ability to convert aflatoxins to form glucuronide or sulphate conjugation products to be excreted (60). Aflatoxin contaminated feed may cause serious illness and even death in animals (58).

Table 4. Action levels for aflatoxin to control contamination in human food and animal feed, as determined by the FDA (61).

Commodity	Action Level (ppb)
Peanuts and peanut products	20
Pistachio nuts	20
Brazil nuts	20
Human foods	20
Milk	0.5 (aflatoxin M ₁)
Animal Feed	
Peanut products intended for finishing beef cattle	300
Peanut products intended for finishing swine of 100 pounds or greater	200
Peanut products intended for breeding beef cattle, breeding swine, or mature poultry	100
Peanut products intended for immature animals	20
Peanut products intended for dairy animals, for animal species or uses not specified above, or when the intended use is not known	20

A study was conducted in which rats were fed aflatoxin as a natural food contaminant for 104 weeks (62). The diets were nutritionally balanced and included 35% ground roasted peanut contaminated primarily with aflatoxin B₁ at 0, 1, 10 and 80 ppb levels and a control diet containing no peanut components. Rats that were fed the aflatoxin at 1 and 10 ppb grew as much as the control rats. There was a higher survival rate for the rats fed the 1 and 10 ppb aflatoxin diets than the control or 0 ppb aflatoxin diet. The liver is generally the first organ to show pathological changes from aflatoxin toxicity (62). Liver tumors were not found in any of the animals, however, rats fed the 80 ppb aflatoxin diet had higher liver lipids, liver cholesterol and a higher incidence of liver lesions than animals fed the 0 ppb diet (62).

Monteiro and Prakash (20) studied the effects of aflatoxin on the extent of protein hydrolysis. Protein solutions (1%) of arachin, conarachin I and conarachin II were mixed with 1×10^{-5} M aflatoxin B₁ in 0.1 M pH 8.1 borate buffer and incubated at 37°C for 2 hours.

After incubation, the solutions underwent chymotrypsin hydrolysis. This research indicated that the addition of aflatoxin B₁ to each peanut protein fraction reduced the extent and rate of hydrolysis. Therefore, it would be beneficial to remove the aflatoxin from the peanut meal prior to enzymatic hydrolysis.

Aflatoxin Binding

Research is being conducted on detoxifying or inactivating aflatoxins to protect food and animal feed from the toxic effects. Irradiation, solvent extraction, density segregation, microbial inactivation, ammoniation, adsorptive materials, and thermal inactivation, including conventional nut processing procedures, may be used to reduce aflatoxin concentration (63). Adsorptive materials, or sequestering agents, such as activated charcoal, bentonite and aluminosilicates can be mixed into contaminated animal feed to bind aflatoxins, enabling them to pass through the animal gastro-intestinal tract, guarding against aflatoxicosis (55, 58). The ideal toxin-binder complex should not dissociate internally and should be expelled in the animal feces (64). Zeolites, hydrated sodium calcium aluminosilicates (HSCAS) and aluminosilicate-containing clays are the most commonly studied mycotoxin adsorbents. Aluminosilicate clays are generally recognized as safe (GRAS) and the U.S. FDA approved their use as anticaking agents in animal feed up to 2% dry weight basis under title 21, sections 582.2727 and 582.2729 in the Code of Federal Regulations (65). *In vitro* aflatoxin binding experiments may not give an accurate prediction of *in vivo* animal protection.

Activated charcoal is a non-soluble powder formed by pyrolysis of organic materials (58). This substance is very porous with a high surface area which provides for adsorption of

numerous toxic materials, including aflatoxins, making them unavailable for gastrointestinal absorption (55). Historically, activated charcoal has been used in the medical field for treating poisoning and drug overdoses. Although activated charcoal is odorless, tasteless and non-toxic, it will adsorb nutrients, vitamins and minerals, making it unsuitable for use in animal feed.

Bentonite, a layered crystalline microstructure comprised primarily of montmorillonite, can also be used to adsorb molecules such as aflatoxins (55). This clay substance is GRAS approved as a direct food additive and is currently used to remove the protein in white wine processing and to sequester aflatoxins in animal feed.

HSCAS has positive charge deficiencies which create the potential for adsorbing cationic compounds and positively charged molecules, such as aflatoxins (55). Similarly, zeolites are very porous with a high surface area and a high cation exchange capacity (58). The surface is polar and binds polar mycotoxins. Zeolite is GRAS and the FDA approves its use as a feed additive and an anti-caking agent (65). This substance is currently used by beef and dairy cattle, broiler, commercial egg, swine, sheep and turkey producers (66).

An animal feeding study investigated the efficacy of HSCAS and bentonite to prevent the developmental toxicity of aflatoxin in pregnant rats (67). The aflatoxins used in this study were produced through the fermentation of corn by *Aspergillus parasiticus*. Pregnant rats were fed one of six diets: (1) control diet; (2) diet containing 2.5 parts per million (ppm) aflatoxin in the feed; (3) diet containing 0.5% bentonite; (4) diet containing 0.5% HSCAS; (5) diet containing 2.5 ppm aflatoxin and 0.5% bentonite; (6) diet containing 2.5 ppm aflatoxin and 0.5% HSCAS. The diet was fed to them for 10 days during their gestation

period. After 20 days of gestation, the rats were killed, uteri were removed and litters were weighed. No maternal mortality occurred except one of the rats in the aflatoxin diet group (2) died on day 14 of gestation. The rats in the aflatoxin diet group (2) had a significantly lower feed intake and body weight gain than the other diet groups and none of the fetuses survived. Significantly lower fetal body weights were observed in the group treated with aflatoxin plus bentonite. The maternal body weights, litter weights and feed intake from the HSCAS or bentonite plus aflatoxin groups were comparable to the control group. The aflatoxin group also exhibited increased liver enzyme activity than the control group, but the diets containing HSCAS and bentonite had decreased liver enzyme levels. HSCAS and bentonite reduced the bioavailability and associated toxicity of aflatoxin in the pregnant rats (67).

Peanut Allergy

The FDA identifies peanuts as one of the top eight allergenic foods (68). The allergenic portion of the protein is present in the cotyledon and does not differ significantly across peanut varieties (32). A peanut allergic reaction is elicited when antibodies called immunoglobulin-E react with allergenic peanut proteins (69). The 3 major peanut allergens, Ara h 1 (conarachin, 7S, vicilin), Ara h 2 (conglutin), and Ara h 3/Ara h 4 (arachin, 11S, glycinin), account for more than 50% of peanut allergic reactions (70). Amylase, which is present in human saliva, first solubilizes peanut proteins in the mouth (32). This reaction may initiate the immediate itching of the tongue and throat in individuals with peanut allergies.

Research has been done to study the prevalence of peanut allergies. Rodent studies have suggested that consuming large quantities of an allergenic food may lead to a decrease in sensitization, thus if children are exposed to large quantities of peanuts they may be less prone to develop an allergy (69). Some studies have indicated that peanut cooking methods influence the degree of allergenicity. Boiling or frying peanuts, which are common cooking practices in Asian culture, reduces the amount of allergenic peanut proteins and lowers the immunoglobulin-E antibody binding intensity compared to roasted peanuts (69, 71).

Roasting peanuts, the most common American practice, seems to increase the reactivity with immunoglobulin-E thus increasing the likelihood of an immune response (69, 71). These findings may explain why the U.S. has a much higher prevalence of peanut allergy than Asian countries.

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CHAPTER 2:
Enzymatic Hydrolysis of Defatted Peanut Meal to Improve
Functional and Nutritional Properties

Abstract

Functional properties and antioxidant capacity of defatted peanut meal (48% protein) were evaluated after hydrolysis with commercial proteases (Alcalase, pepsin and Flavourzyme). Dispersions (10% solids) were hydrolyzed at the optimal conditions for each protease and soluble fractions (hydrolysates) were collected for subsequent testing. Degree of hydrolysis ranged from approximately 20-60% for Alcalase, 10-20% for pepsin and 10-70% for Flavourzyme from 3-240 min. Low molecular weight peptides (<14 kDa) were observed in all hydrolysates as determined by SDS-PAGE. Unique banding patterns revealed the different proteolytic activities of each enzyme. Total soluble material increased a minimum of 30% for all hydrolysates. Antioxidant capacity of all hydrolysates was greater than unhydrolyzed controls, with Alcalase hydrolysates exhibiting the most significant increases. These results suggest that peanut meal could be made more valuable via enzymatic hydrolysis to create small peptides with improved functional and nutritional properties.

Introduction

Peanut meal is defined as the non-food grade material remaining after oil extraction from peanut (*Arachis hypogaea* L.) seed (1). Oil is extracted from peanuts that are considered not suitable for human consumption due to discolored, broken, or aflatoxin contaminated seed. Aflatoxin contaminated peanuts are designated for oil production because the toxic, carcinogenic aflatoxin compounds are not oil soluble. When the oil is pressed and extracted from the peanuts, the relative percentages of all remaining solids in the meal increase, including the remaining protein and residual aflatoxin. Technologies are needed to expand the applications for peanut meal, which is currently sold at a low economic value as either animal feed or fertilizer, dependent upon its aflatoxin concentration.

Plant proteins can serve as a less expensive alternative to animal proteins for fulfilling basic nutritional requirements. Peanut meal is a rich source of protein (typically 45-60%). Aside from providing fundamental nutrition, proteins also play a vital role in food functionality and quality. Protein functionality within a food system is highly dependent on its solubility and extent of protein denaturation. Enzymatic hydrolysis is an established method to create low molecular weight peptides with enhanced functional properties such as solubility, foaming and emulsification, as well as produce bioactive peptides (2).

Bioactive peptides are short-chain amino acids which exhibit specific biological effects, such as antioxidant capacity, upon consumption (3). Bioactive peptides can be generated outside the body through hydrolysis, and then consumed, or digested and released naturally inside the body. Currently established sources of bioactive peptides include: chickpea (4), sunflower (5), corn (6), canola (7), soybean, wheat, rice, barley, and buckwheat

(8). Recent studies have suggested that peanut protein hydrolysates could be used as a natural antioxidant. The effect of roasting time coupled with enzymatic hydrolysis of roasted defatted peanut seeds on antioxidant capacity was studied (9). It was concluded that antioxidant capacity increased with roasting time from 0 to 60 min at 180°C and increased further when hydrolyzed with either Esperase or Neutrase. More recently, Chen et al. (10) reported the antioxidant capacities of peanut protein hydrolysates by measuring the inhibition of linoleic acid autoxidation, scavenging effect on free radicals, reducing power and inhibition of liver lipid autoxidation. Peanut protein hydrolyzed with Alcalase had increased antioxidant capacity over unhydrolyzed peanut protein, but slightly less antioxidant capacity than butylated hydroxytoluene, a synthetic antioxidant (w/v basis) (10).

There is a limited amount of published research regarding methods to expand the use of this high protein peanut oil by-product. In an extension to the previously mentioned studies, it is hypothesized that enzymatic hydrolysis could be used as a method to improve the economic value of peanut meal. The present study evaluates both the antioxidant capacity and the functional properties of commercially available, defatted peanut meal after hydrolysis with different commercial proteases. In conjunction with this work, experiments are simultaneously being conducted to eliminate the aflatoxin from defatted peanut meal to improve both food and feed applications. Advances in these technologies, including process commercialization, can be used to increase the value of this commodity.

Materials and Methods

Materials. Defatted peanut meal was provided by Golden Peanut Company (Alpharetta, GA). The peanut meal was found to be 48.9% protein, as determined by the Dumas

combustion method using an Elementar Rapid N III Nitrogen-Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). Samples were oven dried overnight at 80°C. Samples (0.2000 ± 0.0200 g) were prepared in tin foil packets for combustion analysis. Protein was calculated from N values using a Kjeldahl factor of 6.25. Pepsin (EC 232-629-3, Porcine stomach mucosa, 1020 units/mg protein), 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) 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). Fluorescein sodium salt was obtained from Riedel-de Haën (Seelze, Germany). AAPH [2,2'-Azobis (2-amidino-propane) dihydrochloride] was purchased from Wako Pure Chemical (Richmond, VA). HCl and NaOH were obtained from Fisher Scientific (Fair Lawn, NJ).

Enzymatic Hydrolysis. Dispersions of defatted peanut meal (10% w/w) were prepared in deionized water. Enzymatic hydrolysis was carried out in a water bath under constant stirring using a RXR 1 overhead stirrer (Heidolph Instruments, Schwabach, Germany). The 10% peanut meal dispersions were equilibrated by stirring for 20 min at 37°C, 60°C, or 50°C, the respective optimal temperatures for pepsin, Alcalase, and Flavourzyme hydrolysis. After the 20 min incubation period, the pH was adjusted to 2.0, 8.0, or 7.0, the optimum pH values for pepsin, Alcalase, and Flavourzyme hydrolysis, respectively. Immediately prior to enzyme addition, aliquots of the dispersions were collected to serve as the appropriate unhydrolyzed control samples.

The proteases chosen for this study are commercially available, water soluble, and food-grade. Pepsin is the first in a series of enzymes to begin protein digestion in the human digestive tract. It was chosen to partially simulate how peanut meal would be naturally digested within the body. Pepsin was used at an enzyme/substrate ratio of 19000 units/g peanut meal protein, roughly ten times higher than typically found in the human digestive tract. Alcalase and Flavourzyme were chosen from a commercial processing standpoint. Alcalase is widely used for extensive hydrolysis reactions and was added at an enzyme/substrate ratio of 0.6 Anson Units (AU)/g peanut meal protein. One AU is the amount of enzyme that digests hemoglobin at an initial rate that produces an amount of trichloroacetic acid soluble product which gives the same color with Folin-Ciocalteu Phenol reagent as 1 milliequivalent of tyrosine per minute. Flavourzyme is known to produce less bitter tasting peptides and was used at an enzyme/substrate ratio of 50 Leucine Amino Peptidase Units (LAPU)/g peanut meal protein. One LAPU is the amount of enzyme that hydrolyzes 1 μ mol of L-leucine-p-nitroanilide per minute.

Samples were collected after 3, 5, 10, 15, 30, 45, 60, 120, 180 and 240 min of hydrolysis. Enzymes were heat inactivated by submerging 12 mL aliquots of the dispersions in sealed 15 mL conical tubes in a water bath at 90°C for 15 min. Unhydrolyzed controls were also heat treated. Dispersions were centrifuged at 11000 x *g* for 15 min to separate insoluble material. Centrifugation conditions were chosen empirically based on initial experiments and good visual separation between the pellet and the supernatant. Supernatants (hydrolysates) were collected and frozen at -15°C prior to further analyses. Hydrolysis was carried out in triplicate for each enzyme.

Degree of Hydrolysis. DH was determined spectrophotometrically using the trinitrobenzenesulfonic (TNBS) acid method with slight modifications (2). Hydrolysates (0.16 mL) were added to 3.84 mL of 1% SDS and vortexed. Then, 0.25 mL aliquots were transferred into test tubes containing 2.0 mL 0.2125 M sodium phosphate buffer (pH 8.2), followed by the addition of 2 mL of 0.1% TNBS to each tube. Tubes were then vortexed and incubated for 60 min at 50°C in the dark. The reaction of TNBS with the primary amines was quenched by adding 4 mL of 0.1 N HCl. Tubes were cooled to room temperature for 30 min and the absorbencies were read at 340 nm using a UV-1700 UV-Visible Spectrophotometer (Shimadzu Corp., Kyoto, Japan). Concentrations of 0-6 mM L-Leucine were prepared equivalently and used to create a standard curve.

DH values were calculated using the following formula:

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

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 in the peanut protein substrate was determined by fully hydrolyzing 10% meal dispersion with 6N HCl for 24h at 90°C. The hydrolysis equivalents, h , were determined by reference to the L-Leucine standard curve.

Protein Solubility. A BCA™ Protein Assay Kit (Pierce, Rockford, IL) was used to determine protein concentration of the soluble hydrolysates. Bicinchoninic acid (BCA) forms a complex with cuprous cation (Cu^{+1}) in an alkaline environment. The resulting complex exhibits a purple color that has a strong absorbance at 562 nm. The color produced from this reaction is linear over a broad range of increasing protein concentrations. The

protein concentration of the hydrolysates are determined by reference to a standard curve produced from a common protein, bovine serum albumin (BSA). The BSA standard curve ranges from 0 to 2000 µg/mL protein.

Hydrolysates were diluted 1:20 with deionized water. Then 0.1 mL of all diluted hydrolysates and BSA standards were mixed in test tubes with 2 mL working reagent. Working reagent is a 1:50 mixture of 4% cupric sulfate:sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M sodium hydroxide. The test tubes were incubated for 30 min in a 37°C water bath, cooled to room temperature and the absorbance was read at 562 nm.

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

Hydrolysates were diluted using Novex® Tricine SDS Sample Buffer (2X), NuPAGE® Reducing Agent (10X) and deionized water. Protein concentrations of 10 µg were loaded per well in Novex® 1mm x 10 well pre-cast 16% Tricine Gels (Invitrogen, Carlsbad, CA) which are used for resolving low molecular weight proteins and peptides. See Blue® Plus2 Prestained Standard molecular weight marker with 2.5, 3.5, 6, 14, 21, 31, 36, 55, 66, 97, 116 and 200 kDa was used as a reference. As an additional control, protein extracted at pH 8.0 from defatted raw Runner peanuts (variety Georgia Green) was analyzed in an equivalent manner. Electrophoresis was run at 130 V for 90 min. SimplyBlue™ SafeStain was used to stain the gel for 60 min. The gels were destained in deionized water overnight and then dried using Gel-Dry™ Drying Solution.

Total Soluble Material. Approximately 2g of hydrolysates were analytically weighed in an aluminum dish and heated in a vacuum oven (VWR Scientific, Inc., West Chester, PA) at

115°C for 16 hr. Dried samples were cooled to room temperature in a desiccator prior to final mass determination.

Hydrophilic - Oxygen Radical Absorbance Capacity (H-ORAC) Assay. Antioxidant capacity of the hydrolysates was determined using an adapted H-ORAC procedure (11-13). Assays were prepared in Costar polystyrene flat-bottom black 96 microwell plates (Corning, Acton, MA). A sodium salt solution of Fluorescein was prepared daily at a final concentration of 70 nM in 75 mM phosphate buffer. Trolox standards were prepared daily from 50 to 3.12 μ M in phosphate buffer. AAPH was prepared daily at a final concentration of 153 mM in phosphate buffer immediately prior to usage. Fluorescence was measured using the SAFIRE² monochromator based microplate reader equipped with Magellan (v. 6.1) reader software (Tecan USA, Raleigh, NC). Excitation and emission filter wavelengths were set at 483 +/- 8 and 525 +/- 12 nm, respectively.

The reaction was carried out in 75 mM phosphate buffer at pH 7.4 with a final reaction volume of 250 μ L. Alcalase and Flavourzyme hydrolysates 3 through 240 min were diluted 1:2000 in phosphate buffer prior to measurement. The pH of pepsin hydrolysates had to be altered to be compatible with the H-ORAC assay. Ten mL aliquots of the unhydrolyzed control, 3, 60 and 240 min hydrolysate samples were adjusted from pH 2 to pH 7.4 using 1 N NaOH. After pH adjustment, pepsin hydrolysates 3, 60 and 240 min were diluted 1:2500 in phosphate buffer (pH 7.4). All unhydrolyzed control samples were diluted 1:1000 in phosphate buffer. Diluted hydrolysate samples and Trolox standards, both at 130 μ L, were added to the wells followed by 60 μ L of the Fluorescein solution, which was rapidly added via a multi-channel pipette. The plate containing only the samples,

standards, and Fluorescein was incubated in the SAFIRE² for 15 min at 37°C. Following incubation, 60µL of the AAPH solution was rapidly added via a multi-channel pipette. Prior to the first measurement plates were mixed with a 5 s medium intensity orbital shaking, and data points were acquired over 80, 1 min kinetic cycles with a 5 s medium intensity orbital shaking between cycles. Data was reported as relative fluorescent units (RFU) ranging from 0-50000 RFU and exported into Microsoft Excel (Microsoft, Roselle, IL) for further analysis. ORAC values were calculated using a linear regression equation between Trolox concentration in µM and the net area under the fluorescence decay curve (16). Antioxidant capacity was reported in µM Trolox equivalents (TE) per milligram of soluble protein in the hydrolysates as determined by the BCA assay.

Statistical Analyses. Statistics were performed using a mixed model with fixed factorial effects for enzyme and time and a random beaker (replication) effect to analyze the DH and protein solubility data from this repeated measures design. Means separation was conducted for total soluble material and antioxidant capacity using Tukey's honest significant difference test. All statistics were performed using SAS (Cary, NC).

Results and Discussion

DH is the relative amount an enzyme is able to digest a protein into smaller peptide fragments. DH increased with increasing time for all enzymes, with the most notable increase occurring within the first 3 min of enzyme addition (Figure 1). Statistical analyses revealed that the enzyme used and duration of hydrolysis significantly ($p < 0.0001$) affected DH. DH ranged from approximately 20-60% for Alcalase, 10-20% for pepsin and 10-70% for Flavourzyme over a 3-240 min period. This data reflects the differing specificities and

concentration of each enzyme. Alcalase, a slightly specific endoproteinase that preferentially cleaves large uncharged residues and terminal hydrophobic amino acids (14), yields hydrolysates with higher DH values than pepsin, a more specific single acidic endopeptidase that preferentially cleaves hydrophobic, aromatic residues (15). Accordingly, Alcalase resulted in the most rapid initial rate of hydrolysis, reaching 20% DH after only 3 min, which exceeds pepsin DH after the full 240 min hydrolysis. The rate of pepsin and Alcalase hydrolysis began to slow after 2 and 3 hours, respectively, which is indicative that all peptide bonds susceptible to enzymatic hydrolysis under the given conditions, have been cleaved. Flavourzyme possesses both endoprotease and exopeptidase activity; however, it was used at an enzyme/substrate ratio for extensive hydrolysis by the exopeptidase activity. Flavourzyme activity accelerated steadily throughout the entire hydrolysis, surpassing Alcalase hydrolysis after 4 hours.

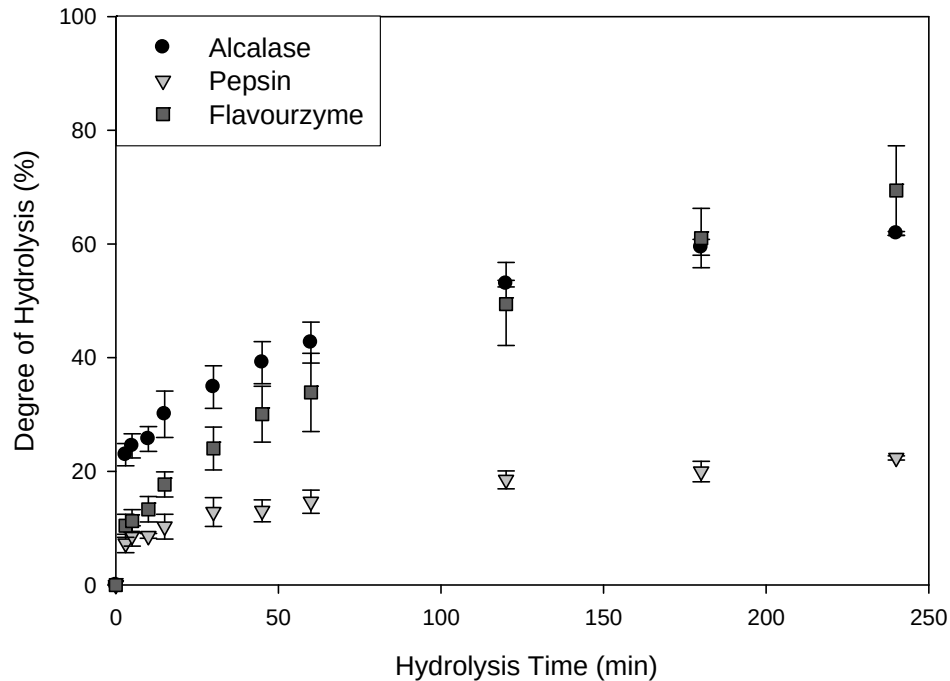


Figure 1. Degree of hydrolysis for the Alcalase, pepsin, and Flavourzyme soluble fractions.

In a similar legume study, chickpea protein isolate was hydrolyzed individually with either Alcalase or Flavourzyme, or in a sequential treatment with both enzymes (4). The enzyme/substrate ratio during Alcalase hydrolysis was 0.4 AU/g protein, slightly less than the ratio used for the current study. Flavourzyme hydrolysis was conducted at an enzyme/substrate ratio of 100 LAPU/g protein, which is double the concentration of Flavourzyme used in the present study. DH (also determined by the TNBS method) of both Alcalase and Flavourzyme hydrolysates reached a plateau at 27% after approximately 120 min of hydrolysis (4). In comparison to the present study, higher DH values were achieved after 240 min of peanut meal hydrolysis with either Alcalase or Flavourzyme. However, when the chickpea isolate was hydrolyzed sequentially with Alcalase followed by Flavourzyme, it reached a DH of 50% after 8 hr (4).

A sequential treatment of Alcalase (0.3 AU/g, 60 min) followed by Flavourzyme (100 LAPU/g, 120 min) on protein isolates prepared from defatted sunflower meal yielded similar DH values to the present work (5). A DH (also determined by the TNBS method) of approximately 65% resulted after 180 min of hydrolysis (5). Although the enzyme/substrate ratio used was half the concentration as the present study, these researchers also observed that the most rapid rate of hydrolysis occurred within the first 5 min of Alcalase addition, nearing 20% DH.

Increasing hydrolysis time minimally affected protein solubility as compared to unhydrolyzed controls for Alcalase and pepsin (Figure 2). A significant increase in protein solubility over time ($p < 0.05$) was only observed for the Flavourzyme hydrolysates which accelerated through 60 min then reached a plateau. Pepsin hydrolysates had more soluble protein than either Flavourzyme or Alcalase hydrolysates across all time points. The observed protein solubility is related more to the adjusted pH of the peanut meal dispersions, than to the DH. When preparing the peanut meal dispersion for pepsin hydrolysis, the pH is lowered from pH ~6.8 to pH 2.0, passing through the isoelectric point of peanut protein (pI 4.5) (16). At this point, the protein is precipitated, noted by a change in the color and clarity of the dispersions from a translucent brown, to an opaque light tan. Previous research has shown that peanut protein is more soluble at pH 2.0 (pepsin) than at pH 7.0 (Flavourzyme) or pH 8.0 (Alcalase) when extracted in water (17). The BCA assay is not able to detect single amino acids and dipeptides because they do not catalyze the biuret reaction which is necessary for this spectrophotometric assay. Therefore, any dipeptides or free amino acids generated during extensive hydrolyses are beyond the detection limits of this assay.

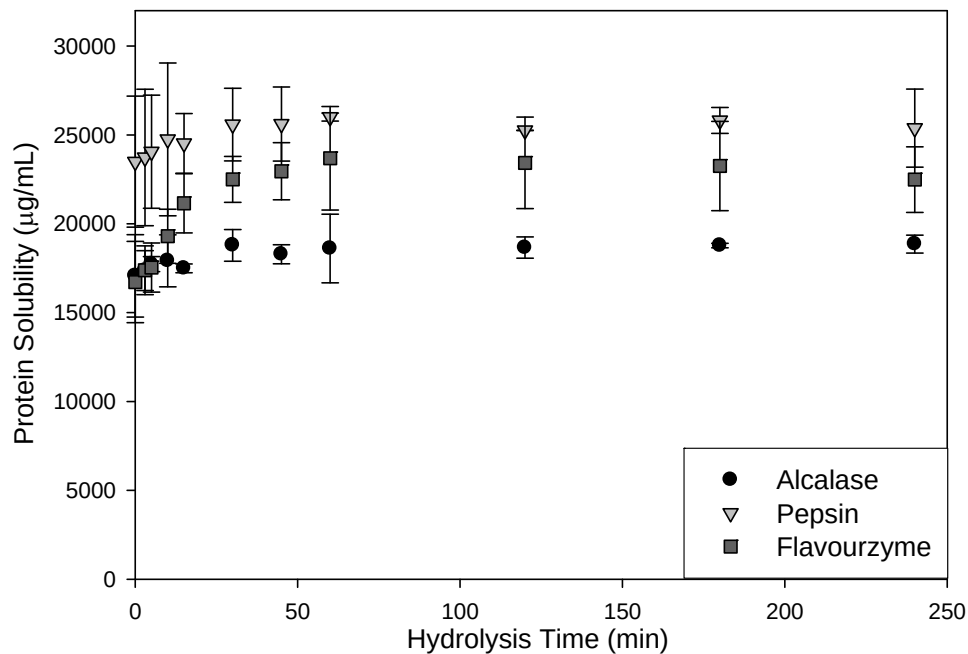


Figure 2. Protein solubility of the Alcalase, pepsin, and Flavourzyme hydrolysates.

SDS-PAGE was used to study the molecular weight distributions of peanut meal proteins and peptides before and after hydrolysis. The molecular weight marker, raw peanut protein at pH 8.0, and the unhydrolyzed control sample at the optimum pH for each enzyme was run alongside hydrolysates on each gel (Figure 3). The raw peanut protein and the unhydrolyzed controls had similar banding patterns on the Flavourzyme (pH 7.0) and Alcalase (pH 8.0) gels, but were slightly different on the pepsin gel due to the lower pH of the unhydrolyzed control. Table 1 defines the molecular weight regions of the main peanut seed storage protein (conarachin and arachin) subunits (18). Specifically, Ara h 2 protein, the predominant peanut allergen, migrates as a doublet to ~13 kDa and can be visualized in the reference and time 0 lanes (Figure 3) (19).

Table 1. Molecular weight regions of the 5 main classes of peanut protein subunits, as determined by SDS-PAGE (18).

Peanut Protein Region	Molecular Weight (kDa)
Conarachin	>50
Acidic arachin	38 - 49.9
Intermediate	23 - 37.9
Basic arachin	18 - 22.9
Low molecular weight protein	14 - 17.9

Generally, the intensity of the protein bands present in the unhydrolyzed control samples deteriorated with increasing hydrolysis time; however, unique banding patterns were observed for each of the enzyme digestions. SDS-PAGE confirmed that the majority of the protein bands were digested after 3 min of hydrolysis for each enzyme, in agreement with DH data (Figure 1). Conarachin, acidic arachin, and intermediate MW proteins were digested after 3 min of Alcalase hydrolysis (DH 22.9%). However, one distinct band in the basic arachin region persisted until 30 min of Alcalase hydrolysis (DH 34.8%). Two clusters of low MW peptide bands <14 kDa were still evident after 4 hours of Alcalase hydrolysis (DH 61.8%), although their intensity was disintegrating, indicative of extensive hydrolysis into smaller peptides.

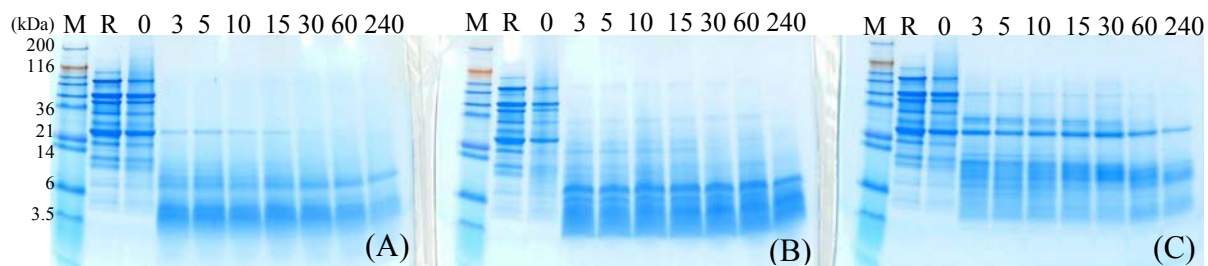


Figure 3. SDS-PAGE of (A) Alcalase, (B) pepsin, and (C) Flavourzyme hydrolysates. “M” is the molecular weight marker. “R” is the reference peanut protein (pH 8.0). Subsequent lanes are marked 0-240 min of hydrolysis.

Conarachin and acidic arachin proteins were also completely digested after 3 min of pepsin hydrolysis (DH 7.3%). Subunits in the intermediate and basic arachin regions persisted through 60 min of hydrolysis (DH 14.6%). Throughout the duration of pepsin hydrolysis (DH 22.3%), two distinct bands between 6 and 14 kDa were visible along with a cluster of low MW peptides <6 kDa. These results are consistent with previous research by Sen, et al. (19) which revealed that the allergenic Ara h 2 protein was resistant to enzymatic digestion. The intense band that occurs at ~10 kDa is a pepsin-resistant Ara h 2 fragment (Figure 3B) that contains many of the same allergenic amino acid sequences as the unhydrolyzed protein (19).

Flavourzyme hydrolysis resulted in more visible bands throughout the 4 hr digestion. Unlike Alcalase or pepsin, one band in the conarachin region was not fully digested until 10 min of hydrolysis (DH 13.3%). One band in the acidic arachin region and two bands in the intermediate MW region persisted through 60 min of hydrolysis (DH 33.9%). The same distinct band that was visible in the basic arachin region after 10 min of Alcalase digestion was also evident through the entire 240 min of Flavourzyme hydrolysis (DH 69.4%). This is indicative that the low pH, in conjunction with pepsin, aided in digestion of that particular basic arachin protein band. Although Flavourzyme had numerous visible bands throughout

hydrolysis, the majority of the banding occurred as low MW peptides <14 kDa. The intensity of the Flavourzyme low MW peptides was notably less than that of the pepsin peptides (<14 kDa), suggesting that Flavourzyme resulted in a more extensive hydrolysis than pepsin.

All digestions resulted in an accumulation of low MW peptides less than 14 kDa. The relative intensity of the protein bands confirms that Alcalase and Flavourzyme resulted in the most digestion after 240 min of hydrolysis which reinforces the DH data provided in Figure 1. These SDS-PAGE results are comparable to those found after another legume, chickpea protein isolate, was hydrolyzed with Alcalase and Flavourzyme (4). In that study, although individual treatment with Alcalase (0.4 AU/g) or Flavourzyme (100 LAPU/g) reached the same DH (27%), the treatments resulted in different electrophoretic banding patterns and Flavourzyme had more visible bands persisting through 27% DH (4). The effects of Alcalase and Flavourzyme on minced yellow stripe trevally fish protein were also studied (20). Similar to our results, SDS-PAGE revealed that Flavourzyme hydrolysis yielded larger molecular weight peptides after 5% and 15% DH than Alcalase hydrolysis. However, all bands deteriorated after 25% DH with either enzyme which is contrary to the present data. Here, low molecular weight peptide bands persisted through 60% Alcalase hydrolysis and 70% Flavourzyme hydrolysis of defatted peanut meal which may be a result of different protein substrates or enzyme concentrations used.

Total soluble material for all hydrolysates increased from approximately 3-7% with increasing hydrolysis time (Figure 4). Unhydrolyzed Alcalase and Flavourzyme samples (0 min) had lower total solids than the unhydrolyzed pepsin samples which is in agreement with

the higher pepsin protein solubility data. However, after 60 min, Alcalase hydrolysates total solids were greater than that of pepsin, which may be attributed to enzymatic activity on other soluble material (aside from protein) such as carbohydrates and fiber accumulating in the hydrolysates. Total soluble material increased a minimum of 30% for all hydrolysates and over 100% for Alcalase hydrolysates after 240 min. It is interesting to observe that the pepsin total solids reached a plateau after 60 min, which is consistent with the DH data. Flavourzyme hydrolysate total solids were the lowest throughout hydrolysis. The total solids data is useful when considering commercial applications such as large batch spray drying for further use in functional foods or nutraceuticals.

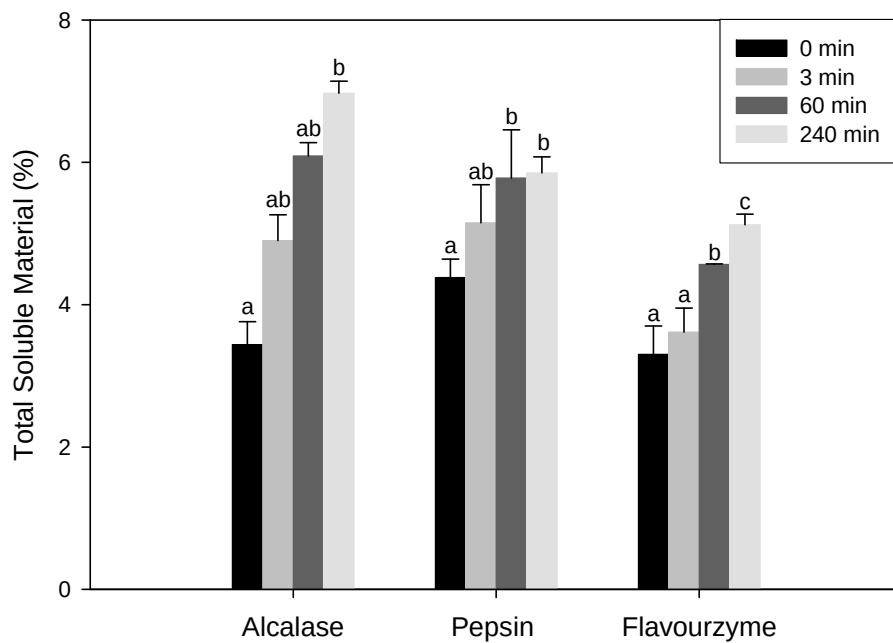


Figure 4. Total solids in the Alcalase, pepsin, and Flavourzyme soluble fractions as determined at 0, 3, 60 and 240 min. Means within a group followed by different letters are significantly different ($p < 0.05$).

Some peptides generated through enzymatic hydrolysis are known to have bioactive properties (5). Antioxidant capacity was measured using ORAC, a well established and reproducible method of antioxidant capacity quantification (21). Specifically, ORAC measures an antioxidants capacity to quench free radicals by hydrogen donation. Antioxidant capacity increased significantly ($p<0.05$) throughout hydrolysis for all enzymes (Figure 5). Alcalase hydrolysates exhibited the highest antioxidant capacity, increasing significantly after only 3 min of hydrolysis. A significant increase ($p<0.01$) in Flavourzyme hydrolysate antioxidant capacity was not observed until 4 hr of hydrolysis. Pepsin hydrolysates exhibited the lowest antioxidant capacity across all time points, consistent with its DH. However, the pepsin hydrolysate values may have been negatively affected by the necessary increase in pH (from 2 to 7.4) to be compatible with the H-ORAC assay. This data is indicative that although the 3 enzymes studied produced unique peptides, and the antioxidant capacity of a peptide is dependent upon its composition, all hydrolysates produced in this study are capable of hydrogen atom transfer. While different peanut protein substrates and alternative methods of measuring antioxidant activity were used, these results are consistent with the previously discussed research performed by Hwang, et al. (9) and Chen, et al. (10), indicating that peanut protein hydrolysates are suitable natural antioxidants.

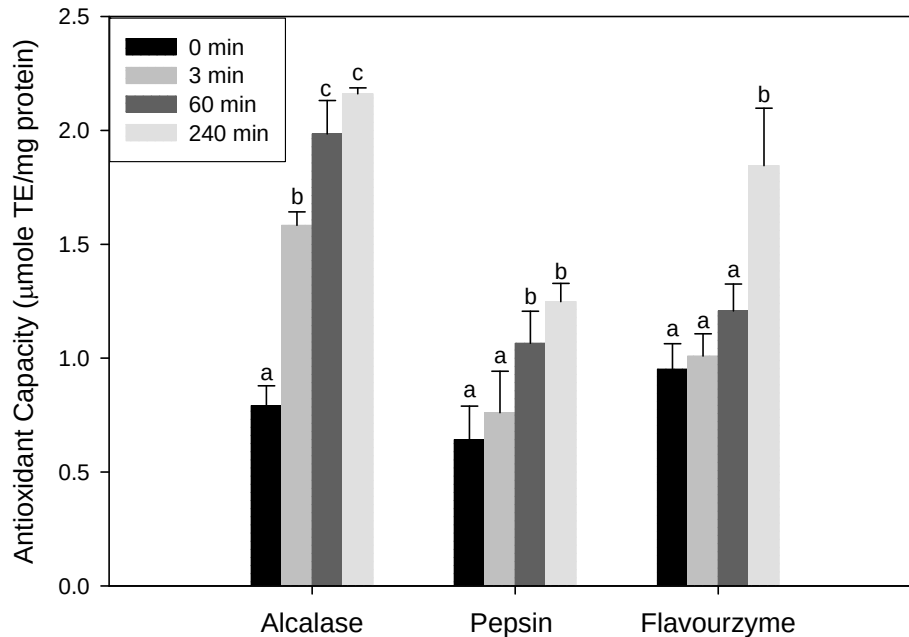


Figure 5. Antioxidant capacity of Alcalase, pepsin, and Flavourzyme hydrolysates. ORAC values were normalized to the amount of soluble protein in the samples. Means within a group followed by different letters are significantly different ($p < 0.05$).

Numerous studies have shown increases in antioxidant activity post hydrolysis; however, past results vary from the present patterns in antioxidant capacity. Defatted canola meal, a protein-rich oil byproduct similar to peanut meal, was hydrolyzed individually with either Alcalase (0.3 AU/g, 60 min, DH 20.6%) or Flavourzyme (50 LAPU/g, 120 min, DH 6.33%) or in a sequential treatment with both enzymes (DH 18.9%) (7). In contrast to the present results, the hydrolysates prepared using Flavourzyme exhibited the highest antioxidant activity, as determined by reducing power and radical scavenging capacity (7). Comparable results were observed when Klompong et al. (20) studied the antioxidant activity of minced yellow stripe trevally fish hydrolyzed with either Alcalase or Flavourzyme.

Similar to the defatted canola meal study, Klompong et al. (20) also found that at the same DH and concentration, hydrolysis with Flavourzyme resulted in significantly higher antioxidant capacity than Alcalase. The differences in results can be attributed to variations in enzyme concentration, duration of hydrolysis, and protein substrate.

Amino acid analyses have shown that peptides exhibiting antioxidant activity are typically rich in histidine and hydrophobic amino acid residues (22, 23). For instance, Megias, et al. (5) found a positive correlation between antioxidant activity and histidine content in peptides of Alcalase hydrolysates. Other researchers determined that the active antioxidant fractions in corn gluten meal hydrolysates were in the peptides ranging 500-1500 Da by using gel filtration chromatography (6). Further studies should be conducted to identify the peptide fractions and amino acid compositions responsible for the antioxidant potential of these hydrolysates.

This research revealed that peanut meal could be made more marketable via enzymatic hydrolysis to create low molecular weight peptides with improved functional characteristics and nutritional value. Alcalase would be recommended for future applications because it resulted in hydrolysates with the greatest total soluble material and antioxidant capacity. The increase in total solids observed with hydrolysis time should improve spray drying efficiency. Finally, the increased antioxidant capacity suggests the hydrolysates could be used as components in functional foods and nutraceuticals. The value of this commodity could be further improved by removing the aflatoxins, which is being studied in conjunction with this work. It would also be worthwhile to investigate the allergenicity of the

hydrolysates to determine if the allergenic protein sequences are still intact. Previous research has indicated that hydrolyzed peanut proteins had reduced allergenicity (24).

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CHAPTER 3:

Effect of Astra-Ben 20™ on Aflatoxin Contaminated Peanut Meal

Abstract

The *in vitro* efficacy of sodium bentonite clay, Astra-Ben 20™ (AB20), to sequester aflatoxin from contaminated peanut meal was studied. Peanut meal dispersions (10% solids) were adjusted to pH 2 and 8 and randomly assigned to one of three treatments: control (no clay), 0.2% AB20 (w/w), or 2% AB20 (w/w). Samples underwent a series of separation steps and both the soluble and insoluble fractions were subjected to subsequent testing. The addition of clay significantly reduced the aflatoxin concentration in both soluble and insoluble fractions. The pH of the soluble samples did not significantly affect the ability of clay to bind aflatoxin. Soluble fractions subjected to 2% AB20 treatment also had significantly lower protein solubility and total soluble material than their respective pH 2 and 8 controls. These results suggest that AB20 could be used as a processing aid to reduce or eliminate the aflatoxin from peanut meal, enabling this high protein material to be used in human food applications.

Introduction

Peanut meal is characterized as the non-food grade material that remains after the extraction of oil from peanuts (*Arachis hypogaea* L.) (1). Oil is produced from peanuts which are not suitable for human consumption due to discolored, broken, or aflatoxin contaminated seed. Aflatoxins are toxic compounds which are produced by the fungi *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare (2). The four major naturally occurring aflatoxins are aflatoxin B₁, B₂, G₁ and G₂ (3). Aflatoxin is an unavoidable contaminant in peanut crop, but can be minimized by harvesting at maturity, low moisture and storing the product in cool and dry conditions (4). Ingestion of aflatoxins from contaminated food or animal feed, called aflatoxicosis, can lead to acute liver damage, edema, hemorrhage, alteration in digestion and possibly death (5). Aflatoxin B₁ is the most predominant, naturally occurring aflatoxin and has been identified as a potent carcinogen (5). The U.S. FDA has issued regulatory guidelines limiting the contamination level of aflatoxin to 20 ppb or less in peanut products intended for human consumption (6).

Effective techniques to guard humans and animals from the toxic effects of aflatoxin in food and feed products would be of great value to the peanut industry. Peanut meal is currently a low economic value commodity that is primarily used as either animal feed or fertilizer, dependent upon the aflatoxin concentration. However, peanut meal is a rich source of protein (typically 45-60%) and the applications for this material could be expanded if the aflatoxin were removed. Decreasing or eliminating aflatoxin in this high protein by-product has the potential to bring the peanut oil industry more profit by using the meal in applications beyond animal feed.

A variety of physical, chemical, and biological methods, including thermal inactivation, adsorptive materials, density segregation, irradiation, ammoniation, solvent extraction, and microbial inactivation may be used to reduce aflatoxin concentration (7). However, the focus of the present study is a physical method to detoxify peanut meal using an adsorptive material. Bentonite, a layered crystalline microstructure comprised primarily of montmorillonite, can be used to adsorb molecules such as aflatoxins (3). This clay substance is generally recognized as safe (GRAS) and approved as a direct food additive under title 21, section 184.1155 in the Code of Federal Regulations (8). Bentonite is currently used as a feed additive as an anti-caking agent (9), as well as in the food industry for wine treatment and for the clarification of vinegar and fruit juices (10). Studies have demonstrated that bentonite can effectively adsorb aflatoxin both *in vitro*, from milk (11), methanol solutions (12), and buffer solutions (13), as well as *in vivo*, to protect animals against the effects of aflatoxin by inclusion in animal feed (12). In the latter application, adsorptive materials are mixed into contaminated animal feed to form a complex with the aflatoxins, enabling them to pass through the gastro-intestinal tract, protecting the animal against aflatoxicosis (3, 4). The ideal toxin-binder complex should not dissociate internally and should be expelled in the animal feces (14).

There is currently no published research regarding the use of sodium bentonite clay as a processing aid to eliminate detectable aflatoxin from peanut meal. The objective of this study was to determine if Astra-Ben 20A™ (AB20) adsorbed aflatoxin from pH 2.0 and pH 8.0 aqueous peanut meal dispersions at levels of 0.2 and 2% clay. It is hypothesized that the clay will effectively sequester the aflatoxins, making the soluble and insoluble peanut meal

fractions suitable for use in both food and feed applications. The present study evaluated the aflatoxin concentration and protein solubility of peanut meal dispersions before and after the addition of sodium bentonite clay. Enzymatic hydrolysis of peanut meal is being studied in conjunction to this work to improve the nutritional and functional properties of this high protein commodity.

Materials and Methods

Materials. Defatted peanut meal (~110 ppb) was provided by Golden Peanut Company (Alpharetta, GA). Astra-Ben 20A™ sodium bentonite clay was provided by Prince Agri Products, Inc. (Quincy, IL). Methanol, potassium bromide, hydrochloric acid (HCl), acetic acid, and sodium hydroxide (NaOH) were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium chloride (NaCl) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Nitric acid was obtained from VWR International (West Chester, PA). AflaTest Developer, AflaTest columns, and mycotoxin standards were acquired from VICAM (Watertown, MA). Aflatoxin Mix Kit–M was purchased from Supelco (Bellefonte, PA).

Sample Preparation. Dispersions of defatted peanut meal (10% w/w) were prepared in deionized water and adjusted to either pH 2.0 or 8.0 using 2N HCl or 2N NaOH, respectively. Each pH had a control (no clay) and 2 levels of AB20 sodium bentonite clay: 0.2% (w/w) or 2% (w/w), totaling six treatments. Dispersions were stirred at room temperature for 60 min. The samples then went through a series of separation steps to partition the soluble and insoluble fractions. First, dispersions were centrifuged at 25000 x *g* for 20 min. The pellet (insoluble fraction) was collected for further testing. Then, the supernatant (soluble fraction) was poured through two layers of cheese cloth to exclude any

additional insoluble matter. Lastly, the filtered supernatants were centrifuged at $8000 \times g$ for 10 min and the soluble fraction was collected for further testing. Soluble and insoluble fractions were frozen at -15°C prior to further analysis. Each treatment was carried out in triplicate.

Detection of Aflatoxin.

VICAM Procedure for Insoluble Fractions. Aflatoxin concentration of the insoluble fractions was determined using the AflaTest[®] Procedure for Peanuts and Treenuts (0-50 ppb) on a Series-4 VICAM Fluorometer (VICAM, Watertown, MA). The fluorometer was calibrated according to the instruction manual. A 25 g sample of the insoluble fraction was added to a blender jar along with 5 g NaCl and 125 mL 60% methanol:40% water and mixed on high speed for 1 min. The extract was poured into fluted filter paper and filtrate collected in a clean beaker. Samples were then diluted by mixing 20 mL filtered extract with 20 mL distilled water and stirred. Dilute extract was then filtered through a glass microfiber filter to collect 10 mL in a glass syringe barrel. Column chromatography was conducted by passing 10 mL filtered extract completely through the AflaTest column at a rate of 1-2 drops/sec. The column was washed twice with 10 mL distilled water at a rate of 1-2 drops/sec. The AflaTest column was eluted with 1 mL HPLC grade methanol at a rate of 1-2 drops/sec and the sample eluate was collected in a glass cuvette. One mL AflaTest Developer solution was added to the eluate in a cuvette, vortexed, and the fluorescence was measured. Accuracy for this method is 0-50 ppb.

Modified VICAM Procedure for Soluble Fractions. Aflatoxin concentration of the soluble fractions was determined using a modification of the *VICAM Procedure for Insoluble*

Fractions described above. This modified procedure (found in Appendix A) was developed in conjunction with a technical scientist at VICAM to account for the high water content in the soluble fractions. A 25 mL sample of the soluble fraction was added to a blender jar along with 5 g NaCl and 50 mL pure methanol and mixed on high speed for 1 min. The extract was poured into fluted filter paper and filtrate collected in a clean beaker. Samples were then diluted by mixing 10 mL filtered extract with 20 mL distilled water and stirred. Dilute extract was then filtered through a glass microfiber filter to collect 9 mL in a glass syringe barrel. Column chromatography was conducted by passing 9 mL filtered extract completely through the AflaTest column at a rate of 1-2 drops/sec. The remaining procedure is equivalent to that described for insoluble fractions.

High Performance Liquid Chromatography (HPLC). Soluble and insoluble samples were prepared according to the VICAM procedures above, except HPLC was used for aflatoxin detection instead of column chromatography. HPLC was conducted according to the Association of Official Analytical Chemists (AOAC) method 991.31 (15). A KOBRA Cell (R-Biopharm Rhone Ltd., Glasgow, Scotland) was used instead of an iodine pump for post-column derivatization (16).

Total Soluble Material. Approximately 2 g samples were analytically weighed in an aluminum dish and heated in a vacuum oven (VWR Scientific, Inc., West Chester, PA) at 115°C for 16 hr. Dried samples were cooled to room temperature in a desiccator prior to final mass determination.

Protein Solubility. Protein concentration of soluble hydrolysates was determined via the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) using bovine serum albumin as the reference protein. All hydrolysates were diluted 1:20 with deionized water prior to analysis.

Statistical Analyses. Statistics were performed using a general linear model with fixed factorial effects for pH and clay to analyze the data from this randomized complete block design. Means separation was conducted for aflatoxin concentration, total soluble material and protein solubility using Tukey's honest significant difference test. All statistics were performed using SAS (Cary, NC).

Results and Discussion

An initial experiment was conducted to test the *in vitro* efficacy of sodium bentonite clay on sequestering the aflatoxin from peanut meal. Using the soluble and insoluble fraction VICAM methods of aflatoxin detection, results revealed that 2% AB20 was able to reduce or eliminate the available aflatoxin from a pH 8.0, 10% peanut meal dispersion (Table 1). The majority of the aflatoxin in the control sample was found in the insoluble portion after centrifugation. After 2% AB20 addition, there was no detectable aflatoxin in the soluble fraction and a greatly reduced quantity (<1 ppb) in the insoluble fraction. However, the limit of detection for the VICAM fluorometer is 1 ppb so that number, 0.9 ppb, may not be accurate. These promising results led us to expand this study to determine the pH and concentration effects of this sodium bentonite clay on aflatoxin adsorption.

Table 1. Aflatoxin concentration (ppb) of pH 8 soluble and insoluble peanut meal fractions after treatment with 2% AB20. Results determined using VICAM detection methods.

Sample	Control	2% AB20
Soluble	1.9	0
Insoluble	21.0	0.9

Aflatoxin concentration of all samples in the six different treatments was then quantified using the HPLC method of detection. Aflatoxin in both the soluble and insoluble fractions was adjusted for the total soluble material (Figures 1 and 2) in each sample leading to the aflatoxin concentration on a dry weight basis (Table 2). Results were as hypothesized; the addition of AB20 significantly reduced the detectable aflatoxin for all samples. The pH of the soluble samples did not significantly affect the clay efficacy. Both 0.2 and 2% AB20 treatments were able to significantly reduce the aflatoxin concentration to levels which are suitable for use in human food products (<20 ppb). The majority of the aflatoxin was found in the insoluble fractions after centrifugation. AB20 was slightly more effective on the pH 8 insoluble samples than pH 2 at 0.2% clay. However, like the soluble samples, there was no impact of pH on insoluble fraction aflatoxin when 2% clay was used. Furthermore, HPLC revealed that aflatoxin B₁ was the most predominant of the total aflatoxin in both soluble and insoluble fractions (data not shown). Physical adsorption by the sodium bentonite clay was responsible for the elimination of aflatoxin from the peanut meal dispersions. The layered crystalline microstructure and interchangeable cations have the ability to adsorb aflatoxin (17). More specifically, it has been suggested that the adsorption is electrostatically dominated; the negatively charged bentonite clay can adsorb organic substances onto its external surfaces or within its interlaminar spaces by interaction with the partially positive

dicarbonyl in aflatoxins or substitution of exchange cations (18, 19). The aflatoxin/clay complex is not detectable by HPLC and will not illicit any harmful effects.

Table 2. Aflatoxin concentration (ppb) of soluble and insoluble peanut meal fractions before and after clay treatment. All numbers are corrected for the total soluble material and reported on a dry weight basis. Results determined using HPLC method of detection. Means within the soluble or insoluble groups followed by different letters are significantly different ($p < 0.05$).

Sample	pH	Control	0.2% AB20	2% AB20
Soluble	2	51.0 ^A	11.6 ^B	0 ^B
	8	50.0 ^A	4.8 ^B	0 ^B
Insoluble	2	80.6 ^A	39.8 ^{BC}	0.9 ^D
	8	90.7 ^{AB}	16.3 ^{CD}	1.5 ^D

Previous studies have shown similar results to the current study. Masimango et al. (13) demonstrated that various bentonite clays (added at 2% w/v) were able to adsorb aflatoxin B₁ from contaminated liquid media so well that only traces remained in the supernatant. Likewise, another *in vitro* study found that 2% AB20 was able to absorb at least 97% of aflatoxin B₁ from pH 3.0, 7.0, or 10.1, 10% methanol solutions (12). In the current study, 2% AB20 was able to adsorb 100% of the total aflatoxin from the soluble samples and 98% from the insoluble samples.

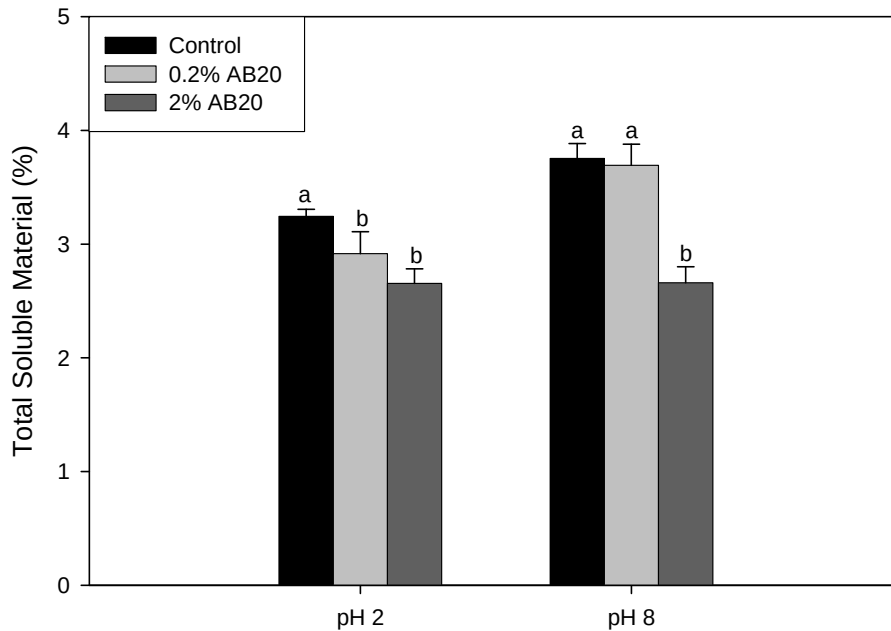


Figure 1. Total solids in the pH 2 and 8 soluble fractions. Means within a group followed by different letters are significantly different ($p < 0.05$).

Total soluble material in the soluble portions ranged from approximately 2.5-4% (Figure 1). A significant decrease ($p < 0.05$) in total solids was observed after the addition of 0.2 and 2% AB20 in the pH 2 samples and only with the addition 2% AB20 at pH 8. Although the pH 2 samples had a significant decrease at 0.2% AB20, the range of soluble material in the samples suggests that adding clay has more of an impact on samples at pH 8. Fractions with the highest total solids and the lowest aflatoxin concentration are the most desirable for future spray-drying applications because the end product will have < 20 ppb aflatoxin, which is upper limit for human consumption (20).

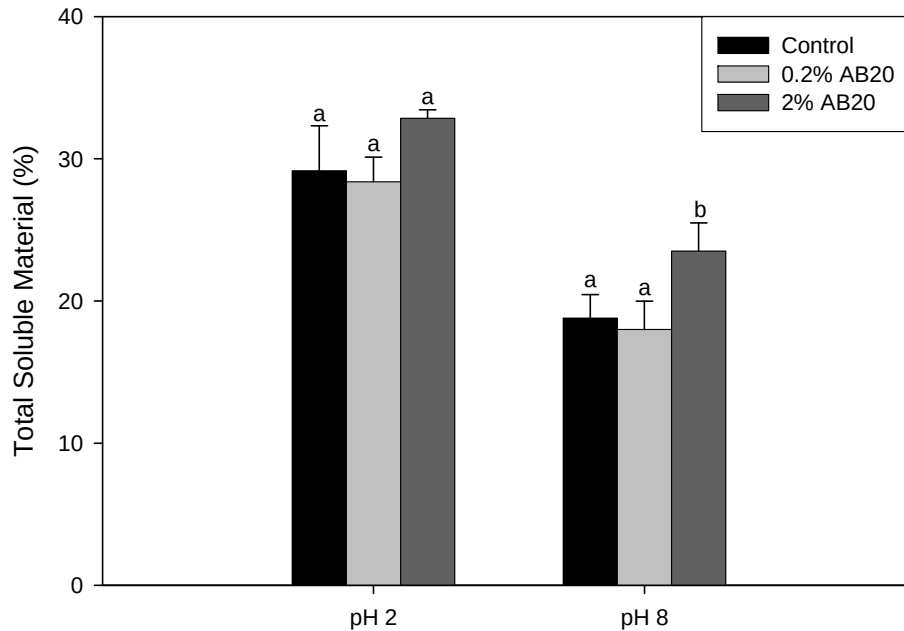


Figure 2. Total solids in the pH 2 and 8 insoluble fractions. Means within a group followed by different letters are significantly different ($p < 0.05$).

The insoluble fractions ranged between 16-34% total soluble materials (Figure 2). It is hypothesized that the aflatoxin/clay complex is mostly contained in the insoluble portion after centrifugation. The addition of clay did not have any not significant affects on the pH 2 insoluble samples. However, 2% AB20 did significantly ($p < 0.05$) increase the total soluble material in the insoluble pH 8 samples. The pH 8 results are consistent with both fractions tested; the total solids in the soluble portions experienced a significant decrease (Figure 1), simultaneously resulting in a significant increase in the insoluble portion. The pH 2 samples did not follow the same pattern.

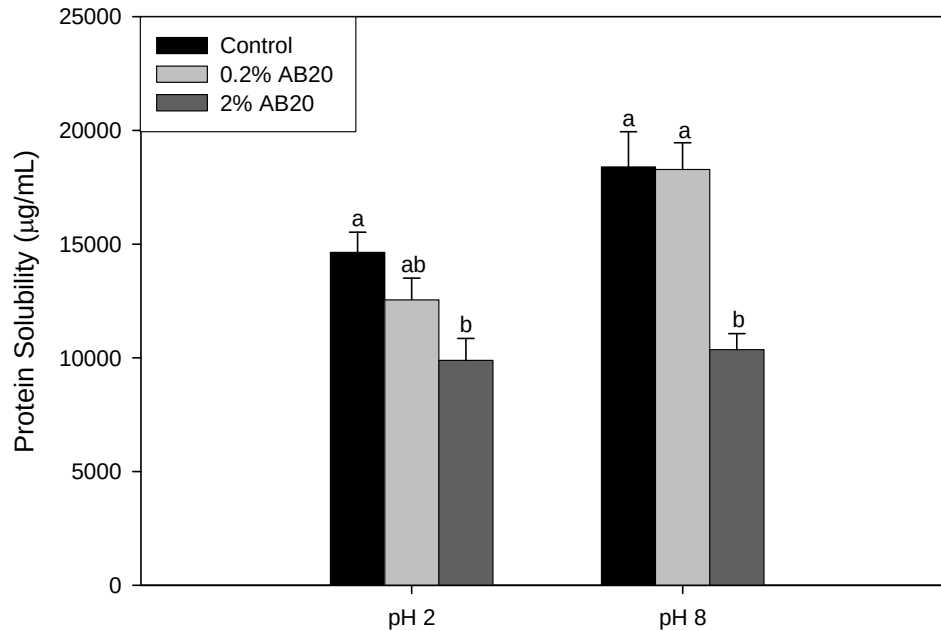


Figure 3. Protein solubility of the pH 2 and 8 soluble fractions. Means within a group followed by different letters are significantly different ($p < 0.05$).

Control and 0.2% AB20 pH 8 soluble samples had more soluble protein than at pH 2 (Figure 3). A significant decrease ($p < 0.05$) in both the pH 2 and pH 8 samples is observed after the addition of 2% AB20 and both of the 2% clay samples had nearly the exact same protein content. Previous studies have indicated that aflatoxin binds protein (21, 22) and the current data suggests that protein is also bound to the aflatoxin/clay complex and pulled into the insoluble fraction after centrifugation. The protein solubility data is consistent with the soluble portion total solids data (Figure 1). In fact, the pH 8 samples have the exact same significance pattern which is indicative that protein is the main component in the total soluble material and supports the theory that protein is bound to the aflatoxin/clay complex. Total protein content is reduced by 32% for the pH 2 and 44% for the pH 8 soluble fractions in the current study (Figure 3). Conversely, Applebaum and Marth (11) found that using bentonite

to adsorb aflatoxin from milk did not decrease the total protein content more than 5% when using up to 2% bentonite (w/v).

Based on these results, the use of 0.2% AB20 on pH 8 peanut meal dispersions would be recommended. This treatment resulted in a reduction of aflatoxin in both the soluble and insoluble fractions below the legal limit, a high amount of soluble material which is optimal for spray-drying, and the greatest protein solubility. If these peanut meal soluble fractions are used in human food products, the GRAS clay could be used as a processing aid to remove the aflatoxin and would not have to be declared on a nutritional label. The majority of the clay/aflatoxin complex would be in the insoluble portion which could still be sold as animal feed, but would have a higher market value due to the sequestered condition of aflatoxin.

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CHAPTER 4:
Conclusions and Future Work

This research suggested two unique methods to improve the potential value of peanut meal: 1) through enzymatic hydrolysis to improve protein functionality, and 2) through clay treatment to reduce the aflatoxin concentration. Results indicated that enzymatic hydrolysis of defatted peanut meal creates low molecular weight peptides with improved functional characteristics and nutritional value. Specifically, Alcalase hydrolysis resulted in hydrolysates with the greatest antioxidant capacity and total soluble material. These findings may help make peanut meal a more desirable source of animal feed.

Additional studies on peanut meal revealed that sodium bentonite clay, AB20, can be used to sequester the aflatoxin in peanut meal dispersions, significantly reducing or eliminating the detectable toxin level. This novel research not only provided an avenue for high protein peanut meal soluble fractions to be used safely in human food applications, but also for the insoluble fractions to be sold as animal feed at a higher price due to the decreased level of detectable aflatoxin.

Future work on improving the value of peanut meal should merge the aforementioned studies. Peanut meal dispersions would be subjected to clay treatment and enzymatic hydrolysis simultaneously. Then, the hydrolysates should be spray-dried to concentrate the solids. The effects of enzymatic hydrolysis on sodium bentonite clay efficacy could be studied by testing for residual aflatoxin and protein solubility. Accordingly, the nutritional and functional properties will have to be re-examined to determine if the presence of clay inhibits the previously established benefits of peanut meal hydrolysis. Finally, the effects of the dried hydrolysates along with the aflatoxin sequestering agent should be tested *in vivo* and compared with the current *in vitro* results. Aflatoxin-free dried soluble fractions could

be used in functional foods, while dried insoluble fractions could be sold as a higher value animal feed. It would also be worthwhile to investigate the allergenicity of these hydrolysates if they are to be used in human foods, to determine if the allergenic protein sequences are still intact.

APPENDICES

Appendix A. Modified AflaTest procedure for peanut meal supernatants

*Developed in conjunction with a technical scientist at VICAM

1.0 Sample Type: Peanut meal supernatant (soluble fraction)

2.0 Detection Instrument: Series 4 Vicam Fluorometer

3.0 Calibration Setting:

Green -1
Red 22
Yellow 11 ± 1

4.0 Set Up:

- 4.1 Calibrate fluorometer daily.
- 4.2 Make AflaTest developer solution daily.
- 4.3 Make sure that reagent blank (1 mL methanol + 1 mL developer in a cuvette) reads 0 ppb.
- 4.4 Make sure 2 mL wash water in a cuvette reads 0 ppb on a calibrated fluorometer.

5.0 Sample Extraction:

- 5.1 Measure 25 mL of supernatant sample with 5 g NaCl and place in a blender jar.
- 5.2 Add 50 mL pure methanol to the jar.
- 5.3 Cover blender jar and blend at high speed for 1 minute.
- 5.4 Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

6.0 Extract Dilution:

- 6.1 Pour 10 mL of filtered extract into a clean vessel.
- 6.2 Dilute extract with 20 mL distilled water. Mix well.
- 6.3 Filter dilute extract through glass microfiber filter into glass syringe barrel using the marking on the barrel to measure 9 mL.

7.0 Column Chromatography:

- 7.1 Pass 9 mL of filtered extract completely through the AflaTest column at a rate of 1-2 drops/second (9 mL = 1 g sample equivalent).

$$\frac{25 \text{ mL supernatant}}{25 \text{ mL supernatant} + 50 \text{ mL methanol}} * \frac{10 \text{ mL filtrate}}{10 \text{ mL filtrate} + 20 \text{ mL water}} * 9 \text{ mL} = 1 \text{ g}$$

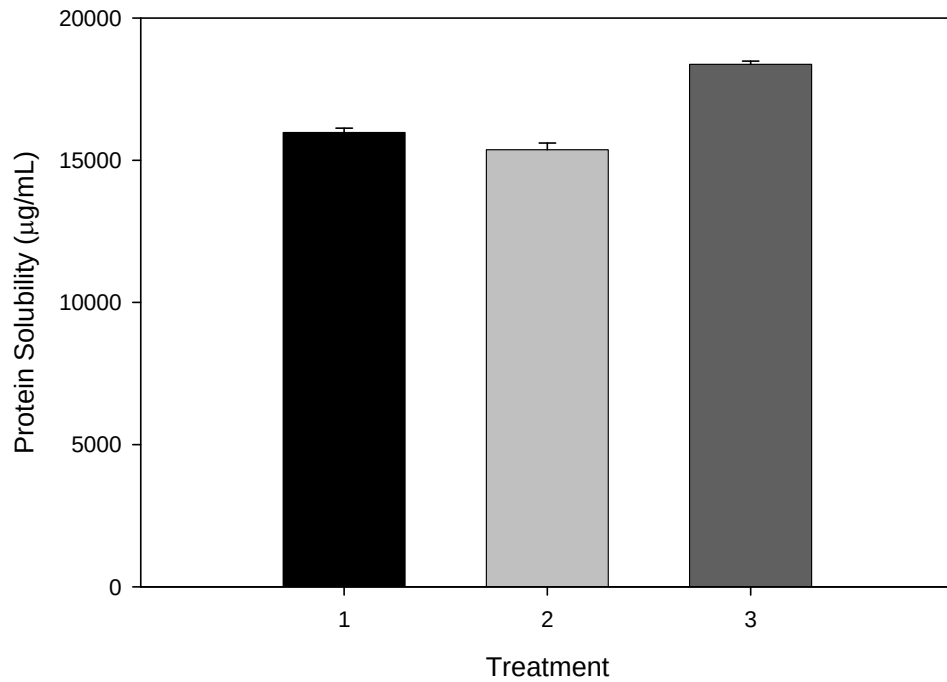
- 7.2 Wash the column with 10 mL distilled water at a rate of 1-2 drops/second.
- 7.3 Wash the column again with 10 mL distilled water at a rate of 1-2 drops/second.

- 7.4 Elute AflaTest column with 1 mL HPLC grade methanol at a rate of 1-2 drops/second and collect all of the sample eluate (1 mL) in a glass cuvette.
- 7.5 Add 1 mL of freshly made AflaTest developer to eluate in the cuvette. Mix well and measure fluorescence in a calibrated fluorometer. Read aflatoxin concentration in parts per billion after 60 seconds.

8.0 Assay Range: 0-50 ppb

9.0 Limit of Detection: 1 ppb

Appendix B. The effects of centrifuge conditions and heat treatment on protein solubility of pH 2 peanut meal dispersion soluble fractions



Treatment 1: centrifuge: 25000 x *g*/20 min, filter through two layers cheesecloth, centrifuge: 8000 x *g*/10 min

Treatment 2: centrifuge: 11000 x *g*/15 min

Treatment 3: heat: 90°C/15 min, followed by centrifuge: 11000 x *g*/15 min

This data indicates that the differences in the pepsin control (at pH 2) protein solubility values (found in Chapter 2, Figure 2) and the pH 2 no clay samples (found in Chapter 3, Figure 3) are due to the heating step applied in the hydrolysis study, as opposed to the centrifuge conditions used.

Appendix C. Acronyms

AAPH	2,2'-Azobis (2-amidino-propane) dihydrochloride
AB20	Astra Ben 20A™
AOAC	Association of Official Analytical Chemists
AU	Anson Units
AUC	area under the curve
BCA	bicinchoninic acid
BHA	butylated hydroxyanisol
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
DH	degree of hydrolysis
FDA	Food and Drug Administration
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
HSCAS	hydrated sodium calcium aluminosilicates
kDa	kilodalton
LAPU	Leucine Amino Peptidase Units
min	minute
MW	molecular weight
NaCl	sodium chloride
NaOH	sodium hydroxide
OPA	o-phthaldialdehyde
ppb	parts per billion
ppm	parts per million
rev	revolutions
RFU	relative fluorescent units
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TE	Trolox equivalents
TNBS	trinitrobenzenesulfonic acid
w/v	weight per volume
w/w	weight per weight