

PEANUT (*Arachis hypogaea* L.) AS A SOURCE OF ANTIHYPERTENSIVE AND
ANTIMICROBIAL PEPTIDES

by

EKUWA ENYONAM QUIST

(Under the direction of Robert Dixon Phillips)

ABSTRACT

To optimize the production and yield of bioactive peptides, defatted raw and roasted peanut flours were subject to varied durations of hydrolysis. Alcalase and sequential hydrolysis with pepsin and pancreatin were the enzyme systems used. Alcalase provided a more extensive hydrolysis ($p \leq 0.05$). The hydrolysates were shown to be a potent source of hypotensive peptides. HPLC fractions for each peanut treatment and enzyme system were assayed for ACE inhibitory activity. IC_{50} for inhibiting ACE activity from the potent hydrophobic end of the chromatograms ranged from 8.7 μ g/ml to 235 μ g/ml for the alcalase system and 7.9 μ g/ml to 65.9 μ g/ml for the pepsin-pancreatin system. The digests also exhibited lethality against pathogenic *Listeria monocytogenes* and *Escherichia coli* O157:H7 with the radii of inhibition ranging from 4 to 13mm and from 2 to 5mm respectively with digests protein concentration of 0.1 to 2mg.

INDEX WORDS: Alcalase, Angiotensin converting enzyme (ACE), ACE inhibition, bioactive peptides, *Escherichia coli* O157:H7, Hydrolysis, Hydrolysates, IC_{50} , *Listeria monocytogenes*, Peanut protein hydrolysates, Pepsin-Pancreatin.

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EKUWA ENYONAM QUIST

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EKUWA ENYONAM QUIST

Major Professor: Robert Dixon Phillips

Committee: Jinru Chen
Louise Wicker

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2005

DEDICATION

“The nights of crying your eyes out give way to days of laughter” (Psalm 30:5b; The Message).

This piece of work is dedicated to **God Almighty**, in whom I live and move and have my being.

It is also dedicated to my parents, **Patrick Quist, MD and Esi Quist, MD** and my Uncle and professional mentor **Prof. Samuel Kofi Sefa-Dedeh**, for their support, encouragement and unfailing love.

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CHAPTER 1
INTRODUCTION

Bioactive peptides have physiologic properties and in recent times several studies have been done on identifying and optimizing the isolation of biopeptides from both plant and animal sources. These peptides are generated both *in vivo* and *in vitro* from proteolytic hydrolysis of food proteins. Peptides with a wide range of regulatory effects have been discovered; some of these effects include immune defense, increased nutrient uptake, neuro-endocrine information transfer, antihypertensive, antithrombotic, antimicrobial, antigastric, and opioid activity. These peptides have been discovered in a varied array of sources including snake venom, spinach, whey proteins and mushrooms. However, the only legumes that have been investigated for biopeptides to the best of our knowledge are chickpeas, peas, cowpeas and soybeans, and it is assumed that since peanut has a similar protein profile as these it will have some biological activity too.

Plant proteins, which provide the bulk of protein sources in developing countries, are being increasingly used as alternative to animal proteins in developed countries in recent years. Legumes provide a good balance of nutrients, and soybean is the most used for plant protein products. Peanut is the second leading legume in terms of world production, and is also a major source of dietary proteins. The value of peanuts can be increased if found to contain bioactive peptides.

The aim of this research therefore is to identify, isolate and optimize yield of bioactive peptides with antihypertensive effects against Angiotensin Converting Enzyme (ACE) which is responsible for increased blood pressure, and antimicrobial effects against *Escherichia coli* O157:H7 and *Listeria monocytogenes*, from peanut protein since peanut has never been tested from this point of view. Alcalase (prepared from *Bacillus licheniformis*) and pepsin-pancreatin

were used as enzymatic sources of hydrolysis for both raw and roasted peanut flours, as they serve as models for *in vitro* and *in vivo* digestion respectively.

For optimal bioactive peptide activity, the influence of parameters such as enzyme concentration, hydrolysis time, temperature and pH, and the interaction between them were determined. All these factors are related to the degree of hydrolysis which significantly determines the generation and availability of biopeptides.

Chapter 2 of this study is the literature review. Chapter 3 looks at proteolytic hydrolysis of both raw and roasted peanut flour with time, the distribution of their protein subunits and the corresponding degree of hydrolysis. Chapters 4 and 5 look at antihypertensive and antimicrobial activities respectively of different hydrolysates and at different concentrations.

CHAPTER 2
LITERATURE REVIEW

Peanut History, Production and Consumption

Peanuts (*Arachis hypogaea* L.) are an annual soil enriching, nitrogen fixing legume. They are grouped into four main types: Spanish, Runner, Virginia and Valencia (Woodroof, 1983a). The peanut originates in South America and was introduced by early explorers and missionaries to other continents (Higgins, 1951; Woodroof, 1983a). It was introduced to Africa by the Portuguese where it became an important food (Rosengarten, 1984; Nwokolo and Smartt, 1996) and from where it was introduced to North America by slave traders who used it as dietary supplements for slaves on slave ships (Higgins, 1951; Woodroof, 1983a).

Peanut is the fourth important oilseed crop of the world in production after soybean, cottonseed and rapeseed (USDA, 2004), and is also the second important legume (Anonymous, 2005). Approximately 75% of the world's peanut is produced in India, China and the United States of America though it is grown on all continents (Salunkhe *et al.*, 1992). About 55% of US peanut are grown in the southeast with Georgia growing about 39% of the total US production (Table 2.1).

Peanut is a source of dietary proteins, oils, minerals and vitamins and it is consumed in different processed forms all over the world. In the United States, about 50% of the peanut produced is consumed as peanut butter and spread (www.aboutpeanuts.com). Peanuts are eaten raw, boiled, roasted, and made into confectionaries and snacks. Oil extracted from peanuts is used in cooking and the oilcake after product is used as animal feed (Woodroof, 1983b), protein isolates from peanuts are used as extenders to dairy products and peanut flour is used as an extender in meat products for use in many recipes (Nwokolo and Smartt, 1996).

Table 2.1 Peanut: Area Harvested, Yield, and Production by State and United States, 2001-2003

	Area Harvested (1,000 Acres)			Yield (lbs)			Production (1, 000 lbs)		
	2001	2002	2003	2001	2002	2003	2001	2002	2003
Alabama	199.0	185.0	189.0	2,675	2,050	2,900	532,325	379,250	548,100
Florida	82.0	86.0	107.0	3,050	2,300	2,700	250,100	197,800	288,900
Georgia	514.0	505.0	535.0	3,330	2,600	3,200	1,711,620	1,313,000	1,712,000
New Mexico	22.2	18.0	17.0	3,020	3,000	2,900	67,044	54,000	49,300
North Carolina	122.5	100.0	100.0	2,910	2,100	2,900	356,475	210,000	290,000
Oklahoma	77.0	57.0	38.0	2,570	2,800	2,900	197,890	159,600	1,110,200
South Carolina	10.2	8.7	18.0	3,000	2,200	3,200	30,600	19,140	57,600
Texas	310.0	280.0	240.0	2,890	3,100	3,400	895,900	868,000	816,000
Virginia	75.0	57.0	33.0	3,130	2,100	2,700	234,750	119,700	89,100
US Total	1,411.9	1,296.7	1,277.0	3,029	2,561	3,102	4,276,704	3,320,490	3,961,200

Source: www.aboutpeanuts.com

The early 1990s saw a decline in the consumption of peanuts due to concerns about contamination with aflatoxins, health and dietary issues about peanut fat content, and allergenicity issues. However with the popularity of a low carbohydrate diet, the discovery of the high satiety effect of peanut and its link to improved health, consumption has soared (USDA, 2002). In the case of peanut allergens, Burks *et al.*, 1992 discovered that enzymatic treatment of the protein results in a moderate change in the allergens whereas heat treatments show no significant changes. Additionally, it is recognized by most health advocates that the allergy issue is currently best addressed by careful processing to exclude allergens from unlabeled foods and conscientious labeling of foods containing peanut and other allergens.

Peanut Nutritional Composition

Peanuts and its products have been a component of the world's diet for years. The geographical location, cultivar type and cultivation conditions influence the nutritional profile of the nuts. Peanut kernel contains about 16.2 – 36% protein. These proteins are classified into albumin (water soluble), globulins (salt soluble) and glutelins (acid/alkaline soluble); the globulins constitute about 87% and consists of arachin and conarachin (Salunkhe *et al.*, 1992). Peanuts are known to be rich in acidic amino acid, but however deficient in essential amino acids lysine, methionine and threonine (Salunkhe *et al.*, 1992).

The oil content of peanuts is 47 - 50%, of which about 76 – 80% is unsaturated fatty acids. Oleic acid makes up 40 – 45%, and linoleic acid makes up 30 – 35% of the composition of unsaturated fatty acids (Woodroof, 1983b). Peanut is an excellent source

of mono- and polyunsaturated fatty acids, with levels exceeding that of soybean (Nwokolo and Smartt, 1996). Though the oils have a high caloric value, they have been shown to have links to improved cardiovascular health. A diet high in monounsaturated fatty acid (MUFA) – oleic acid – is known to reduce low density lipoprotein (LDL) cholesterol whilst increasing high density lipoprotein (HDL) cholesterol. LDL is considered ‘good’ cholesterol in contrast to HDL which is considered ‘bad’ cholesterol. A study by Alper and Mattes (2003) reports that regular consumption of peanuts improve cardiovascular health. In addition to MUFA, peanuts are a rich source magnesium, fiber, folate, vitamin E, copper and arginine, all of which have cardiovascular disease risk reducing properties (Mattes, 2003).

According to Woodroof (1983b) peanut contains 18% carbohydrates with a starch content of 0.5 – 5%, and sucrose content of 4 – 7%. Peanuts have also been said to contain 3% ash which is composed of 26 inorganic constituents of which phosphorus, potassium, magnesium and sulfur are high and virtually unaffected by heat while the remaining 22 though also heat stable are deficient from a nutritional viewpoint (Woodroof, 1983b).

Woodroof (1983b) also reports the vitamin profile of peanuts to include riboflavin, thiamin (which is destroyed to a great degree by roasting and blanching), nicotinic acid and Vitamin E, with appreciable amounts of B complex vitamins and Vitamin K, but practically no Vitamins A, C or D.

Peanut Protein

The concentration and availability of individual essential amino acids determine the nutritive value of a protein source (Nwokolo, 1996). Peanuts contain about 26% protein. Of the 9.1% nitrogen in peanut, 8.74% has been identified as albuminous components – albumen, gluten and globulin. Eighty seven percent (87%) of peanut nitrogen is present as conarachin and arachin which contain 18.3% nitrogen, and are the two isolated globulins of peanut protein (Woodroof, 1983b). Arachin constitutes about 63% with conarachin contributing about 33% of the total kernel protein (Salunkhe *et al.*, 1992; Fontaine *et al.*, 1945). SDS gel electrophoresis shows peanut having 5 minor and major components each with molecular weights between 130, 000 and 30, 000. Both arachin and conarachin have been investigated to have 5 different components each with molecular weights between 81, 000 and 20, 000, and between 84, 000 and 23, 000 respectively (Cherry, 1983; Basha and Cherry, 1976). Conarachin contains as much as three times sulfur than arachin (Woodroof, 1983b) but is poor in phenylalanine and tyrosine whereas arachin is poor in total sulfur, lysine and methionine but rich in threonine and proline (Salunkhe *et al.*, 1992; Kaneko and Ishi, 1978).

Peanut proteins are relatively rich in the acidic amino acids with aspartic acid, glutamic acid and arginine accounting for about 45% of the amino acids (Salunkhe *et al.*, 1992; Young *et al.*, 1973). There is however an identified flaw in the amino acid profile of peanut which is the low content of lysine and methionine which are two of the essential amino acids to human nutrition.

Table 2.2 Amino Acid Composition of Raw Peanut and Defatted Flour

Amino Acids	Values (g/100g edible portion)	
	Raw Peanut	Defatted Flour
Tryptophan	0.25	0.51
Threonine	0.88	1.79
Isoleucine	0.91	1.84
Leucine	1.67	3.38
Lysine	0.93	1.87
Methionine	0.32	0.64
Cystine	0.33	0.67
Phenylalanine	1.34	2.71
Tyrosine	1.05	2.12
Valine	1.08	2.19
Arginine	3.09	6.24
Histidine	0.65	1.32
Alanine	1.03	2.07
Aspartic Acid	3.15	6.37
Glutamic Acid	5.39	10.91
Glycine	1.55	3.14
Proline	1.14	2.30
Serine	1.27	2.57

Source: USDA, 2005

Roasting

Roasting is the rapid cooking with radiant heat or by surrounding the food in question with hot media which is usually oil. Peanuts may be roasted in hot sand, ashes or embers (Woodroof, 1983c). They may be also roasted in their shell or decorticated, and either salted or unsalted prior to roasting (Nwokolo, 1996). There are specific times and temperatures settings for optimal roasting using various procedures (Nwokolo, 1996). Peanuts are either dry or oil roasted. Dry roasting occurs at 160°C for 20 – 30 minutes, may be glazed with 1.5 – 2% oil, and mixed with 2.5% salt without blanching. In cases where the peanuts are blanched, blanching is prior to the application of oil and salt. The peanuts are immersed in heated coconut, peanut or cottonseed oil at 147°C for 3 – 5 minutes (Salunkhe *et al.*, 1992). Color becomes darker with roasting and is used as a visual indication and quality control (Woodroof, 1979). The heat process improves the aroma, flavor and texture of peanuts.

The characteristic flavor of roasted peanuts is from the interaction of sugars with amino acids such as glutamic acid, aspartic acid, phenylalanine and histidine (Salunkhe *et al.*, 1992; Cobb and Swaisgood, 1971). The proteins, minerals and most vitamins are stable to processing. However the heat destroys natural antioxidants and thiamine. Thiamine is concentrated in the skin and is further reduced when the peanuts are blanched (Woodroof, 1983c). In terms of nutritional quality and storage stability, oil roasting is found to be more detrimental than dry roasting (Salunkhe *et al.*, 1992).

Table 2.3 Nutritional Composition of Raw and Roasted Peanuts

Parameter	Raw	Roasted
<i>Proximate Composition (%)</i>		
Moisture	5.6	1.8
Protein	26.0	26.2
Fat	47.5	48.7
Carbohydrates	18.6	20.6
Fiber	2.4	2.7
<i>Minerals (mg/100g)</i>		
Calcium	69	72
Phosphorous	401	407
Iron	2.1	2.2
Sodium	5.0	5.0
Potassium	674	701
<i>Vitamins ($\mu\text{g}/100\text{g}$)</i>		
Thiamin	1.14	0.32
Riboflavin	0.13	0.13
Niacin	17.2	17.1

Source: Woodroof, 1945 in Salunkhe, 1992.

Proteins, Peptides and Amino Acids

A protein is a chain of amino acids joined by peptide bonds in a specific sequence. Proteins play a wide role from functioning as enzymes to hormones to being physiological carries, to providing support and shape among many others. Twenty standard amino acids exist from which an array of polypeptides arise. Based on their side chains, amino acids are classified into 6 groups: aliphatic, aromatic, sulfur-containing, acids with their amides, bases and alcohols. They are further classified as hydrophilic or hydrophobic. The properties of the side chains of amino acids influence the conformation of a protein (Horton *et al.*, 2002a).

Proteins come in different shapes and conformations and are composed of one or more polypeptide chains. Protein structures are described as primary, secondary, tertiary and quaternary. The primary structure is the linear sequence of the amino acid residues, and determines to a large extent protein folding conformations. Protein folding is a sequential process where the formation of the first structural element assists in the alignment of the subsequent features. Chaperones which are proteins are used to ensure correct folding conformations (Horton *et al.*, 2002b).

The secondary structure “consists of regions of regular repeating of conformations of the peptide chain such as α helices and β sheets”. These regularities are maintained by hydrogen bonds between amide hydrogen and carbonyl oxygen of the peptide backbone. Protein folding and stability depend on hydrophobic effect, hydrogen bonding, van der Waals forces, and charge-to-charge interaction which are all non covalent interactions. Non-polar side chains aggregate in the interior whereas polar side chains remain in contact with water molecules. This association leads to the collapse of the polypeptide

backbone and the elements of secondary structure begin to form. Secondary structures are also generated when sections of the polypeptide are forced into the interior neutralizing their polarity by forming hydrogen bonds with each other. Hydrogen bonds give rise to defined regions of the secondary structure as they are the first to form in α helices, β sheets, and turns. The bonds in the interior are more stable than those on the surface since they are not in competition with water molecules. Due to the closely packed nature of the interior of a protein, van der Waals forces may be of a considerable magnitude. Charge-charge interactions between opposite charged side chains may contribute to protein stability. In the interior, these ion pairs are more stronger than those in association with water (Horton *et al.*, 2002b).

The tertiary structure “describes the structure of a fully loaded polypeptide chain”. Many polypeptide chains are composed of several domains linked by a short stretch of amino acid residues. Distinct portions of the primary and secondary structures are brought together in the formation of the tertiary structure. Biochemical efforts are underway to predict the tertiary structure of a protein from its sequence. The quaternary structure is the “arrangement of two or more polypeptides into a multi subunit molecule” (Horton *et al.*, 2002b).

Bioactive Peptides

Bioactive peptides are biologically active peptides derived from the proteolysis of proteins. These peptides have been discovered to have physiologic activity in the living organism including antihypertensive, antimicrobial, antithrombotic, and antiwrinkle, among many others. A varied array of plant and animal proteins provide the source of

these biopeptides. These biopeptides have been discovered in whey, fermented foods, plant seeds, animal muscles, just to mention a few.

Proteolytic Hydrolysis

Proteins are an important group of biomacromolecules responsible for a variety of physiological properties. In food, these properties are essential for producing and maintaining good product quality, and also serve to meet nutritional needs. Protein solubility and hydrolysis play a very important role in the extent of their functionality (Nakai, 1996; Spellman *et al.*, 2003). Hydrolysis of food proteins enhances properties like foaming, emulsification, solubility, nutritional profile and the release of bioactive peptides. Proteins are quite stable to hydrolysis in aqueous solution under benign conditions of temperature and pH. At extreme temperatures and pHs, the rate of hydrolysis increase as the stability of the peptides bonds decrease dramatically; proteolytic enzymes have an even more dramatic effect on the rate of hydrolysis (Ludescher, 1996).

Peptides can be obtained from either chemical or enzymatic synthesis. However from the food safety point of view, enzymatic synthesis is preferred to chemical synthesis (Nakai, 1996). Chemical hydrolysis can destroy L-amino acids, produce D-amino acids and form toxic substances like lysino-alanine (Lahl and Grindstaff, 1989). Enzymatic hydrolyses is more specific since specific linkages are targeted. Enzymatic hydrolyses have been indicated in various researches as a means of increasing the functionality of proteins including the release of biologically active peptides. Enzymatic hydrolyses dates back to centuries ago in food applications such as cheese and fermented food production.

Enzymatic hydrolysis occurs under mild conditions of pH 6-8 and temperature range of 40°C – 60°C compared to the extreme conditions required for chemical and physical treatments and thus minimizes side reactions, with the overall amino acid composition being similar to that of the starting material (Clemente, 2002). These conditions are described as mild and are similar to that in the biological system and thus provide a convenient means of improving the functional properties of proteins whilst retaining nutritive value (Alder- Nissen, 1977).

Enzymatic plant protein digests have been used widely in specified formulation to improve functional and nutritional properties. They are used in special medical diets such as hypoallergenic formulas, geriatric products, high energy supplements, and enteric diets; this functionality is attributed to peptides being more hypotonic and thus providing reduced osmotic problems and improved gastrointestinal absorption efficiency compared to either intact protein or free amino acids (Clemente *et al.*, 1999; Clemente, 2000; Mahmoud, 1994). Plant protein hydrolysates have gained a lot of attention in recent years. However there is a major disadvantage in their use as nutrients, compared to that derived from casein or whey sources, since they lack the superior amino acid profile found in animal sources for example, such as the low levels of essential sulphur-containing amino acids in legumes (Clemente, 2002). Legumes however, of which soybean is the most widely used, in addition to peas and chickpeas, have been used successfully to generate protein hydrolysates (Clemente *et al.*, 1999).

An extensive list of procedures exists for terminating hydrolysis of which acid-inactivation, heat inactivation and enzyme removal by filtration are inclusive. The preferred method according to Lalh and Braun (1994) is to simultaneously adjust the pH

and temperature to levels where the enzymes are most sensitive and thus destroying enzyme activity and terminating the hydrolysis process.

Post-hydrolysis processes have been put in place to enhance the suitability of hydrolysates. Some of these processes control molecular sizes of resulting peptides and eliminate bitterness (Clemente, 2002). The bitterness related to peptides is a result of the hydrophobic content of the amino acids (Clemente, 2002; Alder-Nissen, 1977). Ultrafiltration, precipitation and boiling are some of the known post-hydrolysis procedures just to mention a few; centrifugation and the degree of hydrolysis (DH) were used in this research. According to Alder-Nissen (1977), the degree of hydrolysis serves as a controlling parameter for the reaction to prevent the formation of bitter peptides and to maintain uniform product quality. Various methods such as the pH Stat and o-phthaldialdehyde (OPA) (Peñas *et al.*, 2004) and Trinitrobenzenesulphonic acid (TNBS) (Alder-Nissen, 1979) exist for the determination of DH of proteins, but of these listed the TNBS has been determined as the excellent method for quantifying DH regardless of the enzyme activity used for hydrolysis in a study by Spellman *et al.* (2003).

Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis is a size-based separation technique. It depends on the restriction of charged species moving in an electric potential field by gels with specific pore sizes, created by the concentration of total polymer or crosslinking. For protein separation, polyacrylamide gel electrophoresis is commonly used. The use of detergents, such as sodium dodecyl sulfate (SDS) allows dissociation of non-covalently bonded

subunits into smaller species and prevents association of peptide chains during analysis. SDS also causes the proteins to possess negative charges.

In SDS-PAGE, molecular weight markers of known sizes are run alongside the sample to be characterized; this enables the measuring of the relative sizes of the various protein bands separated in a sample. Proteins of the same group migrate to the same distance on the gel; this makes possible the analyses and characterization of protein composition before and after processing.

Proteolytic Enzymes

Enzymes are highly specific and are classified according to the specificity of the peptide bond they cleave and possess the properties of proteins (Clemente, 2002; Horton *et al.*, 2002c). Protease enzymes possess a varying ability to catalyze hydrolysis of proteins (Clemente, 2002), at reaction rates $10^3 - 10^{17}$ faster than occurs in their absence (Horton *et al.*, 2002c). It is therefore important that the end use of the hydrolysate be considered whilst choosing the enzyme system in regards to specific action on the protein in question. Enzymatic reactions are carried out in optimal conditions that simulate biological systems to optimize the yield of functional proteins.

According to literature (Horton *et al.*, 2002c), there are 6 main classes of enzymes namely:-

- Oxidoreductases/dehydrogenases which catalyze oxidation-reduction reactions.
- Transferases which catalyze group transfer reactions.
- Hydrolases catalyze hydrolysis and are a class of transferases. Water is the acceptor of the transfer group in this case.

- Lyases which catalyze lysis of a substrate to form a double bond. In the reverse reaction where there is an addition of a substrate to the double bond of a second substrate the enzyme is called synthase.
- Isomerases catalyze isomerization reactions where there are structural changes within a molecule.
- Ligases catalyze ligation of two substrates. They are also referred to as synthetases and usually require the chemical potential energy of nucleoside triphosphate.

Proteolytic enzymes are classified as either endopeptidases or exoproteases depending on their mechanism of hydrolysis. “Endopeptidases hydrolyze the peptide bonds within protein molecules at random to produce relatively large peptides. Exoproteases systematically remove amino acids from either the N terminus or C terminus by hydrolyzing the terminal peptide bonds” (Clemente, 2002).

Pepsin and Pancreatin

Pepsin which is an acidic protease in the stomach is the first in a series of enzymes to begin the digestion of proteins. “Pepsin hydrolysis would improve pancreatic digestion by increasing the protein solubility and opening the structure of the molecule, adding to the availability of its peptide bonds to other enzymes” (Gauthier *et al.*, 1986). Protein is further hydrolyzed by pancreatin in the small intestine when it leaves the stomach to produce a combination of free amino acids and small peptides (Schmidl *et al.*, 1994). Pancreatin is a mixture of digestive enzymes (trypsin, chymotrypsin) produced by the pancreas. While pepsin is single enzyme with low specificity and cleaves a large

number of peptide bonds in a polypeptide chain, pancreatin, containing a number of enzymes, has broad specificity in total, and has both endo- and exopeptidase activity.

Alcalase

Alcalase is a protease prepared from submerged fermentation of *Bacillus licheniformis* and is an endoproteinase with optimal temperature between 55°C and 70°C and pH between 6.5 and 8.5 (Novozymes North America, Inc., Franklinton, NC). This enzyme has broad specificity with some preference for terminal hydrophobic amino acids. Literature has it that hydrolysates produced with enzymes that have hydrophobic amino acid specificity have a less bitter taste (Lahl and Braun, 1994; Alder-Nissen, 1986).

Angiotensin Converting Enzyme (ACE) and Hypertension

Hypertension is a major health problem. It results from diverse genetic and environmental factors (Egan *et al.*, 2004; Opie, 1994). In the US alone about 60, 000, 000 individuals have blood pressure conditions that require medical attention; and due to the burgeoning epidemic of obesity and sedentary lifestyles, hypertension no longer predominantly affects the middle aged and older adults, but also young adults and teenagers (Egan *et al.*, 2004).

Several studies have reported antihypertensive activity in snake venom (Ondetti *et al.*, 1971); spinach (Yang *et al.*, 2003); rapeseed (Marczak *et al.*, 2003); whey proteins (Vermeirssen *et al.*, 2005); soy (Wu and Ding, 2002; Gibbs *et al.*, 2004); chickpeas (Yust *et al.*, 2003) and peas (Vermeirssen *et al.*, 2005;) among many others.

Angiotensin Converting Enzyme (ACE) a dipeptidyl carboxypeptidase is known to play a vital role in the regulation of blood pressure. It achieves this by converting Angiotensin I (an inactive decapeptide) into Angiotensin II (a salt retaining octapeptide and vasoconstrictor) (Opie, 1994) by cleaving a dipeptide from the carboxyl terminal of Angiotensin I (Kostis *et al.*, 1987), and also inactivates bradykinin (a vasodilator nonpeptide) by the same mechanism (Yang *et al.*, 1970). Angiotensin I originates from angiotensinogen, a glycoprotein in the liver under the influence of renin, an aspartyl protease which is formed in the kidney (Opie, 1994 and Kostis *et al.*, 1987).

Angiotensin II stimulates the release of aldosterone from the adrenal cortex which in turn produces kaliuresis and increases sodium and water retention through its direct effect on the distal renal tubule. Sodium and water retention produces an increase in blood pressure (Frishman, 1987). Fig 2.1 shows an outline of the formation of angiotensin II with corresponding vasoconstriction and stimulation of aldosterone leading to an increase in blood pressure.

ACE has a molecular weight of 135, 000 to 150, 000 daltons, contains approximately 25% carbohydrate and is localized in the vascular endothelium of the lungs, blood vessels, kidneys, brain, male reproductive system and plasma (Kostis *et al.*, 1987). Angiotensinogen is synthesized primarily in the liver but is also found in the brain and kidneys with a molecular weight of 58, 000 to 61, 000 daltons and a 14% carbohydrate content (Kostis *et al.*, 1987). Renin with a molecular weight of 35, 000 to 45, 000 daltons is produced predominantly in the kidneys but is also found in the brain, salivary glands, blood vessel walls, genital tracts, adrenal glands and tumor tissues (Kostis *et al.*, 1987).

ACE Inhibitors

“An inhibitor is a compound that binds to an enzyme and interferes with its activity by preventing either the formation of the enzyme-substrate complex or its breakdown” (Horton *et al.*, 2002). According to the same authors, inhibitors are classified as either reversible or irreversible. There are three basic reversible inhibition reactions: Competitive, Uncompetitive and Non-competitive.

In classical competitive inhibition, the inhibitor and substrate compete for and bind to the same enzyme active site. In non-classical competitive inhibition, both the substrate and inhibitor bind at different active sites. In this case, the complexes formed depend on the concentration of each in the solution and also on the relative affinities of the enzyme for them. In uncompetitive inhibition, the inhibitor binds to the enzyme-substrate complex whereas in non-competitive inhibition the inhibitor binds to either the enzyme or the enzyme-substrate complex.

Most ACE inhibitors are competitive (Vermeirssen *et al.*, 2002; Meisel, 1997). ACE Inhibitors inhibit ACE activity and thereby prevent the formation of Angiotensin II. They also prevent the breakdown of bradykinin. According to Ram and Fenves (2002), the inhibition of Angiotensin II formation and bradykinin breakdown relieves vasoconstriction and promotes vasodilation respectively. Hypertension is one of the leading indications for the use of drugs in North America, and ACE Inhibitors are known to be renoprotective by reducing intraglomerular pressure. They also improve insulin sensitivity, making them a better choice for diabetics (Ram and Fenves, 2002).

ACE inhibitors are a relatively new class of cardiovascular drugs with captopril, the original member of this group, and enalapril, the second to become commercially

available in the US being marketed in the 1980s (Kostis and DeFelice, 1987). According to the same authors, the clinical success of these two drugs has led to the synthesis of new ACE inhibitors now in pre-clinical and clinical development stages.

Microbial Quality and Outbreaks

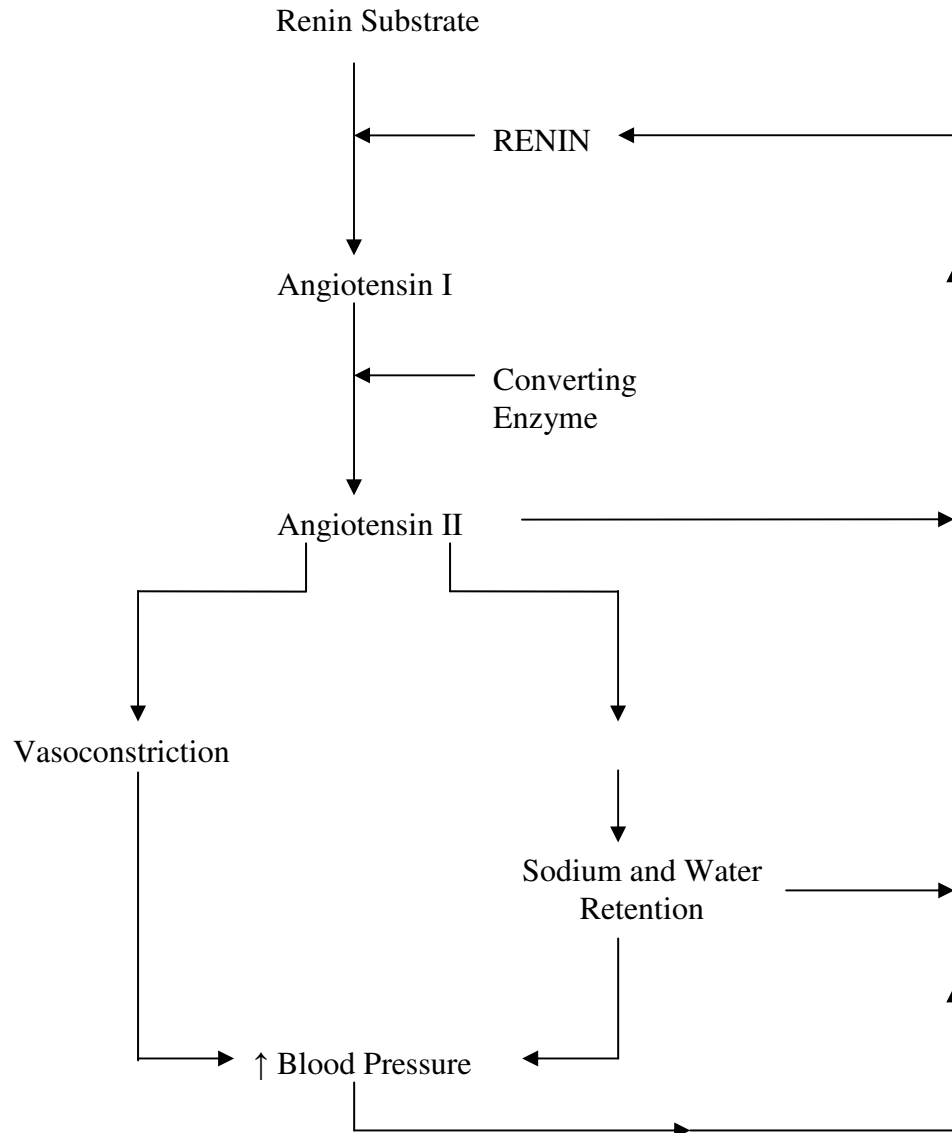
The presence of microorganisms in foods may be desirable or undesirable depending on the end use. Their presence in food may lead to enhancement in food quality, result in food spoilage and/or cause food poisoning on consumption.

The CDC estimates that 76 million Americans get sick, more than 300,000 are hospitalized, and 5,000 people die from foodborne illnesses each year (<http://www.cdc.gov/ncidod/diseases/food/index.htm>). Research indicates the adaptation and resistance of pathogens to antibiotics (Sofos, 2002, Davidson and Harrison, 2002, and McDonnell and Russell, 1999).

***Escherichia coli* O157:H7 and associated outbreaks**

Escherichia coli is a common part of the normal facultative anaerobic microflora in the intestinal tract of humans. Most of the strains are harmless but some are pathogenic and cause diarrheal diseases (Meng *et al.*, 2001).

E. coli O157:H7 is the predominant cause of enterhemorrhagic associated disease worldwide and was first discovered in 1982 in Oregon in an outbreak associated with undercooked hamburgers (Meng *et al.*, 2001). Undercooked ground beef has been the major vehicle associated with *E. coli* O157:H7 outbreaks; unpasteurized milk and juices are now known vehicles.



Source: Adapted from Weber, 1987 in "Angiotensin Converting Enzyme Inhibitors".

Fig 2.1 Scheme of the Renin-Angiotensin System Indicating Angiotensin II Formation with Corresponding Vasoconstriction and Stimulation of Aldosterone.

The first *E. coli* outbreak from apple cider was in 1991 in Southeastern Massachusetts (Besser *et al.*, 1993). This kind of outbreak is usually associated with apple contaminated by contact with soil manure (Meng *et al.*, 2001), in instances where ruminants frequented the orchard (Cody *et al.*, 1999) or where cattle fields were close to the cider press (Besser *et al.*, 1993) and contamination could occur during processing. Since apple cider was traditionally unpasteurized, pathogens that were present on the fruits were likely to be present in the cider. In a study by Zhao *et al.* (1993), they discovered that the use of 0.1% sodium benzoate, which is an approved preservative, substantially increased the safety of apple cider by decreasing the number of *E. coli* to undetectable population (reduction of $> 4\log_{10}$ CFU/ml).

E. coli O157:H7 is responsible for approximately 73, 000 cases of food borne illnesses in the U.S. annually, with an estimated 2, 100 hospitalizations, and 3-5% of these cases are fatal as a result of patients developing hemolytic uremic syndrome (HUS) (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/escherichiacoli_t.htm).

***Listeria monocytogenes* and associated outbreaks**

Listeriosis according to literature was mentioned in early medical literature but was definitively documented in 1981 during the Nova Scotia, Canada outbreak from coleslaw (Swaminathan, 2001 and Schlech *et al.*, 1983). Food has been investigated as a major source of transmission of listeriosis since the 1981 outbreak (Swaminathan, 2001), and these foods include dairy products, sea foods and meat. Poultry supports the growth of *L. monocytogenes* better than other meats and contamination of animal muscle tissue occur either from symptomatic or asymptomatic carriage of the organism before

slaughter or contamination of the carcass after slaughter (Swaminathan, 2001). The organism, according to Swaminathan (2001), attaches strongly to the surface of raw meat and is difficult to remove or inactivate.

As of 1997, there were 2, 500 cases with 500 fatalities from listeriosis annually in the US. Though there is a reported 38% decline of cases between 1996 and 2002, outbreaks continue to occur

(http://www.cdc.gov/ncidod/dbmd/diseaseinfo/listeriosis_t.htm).

Hydrolysates from peanuts have not been generated for use in specialized formulations. There is also no known publication on peanut in regards to the generation of biologically active peptides with antihypertensive activity against ACE, and antimicrobial effects on *E. coli* O157:H7 and *L. monocytogenes*. Thus this research aims at adding peanut to the legume protein hydrolysate sources list.

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CHAPTER 3
THE EFFECT OF ENZYME SYSTEMS AND PROCESSING ON THE
HYDROLYSIS OF PEANUT PROTEIN¹

¹ E. E. Quist, R. D. Phillips and F. K. Saalia. To be submitted to the Journal of Food Science.

ABSTRACT

In vitro protein digestion studies were carried out on raw and roasted peanut. Defatted raw and roasted peanut flour was used as starting material in the production of peanut protein hydrolysate. Peanut flour was hydrolyzed with alcalase in one batch, and a sequential digestion with pepsin and pancreatin in another batch for up to 24 hours. The degree of hydrolysis (DH) at different times of hydrolysis was determined using the Trinitrobenzenesulfonic Acid (TNBS) method. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to indicate destruction of native protein units in the enzymatic digests.

Hydrolysis with alcalase was very rapid for the first 6 hours after which a plateau was reached, whereas that with pepsin-pancreatin was very gradual reaching a plateau after 12 hours of hydrolysis. Raw peanut hydrolyzed with alcalase and pepsin-pancreatin had 23% and 21% DH after 24 hours respectively, whilst roasted peanut hydrolyzed with alcalase had 21% DH, with the pepsin-pancreatin hydrolysate recording the highest value of 25% after 24 hours of hydrolysis.

SDS-PAGE results showed that raw peanut samples behaved differently from the roasted samples. Increasing hydrolysis time reduced larger peanut protein subunits, with only peptides of < 20 KDa visible after hydrolysis for raw peanut, and virtually no distinct visible bands for the roasted peanut after 3 hours of hydrolysis.

Keywords: Alcalase, Hydrolysis, Peanut, Pepsin-Pancreatin, Protein, Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE), Trinitrobenzenesulfonic Acid (TNBS).

INTRODUCTION

Enzymatic plant protein digests have been used widely in specified formulation to improve functional and nutritional properties. They are used in special medical diets such as hypoallergenic formulas, geriatric products, high energy supplements, and enteric diets; this functionality is attributed to peptides being more hypotonic and thus providing reduced osmotic problems and improved gastrointestinal absorption efficiency compared to either intact protein or free amino acids (Clemente *et al.*, 1999; Clemente, 2000a; Mahmoud, 1994).

Protein solubility and hydrolysis play a very important role in the extent of their functionality (Nakai, 1996; Spellman *et al.*, 2003). Peptides can be obtained from either chemical or enzymatic hydrolysis of proteins, but enzymatic hydrolysis is preferred to chemical hydrolysis from the food safety point of view (Nakai, 1996). This preference can be attributed to the fact that chemical hydrolysis can destroy L-amino acids, produce D-amino acids and form toxic substances like lysinoalanine (Lahl and Braun, 1994, Lahl and Grindstaff, 1989) and also because enzymatic hydrolysis is moderately cheaper (Clemente, 2002a), more specific and less destructive than chemical hydrolysis which ultimately destroys all peptide bonds.

Enzymatic hydrolysis is employed in various food applications for example, the production of fermented foods like cereal and tuber dough. The process has been indicated in numerous researches as a means of increasing the functionality of proteins. It has also been indicated as a means to generate biologically active peptides. The protein composition and hydrolysis conditions have a direct bearing on the functionality of hydrolysates. Enzymatic reactions are carried out in optimal conditions that simulate

biological systems to enhance the yield of functional peptides. The enzyme substrate ratio used in digestion is the most variable factor as the enzymatic release of amino acids varies with the ratio (Gauthier *et al.*, 1986; Robbin, 1978).

According to Clemente (2002a) the most effective way to produce protein hydrolysates with defined characteristics is by sequential hydrolysis with endopeptidases and exoproteases coupled with the development of post hydrolysis procedures.

The degree of hydrolysis is a measure of protein degradation and a controlling parameter for the process, as well as a means of determining protein hydrolysate properties (Alder-Nissen, 1979; Mahmoud *et al.*, 1992).

Though there is no known published information on generating peanut hydrolysates for various end use peptides, it is believed that since other legumes like soybeans and chickpeas have proved a suitable source of hydrolysates for these purposes, peanuts will also yield a resourceful source. In addition to the limitation on peanut utilization as a result of allergens will be addressed during the generation of hydrolysates; since there are moderate reductions in peanut allergenicity with enzymatic proteolysis (Burks *et al.*, 1992).

MATERIALS AND METHODS

1. MATERIALS

Roasted peanut flour was purchased from Golden Peanut Company, Alpharetta, GA. Raw peanut flour was prepared from blanched peanuts purchased from Tara Foods, Albany, GA. Both Pepsin (EC 3.4.23.1, Porcine stomach mucus, 2 174units/mg); Pancreatin (EC No. 232-468-9, Porcine Pancreas, Activity equivalent to 4 x U.S.P);

L-Leucine and Thimerosal were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Trinitrobenzenesulfonic Acid (TNBS) was also purchased from Sigma-Aldrich Chemical Co. Alcalase 2.4L FG (Batch PLN05303) was donated by Novozymes North America, Inc. (Franklinton, NC).

2. METHODOLOGY

The raw peanut flour was produced by grinding the blanched kernels using a coffee bean grinder (Series CBG5, Black & Decker Co., Towson, MD). The flour was then defatted by a series of extractions with hexane at room temperature, followed by grinding using the same grinder.

Proximate Analyses of peanut flours

Fat content was determined using the goldfish apparatus (Model 35001, Laboratory Construction Co., Kansa City, MO). Nitrogen was determined using LECO FP2000 (Model 602-600, LECO Co., Warrendale, PA). Protein was calculated from the Nitrogen value using a Kjeldahl factor of 5.46. Moisture/solid content was determined using the Isotemp Vacuum Oven (Model 281A, Fisher Scientific Co., Suwanee, GA).

Enzymatic Hydrolysis

Prior to hydrolysis, both roasted and raw peanut flours were defatted with hexane to fat content of 0.8% and 1.5% respectively. The defatted peanut flour was used as starting material for hydrolysis.

In this *in vitro* study, pepsin-pancreatin was used to simulate human digestion, and alcalase was used from the industrial point of view. The pH of the reaction was kept at the required value by adding base. It is known that the number of peptide bonds cleaved is proportional to the base consumed at a constant pH (Alder-Nissen, 1977).

Two hundred and fifty (250) mg of peanut protein was hydrolyzed sequentially with pepsin and pancreatin according to the method of Gauthier *et al.* (1986) with modifications in a water bath. The peanut flour was suspended in 20ml of 0.1M HCL and stirred for 5min using a Burrel Wrist Action Shaker (Model 75 – Burrel Co., Pittsburg, PA). The pH was adjusted to 1.9 using 1N NaOH and preincubated to 37°C for 20min before the enzyme was added. 1ml pepsin solution (1mg/ml in 0.1NHCL; 2 174units/mg protein) was used to initiate hydrolysis which was terminated after 30min by raising the pH to 7.5 using 1N NaOH. The second step of the proteolysis was carried out using 10ml of pancreatin solution (1mg/ml in 0.01M pH 7.5 sodium phosphate buffer, NaPO₄) was added to initiate the next phase of hydrolysis. Hydrolysis was carried out for 24 hours; drops of 1N NaOH were used to adjust and maintain the pH at 7.5 throughout the reaction. Thimerosal (1% of total solids) was added to prevent microbial growth.

Using alcalase as enzymatic source, the peanut flour was suspended in 20ml of 0.01M pH 7.5 sodium phosphate buffer. It was stirred and pre-incubated at 60°C for 20mins. Alcalase was used at an enzyme – substrate ratio of 0.3AU/g peanut flour. pH was monitored and maintained at 7.5 by adding drops of 1N NaOH.

All hydrolysis was carried in a water bath maintained at 37°C for the pepsin-pancreatin, and 60°C for the alcalase hydrolyses. A pH meter was used intermittently to monitor pH. Hydrolysis was terminated by boiling the hydrolysate for 15min.

Hydrolysates were centrifuged at 27 000g for 15min at 10°C. The supernatant was decanted and used for further analyses. A flow chart of the procedure is outlined in fig 3.1.

Degree of Hydrolysis

Degree of Hydrolysis (DH) which is the percentage of peptide bonds cleaved was measured using the method of Alder-Nissen *et al.* (1979), which relies on the spectrophotometric assay of a chromophore formed by the reaction of Trinitrobenzenesulfonic Acid (TNBS) with primary amines under alkaline conditions with maximum absorption at 340nm. A stock solution of 7.62mM of L-Leucine (0 – 3mM) was used to generate a standard curve of absorbance at 340nm against amino nitrogen (mg/L) to calculate the amount of free amino groups.

Two (2) ml of hydrolysate from specified hydrolysis times were added to test tubes containing 10ml of hot 1% SDS, shaken and kept at 75°C by immersing in a water bath for at least 15mins. The contents of the test tube were transferred to a 50ml volumetric flask and the volume made up using 1% SDS solution. Aliquots of 0.25ml were then transferred to test tubes containing 2.0ml of 0.2125M pH 8.2 sodium phosphate buffer, to which 2ml of 0.1% TNBS was added, shaken and incubated at 50C in the dark for 60minutes. The reaction was terminated by lowering the pH using 4ml of 0.1N HCL. It was then allowed to cool to room temperature for about 30minutes before absorbencies at 340nm were read using Thermo Spectronic Spectrophotometer (Genesys 6, Thermo Electron Co., Madison, WI).

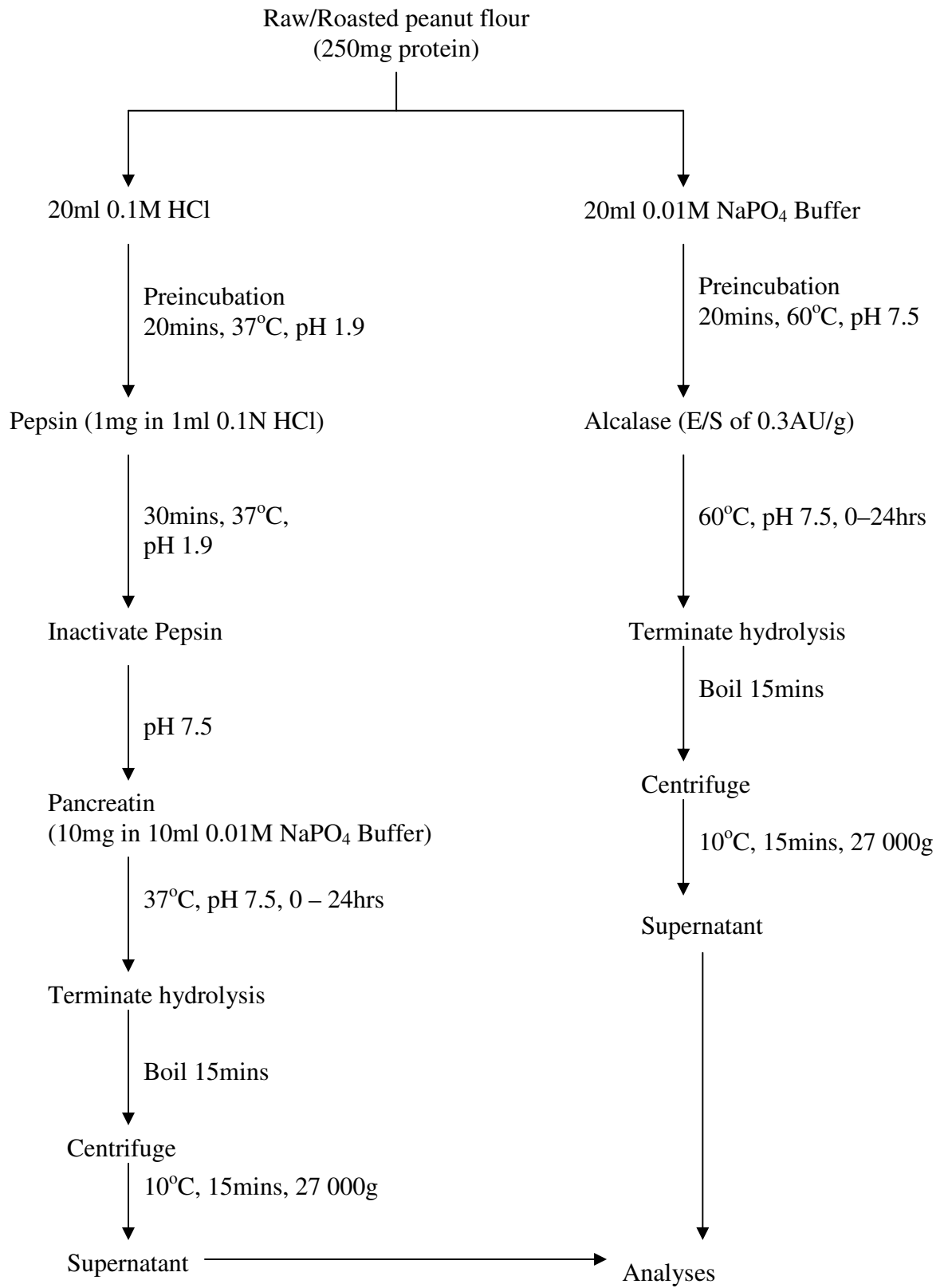


Fig 3.1 Flow Chart for the Enzymatic Hydrolysis Process

DH values were calculated from the formula

$$\%DH = ((AN_2 - AN_1)/Npb) 100$$

where AN_1 and AN_2 are the amino nitrogen content (mg/g protein) before and after hydrolysis respectively. Npb is the nitrogen content of the peptide bond (mg/g protein) in the protein substrate; a value of 183.15 is used for peanut protein. AN_1 and AN_2 values were obtained by reference to the L-Leucine standard curve and divided by the protein content of the sample.

Triplicate determinations were used for %DH values.

SDS-PAGE

The method of Laemmli (1970) with modifications was used, with Bio-Rad protean II system (Bio-Rad, Hercules, CA). Bio Rad pre-cast 16 X 16cm 12% Tris- HCl gels were used. Molecular weight markers with 6.9, 20, 29, 37, 53.5, 97, 115.7, and 194 kDa weights were used as reference. The protein hydrolysates were diluted in a 1:1 ratio with sample buffer constituted with Laemmli sample solution to which 10% mercaptoethanol was added. The denaturation of the protein was enhanced by boiling the resultant mix for 4minutes. 27 μ l of the resulting solutions (100 μ g peptide from the pepsin-pancreatin digests and 190 μ g from the alcalase digests) were then loaded into the wells. Electrophoresis was run at 35mA for stacking and 45mA for separation. Stain solution containing 0.08% Comassie Brilliant Blue G-250, 1.6% Phosphoric Acid, 8% Ammonium Sulfate and 20% Methanol were used to stain the gels overnight. The gels were dried after destaining in stain wash containing 1% acetic acid and 1% glycerol in water.

RESULTS AND DISCUSSION

Amino Nitrogen equivalent curve (Fig 3.2) was generated from the (0-3mM) L-Leucine standard curve (Fig 3.3). 1mmol Leucine is equivalent to 1mmol (14mg) Amino Nitrogen.

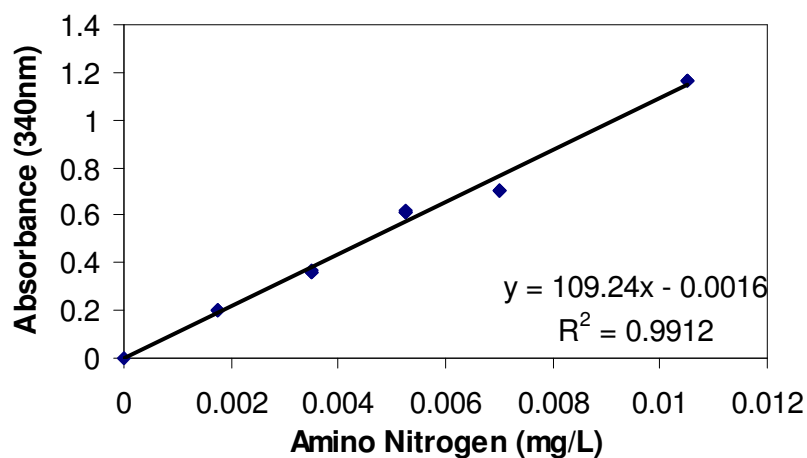


Fig. 3.2 Amino Nitrogen Standard Curve

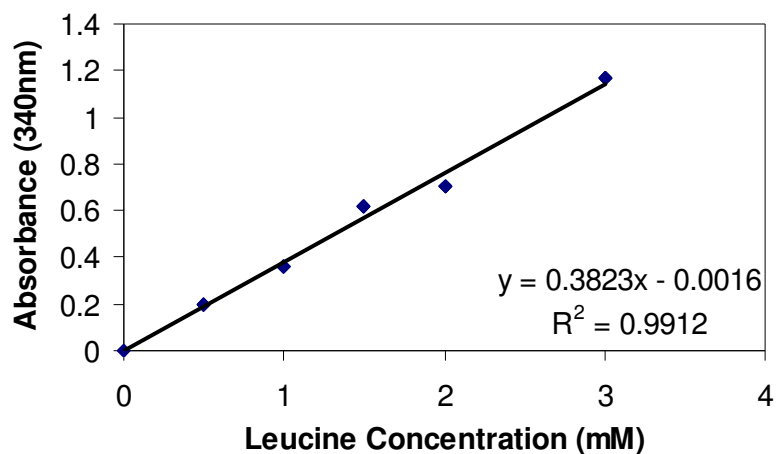


Fig. 3.3 (0-3mM) L-Leucine Standard Curve

The enzymes were inactivated by the treatment with hot 1% SDS, and the prolonged heat treatment not only inactivated the enzymes but also served to disperse the protein hydrolysate (Alder-Nissen, 1979).

DH helps to monitor the rate and extent of protein breakdown. The slope of the hydrolysis curve represents the rate of the reaction. The degree of hydrolysis increased with time (Fig 3.4 – 3.7). That of raw peanut and roasted peanut hydrolyzed with alcalase increased from 0% to 22.6% over a 24 hour period, and from 0% to 20.6% respectively. Raw and roasted peanut hydrolyzed with pepsin-pancreatin records an increase in hydrolysis from 0% to 21.4%, and 0% to 25.1% respectively.

The shape of the hydrolysis curve is characteristic of the protein-enzyme system and hydrolysis parameters which includes enzyme and substrate concentrations, pH and temperature (Alder-Nissen, 1977).

The general trend of hydrolysis for the roasted flour is noted to be more rapid than that of the raw flour (Fig. 3.6 and 3.7). Hydrolysis with alcalase is noted to be accelerated for the first six hours after which it begins to plateau (Fig 3. 4 and 3.6), whereas that with pepsin-pancreatin proceeds at a relatively slower rate and plateaus off 12 hours (Fig 3.5 and 3.7). The plateaus are characteristic of the rate of the reactions which are represented by the slope of the curves which level off after a certain DH is attained. At this point the constant rate of reaction observed is due to the fact that all peptide bonds susceptible to enzymatic hydrolysis have been cleaved.

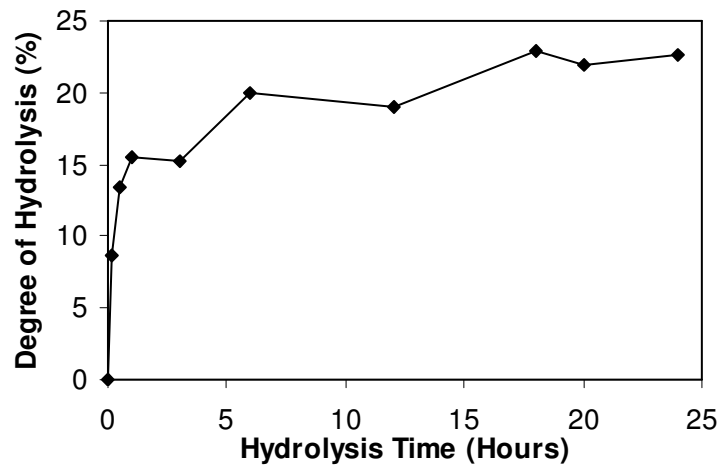


Fig. 3.4 Hydrolysis with Time of Raw Peanut Flour with Alcalase

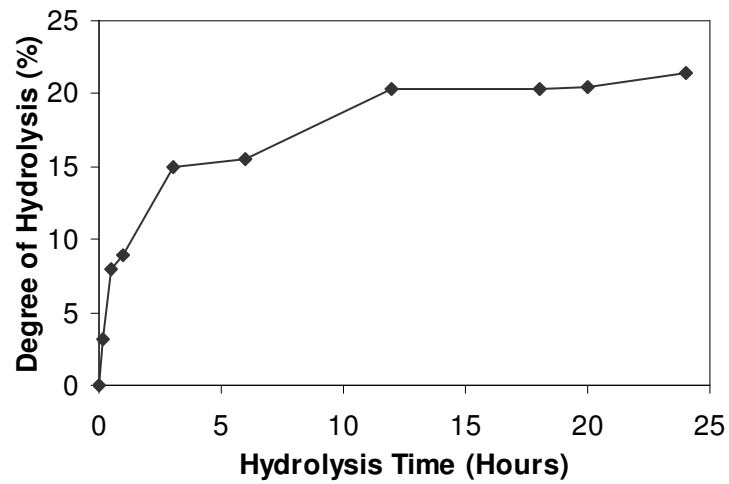


Fig. 3.5 Hydrolysis with Time of Raw Peanut Flour with Pepsin-Pancreatin

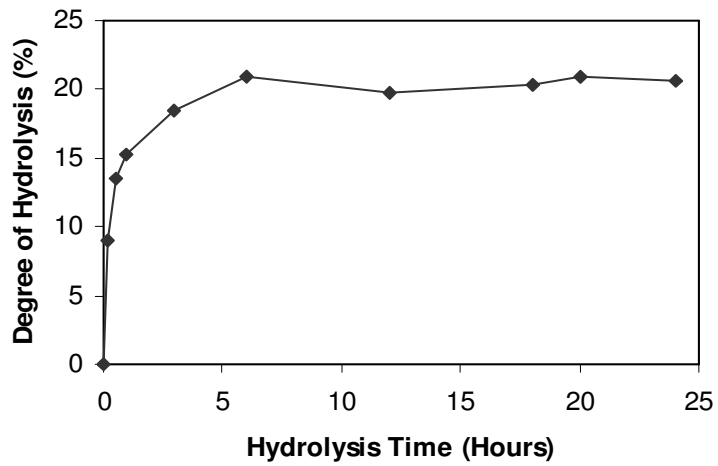


Fig. 3.6 Hydrolysis with Time of Roasted Peanut Flour with Alcalase

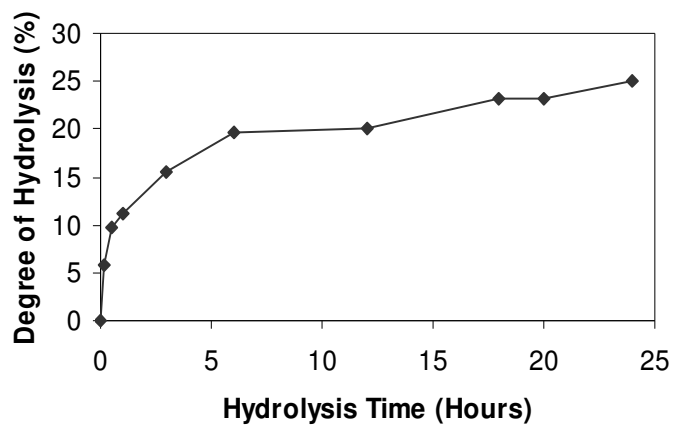
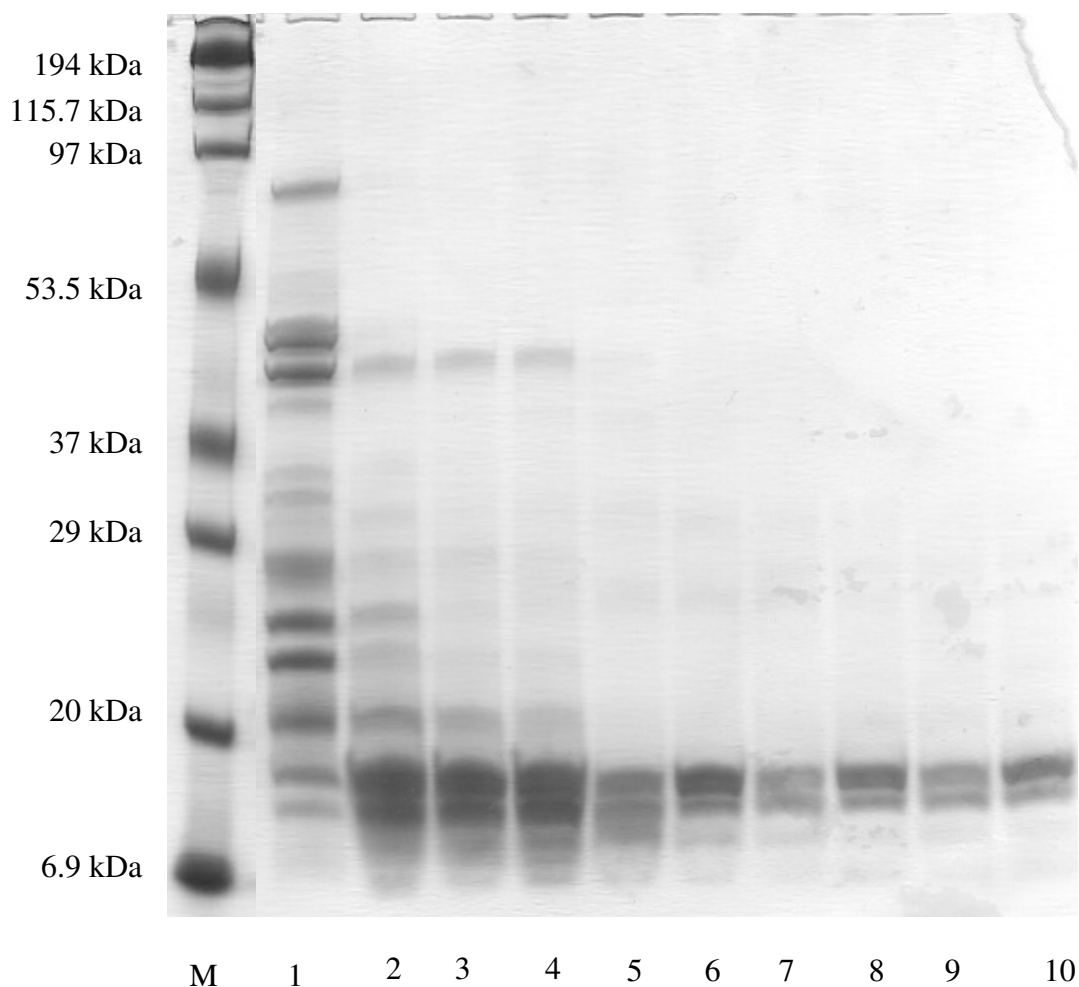


Fig. 3.7 Hydrolysis with Time of Roasted Peanut Flour with Pepsin-Pancreatin

When subject to SDS-PAGE, peanut protein subunits are divided into 5 main classes namely the conarachin region (MW > 50kDa); acidic arachin region (MW 38 – 49.9kDa); the intermediate MW region (23 – 37.9kDa); basic arachin region (MW 18 – 22.9kDa) and the low MW protein (14 – 17.9kDa) (Bianchi-Hall *et al.*, 1993).

SDS-PAGE zymograms were used to study the molecule weights distribution of proteins and peptides before and after hydrolysis and it shows that most bands corresponding to peanut protein had disappeared after 3 hours of hydrolysis. Progressive and complete disintegration of several protein bands were observed with increasing hydrolysis time. The zymograms of the intact raw peanut proteins show 7 bands in the conarachin region which also represents subunits of the 7S globulins, 5 in the acidic arachin region, and 4 distinct bands and a cluster in the basic arachin (basic 11S globulins) and the low MW proteins (Figs. 3.8 and 3.9). The intact roasted peanut pattern is not much different from the raw. There was a reduction in number of the subunits of 7S proteins; 4 distinct bands with a cluster. It shows 4 distinct bands each together with a cluster in both the acidic and basic arachins regions (Figs. 3.10 and 3. 11). Similar patterns of peptides were observed for the 2 treatments and enzymes used (Fig. 3.8 – 3.11). The bands of <20KDa which represents subunits of the low MW proteins in the case of the pepsin-pancreatin system however persisted to the end of hydrolysis. The bands from the raw peanut system persisted longer than those of the roasted system and this could be attributed to the fact that heat treatment degrades certain anti nutritional factors that may have slowed down hydrolysis, and thus the rapid observed degradation of protein subunits of the roasted peanut samples. Generally, the number of subunits and their intensities in each region decreased with increasing hydrolysis time.

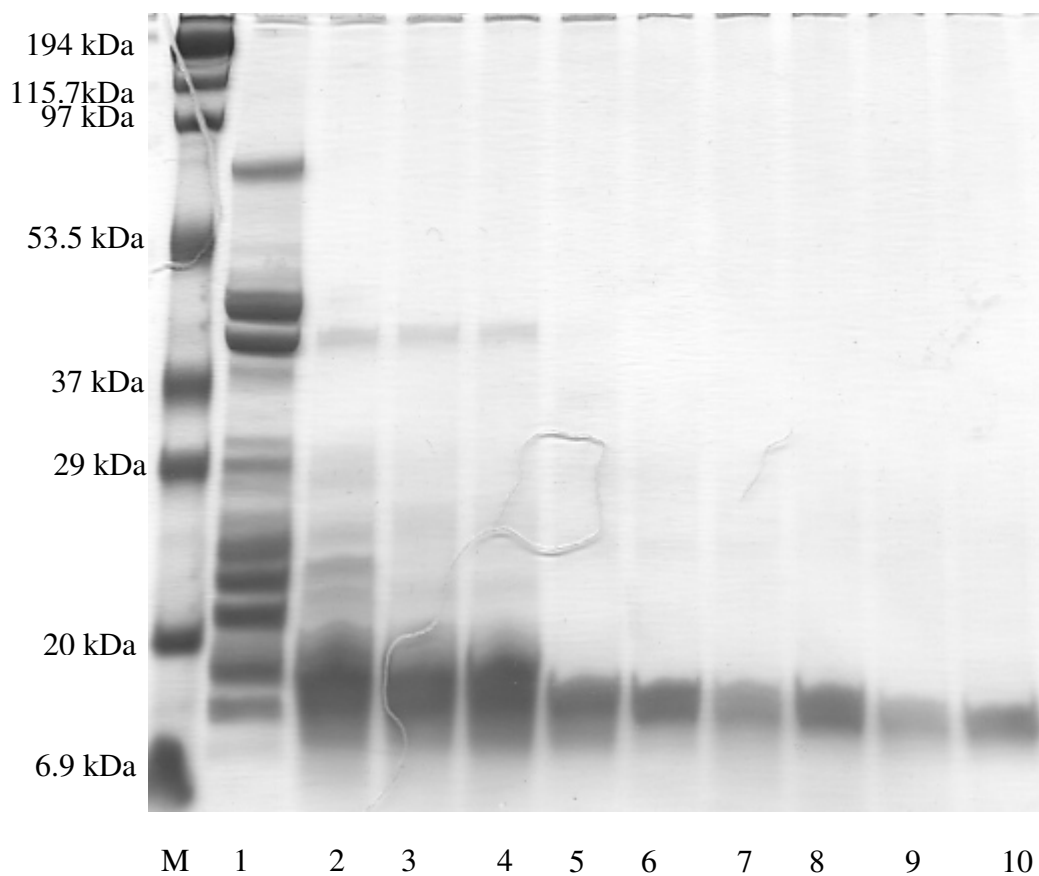
At 10mins of hydrolysis with DH of 8.7%, 1 distinct visible band was observed in the conarachin, 2 in the acidic 11S, 5 in the intermediate region, and 1 distinct together with a cluster in the basic 11S and low MW proteins regions for raw peanut flour hydrolyzed with alcalase. Up to 3 hours of hydrolysis (15.2% DH), 1 band persisted in the acidic 11S region. Subunits in the intermediate and basic arachin regions persisted until 12hours of hydrolysis. At the end of hydrolysis at 24hours (22.9% DH) visible bands were those <20kDa (Fig. 3.8).



Lane Legend: M (Molecular Marker); 1 (Unhydrolyzed Sample); 2 (10mins); 3 (30mins); 4 (1hour); 5 (3hours); 6 (6hours); 7 (12hours); 8 (18hours); 9 (20hours); 10 (24hours). 190 μ g peanut protein loaded into each well.

Fig 3.8 SDS PAGE of Raw Peanut Hydrolyzed with Alcalase

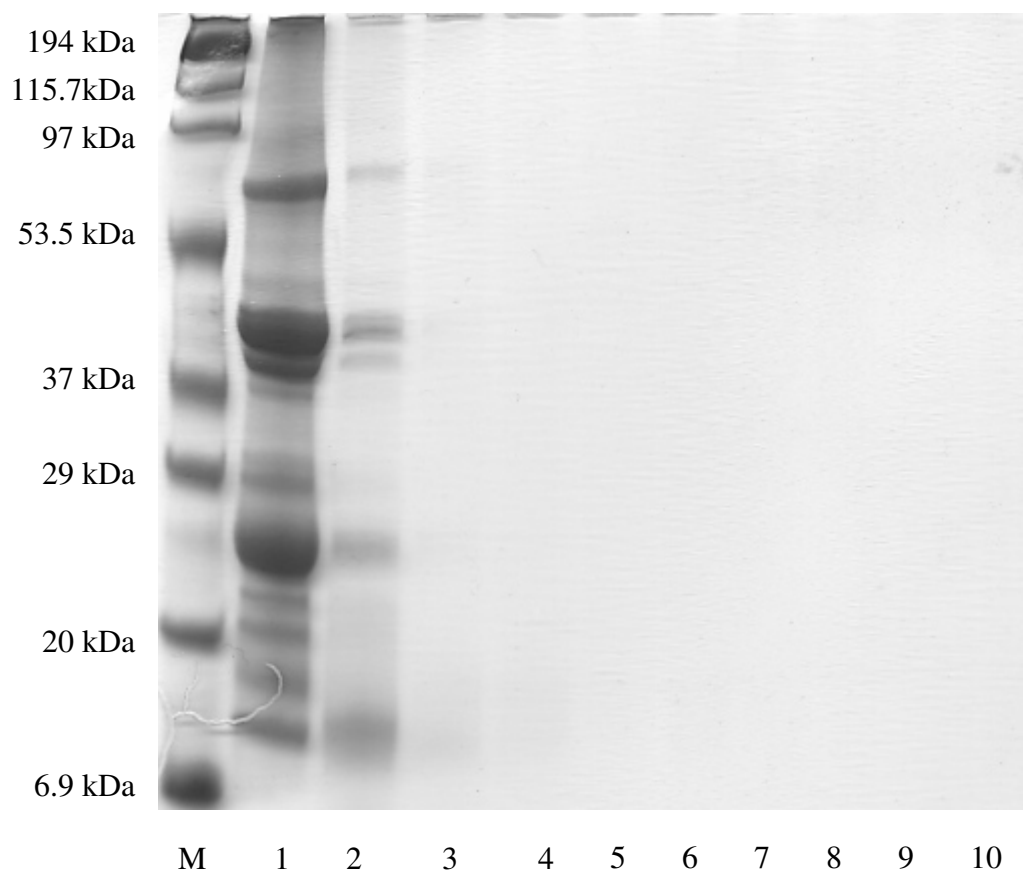
The SDS-PAGE bands in the case of raw peanut hydrolyzed with pepsin-pancreatin is similar to that of raw peanut hydrolyzed with alcalase. The conarachin subunits disintegrated at 10mins with the subunits of the acidic arachins persisting until after 1 hour of hydrolysis (8.9% DH). At 12 hours of hydrolysis with recorded DH of 20.3% protein subunits in the intermediate-basic arachin were visible with decreasing intensities as hydrolysis proceeded. At the end of the 24 hours of hydrolysis, only bands of <20kDa persisted representative of the low MW peptides and a residual of the basic arachins were observed (Fig. 3.9).



Lane Legend: M (Molecular Marker); 1 (Unhydrolyzed Sample); 2 (10mins); 3 (30mins); 4 (1hour); 5 (3hours); 6 (6hours); 7 (12hours); 8 (18hours); 9 (20hours); 10 (24hours). 100 μ g peanut protein loaded into each well.

Fig 3.9 SDS PAGE of Raw Peanut Hydrolyzed with Pepsin-Pancreatin

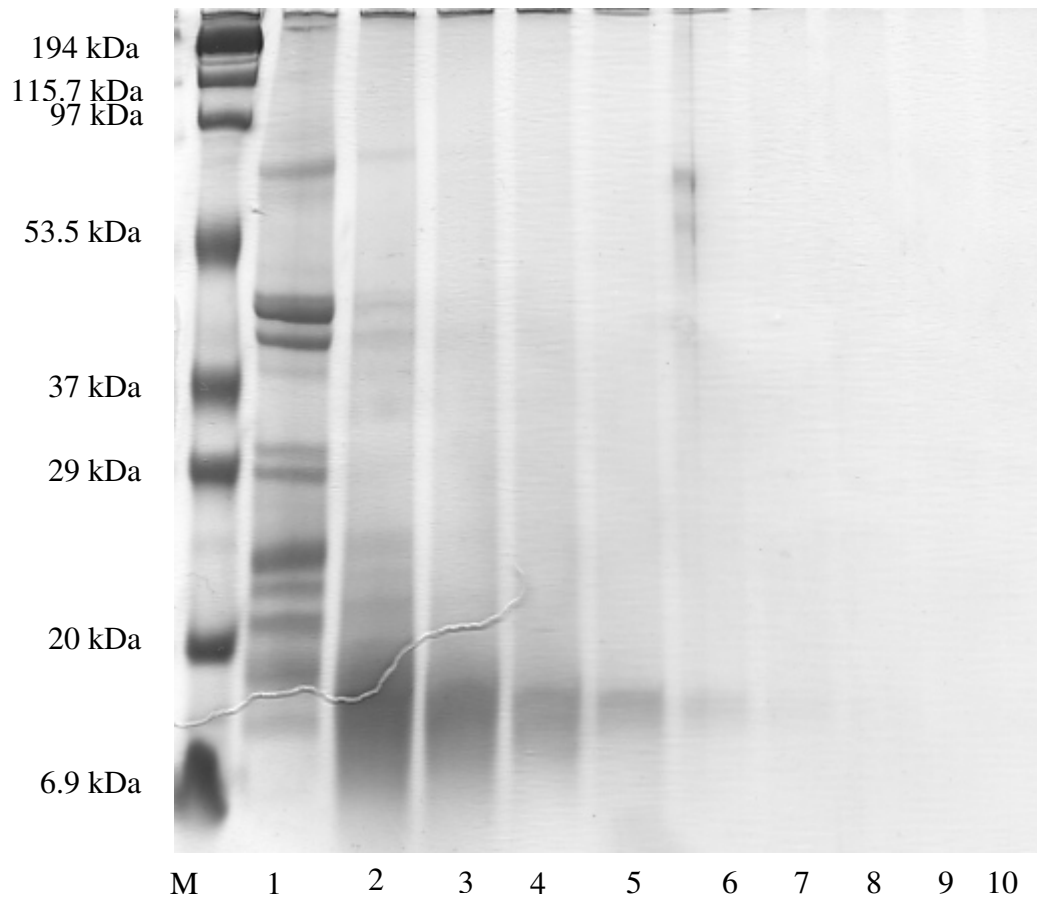
In the case of roasted peanut hydrolyzed with alcalase, there were no visible bands after an hour of hydrolysis (9.1% DH). A residual cluster of the low MW peptides were observed till 1 hour of hydrolysis. At 10mins of hydrolysis, there was 1 identified subunit in the conarachin region and 3 each in the acidic and basic arachin regions (Fig. 3.10).



Lane Legend: M (Molecular Marker); 1 (Unhydrolyzed Sample); 2 (10mins); 3 (30mins); 4 (1hour); 5 (3hours); 6 (6hours); 7 (12hours); 8 (18hours); 9 (20hours); 10 (24hours). 190 μ g peanut protein loaded into each well.

Fig 3.10 SDS PAGE of Roasted Peanut Hydrolyzed with Alcalase

At 10mins of hydrolysis with DH of 5.8%, there was 1 observed subunit in the conarachin region and 2 subunits of the acidic arachins. Protein bands were disintegrated to bands of less than 29kDa which were minimized to less than 20kDa corresponding to subunits of the basic arachins and low MW peptides, at 30mins of hydrolysis (9.6% DH) and persisted until 12hours (20.1%DH) after which no bands were visible (Fig. 3.11).



Lane Legend: M (Molecular Marker); 1 (Unhydrolyzed Sample); 2 (10mins); 3 (30mins); 4 (1hour); 5 (3hours); 6 (6hours); 7 (12hours); 8 (18hours); 9 (20hours); 10 (24hours). 100 μ g peanut protein loaded into each well.

Fig 3.11 SDS PAGE of Roasted Peanut Hydrolyzed with Pepsin-Pancreatin

More peanut hydrolysate protein from the alcalase system (190µg/ml) was loaded into the wells compared to 100µg/ml from the pepsin-pancreatin system for the SDS-PAGE. This was due to the fact that in loading 100µg/ml hydrolysate protein from the alcalase system, the separated protein subunits were very faint compared to the intensities when 190µg/ml was loaded.

The findings from the zymograms are consistent with other legume protein studies. In a study by Clemente (2000b) it is reported that at a DH >9% of chickpea protein isolate no electrophoretic bands exist in the conarachin region, there is a residual presence in the acidic arachin region, and a diffused enriched pattern in the low MW proteins. In this same study using flavourzyme which is an exopeptidase, it was observed that even at 27% DH, the basic polypeptide chains of the basic arachins remained unaltered which was similar to the patterns representative of the hydrolysis with pepsin-pancreatin. It was also reported that the alcalase hydrolysates did not show electrophoretic bands at 27% DH. In this current research however the absence of electrophoretic bands is observed at 13.6% DH for the roasted-alcalase set up, and 20.1% DH for the roasted-pepsin-pancreatin set up. This difference in observation for the legume proteins could be due to modification of the protein subunits by the heat treatment. The absence of protein bands is attributed to the fact that in that hydrolyzed state there is a high proportion of small peptides and free amino acids with low MW proteins.

Statistical analyses reveal that the type of enzyme used and the duration of hydrolysis significantly ($p \leq 0.05$) affected the degree of hydrolysis, treatment given to the peanut however had no significant effect (Table 3.1).

Six to Twelve hours, and 20 to 24 hours of hydrolysis were not significantly different, however 12 to 18 hours of hydrolysis recorded significant differences (Table 3.1). Hydrolysis with alcalase had statistical significant higher DH values than that with pepsin-pancreatin (Table 3.1). This is confirmed by the fact that SDS-PAGE results reveal that the alcalase mediated hydrolysis show rapid band disintegration. Though there were variation in the degree of hydrolysis of the peanut treatment type and the roasted peanut recorded higher DH values, Duncan grouping shows that roasting had no significant effect ($p \geq 0.05$) on hydrolysis (Table 3.1) which is contrary to what was expected because of the elimination of antinutritional factors and enzyme inhibitors with heat treatment, and also since SDS-PAGE shows a progressive and complete degeneration of protein bands in the roasted peanut systems.

CONCLUSION

The alcalase system showed a more extensive hydrolysis compared to the pepsin-pancreatin system, and will therefore be a better choice for the production of peptides for protein supplementation and other uses. The generation of bitter hydrolysates is a force to reckon with. The bitter taste is due to hydrophobic peptides. Alcalase has broad specificity with some preference for terminal hydrophobic amino acids. Literature has it that hydrolysates produced with enzymes that have hydrophobic amino acid specificity have a less bitter taste (Lahl and Braun, 1994; Alder-Nissen, 1986). This fact reinforces alcalase being a better choice for the generation of hydrolysates.

The trend of hydrolysis is verified in the electrophoresis results where it is observed that the bands from the alcalase system disintegrated faster with time. The basic

arachin and low molecular weight protein subunits of the raw peanuts were resistant to enzyme hydrolysis up to 24 hours.

Peanuts can be added to the legume protein hydrolysates source list for supplementary feeding and other physiologic needs.

FUTURE RESEARCH / RECOMMENDATIONS

It will be interesting to compare the effect on hydrolysis of both defatted and regular peanut flour. In the case of the alcalase system, it will be useful to include flavorzyme to represent the exopeptidase portion of the hydrolysis. It is also recommended that the hydrolysates be run on peptide gels for the separation of peptides <20kDa to enhance analyses of the protein bands.

ACKNOWLEDGEMENT

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Table 3.1 Data Showing the Effect of Time, Treatment and Enzyme Type on the Degree of Hydrolysis*

PARAMETERS			
Time (Hours)	Treatment	Enzyme	
0 ^g	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
0.167 ^f	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
0.5 ^e	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
1 ^e	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
3 ^d	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
6 ^c	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
12 ^{b, c}	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
18 ^{a, b}	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
20 ^{a, b}	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
24 ^a	Raw ¹	Alcalase ¹	
	Roast ¹	Pepsin-Pancreatin ²	
F-Value	97.88 [~]	3.28 [#]	6.44 [~]

*Parameters in same columns not followed by the same letter or figure are significantly different ($\alpha = 0.05$) as determined by Duncan's Grouping. Increasing numeric value signifies decreasing activity or response. [~] Value is significant at 0.05. [#] Value is insignificant at $p \geq 0.05$.

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CHAPTER 4
ANGIOTENSIN CONVERTING ENZYME INHIBITORY ACTIVITY OF
PROTEOLYTIC PEANUT DIGESTS¹

¹E. E. Quist, R. D. Phillips, F. K. Saalia, and L. Wicker. To be submitted to The Journal of Agriculture and Food Chemistry.

ABSTRACT

Angiotensin I converting enzyme (ACE) plays a major role in the regulation of blood pressure. It was determined that proteolytic peanut digests have an inhibitory effect on the activity of ACE. Defatted raw and roasted peanut flours were hydrolyzed with alcalase or sequentially with pepsin and pancreatin and tested for hypotensive potential.

Three fractions from the hydrophobic end of the chromatogram of each hydrolysate were the most potent for inhibiting ACE activity in comparison to 7 other fractions. These potentially potent fractions were then assayed for IC_{50} . Fractions from alcalase system for raw peanut recorded IC_{50} values of 8.7 $\mu\text{g/ml}$ to 122 $\mu\text{g/ml}$, with that of the roasted recording values of 12 $\mu\text{g/ml}$ to 235 $\mu\text{g/ml}$. IC_{50} values of 7.9 $\mu\text{g/ml}$ to 65.9 $\mu\text{g/ml}$, and 11 $\mu\text{g/ml}$ to 36 $\mu\text{g/ml}$ for raw and roasted peanut respectively from the pepsin-pancreatin system were observed. These values are in comparison to the IC_{50} value of 0.36 $\mu\text{g/ml}$ of a known commercial ACE inhibitor (pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro).

KEYWORDS: Angiotensin converting enzyme (ACE), ACE inhibition, bioactive peptides, IC_{50} , Peanut protein hydrolysates.

INTRODUCTION

Hypertension is a major health issue resulting from diverse genetic and environmental factors (Egan *et al.*, 2004; Opie, 1994). In the US alone about 60, 000, 000 individuals have blood pressure conditions that require medical attention; hypertension is no longer relegated to the middle aged and elderly, but also young adults and teenagers due to the epidemic of obesity and sedentary lifestyles in recent years (Egan *et al.*, 2004). Bioactive peptides from both animal and plant sources have been shown to have physiological properties including antihypertensive activity.

ACE a dipeptidyl carboxypeptidase is known to play a vital role in the regulation of blood pressure. It achieves this by converting Angiotensin I (an inactive decapeptide) into Angiotensin II (a salt retaining octapeptide and vasoconstrictor) (Opie, 1994) by cleaving a dipeptide from the carboxyl terminal of Angiotensin I (Kostis *et al.*, 1987), and inactivates bradykinin (a vasodilator nonpeptide) by the same mechanism (Odentti *et al.*, 1977). Therefore inhibiting ACE cumulates in positive hypotensive effect.

One of the major discoveries in cardiovascular pharmacology was the isolation of ACE inhibitor from the venom of Brazilian snake *Bothrops jararaca*. It is said to have been described for the first time by Ferreira in 1965 (Odentti *et al.*, 1971). According to Opie (1994) and Kostis *et al.* (1987), it was this discovery, which led to the systematic search and development of synthetic oral inhibitors. The pharmacologic interference with the renin-angiotensin system and intervention effort led to the development of captopril by Cushman and associates, and enalapril by Patchett and associates in 1977 and 1980 respectively (Kostis *et al.*, 1987). The efficiency of ACE inhibitors reducing blood pressure in hypertensives is 40-50% in the patients when used as a monotherapy,

and 80-90% of patients when used in combination with a diuretic (DeFelice and Kostis, 1987).

ACE inhibitors are a recent addition to cardiovascular drugs. The successes of captopril and enalapril have led to research involving the sequencing and synthesis of amino acids involved in the regulation of cardiac activity (DeFelice and Kostis, 1987). ACE inhibitory activity depends on the peptide's amino acid composition and sequence which are directly related to the specificity of the enzyme used and the hydrolysis conditions. ACE inhibitory peptides are continuously formed and degraded during hydrolysis; maximum ACE inhibition in hydrolysis is an optimum between the two processes (van der Van *et al.*, 2002). Most ACE inhibitors are competitive (Vermeirssen *et al.*, 2002; Meisel, 1997).

The blockade of the renin-angiotensin-aldosterone system is the mode of action of ACE inhibitors to curb hypertension. ACE Inhibitors therefore inhibit the conversion of angiotensin I to angiotensin II and increases bradykinin levels. ACE inhibitors have been identified and isolated from a varied array of plant and animal sources including mushrooms (Lee *et al.*, 2004); skeletal muscle (Arihara *et al.*, 2001); fish scales (Fahmi *et al.*, 2004); fermented foods (Gibbs *et al.*, 2004; Hernández-Ledesma *et al.*, 2004); sunflower seeds (Megías *et al.*, 2004); soy (Wu and Ding, 2002; Gibbs *et al.*, 2004), chickpeas (Yust *et al.*, 2003) and peas (Vermeirssen *et al.*, 2005). The fact that ACE inhibitors are diverse and derived from different proteins by different enzymes and hydrolysis times indicates that a variety of peptides with various amino acid sequences are able to inhibit ACE (van der Van *et al.*, 2002).

Peanut is the fourth most important oilseed in the world (USDA, 2004) and considered a major source of edible oils and protein meals valuable in human nutrition (Nwokolo, 1996) because of its amino acid profile. In this *in vitro* study, pepsin-pancreatin was used to simulate human digestion, and alcalase was used from the industrial point of view.

To the best of our knowledge peanut protein as a source of hypotensive agents has not been studied. The fact that other legumes exhibit such properties led to the design of this research.

MATERIALS AND METHODS

1. MATERIALS

ACE (EC 3.4.15.1) from rabbit lung was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). ACE reagent N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) was purchased from Sigma-Trinity Biotech (Wicklow, Ireland). Synthesized ACE inhibitor was purchased from Sigma. Water and Acetonitrile (purchased from Fisher Scientific Co., Suwanee, GA) were of HPLC grade.

2. METHODOLOGY

General ACE Inhibition

The method of Holmquist *et al.* (1979) with modifications by Vermeirssen *et al.* (2002) was used. Five hundred micro liters each of hydrolysates and ACE substrate, N-[3-(2-furyl)acryloyl]-L-phenylalanylglycine (FAPGG) were mixed together and preincubated for 2min at 37°C. One hundred micro liters of ACE (0.02units; 18.18 units/l

reaction mixture) was added to the mixture and absorbance measured at 340nm after 10mins using a temperature control Thermo Spectronic Spectrophotometer (Genesys 6, Thermo Electron Co., Madison, WI). By Sigma's definition, one unit of ACE produces 1.0 μmol of hippuric acid from Hippuryl-His-Leu per min in 50mM HEPES and 300mM NaCl at pH 8.3 at 37°C. ACE hydrolyzes FAPGG to furylacryloylphenylalanine (FAP) and glycylglycine (GG) which is detected by a decrease in absorbance at 340nm (Sigma-Trinity Biotech).

Chromatographic Analyses

The hydrolysate with the observed maximum inhibition in each batch was analyzed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC). The HPLC system comprised a Waters 2690 Separations Module and a Waters 996 Diode Array Detector, and the chromatographic separations were performed on a reverse phase C-12 Phenomenex preparative column (259 X 10mm Jupiter 4u Proteo 90A) which is optimized by the manufacturer for peptide separation.

The mobile phase was A - Acidified Water (0.1% TFA), B - Acidified Acetonitrile (0.1% TFA) and C- Acetonitrile at a flow rate of 1ml min^{-1} . The gradient used was 100% A at 0mins changing to 30%A with 70% B at 75mins; 60% B with 40% C at 80mins; 100% A at 90mins. This gradient gave great resolution and was optimized by comparing different applied gradients.

Hundred (100) μl volume of each hydrolysate was injected and eluted analytes were detected at 210nm. Chromatographs were divided into 10 fractions which were collected in tubes using a Spectra/Chrom CF-1 fraction collector. Collected fractions

were dried under low heat and a stream of nitrogen and reconstituted in 0.01M sodium phosphate buffer (pH 7.5). Spectrophotometric assay to determine peptide concentrations were performed on Thermo Spectronic Spectrophotometer (Genesys 6, Thermo Electron Co., Madison, WI) at 210nm and 225nm.

Peptide concentration ($\mu\text{g/ml}$) was calculated using the formula

$$\text{Concentration} = (A_{210} - A_{225})144$$

Specific ACE Inhibition

The reconstituted fractions were tested for ACE inhibitory action by mixing equal amounts of peptide (1.2 $\mu\text{g}/500\mu\text{l}$ to 2.5 $\mu\text{g}/500\mu\text{l}$) in each fraction with 500 μl of ACE substrate, incubating with 100 μl of ACE and taking OD readings after 10minutes. The fractions with the corresponding highest activity were then assayed for IC_{50} . The relation between ACE activity and peanut hydrolysate concentration was fitted to a sigmoid curve by plotting absorbance by the log of concentration. IC_{50} which is the inhibitor concentration needed to inhibit 50% of enzyme activity was calculated from the plot by fitting the data to the Marquardt-Levenberg four parameter model;

$$y = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + 10^{(\log \text{IC}_{50} - \log \text{Conc}) b}}$$

where y is the observed absorbance, Min and Max represent the baseline of 0% inhibition and asymptote of 100% activity respectively, and b is indicative of the slope at the inflexion point which is the point of IC_{50} . The commercial peptide was used to set the 0% and 100% limit of ACE inhibitory activity.

RESULTS AND DISCUSSION

The increase in absorbance with increasing inhibitor concentration was used as a measure for enzyme activity. Below a given inhibitor concentration, there is no observed ACE inhibitory activity. At this point inhibitory activity is said to be 0% and ACE activity is said to be 100%. Above a certain concentration ACE inhibitory activity is fairly constant; at this point 100% inhibition is reached and increasing concentration has no effect. These activity-log concentration profile plots results in a sigmoid curve (Fig. 4.1). Fig. 4.1 shows the sigmoid curves obtained from the commercial peptides, and that of fraction VI of two of the samples used in this study.

After about 12 hours of hydrolysis no increase in ACE inhibition was observed. At this point the biopeptide degradation dominates the formation of new peptides, and this is the point of optimal (not maximum) activity (Vermirssen *et al.*, 2003).

The chromatograms from four hydrolysates (either raw or roasted flour hydrolyzed with either alcalase or sequentially with pepsin and pancreatin) were divided into 10 fractions (Fig. 4.2 – 4.5) and assayed for ACE inhibitory activity. The hydrophobic ends of the chromatograms were determined to have greatest inhibitory power. Fractions VI, VIII and IX corresponding to retention times 60 -62mins, 72 – 74mins and 76-78mins respectively from each hydrolysate proved to be most potent and were evaluated for IC_{50} ; Table 4.1 shows the IC_{50} values from the said fractions. The commercial peptide used recorded an IC_{50} value of 0.36 μ g/ml.

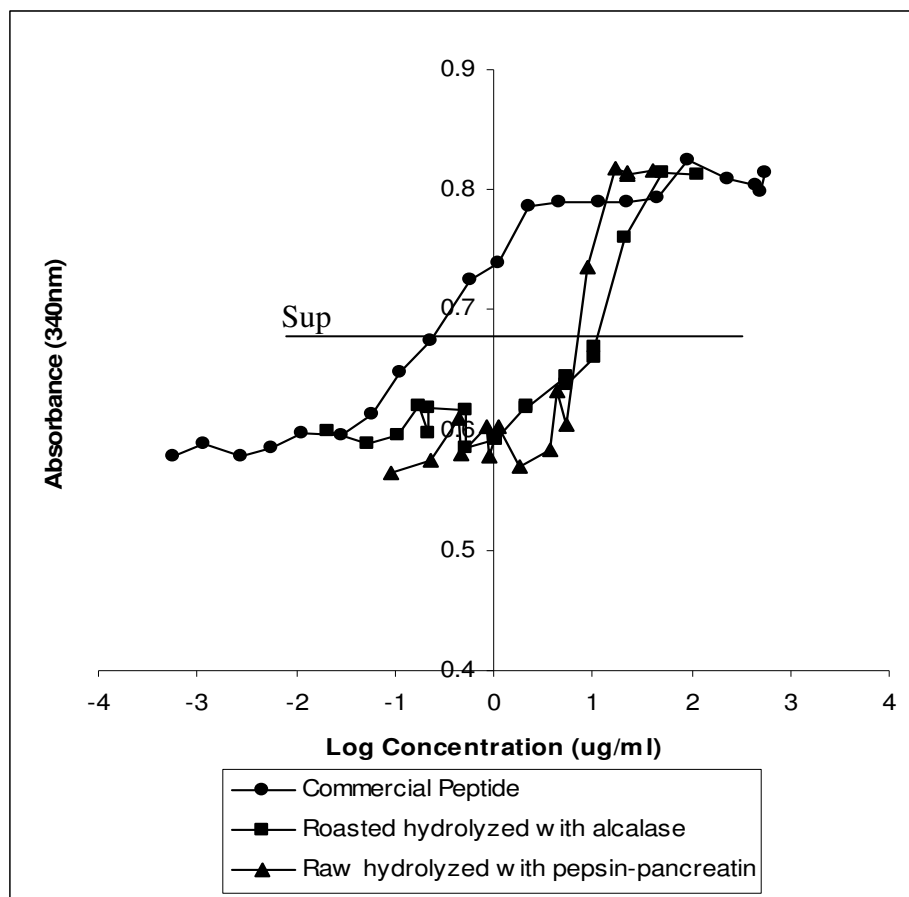


Fig. 4.1 ACE Inhibitory Activity Curves

Table 4.1 IC₅₀ Values of Chromatographic Fractions

Fraction ID	Enzyme System/Peanut Treatment		Enzyme System/Peanut Treatment	
	Alcalase IC ₅₀ (µg/ml)		Pepsin-Pancreatin IC ₅₀ (µg/ml)	
	Raw	Roasted	Raw	Roasted
VI	8.66	11.95	7.93	11.09
VIII	215.33	105.66	188.71	99.82
IX	122.29	235.29	65.86	35.91

The order of potency of ACE inhibitory activity of the fractions is VI > VIII > IX. Statistical analyses reveal that fraction VI is significantly different ($p \leq 0.05$) from VIII and IX, and there is no significant difference in the potency of fractions VIII and XI (Table 4.2).

Table 4.2 Effect of Retention Time, Enzyme System and Peanut Treatment on IC_{50} *

	Parameters		
	Retention Time (mins)	Treatment	Enzyme
60 – 62 ^a	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ¹
		Alcalase ¹	Pepsin-Pancreatin ¹
72 – 74 ^b	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ¹
		Alcalase ¹	Pepsin-Pancreatin ¹
76 – 78 ^b	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ¹
		Alcalase ¹	Pepsin-Pancreatin ¹
F-value	6 [~]	0.27 [#]	1.93 [#]

*Parameters in same columns not followed by the same letter or figure are significantly different ($\alpha = 0.05$) as determined by Duncan's grouping. [~] Value is significant at 0.05. [#] Value is insignificant at $p \geq 0.05$.

The obtained IC_{50} are consistent with that obtained from other legumes, and that of fraction VI is recognized to be most potent among document source of antihypertensive peptides from legume sources. In a study by Yust *et al.* (2003), the IC_{50} values of chickpeas are reported to range from 11 - 21 μ g/ml depending on the enzyme system used, and these potent fractions were derived from the hydrophobic end of the chromatograms. Soy (Wu and Ding, 2002) and peas (Vermeirssen *et al.*, 2005) have been researched to have IC_{50} values of 65 μ g/ml and 70 μ g/ml respectively. Rapeseed is also reported to exhibit antihypertensive activity with IC_{50} values of 160 – 1300 μ g/ml (Marczak *et al.*, 2003).

Statistical analysis on the whole digests show that raw peanuts possess significantly ($p \leq 0.05$) higher inhibitory power on ACE activity than the roasted. It also reveals that the alcalase system produces significantly more potent antihypertensive peptides than those from the pepsin-pancreatin system (Table 4.3). This finding is correlated to the observations in Chapter 3 where the alcalase mediated hydrolysates are observed to have significantly higher rates and degrees of hydrolysis.

Though the whole digests of raw peanut are observed to have higher ACE inhibitory potency, for the chromatographic fractions, statistical analyses indicate that the processing treatment has no significant effect ($p \geq 0.05$) on the ACE inhibitory power. The enzyme system used also had no significant effect on the IC_{50} values of the chromatographic fractions. This observation could be attributed to the fact that the peptide content of the hydrolysates may be identical. This is also observed in the similarity of the chromatogram peaks of all the four hydrolysates analyzed.

Table 4.3 Data Showing the Effect of Time, Treatment and Enzyme Type on ACE Inhibition*

PARAMETERS			
Time (Hours)	Treatment	Enzyme	
0 ^c	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ²
	Roast ²	Alcalase ¹	Pepsin-Pancreatin ²
0.167 ^{a, b}	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ²
	Roast ²	Alcalase ¹	Pepsin-Pancreatin ²
0.5 ^{a, b}	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ²
	Roast ²	Alcalase ¹	Pepsin-Pancreatin ²
1 ^a	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ²
	Roast ²	Alcalase ¹	Pepsin-Pancreatin ²
3 ^b	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ²
	Roast ²	Alcalase ¹	Pepsin-Pancreatin ²
6 ^{a, b}	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ²
	Roast ²	Alcalase ¹	Pepsin-Pancreatin ²
12 ^{a, b}	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ²
	Roast ²	Alcalase ¹	Pepsin-Pancreatin ²
18 ^b	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ²
	Roast ²	Alcalase ¹	Pepsin-Pancreatin ²
20 ^b	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ²
	Roast ²	Alcalase ¹	Pepsin-Pancreatin ²
24 ^{a, b}	Raw ¹	Alcalase ¹	Pepsin-Pancreatin ²
	Roast ²	Alcalase ¹	Pepsin-Pancreatin ²
F-Value	5.36 [~]	35.67 [~]	364.01 [~]

*Parameters in same columns not followed by the same letter or figure are significantly different ($\alpha = 0.05$) as determined by Duncan's grouping. Increasing numeric value signifies decreasing activity or response. [~] Value is significant at 0.05.

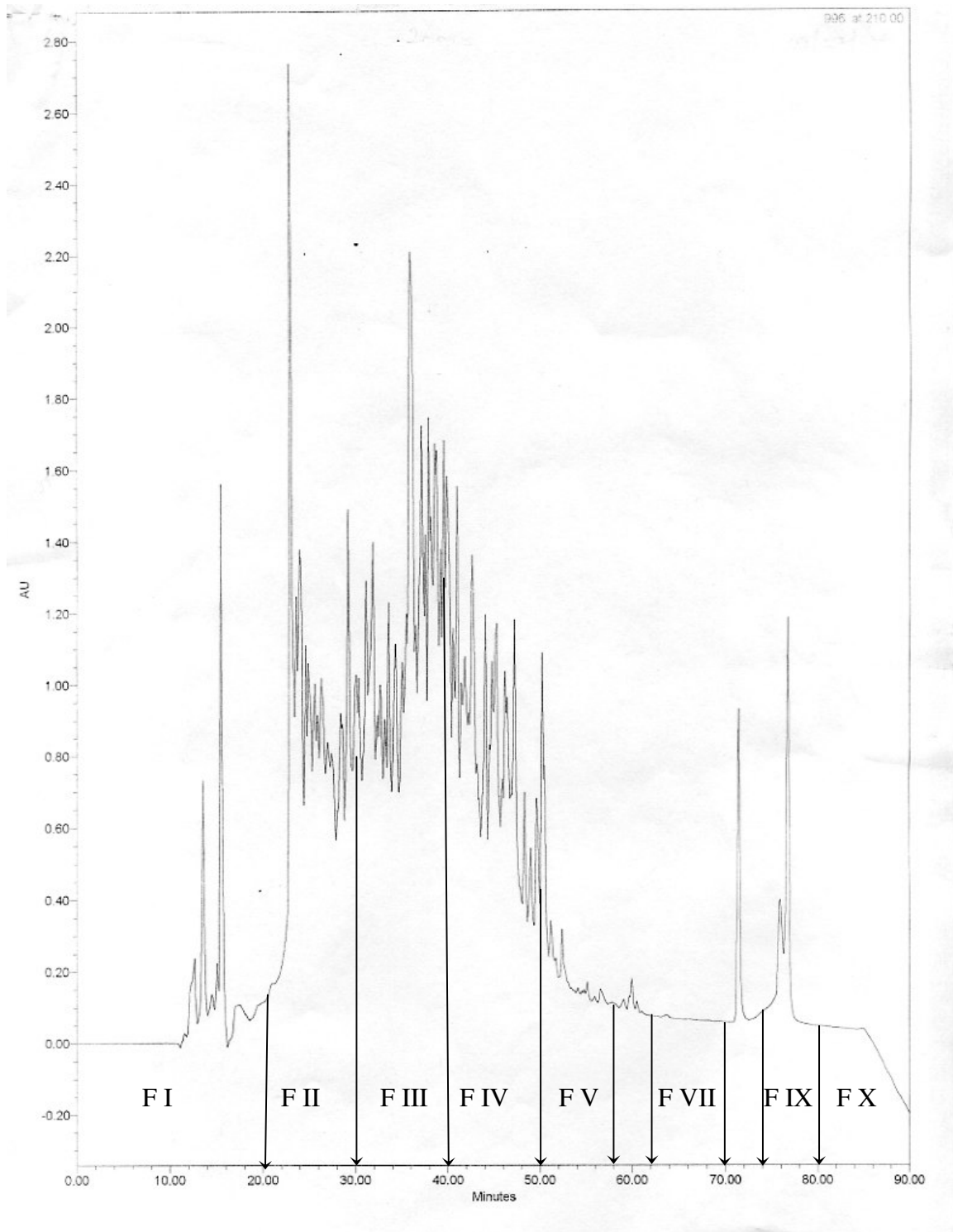


Fig 4.2 Chromatogram of Raw Peanut Flour Hydrolyzed with Alcalase

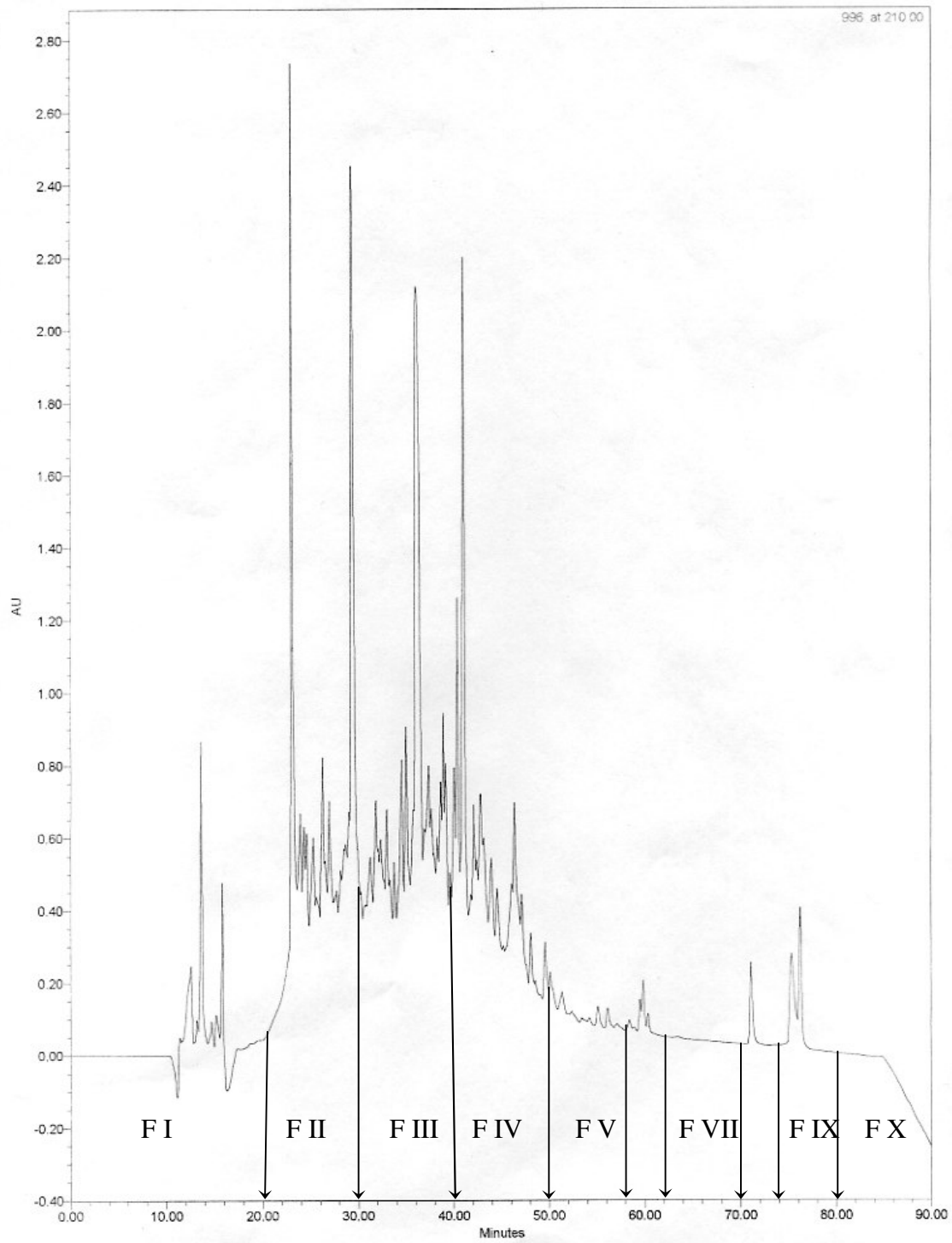


Fig 4.3 Chromatogram of Raw Peanut Flour Hydrolyzed with Pepsin and Pancreatin

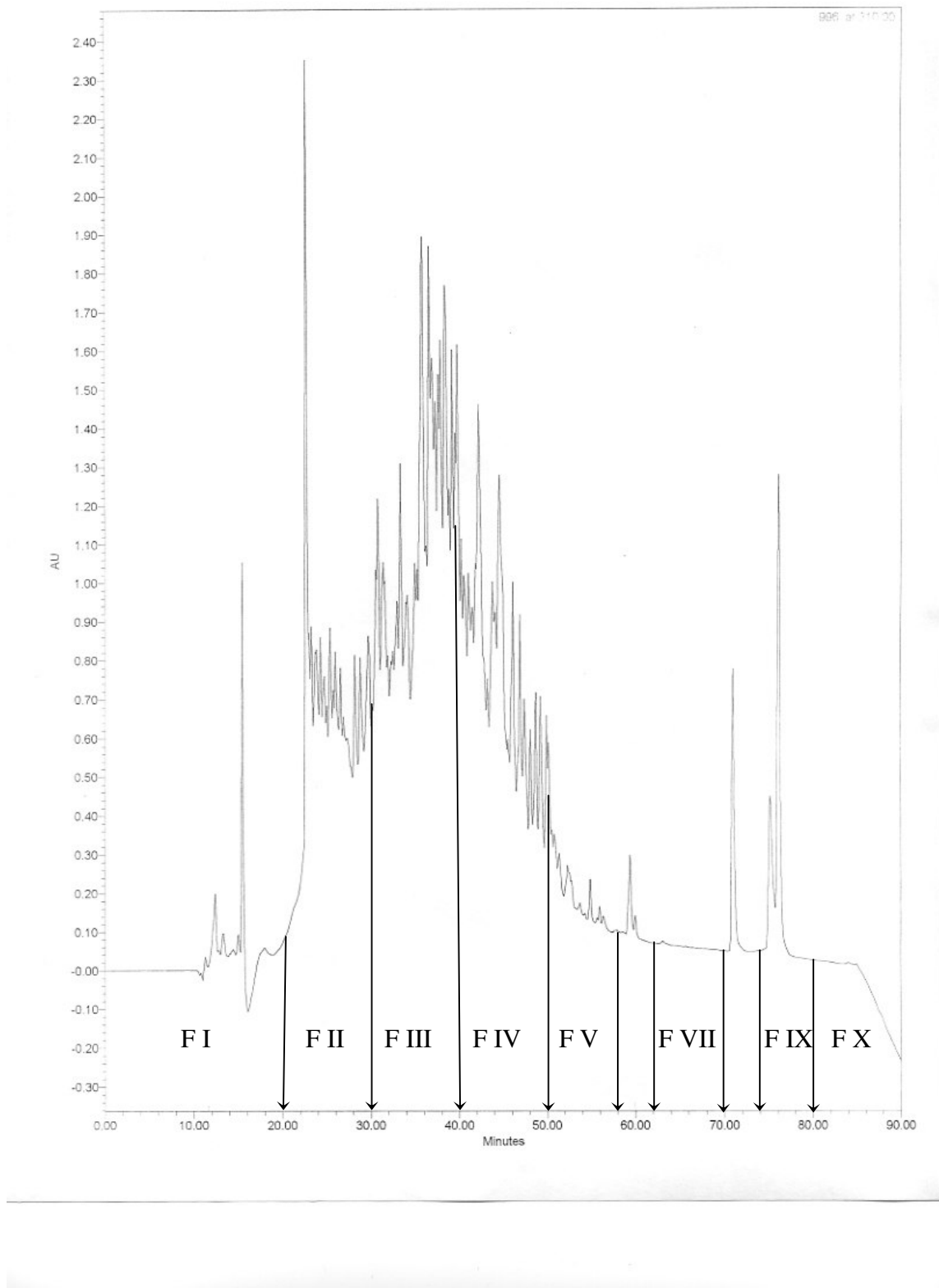


Fig 4.4 Chromatogram of Roasted Peanut Flour Hydrolyzed with Alcalase

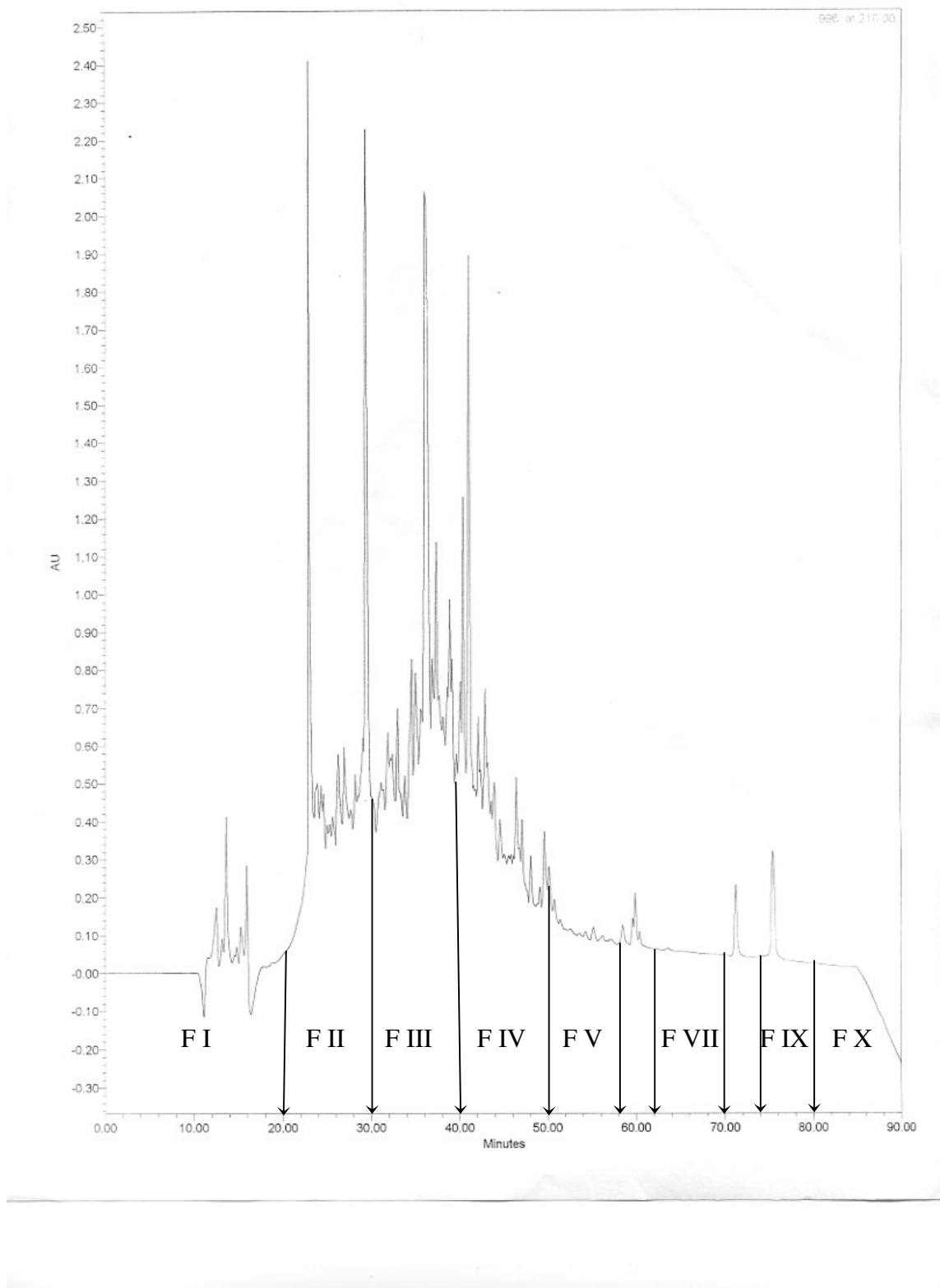


Fig 4.5 Chromatogram of Roasted Peanut Flour Hydrolyzed with Pepsin and Pancreatin

CONCLUSION

Defatted peanut flour may be useful in human nutrition as a source of hypotensive peptides since biologically active peptides are generated during digestion, and may also be utilized as an alternative to synthetic ACE inhibitory medication with undesirable side effects.

Since zymograms in Chapter 3 show the progressive disintegration of peanut protein subunits with hydrolysis leaving no visible bands in the case of roasted peanuts and low MW proteins for the raw at the end of hydrolysis, it can be inferred that small peptide units are responsible for ACE inhibitory activity and resultant decrease in blood pressure.

FUTURE RESEARCH / RECOMMENDATIONS

The antihypertensive activity *in vivo* should be tested. It is recommended that the identified fractions of highly potent ACE inhibitory activity be further purified with HPLC and resulting peaks from that be assayed for IC₅₀. Amino acid assay to identify the composition of the derived peptide is currently underway.

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CHAPTER 5**ANTIMICROBIAL ACTIVITY OF PROTEOLYTIC PEANUT DIGESTS¹**

¹ E. E. Quist, R. D. Phillips and J. Chen. To be submitted to Journal of Food Science.

ABSTRACT

Hydrolysates from raw and roasted peanut flour hydrolyzed with either alcalase or pepsin-pancreatin were tested for bactericidal properties. Antimicrobial activity was observed in a concentration dependant sequence. Concentrations of 0.1 -2mg hydrolyzed protein of each hydrolysate were tested against *Micrococcus luteus* (a susceptible 'indicator species') and pathogenic *Escherichia coli* O157:H7 and *Listeria monocytogenes*. Effectiveness of the hydrolysates decreased with decreasing concentrations, and higher lethality was observed for *L. monocytogenes* compared to *E. coli* O157:H7. The radii of inhibitory zone decreased from approximately 26 – 18mm for *M. luteus*; 13 – 4mm for *L. monocytogenes* and 5 – 2mm for *E. coli* O157:H7 with decreasing proteolytic digests concentrations.

Keywords: Alcalase, *Escherichia coli* O157:H7, Hydrolysates, *Listeria monocytogenes*, *Micrococcus luteus*, Peanut, Pepsin-Pancreatin.

INTRODUCTION

The presence of microorganisms in foods may be desirable or undesirable depending on the end use. Their presence in food may lead to enhancement in food quality, result in food spoilage and cause food poisoning on consumption. Foodborne pathogens cause outbreaks of infections resulting in some fatalities. The Centers for Disease Control and Prevention (CDC) estimates that 76 million Americans get sick, more than 300,000 are hospitalized, and 5,000 people die from foodborne illnesses annually (<http://www.cdc.gov/ncidod/diseases/food/index.htm>). With the onset of microbial resistance to conventional antimicrobials in the recent past, numerous studies have looked into a wide array of natural sources of antimicrobial of which peptides from plant and animal protein are a category. Some of these studies have included peptide sources from human and bovine lactoferrin (Samuelsen *et al.*, 2004); hen egg white lysozyme (Mine *et al.*, 2004); frog skin secretions (Chen *et al.*, 2005); insect venom (Medes *et al.*, 2005); cowpea seeds (Carvalho *et al.*, 2001) among many others. These biopeptides usually have a broad antimicrobial activity spectrum including both gram positives and negatives (López-Solanilla *et al.*, 2003) with an amino acid residue number of between 20 and 60, and function by permeabilization of the cell membranes of organisms (Birkemo *et al.*, 2004).

M. luteus is known to be a sensitive strain and has been used over the years as a model to set the threshold for numerous antimicrobial activities (Beckland *et al.*, 2002; Pérez *et al.*, 1999; and Hornstein *et al.*, 1997). With a resulting concentration based inhibitory activity against *M. luteus*, peanut protein digests from either alcalase and pepsin-pancreatin enzyme systems were tested against *E. coli* O157:H7 and *L.*

monocytogenes. An estimate of 73, 000 cases of *E .coli* O157:H7 infections occur in the US annually with an estimated 2, 100 hospitalization, and 3-5% of these cases are fatal as a result of patients developing hemolytic uremic syndrome (HUS) (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/escherichiacoli_t.htm). As of 1997, there were 2, 500 cases with 500 fatalities from listeriosis annually in the US. Though there is a reported 38% decline of cases between 1996 and 2002, outbreaks continue to occur (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/listeriosis_t.htm).

Escherichia coli is a common part of the normal facultative anaerobic microflora in the intestinal tract of humans. Most of the strains are harmless but some are pathogenic and cause diarrhea diseases. *E. coli* O157:H7 is the predominant cause of enterhemorrhagic associated diseases worldwide (Meng *et al.*, 2001). Undercooked ground beef has been the major vehicle associated with *E. coil* O157:H7 outbreaks, unpasteurized milk and juices are now known vehicles with the first outbreak from apple cider reported in 1991 in Southeastern Massachusetts (Besser *et al.*, 1993). Outbreaks in apple cider is usually associated with apple contaminated by contact with soil manure (Meng *et al.*, 2001), and in instances where ruminants frequented the orchard (Cody *et al.*, 1999) or cattle fields were close to the cider press (Besser *et al.*, 1993) and contamination could occur during processing. Since apple cider was traditionally unpasteurized pathogens that were present on the fruits were likely to be present in the cider. In a study by Zhao *et al.* (1993), they discovered that the use of 0.1% sodium benzoate substantially increased the safety of apple cider by decreasing the number of *E. coli* to undetectable population (reduction of $> 4\log_{10}$ CFU/ml). In the library of antimicrobial peptide studies, nutmeg (Takikawa *et al.*, 2002), garden thyme (Tepe *et al.*,

2004), and Lemon balm (Mimica-Dukic *et al.*, 2004) are among the identified plant sources of antimicrobial peptides against *E. coli*.

Food has been investigated as a major source of transmission of listeriosis since its first documented outbreak in 1981 (Swaminathan, 2001). *L. monocytogenes* is a common contaminant of the raw materials of animal and plant origin used by the food industry (López-Solanilla *et al.*, 2003). Poultry supports the growth of *L. monocytogenes* better than other meats and contamination of animal muscle tissue occur either from symptomatic or asymptomatic carriage of the organism before slaughter or contamination of the carcass after slaughter (Swaminathan, 2001). The organism according to Swaminathan (2001) attaches strongly to the surface of raw meat and is difficult to remove or inactivate. Sources of plant peptides that have shown antimicrobial activity against *L. monocytogenes* include wheat flour thionins and potato snakins (López-Solanilla *et al.*, 2003).

There is however limited information on the susceptibility of *E. coli* and *L. monocytogenes* to biopeptides from plant origins, and this study looks at the antimicrobial effect of hydrolyzed peanut protein on the named pathogens.

MATERIALS AND METHODS

1. MATERIALS

Micrococcus luteus ATCC 10240, *Escherichia coli* O157:H7 isolated from an apple cider outbreak in the United States, and *Listeria monocytogenes* isolated from a ground beef outbreak in the United States were used in this study. The cultures were retrieved from frozen storage and grown on tryptic soy agar (TSA) at 37°C for 18h. The resulting

cultures were transferred into tryptic soy broth (TSB) and incubated under the same conditions. Both agar and broth were purchased from Becton, Dickinson and Co., Sparks, MD.

2. METHODOLOGY

The antimicrobial activity of peanut biopeptides were determined using the procedure of Mine *et al.* (2004) was used with modifications.

Bacteria Purification

A loop full of each tested culture in TSB was transferred onto TSA agar plates and incubated overnight at 37°C of *E. coli* and *L. monocytogenes*, and 25°C for *M. luteus*. Colonies were picked and re-streaked onto fresh agar plates, and the inoculated plates were incubated at the appropriate temperatures. The resulting cultures were inoculated into 10ml TSB and incubated at the appropriate temperatures overnight. The optical densities of the bacterial suspensions were then measured at a wavelength of 600nm using a Novaspec II Spectrophotometer (Model 80-2088-64, Pharmacia Biotech, Cambridge, England). The bacteria cells were harvested by centrifuging at 3000rpm at 5°C for 10min. The harvested cells were washed with 0.1M potassium phosphate buffer (pH 7.0) by centrifuging and re-suspended in the same buffer to an optical density of 0.75 ± 0.05 at 600nm.

Antimicrobial Assay

The bacterial suspensions described above were then streaked onto TSA plates using sterile swabs. Wells of 1cm depth and 9mm diameter were implanted. The bottoms of the wells were lined with soft agar and then filled with appropriate concentrations of hydrolyzed peanut protein. Unhydrolyzed peanut flour in buffer was used as control. The plates were incubated for 24 hours and the radii of inhibition zones measured.

RESULTS AND DISCUSSION

At the initial stage of the research, antimicrobial activities of different concentrations of the peanut hydrolysates against *M. luteus* were evaluated. Given the high bactericidal activity on this strain which is widely used in antimicrobial screening, the hydrolysates were tested on two major foodborne pathogenic organisms, *E. coli* O157:H7 and *L. monocytogenes*.

The results show that for the antimicrobial activity of raw peanut protein hydrolysates of increasing concentration of 0.1mg to 2mg, the radii of inhibition zones increased from 17.8mm to 26mm for alcalase hydrolysates, and 17.5 mm to 25mm for pepsin-pancreatin hydrolysates against *M. luteus* (Table 5.1; Fig 5.1 – 5.5). In the case of roasted peanut, the inhibitory zones increased from 17.7mm to 26.3mm for increasing protein hydrolysates (0.1 – 2mg) of alcalase mediated hydrolyses, and 17.9mm to 24.7mm for the pepsin-pancreatin samples (Table 5.2; Fig. 5.1 – 5.5).

The radii of inhibition zones in the case of *L. monocytogenes* increased from 4.8mm to 13.2mm, and 3.6mm to 12.2 mm for raw alcalase and pepsin-pancreatin digests respectively (Table 5.1; Fig 5.6 – 5.10). Inhibitory zones of 4.5mm to 12.7mm, and 4mm

to 11.9mm were observed for 0.1mg to 2mg of roasted alcalase and pepsin-pancreatin digests respectively (Table 5.2; figure 5.6 – 5.10).

Table 5.1 Antimicrobial Activity of Raw Peanut Hydrolysates

Microorganism	Enzyme System	Diameter of Inhibition Zone ^a (mm)				
		2mg	1mg	0.5mg	0.25mg	0.1mg
<i>M. luteus</i>	Alcalase	26	24.5	22.8	18.9	17.8
	Pepsin-Pancreatin	25	22.3	18.9	18.1	17.5
<i>L. monocytogenes</i>	Alcalase	13.2	10.9	11.5	8.8	4.8 ^b
	Pepsin-Pancreatin	12.2	9.1	8.8	5.8	3.6 ^b
<i>E. coli</i> O157:H7	Alcalase	4.3	2.8 ^b	NZ	NZ	NZ
	Pepsin-Pancreatin	3.8	2.6 ^b	NZ	NZ	NZ

^a The Diameter of Inhibition Zone is the distance from the centre of the well to the outer boarder of the zone, and is representative of replicate analyses.

^b Hazy Zone

NZ: No Zone detected

Table 5.2 Antimicrobial Activity of Roasted Peanut Hydrolysates

Microorganism	Enzyme System	Diameter of Inhibition Zone ^a (mm)				
		2mg	1mg	0.5mg	0.25mg	0.1mg
<i>M. luteus</i>	Alcalase	26.3	24.1	22.6	18.9	17.7
	Pepsin-Pancreatin	24.7	21.8	18.8	17.8	17.9
<i>L. monocytogenes</i>	Alcalase	12.7	10.2	11.6	7.8	4.5 ^b
	Pepsin-Pancreatin	11.9	9.9	9.2	5.3	4 ^b
<i>E. coli</i> O157:H7	Alcalase	5.3	3.2 ^b	NZ	NZ	NZ
	Pepsin-Pancreatin	4.6	2.3 ^b	NZ	NZ	NZ

^a The Diameter of Inhibition Zone is the distance from the centre of the well to the outer boarder of the zone, and is representative of replicate analyses.

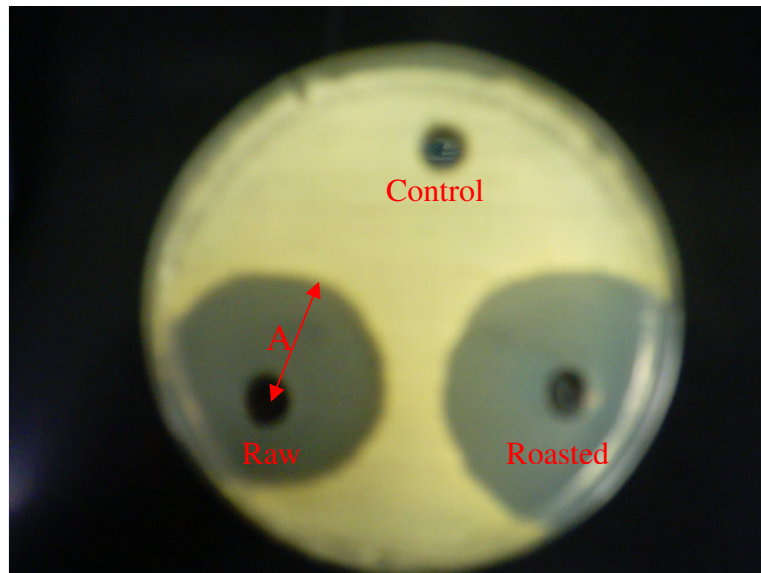
^b Hazy Zone

NZ: No Zone detected

Table 5.3 Bactericidal Effect of Treatment and Enzyme System, and Susceptibility of Microbes to Hydrolysates*

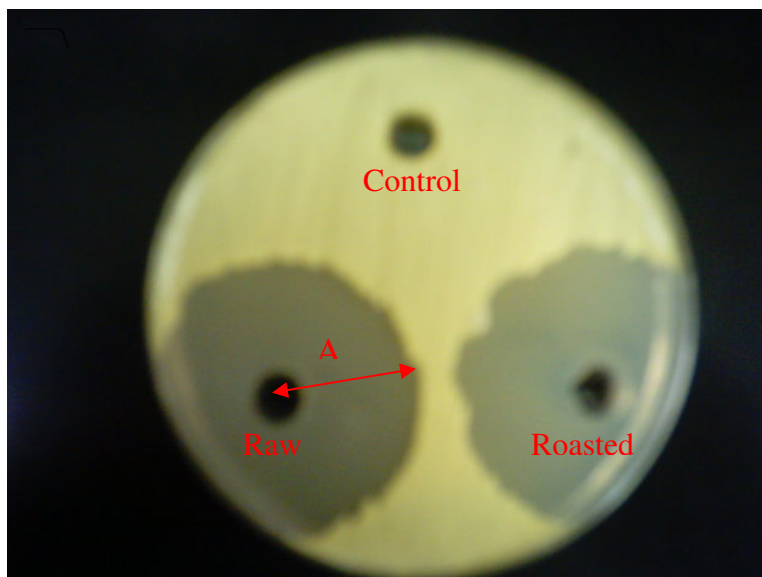
PARAMETERS			
Treatment	Enzyme System	Microorganism	
Raw ¹	Alcalase ¹	<i>M. luteus</i> ¹	
		<i>L. monocytogenes</i> ²	
		<i>E. coli</i> O15:H7 ³	
	Pepsin-Pancreatin ²	<i>M. luteus</i> ¹	
		<i>L. monocytogenes</i> ²	
		<i>E. coli</i> O15:H7 ³	
Roasted ¹	Alcalase ¹	<i>M. luteus</i> ¹	
		<i>L. monocytogenes</i> ²	
		<i>E. coli</i> O15:H7 ³	
	Pepsin-Pancreatin ²	<i>M. luteus</i> ¹	
		<i>L. monocytogenes</i> ²	
		<i>E. coli</i> O15:H7 ³	
F-Value	0.00 [#]	39.42 [~]	3617.56 [~]

*Parameters in same columns not followed by the same letter or figure are significantly different ($\alpha = 0.05$) as determined by Duncan's Grouping. Increasing numeric value signifies decreasing activity or response. The same pattern applies to the 0.1 – 2mg hydrolysate concentrations used. [~] Value is significant at 0.05. [#] Value is insignificant at $p \geq 0.05$.



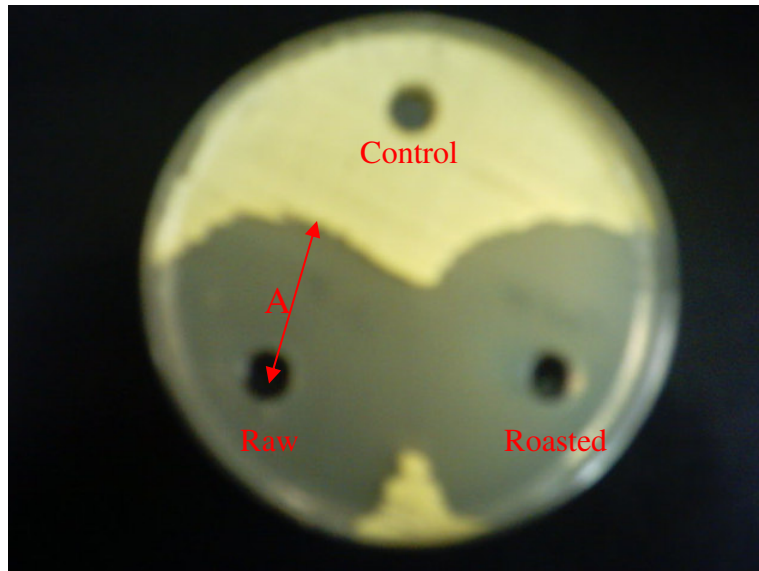
A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.1a Antimicrobial Activity of 0.1mg Peanut Hydrolyzed Protein against *M. luteus*



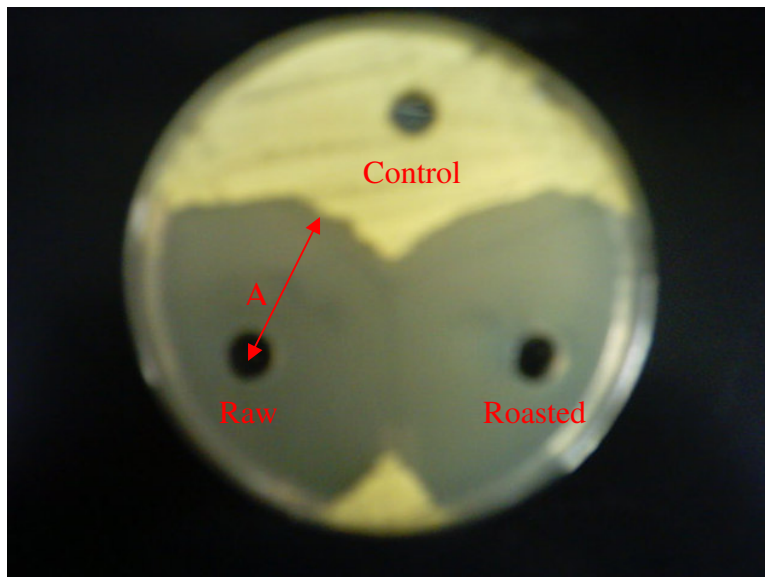
A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.1b Antimicrobial Activity of 0.25mg Peanut Hydrolyzed Protein against *M. luteus*



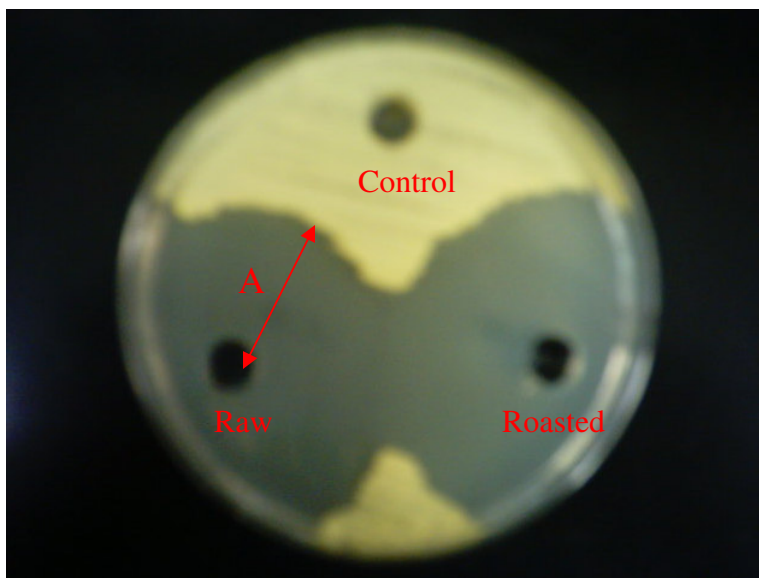
A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.1c Antimicrobial Activity of 0.5mg Peanut Hydrolyzed Protein against *M. luteus*



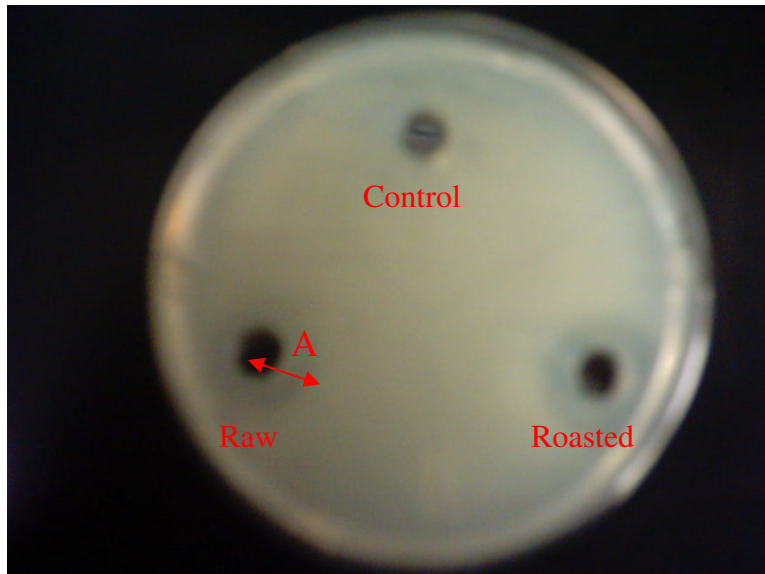
A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.1d Antimicrobial Activity of 1.0mg Peanut Hydrolyzed Protein against *M. luteus*



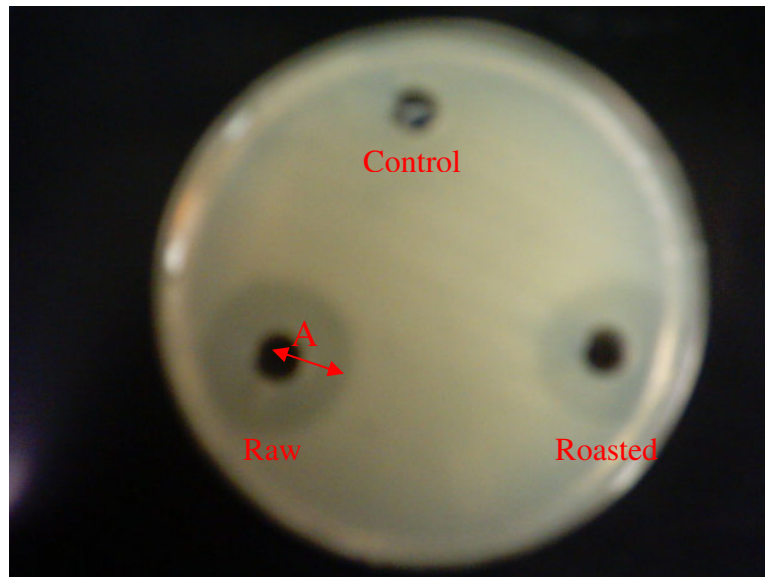
A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.1e Antimicrobial Activity of 2mg Peanut Hydrolyzed Protein against *M. luteus*



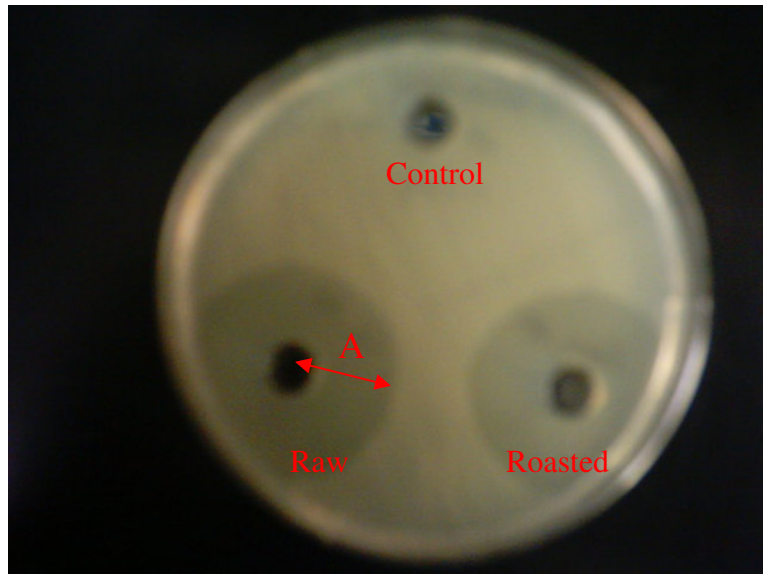
A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.2a Antimicrobial Activity of 0.1mg Peanut Hydrolyzed Protein against *L. monocytogenes*



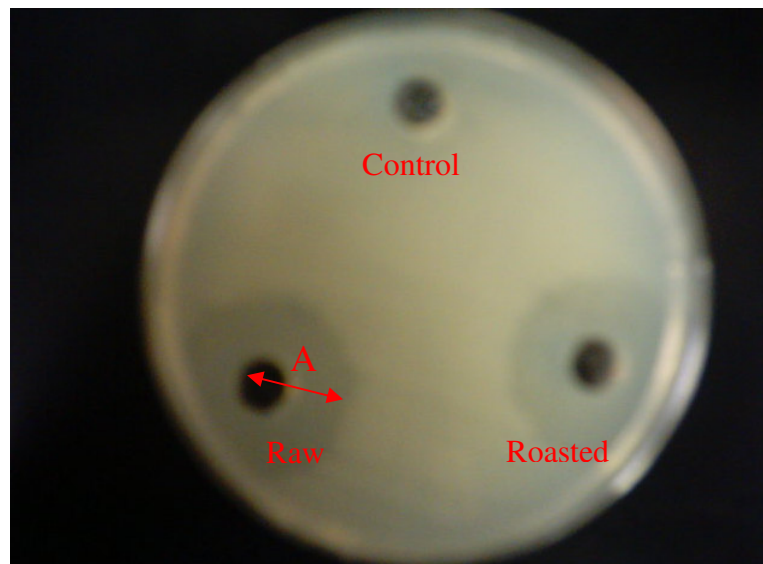
A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.2b Antimicrobial Activity of 0.25mg Peanut Hydrolyzed Protein against *L. monocytogenes*



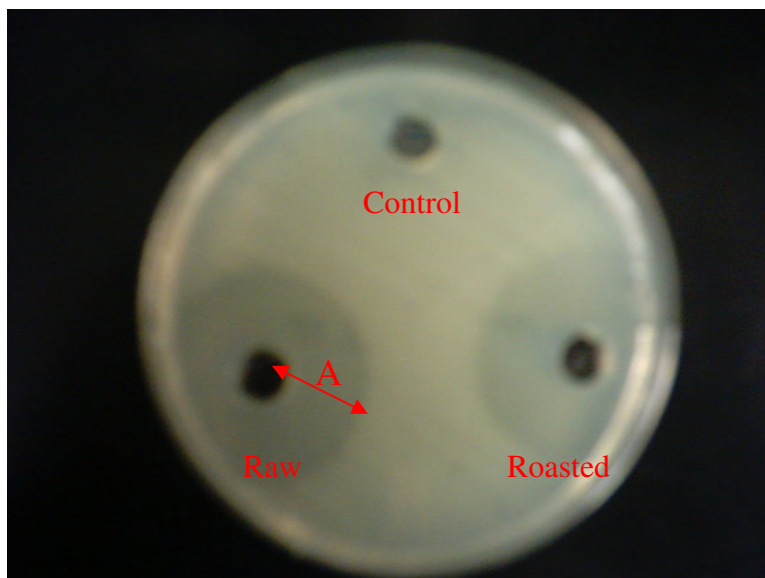
A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.2c Antimicrobial Activity of 0.5mg Peanut Hydrolyzed Protein against *L. monocytogenes*



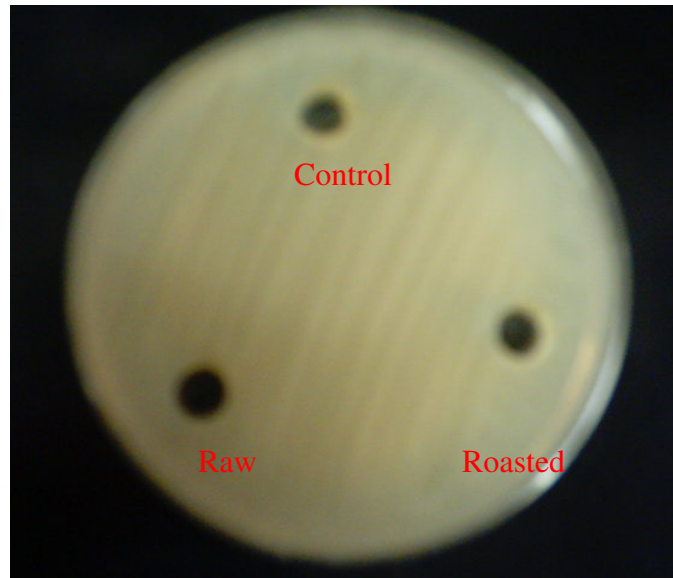
A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.2d Antimicrobial Activity of 1.0mg Peanut Hydrolyzed Protein against *L. monocytogenes*



A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

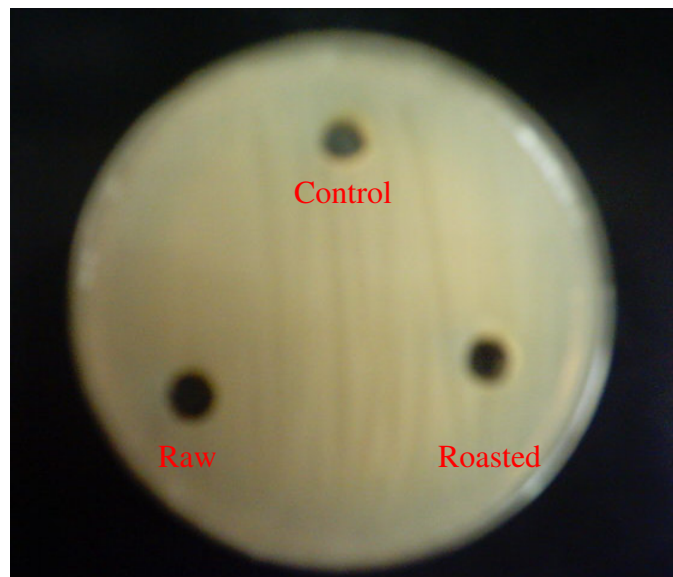
Fig 5.2e Antimicrobial Activity of 2.0mg Peanut Hydrolyzed Protein against *L. monocytogenes*



A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the

Fig 5.3a Antimicrobial Activity of 0.1mg Hydrolyzed Peanut Protein against *E. coli*

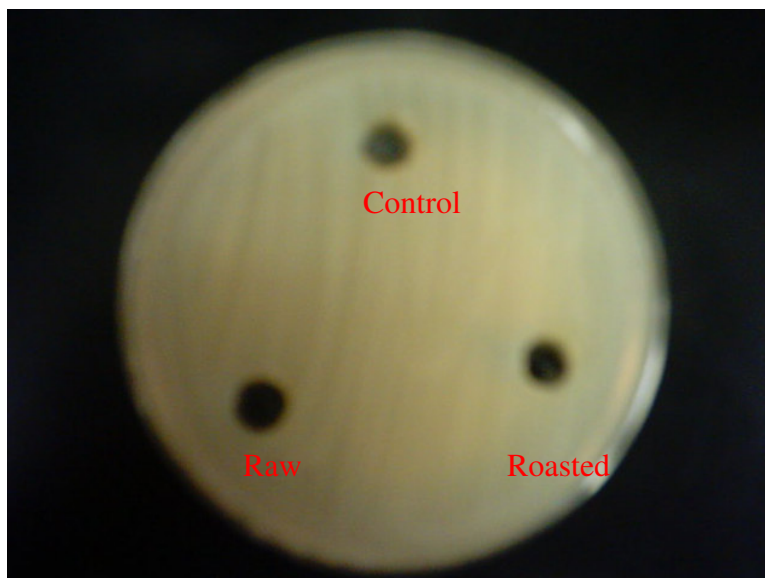
O157:H7



A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.3b Antimicrobial Activity of 0.25mg Hydrolyzed Peanut Protein against *E. coli*

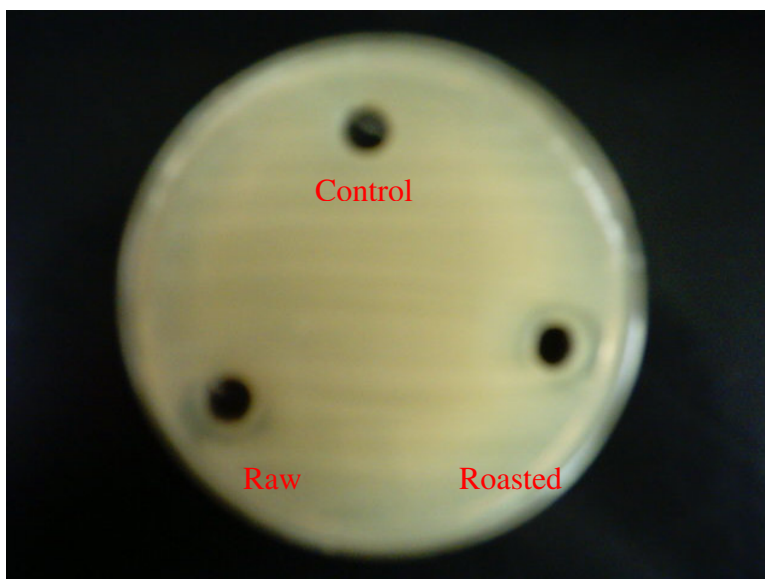
O157:H7



A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.3c Antimicrobial Activity of 0.5mg Hydrolyzed Peanut Protein against *E. coli*

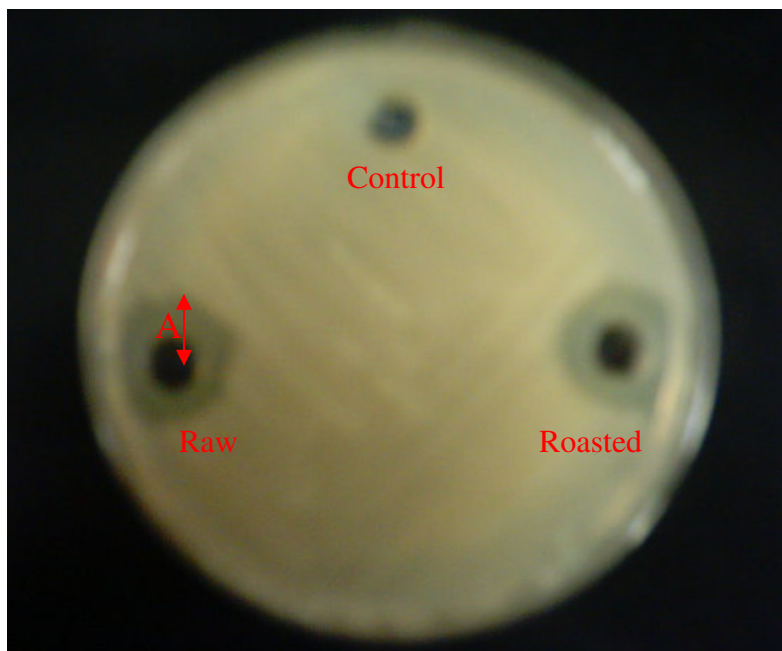
O157:H7



A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.3d Antimicrobial Activity of 1.0mg Hydrolyzed Peanut Protein against *E. coli*

O157:H7



A: The inhibition zone which is the distance from the centre of the well to the outer boarder of the zone

Fig 5.3e Antimicrobial Activity of 2.0mg Hydrolyzed Peanut Protein against *E. coli*

O157:H7

For *E. coli* O157:H7 however, there were no zones of inhibition observed for 0.1, 0.25 and 0.5 mg of protein digests but zones of 2.8mm and 4.3mm were observed for 1mg and 2mg respectively of raw alcalase hydrolysates, and 2.6mm and 3.8mm for 1mg and 2mg respectively of raw pepsin-pancreatin hydrolysates (Table 5.1; Fig 5.11 – 5.15).

No inhibitory zones were observed for 0.1, 0.25 and 0.5mg of both alcalase hydrolyzed proteins against *E. coli* O157:H7 just as was observed in the case of raw peanut protein digests. Inhibitory zones of 3.2mm and 5.3mm were observed for 1mg and 2mg protein hydrolysates from the roasted-alcalase enzyme system, and 2.3 and 4.6mm were recorded for 1mg and 2mg hydrolyzed protein from the roasted-pepsin-pancreatin enzyme system (Table 5.2; Fig 5.11 – 5.15).

M. luteus served as a positive source of reference for antimicrobial activity. *L. monocytogenes* was more susceptible to the hydrolysates with *E. coli* O157:H7 being the least susceptible (Table 5.3); this observation is consistent with finding that gram-positive organisms are more susceptible to antimicrobials from plant sources (Marino *et al.*, 2001).

Statistical analyses show that hydrolyzed protein from the alcalase enzyme system exhibited more inhibitory power against the microorganisms (Table 5.3). This observation is confirmed by the more extensive alcalase hydrolyzed samples reported in Chapter 3. There was however no statistical differences in the inhibitory power of either the raw or roasted peanut sources. Since zymograms in Chapter 3 show the degeneration of larger peanut protein subunits, and the resistance of the basic arachins and low MW proteins in the case of raw peanuts, and no visible subunits in the case of roasted peanuts, it can be inferred that the low MW proteins of peanuts are responsible for antimicrobial

activity. Since the unhydrolyzed peanuts samples showed no lethality against the pathogens, it can be deduced that proteins have to be cleaved to be effective antimicrobial agents.

Peanut protein hydrolysate are potent antimicrobial agents. Antimicrobial activity increased with increasing peptide concentration. This was the expected trend since concentration is proportional to the potency of antimicrobials utilized.

CONCLUSION

The elimination of microorganisms capable of causing foodborne diseases is critical to reducing infections arising from consumption of foods contaminated with such pathogens, and peanut biopeptides have proved to be effective in inhibiting the growth and survival of such pathogens. Hydrolyzed peanut protein may therefore be used as an integral component in food safety. It can also be used in the control and treatment of pathogenic mediated ailments. Antimicrobial activity of the digest is concentration dependant and is more effective against *L. monocytogenes* compared to *E. coil* O157:H7.

FUTURE RESEARCH / RECOMMENDATIONS

It is recommended that further research be done to identify the amino acid profile and to isolate and sequence the peptides involved in the inhibitory activity of the proteolytic fractions. It will also be necessary to study the mechanism of inhibition of the peanut protein, and to determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC).

ACKNOWLEDGEMENTS

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Peanut is the 4th most important oilseed in the world. Its consumption trend suffered a decline due to issues of allergenicity, aflatoxin contamination and dietary issues about peanut fat content. Though the recent years have seen a rise in the consumption trends again, the value of peanuts can be further boosted with the knowledge of it being a potent source of bioactive peptides.

Simulated gastrointestinal digestion was achieved with sequential hydrolysis with pepsin and pancreatin, and hydrolysis from the industrial point of view was achieved with alcalase. Defatted raw and roasted peanut flours were used as starting materials. Peanut treatment showed no significant effect on the degree of hydrolysis. The basic arachins and low MW proteins of the raw peanuts were resistant to hydrolysis. Alcalase showed a more extensive hydrolysis compared to the pepsin-pancreatin digests.

Peanuts have been discovered to be a source of hypotensive peptides. Raw peanut hydrolysates and alcalase mediated hydrolysates proved to be more potent in inhibiting ACE activity compared to roasted peanut and pepsin-pancreatin mediated hydrolysates. However the peanut treatment and enzyme source had no significant effect on the IC₅₀ of the chromatographic fractions analyzed.

Proteolytic peanut digests exhibited inhibitory power against pathogenic *E. coli* O157:H7 and *L. monocytogenes*. The alcalase generated hydrolysates were more lethal to the pathogens than those generated from pepsin-pancreatin.

Peanut protein can be used as an alternate source to synthetic ACE inhibitory medication, and also used as an integral part of food safety and in the treatment of microbial source diseases. Alcalase compared to pepsin-pancreatin will be a better source for the production of peptides for protein supplementation, and the generation of antihypertensive and antimicrobial peptides.

Since zymograms show peptides in the basic arachin and low MW regions at the end of hydrolysis, it is inferred that these residual peptides are responsible for the inhibition of ACE activity and the resultant decrease in blood pressure, and also for the inhibited growth and survival of pathogens.