KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF SCIENCE

Limited Proteolysis, Evaluating the Degree of Hydrolysis and Molecular Weight Profile of Protein Hydrolysates from *Vigna subterranea* and *Cajanus cajan*

A THESIS SUBMITTED TO THE DEPARTMENT OF FOOD SCIENCE AND TECHNOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE IN FOOD SCIENCE AND TECHNOLOGY

BY

AFIA SAKYIWAA AMPONSAH (BSc. FOOD SCIENCE AND TECHNOLOGY)

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CERTIFICATION PAGE

I hereby declare that this submission is my own work toward the award of the MPhil degree and that, to the best of my knowledge, it contains no material previously published by another person or material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

Afia Sakyiwaa Amponsah	……	……
(PG 6126011)	Signature	Date

Certified by:

Mr. Isaac W. Ofosu	……	……
(Supervisor)	Signature	Date
PROF. (MRS) I. ODURO	……	……
(Head of Department)	Signature	Date

ABSTRACT

The protein hydrolysates of two neglected and underutilized legumes; ***Vigna subterranea*** (Bambara groundnut) and ***Cajanus cajan*** (Pigeon pea) were electrophoretically profiled having previously been subjected to limited proteolysis. The limited enzymatic hydrolyses were carried out with alcalase, pepsin and papain while the Ninhydrin protocol was used to follow the degree of hydrolysis. Response surface D-optimal design was used to plan two set of 17 experimental runs one for Bambara groundnut protein and the other for Pigeon pea protein. The initial design which followed a quadratic model was obtained using the two treatment factors: *time of hydrolysis* (10.00-120.00 min), numeric factor and *type of enzyme* (alcalase, pepsin and papain), categoric factor. Bambara groundnut proteins were only marginally vulnerable to hydrolysis just as Pigeon pea proteins were. However, pepsin had the least degree of hydrolysis whereas alcalase which generally has a larger molecular weight had the highest degree of hydrolysis for Pigeon pea protein. Papain's performance in degree of hydrolysis may be due to the low cysteine amino acids in Pigeon pea. On the other hand, alcalase had the least degree of hydrolysis whereas papain had the highest degree of hydrolysis for Bambara groundnut protein. The image J analysis of the electrophoregram of the hydrolysates at the 10^{th} min and 120^{th} min for Bambara groundnut and Pigeon pea proteins confirmed the actions of these enzymes. It also confirmed the presence of numerous polypeptides of relatively low molecular weights especially at the 120^{th} min. It is believed that the technique of limited proteolysis would be mastered for NULs such as Bambara groundnut and Pigeon pea proteins, and subsequently; their surface functional properties and thus, the food industrial applications of their resultant polypeptides would fully be realized.

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DEDICATION

This thesis is dedicated to my family who are most dear to me.

CHAPTER ONE

1.0 INTRODUCTION

The need for new food sources has become essential to combat the global food insecurity. All over the world and most especially in developing countries there is a lot of pressure on a number of food sources to fight hunger and malnutrition. The cost of these few food sources and their inadequacy in supplies make people who cannot afford the purchase tend to be malnourished.

Enzymatic hydrolysis of proteins releases small peptides and free amino acids, and may contribute to increase the nutritional value of food proteins. One method to improve the food utilization of underutilized legumes is therefore through limited proteolysis and this process has been reported to contribute to the nutritional, functional, immunological and biological activity of the protein hydrolysate obtained (Cheison *et al.*, 2009).

It has been reported that the two cereals; maize and wheat satisfies little of the protein and calorie requirement (IPGRI, 2000). In comparison, legume stands to provide quality protein than cereals (Ekanayake *et al*., 2000). In trying to solve the imbalances in food security, one of the major tools is the use of legume protein products with special functional properties. Legumes with high quality protein and calories that are not extensively utilized as food have become a primary target in this effort.

Thus legumes with high nutritional quality which are easy for people with a low income to purchase; foods that are less costly to produce need to be cultivated in the tropics where a large portion of the persons who are affected by food shortages reside (Ekanayake *et al*., 2000).

1.2 PROBLEM STATEMENT

The advancement of science and technology has transformed soya beans into diverse products and enhanced its commercialization and marketing systems leading to the neglect and underutilization of the so called NULs (neglected and underutilized legumes). The problem with plant protein is its insolubility in water. While some scientists have sought to increase the application of plant proteins through chemical modification to increase its hydrophilicity, in order to increase their solubility, others frown on chemical modification protocols due to consumer negative perceptions about artificial chemicals in foods. Hydrolysis of proteins depolymerizes the parent proteins and the resulting polypeptides are more soluble in water. However, the advancement of limited proteolysis has not fully been exploited to increase the processing difficulties of NULs proteins which are already believed to be hardy since more favorable soy bean is available for industrial applications. Limited proteolysis could be used to control the breakdown of the parent protein to such limits that could permit the solubility of the resulting polypeptide so it could evoke the surface functional properties that is desperately desired for food functionalities.

1.3 JUSTIFICATION OF WORK

Generally, limited proteolysis of protein preparations offers a possibility to obtain hydrolysates with enhanced functional properties. As a result of this process, molecular weight is reduced, the number of functional groups capable of ionisation is increased and a change in surface hydrophobicity occurs (Lahl and Braun, 1994), which leads to the change in the functional properties of the system. This depends, however, not only on the enzyme used and conditions of proteolysis, but also on the kind of raw material and the method by which it was obtained.

The degree of hydrolysis according to Cheison *et al.* (2000) is an important parameter to understand and interpret the effect and extent of the hydrolytic process of the proteins and it is useful to establish the relationships between proteolysis and the improvement of the functional, bioactive and sensory properties of these biomolecules. In the current study three enzymes (cysteine, aspartic and serine enzyme groups) would be use to hydrolyse two different legume proteins. The choice of enzymes was influenced by their sources and the influence they have on the protein type.

From the outlines above, it can clearly be seen that limited proteolysis is a promising approach to improve functional properties of legume proteins. The method could be a better way of modifying food proteins to allay any fears or answer regulatory questions that must be raised as a matter of concern when chemical modification of proteins are carried out.

1.4 GOAL

The main goal of the project is to upscale the utility of NULs proteins and thus, the livelihood of the poor forest dwellers who make a living on its cultivation.

1.5 OBJECTIVE

The objectives are:

- To establish the relationship between hydrolysis time and the degree of hydrolyses of Bambara groundnut and Pigeon pea proteins in alcalase, pepsin and papain systems respectively.
- To electrophoretically profile the protein hydrolysates.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The case of Neglected and Underutilized Legumes

The utility of Bambara groundnut: Legume is seen to be one of the inexpensive sources of proteins in the world today. The food utilization of most underutilized legumes such as Bambara groundnut and pigeon pea has caught the attention of most researchers. These legumes are shown to be a powerful tool to fight protein deficiencies in most developing countries in Africa (Bamshaiye *et al*., 2011). Bambara groundnut is placed among one of the protein rich legumes when the food usage, proximate and amino acid compositions were studied. It is a major source of inexpensive protein in sub-Saharan Africa. The crop has high lysine and methionine content. Humans, pigs, consume seeds and poultry while the haulm is used as fodder for livestock. The crop also features prominently in cropping systems in Ghana and makes a significant contribution to soil fertility through symbiotic nitrogen fixation (IPGRI, 2000).

Bambara groundnut is the third most important legume after groundnut and cowpea but its full economic significance has not been determined (FAO, 2007). Though it has global availability especially in hostile tropical environments, the African legume is underutilized and remains one of the crops most abandoned by science. This is because the untreated protein of the Bambara groundnut has some drawbacks with regards to the functional properties despite the high protein content, and because of this it has not gain the desired popularity. The usefulness in the food industry is often limited by the presence of toxic and antinutritive components which include the presence of trypsin inhibitors that slow down digestion (Kato *et al.*, 1980).

Bambara groundnut protein in its untreated form also has reduced foaming and emulsion due to poor interfacial film formation, which is desirable for emulsion, and foam formation (Kato *et al.,* 1980), off flavour development during storage and thus degrading the product, the testa of the bean is very tough and this makes cooking to last for several hours. Many studies has been carried out to determine if most underutilized legumes like Bambara groundnuts and Pigeon pea have high food value as compared to soy and cowpea. Oyeleke *et al. (*2012) determined the chemical composition between different varieties of Bambara groundnut. Ahmed *et al*. (2010) also evaluated the nutritive value of Bambara groundnut (*Vigna subterranean*) pods, seeds and hull as animal feeds.

Protein isolation tends to affect the functional properties, colour and flavor of most processed foods. Bambara groundnut protein isolates are said to have high water holding adsorption, high foaming capacity, foaming stability and emulsion capacity at selected pH.

The utility of Pigeon pea: Pigeon pea is grown locally and also has a limited consumption by some rural population (local farmers) during the period of food scarcity (January - April) when other popular agricultural products (yam, maize, plantain etc) are scarce and very expensive (Nene *et al.,* 1990). The protein content is comparable with those in other legumes like cowpea and groundnut which have been used in complementing maize. It is rich in mineral quality and fibre content. Pigeon pea grows well in Nigeria but the hard-to-cook phenomenon and the presence of anti-nutrients have limited its utilization (Nene *et al.,* 1990).

2.2 Neglected and Underutilized Legumes as an Alternative Food Source

The quest for expanded protein resources: Neglected and Underutilized Legumes has been defined as those legumes that have been overlooked by scientific research and development, and yet play a crucial role in the food security, income generation and food culture of the rural poor. The lack of attention has meant that their potential value is under-exploited and places them in danger of continued genetic erosion and ultimately disappearance (IPGRI, 2000).

Neglected and underutilized legumes occupy a central niche in food basket of the poor. They are a fundamental component of the natural resource available to the poor. They empower the poor to pursue sustainable resources rather than goods-based development (Burgess, 1994; Blench, 1997). An attempt to explore these NULs will be a strategic way to fight food insecurity and would boost the economic needs of the rural poor, as well as secure the food base resource for both producer and consumers (Padulosi 1999). However, even with legumes more attention is given to a few of them, among these are soybean and cowpea leaving Bambara groundnut, pigeon pea, *Canavalia* and others. These legumes which have received little to no attention but have the potential of providing high protein are termed as Neglected and Underutilized Legumes (NUL) (IPGRI, 2000).

2.3 Limitations of Legume Grain Utilization

Consumption of the legume grains also leads to excessive flatulence causing embarrassment to consumers. The principal cause of flatulence, as with all legume grains, is the oligosaccharides in the carbohydrate portion (Lazaroff, 1989). The best-known oligosaccharides in legume grains are raffinose and stachyose. Neither home cooking nor high-temperature industrial heating processes

dispatch raffinose and stachyose since they are stubbornly heat stable. The digestive fermentation that takes place always results in gas and sometimes in odour (Lazaroff, 1989) leading to the excessive flatulence which causes embarrassment. Thus, making consumers less interested in the use of such legumes. Nonetheless, experiential evidence and fragmentary research results suggest that it is a crop with great potential and hence there is the need to find ways to improve their functionality. To improve the functionality, the chemistry of the proteins and their protein functionality must therefore be brought to the fore.

2.4 Food Industry Uses of Proteins

Proteins are often used in the food industry as functional ingredients, thus imparting some desirable trait to a food product. One potential application within the food industry includes gelation, or the forming of a three-dimensional network by making a fluid into a solid or semi-solid. This attribute contributes to the textural properties of food, flavor release, moisture holding, and water holding capacity. Other potential applications include foam formation and stability, which influences texture in products such as angel food cake. Proteins are also used in emulsions (defined as a dispersion of one liquid into another liquid or solid), as stabilizers due to interfacial properties with polar groups; thus allowing for interaction within a liquid such as water and a non-polar group that allows for interaction within a liquid such as oil (Lazaroff, 1989).

Aside traditional food usage and forage uses which are common for legume application, legumes can be milled into flour to make bread, doughnuts, tortillas, chips, spreads, and extruded snacks or used in liquid form to produce milks, yogurt, and infant formula (Garcia *et al*., 1998). Pop

beans (Popenoe *et al*., 1989), licorice (Kindscher, 1992), and soybean candy (Genta *et al*., 2002) provide novel uses for specific legumes.

The trend today is that many healthy foods are largely made from soybeans in the form of '*tofu'* a curd made in a process similar to that used for making cheese. Soy milk is also made by grinding soybeans and mixing them with water to form a milk-like liquid whereas '*tempeh"* is a food made from fermented soybeans which has a meaty texture and nutty flavour and used frequently as a substitute for meat. The animal proteins, whey and egg whites are currently the favourites used by strength athletes whereas a good number of endurance athletes use plant proteins from soy, peanut, corn, rice and wheat (Genta *et al*., 2002). The time has therefore come for other legume protein resources to be exploited for similar applications as soy.

2.6 Technologies for Improving the Food Utilization of NULs

Dehulling of legume as a physical method of processing has been studied and identified to increase the foaming and emulsifying properties of bean flour (Desphande *et al.,* 1982). Fermentation has been another method which has been used to modify the chemical composition and functional properties of pigeon pea flour (Adebowale and Maliki, 2011).

Studies of transglutaminase have been reported by Larre (1993) .The transglutaminases catalyse the binding of primary amines to glutaminyl residues, the reticulation of peptic chains by the formation of α - (y-glutamy1) lysyl bonds and also, they may deaminate glutaminyl residues. Without available amine in the medium, water can play the role of acyl acceptor and the carboamide group is then deaminated. Protein fragmentation with proteases and food protein

modification and ligation by transglutaminases are used in order to improve the functional properties of some food proteins. The transglutaminase of guinea pig liver largely has been used to modify many food proteins (Larre, 1993).

2.7 Proximate Composition of Bambara Groundnut and Pigeon Pea

Upon the comparative analysis of two varieties of Bambara groundnut (cream and white red), Aremu *et al.* (2006) reported that the total ash, crude protein and carbohydrate were 4.28 and 3.89 %, 11.56 and 11.05 %, 73.3 % and 73.8 % respectively. The two varieties were reported to have significant difference in moisture, crude fibre and crude fat. This work showed lower percentage in protein the two varieties as compared to other works and could be attributed to the genotype and environmental conditions under which they were grown.

It has been recognized that protein isolate offers immense possibility in the development of new class of formulate. Bambara groundnut protein, fat, ash, crude fiber and total carbohydrates were 17.70, 6.58, 4.22, 3.50 and 86.0 % in flour and 85.97, 0.0, 3.37, 0.02 and 10.64 % in protein isolate (Larre, 1993).

2.8 The Chemistry of Limited Proteolysis

2.8.1 Limited proteolysis

Limited proteolysis is a process by which peptides are produced by partial hydrolysis of proteins with enzymes to obtain smaller molecular size and less quaternary structure than the original proteins (Bond, 1990). A schematic diagram of the process is as shown in Figure 2.1 below:

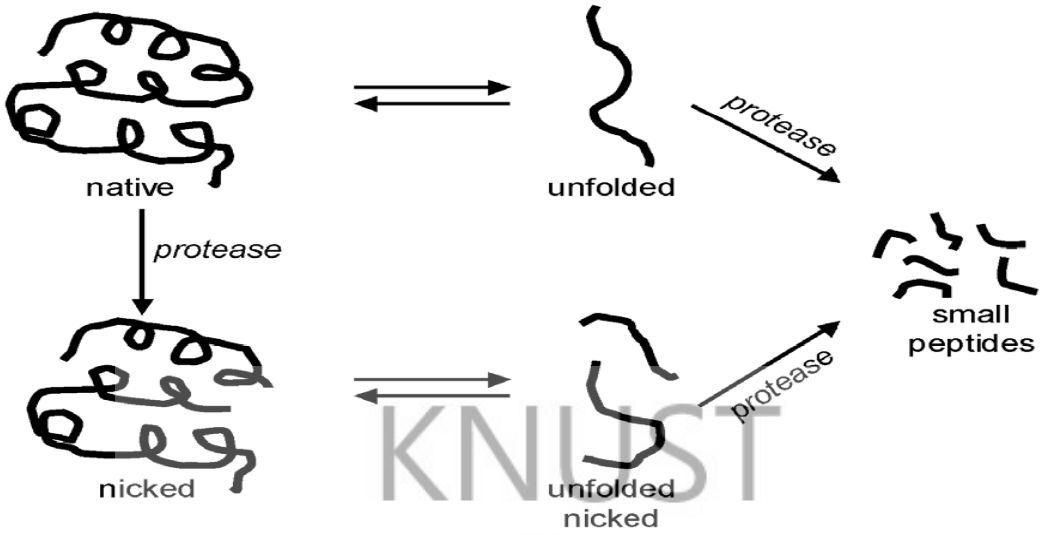

Figure 2.1: Schematic view of the mechanism of proteolysis of a globular protein (Fontana *et al.*, 2004).

A dual mechanism of protein degradation is shown in figure 2.1. This involves a substrate for proteolysis as the unfolded protein and the native form of the protein. In this last case proteolysis is limited and occurs at flexible site(s), leading to a nicked protein species that can unfold and then be degraded to small peptides. The fundamental principle of limited proteolysis is that the protease can bind to the protein substrate at sites whose conformations are complementary to its active site (Fontana *et al.*, 1993, 1997a,b). Since a rigid protein structure has a specific conformation, the chances of conformational complementarily are relatively small and thus native globular proteins are usually quite resistant to proteolysis.

On the other hand, the flexible regions of the native protein imply existence in a range of local conformational isomers (Fontana *et al*., 1993, 1997a,b). This correlation was first demonstrated with thermolysin (a thermostable neutral metalloproteinase enzyme) (Fontana *et al.*, 1986) and subsequently with other proteases (Fontana *et al*., 1993, 1997b). These regions have also shown higher flexibility in molecular dynamics simulations, for example, in apomyoglobin (Fontana *et*

al., 1997b). As expected, the sites of limited proteolysis in proteins are sufficiently exposed to be able to bind to the protease's active site. But the exposure which is a required characteristic of the cleavage site is not sufficient to explain the *specific* proteolysis. Indeed, flexibility of the chain segment suffering proteolytic attack is the key parameter dictating limited proteolysis (Fontana *et al*., 1986).

It has been shown in several studies that there is a clear-cut correlation between sites or regions of enhanced segmental mobility and sites of limited proteolysis (Fontana *et al*., 1986, 1993; Polverino de Laureto *et al*., 1995). Proteolysis takes place at a helical segment of the proteins and the helix is likely to have been destroyed by end-effects and loss of the cooperative hydrogen bonds that stabilize it.

The loss of hydrogen bonds creates charges and as a result the ends that are newly created become charged and if buried, might conceivably destabilize the protein core. Thus, limited proteolysis occurs preferentially at those loops which display inherent conformational flexibility, whereas the protein core remains quite rigid and thus resistant to proteolysis (Fontana *et al*., 1986, 1993, 1997a).

2.8.2 Factors that affect enzyme activity in limited proteolysis

In general, proteins break down and release smaller peptides at optimal hydrolysis conditions. The characteristics of the released peptides are mostly dependant on the type of enzymes and hydrolysis conditions. Specifically, the nature of these released peptides can be affected by many factors, such as the origin of enzymes, enzyme activity and selectivity, enzyme to substrate ratio, and hydrolysis conditions including pH, temperature, and time (Barrett *et al*., 2004).

Paraman *et al.* (2007) reported that the type of enzyme used for protein hydrolysis had a significant impact on emulsifying properties of the hydrolysates. Alcalase is an endo-type serine bacterial protease with broad specificity, having a preference for a large uncharged residue's carboxyl sites. Liquipanol is an endo-type cysteine protease of papaya latex with broad specificity toward peptide bonds. The enzyme pepsin is an aspartic protease obtained from porcine stomach has specificity toward the C-terminal side of tyrosine, phenylalanine, and tryptophan residues.

The differences in reaction conditions of each enzyme such as hydrolyzing temperature and pH can affect the extent of protein unfolding, thus resulting in varying degrees of protein hydrolysis. Physical treatments such as homogenization (2, 4, 6, and 8 min), sonication (5, 10, 15, and 20 min), and thermal treatments (10 min at 40, 60, and 80° C) were evaluated for improvement of rice protein emulsifying properties (Paraman *et al*., 2007).

Physical treatments, in one or several forms did not improve rice protein solubility or emulsifying properties, and neither sonication nor homogenization had an effect on the degree of hydrolysis. Physical treatments may have altered the structural conformation of proteins but did not cleave peptide bonds. Therefore, degree of hydrolysis remained negligible despite pretreatments (Paraman *et al*., 2007).

2.8.3 Functional properties and peptide profile of limited protein hydrolysates

Limited and controlled hydrolysis by enzymes are also applicable in altering the functional properties of vegetable proteins in a desirable way when the proteins have been denatured to concentrates and isolates or lack sufficient functionality in their native state (Adler-Nissen *et al*.,

1983). Again it has been reported that efficient methods of isolation and modification with approved food-grade enzymes and chemicals would be beneficial for enhancing functional properties of cereal protein (Kamara *et al*., 2010). The released peptides after limited proteolysis will lead to enhanced functional properties depending on their physicochemical properties (Tsumura *et al.*, 2005).

Gelation was improved when hydrophobic groups were exposed upon hydrolysis (Kuipers *et al*., 2005; Creusot and Gruppen, 2007; Kuipers *et al*., 2007). Additionally, the exposed hydrophobic groups facilitated interactions between protein and oil, considerably enhancing emulsification property (Qi *et al.,* 1997). Similarly, increased surface hydrophobicity also allows proteins to form more stable foams (Ortiz and Wagner, 2002). Furthermore, hydrolysis can induce changes in the balance of net charges that lead to enhanced solubility (Kuipers *et al*., 2005; Lamsal *et al*., 2007; Zorin and Baiarzhargal, 2009). These studies, however, were carried out at relatively low protein concentrations of 0.2~1% without any thermal treatment.

Since protein solubility is governed by thermal stability and concentration, formulating a stable high protein beverage is one of the biggest challenges in food industry. Therefore, more work needs to be done to investigate how hydrolyzed protein solubility is affected by thermal stability and high protein content. In addition to the enhanced functional properties, food protein-derived bioactive peptides can be released upon limited enzymatic hydrolysis. Biologically active peptides are characterized as protein-derived amino acid sequences that may have regulatory and physiological effects on the human body beyond normal and adequate nutrition (Korhonen and Pihlanto, 2003; Shahidi and Zhong, 2008). Since not all released peptides are bioactive, released

peptides upon hydrolysis play an important role in protein functionality and bioactivity (Wang *et al.,* 2008).

2.8.5 The use of limited proteolysis to improve the functional properties of proteins

The influence of enzymatic hydrolysis of soy protein concentrate (SPC) and soy protein isolate (SPI) on emulsifying activity index was evaluated and the hydrolysis profiles of SPC and SPI hydrolysates were determined with sodium dodecylsulphate-polyacrylamide gel electrophoresis, in order to reveal the relationship between mode of enzymatic hydrolysis using trypsin or neutrase under controlled hydrolysis conditions and corresponding modification in emulsifying activity index.

Using the degree of hydrolysis (DH) of 1 or 2 %, analysis results from SDS-PAGE indicated that these hydrolysates had different peptide compositions due to the differences of enzyme specificity and degree of hydrolysis. SPC hydrolysates or SPI hydrolysates prepared with trypsin had more large peptides than that prepared with neutrase. The hydrolysates prepared with trypsin exhibited better emulsifying activity index than that with neutrase, especially when degree of hydrolysis of hydrolysates was 1 % (Zhao and Hou, 2009). Paraman *et al*. (2007) investigated the effect of controlled degree of hydrolysis on solubility and emulsifying properties of rice endosperm protein. The optimum degree of hydrolysis (DH) was determined for acid, neutral, and alkaline type proteases (pepsin, alcalase and liquipanol).

Solubility and emulsifying properties of the hydrolysates were compared and correlated with degree of hydrolysis and surface hydrophobicity. Degree of hydrolysis was positively associated with solubility of resulting protein hydrolysate regardless of the hydrolyzing enzyme, but

enzyme specificity and degree of hydrolysis interactively determined the emulsifying properties of the protein hydrolysate. The optimum degree of hydrolysis was 6–10 % for good emulsifying properties of rice protein, depending on enzyme specificity. High hydrophobic and sulphhydryl disulphide (SH-SS) interactions contributed to protein insolubility even at high degree of hydrolysis. The exposure of buried hydrophobic regions of protein that accompanied high-temperature enzyme inactivation promoted aggregation and cross-linking of partially hydrolyzed proteins, thus decreasing the solubility and emulsifying properties of the resulting hydrolysate (Adler-Nissen *et al*., 1983) .

In order to achieve a relatively inexpensive, high quality functional additive by the action of enzymes, the applicability of chymosin as agents for improvement of solubility, emulsifying and foaming properties of pea (*pisum sativum*, L.) protein isolates were determined and the protein composition produced by enzymes were as well characterized. A relatively inexpensive, high quality functional additive could be obtained by the action of this enzyme (Barac *et al*., 2011). Neutrase has been used to modify the functional and physico-chemical properties of extruded soy with the aim to select the optimal conditions for enzymic hydrolysis (Surówka *et al*., 2004).

Zhao and Hou (2009) worked on soybean protein products; soybean protein concentrate or soybean protein isolate, were hydrolyzed by neutrase and trypsin under controlled conditions to prepare soybean protein hydrolysates with low degree of hydrolysis after which emulsifying activity index was evaluated. Also Soy protein isolates were hydrolysed at various degrees with one enzyme and the functional properties were evaluated as affected by the proteolytic activity (Puski, 1973). The effects of enzymatic hydrolysis on the functional and physicochemical properties of defatted foxtail millet protein hydrolysate through amino acid analysis, molecular

weight distribution, nitrogen solubility, surface hydrophobicity, foam capacity and stability, emulsifying capacity, water and oil holding and in vitro digestibility was studied by Kamara *et al*. (2010) and a significant improvement was obtained in general after hydrolysis.

Defatted foxtail millet flour was enzymatically hydrolyzed by several commercially available proteases (Protamex, Papain, Alcalase 2.4 L, Flavourzyme and Neutrase), with protein recovery of 59.6 %, 62.8 %, 81.8 %, 78.9 %, and 67.5 %, respectively. The molecular weight ranged below 200 and 5000 Da and was not affected by the time of hydrolysis. Hydrophobicities of 122.75 and 166.05, maximum solubility of 91 % and 83 % at pH 12.0, emulsifying capacity was 82.33 % and 72.32 %, foam capacity and foam stability ranged from 185.33 to 137.80 g/ mL)1 and from 185.33 to 111.1 g/ mL)1 for both defatted foxtail millet protein hydrolysate and defatted foxtail millet protein isolate, respectively. Enzymatic modification was responsible for changes in the protein functionality and defatted foxtail millet protein hydrolysate are potential as functional food ingredient.

The possibility to get peptides with desirable properties is one of benefits of limited proteolysis. Isolation of peptides from food is difficult because they are present in a complex mixture containing various substances such as free amino acids, sugars, salts and acids. Generally, for separation of peptides, classical, preparative, chromatographic methods based on their hydrophobicity, molecular size and net charge (Surówka *et al*., 2004) are used.

2.9 The Protein Profiles of Enzyme Hydrolyzed Proteins

Changes in the proteins, peptides content and lipids profile in enzymatically modified pea and lentil flours and characteristic of isolated peptides by immobilized metal ion chromatography

(IMAC) method and enzymes trypsin and pepsin were the aim of Baraniak and Swieca, (2008). The level of proteins and lipids in hydrolysed flours which were changed significantly after the hydrolysis. Generally, peptides separation profiles, performed on immobilized Zn (II), were dependent on the kind of flour and enzyme used in the hydrolysis process. Both trypsin and pepsin released a large amount of peptides and peptides isolated from the hydrolysed flours which were not significant.

In the lights of this, it is clearly visible that investigated peptides had a weak affinity to the chelated metal ions. It is noteworthy, that in some cases the influences of chelating factor on separation profiles were noticeable (Baraniak and Swieca, 2008).

The molecular size of the proteins and its hydrolysates were determined by SDS-PAGE according to the method of Laemmli (1970) after which electrophoresis was performed at a constant voltage of 200V for 40 min. The result showed that enzymatic-treated samples, the intensity of high molecular weight bands decreased with increasing degree of hydrolysis and low molecular weight bands increased gradually with increasing degree of hydrolysis (Paraman *et al*., 2007).

Work by Paraman *et al*. (2007) showed that as degree of hydrolysis increased, high molecular weight bands disappeared and high intensity bands at low molecular range were generated. This trend was maintained regardless of the enzyme treatments, indicating nonspecific activity of enzymes. Even though a complete disappearance of high molecular size bands at high degree of hydrolysis were observed in the electrophotograms, the protein hydrolysates showed only 30%

solubility. This observation, once again, indicated that a large amount of cleaved peptides remained associated with insoluble proteins through intermolecular hydrophobic interactions and disulfide bonds.

2.8.4 The limitations of limited proteolysis

Although hydrolyzed protein has improved functionality and digestibility and may result in bioactive peptide liberation overall, the bitter taste that can accompany hydrolysis limits the utilization of enzyme hydrolysates as food ingredients. Partially hydrolyzed soy protein isolate tends to have a bitter taste caused by the formation of low molecular weight peptides composed of mainly hydrophobic amino acids. The average hydrophobicity of a peptide and the position of the hydrophobic amino acids are highly related to the bitter taste (Matoba and Hata, 1972; Kim and Lee, 1992).

To minimize the adverse effect on the sensory quality while retaining the desired functionality and bioactivity, controlled and limited degree of hydrolysis (DH) at 2~8 % is strongly recommended. Research on whey protein has shown that a low DH (2~8 %) is sufficient to obtain enhanced functional properties and maximum liberation of bioactive peptides with minimal detection of bitter low molecular weight peptides (Schlothauer *et al*., 2005). Therefore, it is important to maintain a low DH to achieve desired functional properties and bioactivity with reduced adverse effects on sensory quality.

2.10 Methods of Determining the Degree of Hydrolysis

Several methods are available for the determination of the degree of hydrolysis. Techniques for the determination of degree of hydrolysis are based on several principles. The pH stat method determines the quantity of alkaline solution which is measures by automatic titration. This is needed to keep the pH constant throughout the reaction (Spellman *et al*., 2003). Formol titration method and the derivatization with ortophtaladehyde both have the principle of determining the α-amino nitrogen. The degree of hydrolysis of formol titration is calculated using the relationship between α-amino nitrogen (AN) and the total nitrogen (TN);

$$\text{Degree of hydrolysis} = \frac{\alpha\text{-amino nitrogen}}{\text{Total nitrogen}}$$

Formol titration is reported by several authors as properly standardized and represents an efficient, fast and cost-effective method for monitoring the degree of hydrolysis process of proteins (Denis *et al*., 2008). Degree of hydrolysis can also be determined using the method which determines the soluble proteins content in trichloroacetic acid. Where it is measured as the percentage of protein in 10 % (w/v), trichloroacetic acid TCA in reaction to the total protein content of the sample according to Hoyle and Merrit (1994).

In this method, the addition of the trichloroacetic acid results in the total precipitation of non-hydrolyzed proteins and high molecular mass peptides (Carreiers *et al*., 2003), producing a higher concentration of soluble free amino acids and small peptides that are found in the supernatant. Osmometry method also deals with the determination of the degree of hydrolysis base on the depression of the freezing point which is measured using micro-osmometer.

2.11 The Influence of Reaction Time on the Degree of Hydrolysis

Paraman *et al*. (2007) stated that the hydrolysis profiles of three enzymes (alcalase, liquipanol, and pepsin) obtained at optimum conditions of each enzyme showed that the type of enzyme and hydrolysis time had an interactive effect on degree of hydrolysis. Treatment with alcalase, liquipanol, and pepsin enzymes resulted in a quadratic trend for degree of hydrolysis (DH) as a function of hydrolysis time.

Each of the three enzyme treatments resulted in a sharp increase in DH during the first 30 min, followed by a decreased rate thereafter. The decrease in rate of hydrolysis could be due to product inhibition, substrate exhaustion, or formation of inhibiting peptides. Differences in enzyme specificity may have led to differences between the protein hydrolysis curves. The rate of hydrolysis and final degree of hydrolysis were also dependent upon the initial level of protein denaturation. The differences in reaction conditions of each enzyme such as hydrolyzing temperature and pH could have affected the extent of protein unfolding, thus resulting in varying degrees of protein hydrolysis.

Kamara *et al*. (2010) also reported the treatment of defatted foxtail millet protein hydrolysate with Alcalase showed the highest increase in protein content of defatted foxtail millet protein hydrolysate during the first 300 min of hydrolysis which was followed by Flavourzyme and finally Neutrase.

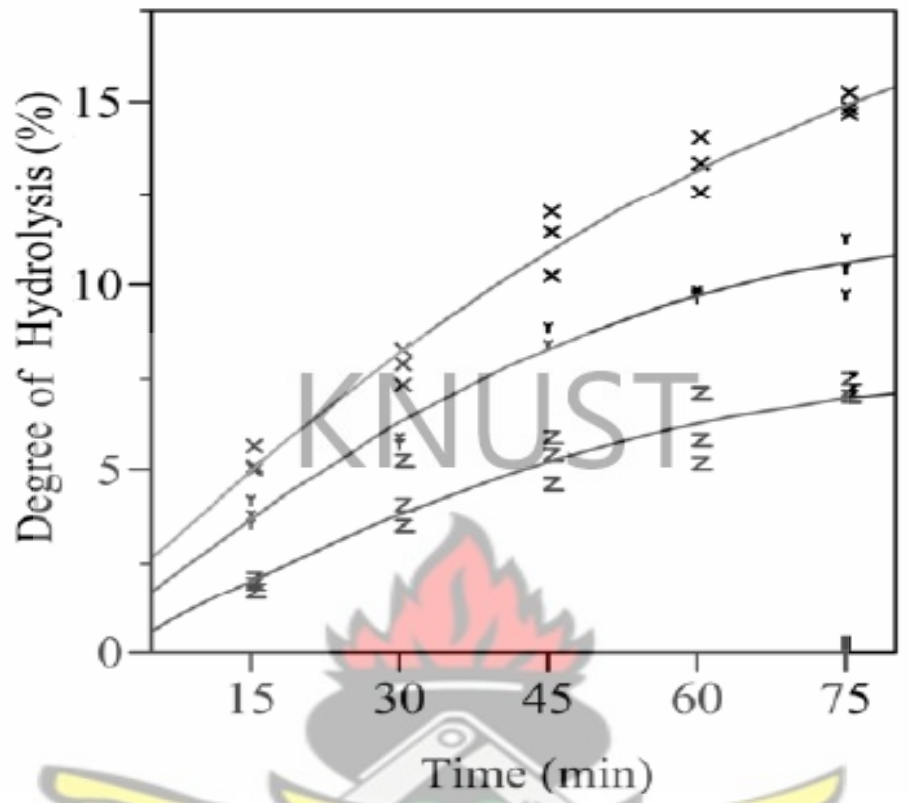

Figure 2.2: Hydrolysis profiles of rice protein treated by alcalase (x), liquipanol (y), and pepsin (z) as a function of hydrolysis time (Paraman et al., 2007).

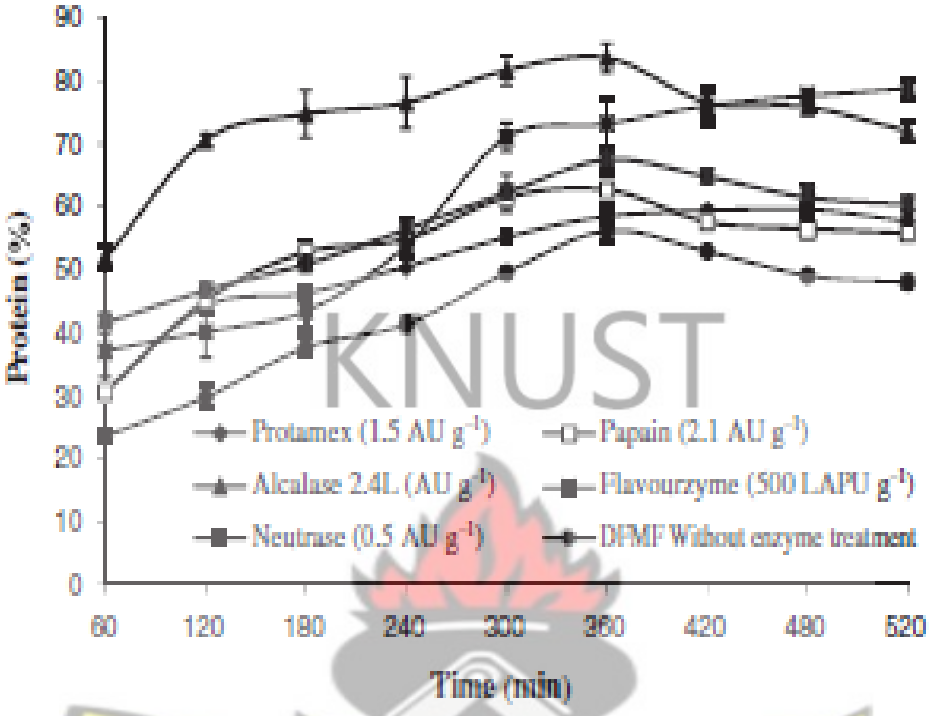

Figure 2.3: Amount of protein solubilised by enzymatic hydrolysis of DFMF by different proteases (Kamara et al., 2010).

2.12 Future Trends in Limited Proteolysis Research

The interesting aspects of chemical modifications such as deamination, acylation or phosphorylation for improvements of the solubility, emulsifying, foaming and gelling properties of animal (milk) and plant proteins has been described (Carreiers *et al.*, 2003). However, this is due to mostly secondary reactions and by-products, chemical modification sometimes presents disadvantages limiting its utilization in food systems.

Enzymatic modification is an alternative way for modifying protein structure and improving their functional properties. Enzymatic modification is an interesting tool for improving the

functional properties of food proteins. Till now, most of the studies are concerned with proteolysis and their industrial applications. However, this method has not been fully explored and it is possible to optimize the composition of hydrolysates by varying or controlling the location of the bond hydrolyzed.

Such an approach will give rise to peptides with high functionality. Moreover, enzymes other than proteases may be used to give proteins new properties (Chobert *et al*., 1988). Even though enzymatic modification of protein isolates are said to be the most convenient and milder method, extensive modification may impair the functional properties of the hydrolysate or cause off-flavour of the hydrolysate. Therefore degree of hydrolysis should be critical in limited proteolysis (Chobert *et al*., 1988).

Experiments from severe and mild thermal enzyme inactivation methods clearly indicated that high-temperature enzyme inactivation significantly decreases the protein solubility and emulsifying properties of resulting hydrolysates. Exposure of hydrophobic regions during high-temperature enzyme inactivation promoted aggregation and cross-linking of partially hydrolyzed proteins (Carreiers *et al*., 2003).

As a consequence, solubility and emulsifying properties decreased significantly. Surface hydrophobicity values decreased with the high-temperature inactivation method (90 °C for 15 min). It is therefore, recommended that the final pH of the protein hydrolysate should always be adjusted to pH 7.0 to maintain the experimental uniformity, even though the hydrolysis and enzyme inactivation were conducted at different pH levels for each enzyme (Paraman *et al*.,2007).

From these, limited proteolysis from enzymes of different origins can also be tested on NULs to see their effect on the molecular weight profile even at lower degree of hydrolysis.

It can therefore be observed from the literature above that there are still grey areas which have not been researched. Even though most work has been done on the assessment on the nutritive value of these legumes, the focus has been on animal food production. However upon modification of the functional properties of these legumes, they can be incorporated into human food; vegetarian diets and complementary foods. Works on the effect on limited proteolysis by proteases from different groups on Bambara groundnut and Pigeon pea has not been studied. The functional properties of these two legumes have not been compared in any work although they both tend to possess good quantity of proteins. Correlation between the degree of hydrolysis and protein profiles has not as well been studied. A study around these areas as well will add to the knowledge base thus improving their utilization.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

Pigeon pea was obtained from an out grower in *Drobo* in the *Brong Ahafo* region Ghana, while the Bambara groundnut was purchased from the *Ayigya* Market in Kumasi. Three enzymes; *pepsin* (aspartic protease obtained from porcine stomach), *alcalase* (bacterial serine protease from *Bacillus licheniformis)* and *papain* (cysteine protease obtained papaya fruit latex) were obtained from Sigma –Aldrich, USA.

3.1.1 Milling and defatting of dried Bambara groundnuts and Pigeon pea

The dried beans were sorted and milled into fine powder and defatted using hexane in a ratio of seed meal-to-solvent, 1:10 w/v, in a cold extraction. The defatted flour was spread on trays and solar-dried for about two to three hours in a solar tent dryer, to expel the volatile extraction solvent and stored in plastic bags for further analysis.

3.1.2 Protein Extraction and Characterization of Isolates

The protein was extracted by using the Gomez –Brenes *et al.* (1983) method where protein extraction from the dried defatted Bambara groundnut flour was done by using 0.01M NaOH. The meal to solvent ratio of 1:10 w/v was stirred in a large plastic container at room temperature for two hours. This mixture was allowed to stand for further 2 h. Proteins and soluble polysaccharides such as oligosaccharides were dissolved while the insoluble polysaccharides and residues settled at the bottom.

The supernatant was decanted and centrifuged (Towson and Mercer G24 Centrifuge, Shimadzu, Tokyo-Japan) at 2500 rpm for 30 min in order to separate any the insoluble residues. The supernatant was later acidified to a pH range of 4.5-5.0 with 0.1 M HCl to precipitate the proteins. The supernatant still containing the soluble polysaccharides was decanted and discarded. The protein suspension which was obtained after precipitation was further subjected to centrifugation at 2500 rpm for 30 min to separate the proteins. The precipitated proteins were thrice washed with distilled water and freeze dried in a freeze dryer(HETO POWER DRY LL300, Thermo Fisher Scientific, Waltham, MA).

The freeze-dried proteins were later characterized by determining their protein concentrations. Aliquots of the protein isolates were taken from the supernatant solutions (from the runs) for protein estimation by means of standard curve according to the method of Bradford (1976). The Coomassie protein assay reagent was used with stock solution of bovine serum albumin (BSA) of concentration 0.5 mg/mL were prepared with phosphate buffer of pH 5.8 being used for the standard. The absorbance was measured at 595 nm using spectrophotometer (UV/VIS 1601, Shimadzu, Tokyo-Japan). The absorbance generated for the runs were converted to amount of protein extracted as using the equation generated from the standard curve.

3.2 METHODS

3.4 Limited Proteolysis Experimental Design

Two factors; *time of hydrolysis* (10 -120 min), numeric factor and *type of enzymes* (alcalase, pepsin and papain) the choice of enzymes for the hydrolysis was influenced by the type of peptide bonds they hydrolyse. Categoric factor were used to treat Bambara groundnut and Pigeon

pea proteins. These factors were studied in a response surface methodology, D-optimal design where 17 experimental runs were projected in a quadratic initial design to study the response; degree of hydrolysis of the limited proteolysis of the two separate proteins. This design was chosen because it is recommended as a choice when there are categoric and numeric factors in a single treatment design or when there are constraints in the design or when there is the need to fit a cubic or higher order model (Myers *et al*., 2004).

Table 3.1: The design summary of response surface D-optimal plan projecting 17 exprimental runs for two factors: *time of hydrolysis* and *type of enzyme* for Bambara groundnut and Pigeon pea proteins

Runs	A: Time (min)	B: Enzymes
1	120	Alcalase
2	10.0	Alcalase
3	65.0	Alcalase
4	37.5	Alcalase
5	120.0	Alcalase
6	10.0	Alcalase
7	120.0	Pepsin
8	65.0	Pepsin
9	10.0	Pepsin
10	37.5	Pepsin
11	120.0	Pepsin
12	10.0	Pepsin
13	120.0	Papain
14	10.0	Papain
15	65.0	Papain
16	37.5	Papain
17	120.0	Papain

3.3.1 Limited Proteolysis Process

The partial hydrolyses of the two protein isolates were monitored using the Ninhydrin technique (Adler-Nissen, 1986). In this protocol, amino acids react with Ninhydrin hydrate at pH 5 and at 100 °C for a standard period of time to give a purple-blue, ammonium salt of diketohydrindylidene-diketohydrindamine as the major product. The absorbance of the purple-blue product is measured at 570 nm. The purities of the Bambara groundnut protein (BGPI) and Pigeon pea protein isolate (PPPI) were determined. They were reconstituted to 8 % (w/v) in deionized water, homogenized, adjusted to optimum pH of each enzyme (table 3.1), and agitated at 50 °C for 15 min before enzyme was added. The dispersions were treated with the proteases under optimum conditions of each enzyme. The optimum incubation was pH 9.0 at 60 °C for alcalase and that for papain was at a of pH 7.0 at 50 °C, whiles pepsin was run at a pH of 3.0 and at a temperature of 37 °C. The hydrolytic reactions were terminated by inactivating pepsin by neutralization with 0.2N NaOH at 50 °C for 5 min whiles alcalase was inactivated by lowering pH to 5.0 at 70 °C for 15 min with 0.2N HCl. Papain was inactivated at 85 °C for 10 min. The resulting hydrolysates were readjusted to pH 7.0.

3.5 Determination of the Degree of Hydrolysis

A series of calibration standard solutions between 0.01 and 0.15 amino acid meq/g was prepared by diluting 1.5 mM leucine standard solution with same solvent that was used to prepare the sample protein solution and 0.2 ml of Ninhydrin reagent added. Another 0.2 ml of the Ninhydrin reagent was then added to 1 ml of the hydrolysate, 1 ml of a blank solution and 1 ml of the unhydrolysed proteins. These were all analyzed at 570 nm using spectrophotometer (UV/VIS 1601, Shimadzu, Tokyo-Japan). A calibration curve was obtained for leucine by plotting A_{570}

versus equivalent amino acid standard concentration. For each time point, the concentration equivalents of amino groups in the samples were determined by extrapolating from each standard curve. For each time point, the amount of released amino groups (*h*) was determined by subtracting the value of the corresponding unhydrolysed control in the corresponding standard curve after the blank has already been accounted for. This gave the number of equivalents of peptide bonds hydrolyzed (h), expressed as meq/g protein from the following equations according to Adler-Nissen (1986):

$$h = (A \times b)/m$$

where A is the absorbance at 570; b and m are the y intercept and slope of the calibration curve respectively.

The degree of hydrolysis at each time point was then calculated from the following equation:

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

where h_{tot} is the total amount of peptide bonds given as 8 (obtained as the reconstituted 8 % (w/v) in deionized of the sample protein prepared in the sample characterization stage).

3.7 SDS-PAGE Analysis

The molecular weights of the hydrolysates from the 10th minute of hydrolysis and the 120th minute of hydrolysis (Table 3.2) for all the three enzymes were determined using polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulphate (SDS). A Tris-HCl buffer (pH 6.8) containing 2 % SDS, 10 % glycerol and 0.002 % bromophenol blue was used as the sample buffer. The samples were all loaded into the 10 welled precast gel (Bio-Rad Laboratories, Richmond, CA) according to the procedure described by Laemmli (1970) and placed into a Mini-Protean II dual slab cell (Bio-Rad Laboratories, Richmond, CA). Electrophoresis was

carried out at a voltage between 80 and 120 V. The separated bands of proteins were stained with Coomassie brilliant blue R 250. Molecular weights were determined by measuring the distances of migration in comparison with the standards.

3.8 Image J analysis of electrophoregram

The electrophoregram from the SDS-PAGE was scanned using HP Scan jet 2,400 at 1,200 dpi (Hewlett- Packard, Glasgow, Scotland, U.K.) and analyzed using the image J software according to the gel analyzer option as described by Ferreira and Rasband (2010). In this analysis, the scanned electrophoregram was opened in image J and the 'analyze>gels>gel analyzer options' were clicked in series and the boxes 'label with percentages, outline lanes and invert peaks' checked. The rectangular tool was then chosen, positioned around the stained bands in the first lane and the 'analyze>gels>select first lane' commands were chosen. Using the arrow keys, the rectangle was moved over the next lane after which the 'analyze>gels>select next lane' selected and measurement of bands done as before. The rest of the bands in the lanes were similarly measured and the profile of each lane were plotted using the 'analyze>gels>plot lanes' commands. The straight line tool was selected with which straight lines were drawn at the base of each peak which enclosed the area of the peak. With the wand command, the inside of the individual peaks were clicked after which the 'analyze>gels>label peaks' were clicked to express each peak size as a percentage of the total size of all the measured peaks in one column. From the results window, the 'edit>copy all' buttons were clicked which copied the results into a spreadsheet. Subsequently the percentage peaks against the logarithm of the molecular weights of the proteins (having calculated them previously from their Rf's) were run in a statistical tool, (GraphPad Prism, 2007) to obtain the graphs.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Data analysis

Essentially, response surface methodology was used to estimate the relationship between a set of controllable experimental factors and observed results (Gao *et al*., 2006). The independent and dependent variables were fitted to the regressional model equations and examined for the goodness of fit to ensure that the best model ($p<0.05$) provided an adequate approximation to the true system and verified that none of the least squares regression assumptions were violated (Myers *et al.*, 2004).

4.2 Data analysis for Bambara groundnut protein hydrolysis

After the DH responses were loaded and run for treatment of the Bambara groundnut proteins, the *sequential model sum of squares table (Table 4.1)* was obtained. This table gave the tool to check for a suggested regressional models that would best fit the response.

A p-value of 0.0001 and 0.0009 were obtained for linear *vs* mean and both quadratic *vs* cubic models, respectively but the design expert tool suggested a cubic model for the response based on the fact that it was the model that had the highest order polynomial where the additional terms were significant and the model was not aliased.

Table 4.1 Sequential model sum of squares table showing the suggested model (*) of the highest order polynomial where the additional terms are significant and the model is not aliased for the treatment of Bambara groundnut proteins

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob> F
Mean vs Total	113.00	1	113.00		
Linear vs Mean	46.86	3	15.62	99.00	0.0001
2FI vs Linear	0.66	2	0.328	2.59	0.1198
Quadratic vs 2FI	1.11	1	1.105	38.18	0.0001
Cubic vs Quadratic*	0.26	3	0.086	19.69	0.0009
Residual	0.03	7	0.004		
Total	161.92	17	9.525		

Table 4.2 ***Lack of fit test table showing the suggested model (*) of the highest order polynomial with the biggest prob> F value the treatment of Bambara groundnut proteins***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
Linear	2.03	8	0.254	62.84	0.0001
2FI	1.37	6	0.229	56.70	0.0002
Quadratic	0.27	5	0.054	13.33	0.0065
Cubic*	0.01	2	0.005	1.30	0.3520
Pure Error	0.02	5	0.004		

The regressional models were studied to track the one that had the highest p value. It was not expected that the DH responses obtained for the treatment of Bambara groundnut proteins would not fit. Thus, it could be seen from the table 4.2 that the p values ranged from 0.0001 for linear regression to 0.352 for the cubic regression meaning the DH responses best fit the cubic model compared to all others.

The model summary statistics was studied from table 4.3 which focused on the model maximizing the "*adjusted r-squared*" and the "*predicted r-squared*". It is aimed at selecting model with "*r-squared*" maximized and approaching 1. From table 4.3 it is clearly seen that there are progressions of the sum of squares from the linear regressions and maximizing with the cubic regressions.

Table 4.3 ***Model summary statistics table showing the suggested model (*) of the highest order polynomial with the maximized r-squared value the treatment of Bambara groundnut proteins***

Source	Std. Dev.	r-squared	adjusted r-squared	predicted r-squared	PRESS
Linear	0.40	0.96	0.95	0.93	3.44
2FI	0.36	0.97	0.96	0.95	2.60
Quadratic	0.17	0.99	0.99	0.98	0.99
Cubic*	0.07	0.99	0.99	0.99	0.34

Table 4.4 ***Analysis of variance table showing the significance(*) of the suggested model as well as the significance of the factors and their interractions in the regressional model that has been obtained for the treatment of Bambara groundnut proteins***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
Model	48.89	9	5.43	1239.60	0.0001*
A-Time of hydrolysis	46.50	1	46.50	10611.78	0.0001*
B-Enzymes	0.04	2	0.02	4.44	0.0569
AB	0.78	2	0.39	88.97	0.0001*
A^2	1.11	1	1.11	252.23	0.0001*
A^2B	0.11	2	0.05	12.21	0.0052*
A^3	0.16	1	0.16	36.49	0.0005*

Table 4.4 shows that the model that has been obtained for the expression of the DH of Bambara groundnut protein hydrolysates was significant ($p < 0.05$). Similarly, the time for hydrolysis was also significant ($p<0.05$). There also seem to be significant interaction between the two factors

($p<0.0001$) though the type of enzymes for the proteolytic reactions did not show significant differences ($p>0.05$). The above analysis was critical since it verified that none of the least squares regression assumptions were violated (Myers *et al.,* 2004). Other constants such as the "*prediction r-squared*" of 0.9929 were obtained which is in good or reasonable agreement with the "*adj r-squared*" of 0.9986. since according to Myers *et al*. 2004, it should be within ±0.2. Thus, the model could be used to navigate the design space which are the limits of the least and highest of the levels of the factors selected for the study.

4.3 Effect of time on the degree of hydrolysis for the three enzymes on Bambara groundnut

From figure 4.1 above there was a gradual increase in degree of hydrolysis for all three enzymes with papain having the highest degree of hydrolysis at start time of 10 min. In the initial period of hydrolysis, a large number of peptide bonds are cleaved, thus showing an increase in degree of hydrolysis. This was similarly reported by Silvestre *et al.* (1994) when whey protein concentrate were hydrolyzed with pancreatin. Vioque *et al.* (2000) also reported similar trend when rapeseed protein isolate was hydrolyzed with alcalase. Differences in enzyme specificity may have led to differences between the protein hydrolyses curves and the degree of hydrolysis.

Around 80 min hydrolysis time, the degree of hydrolysis was quite similar for all the three enzymes. All the DHs of the enzymes for the Bambara groundnut proteins went through a steady increase after which the DHs rose sharply towards the end of their hydrolyses with alcalase, a serine protease which is a nonspecific endoprotease (Vioque *et al.,*2000) and hydrolyzes serine peptide groups had the highest DH value.

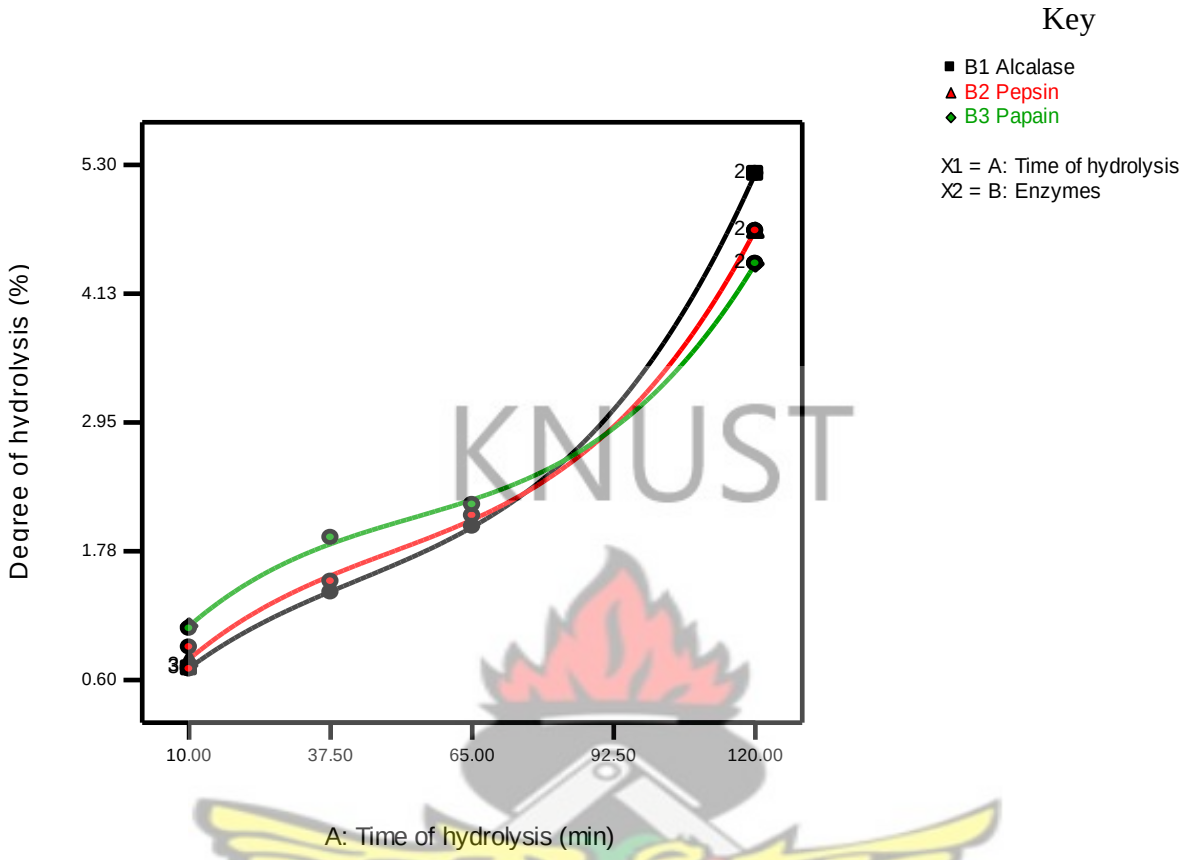

Figure 4.1: Regressional plot showing the time of hydrolysis and the degree of hydrolysis of Bambara groundnut protein hydrolysate as a result of proteolysis (■ Alcalase, ▲ pepsin and ♦ papain)

Vioque *et al.* (2000) reported that alcalase hydrolysates with DH of 7.7 % gave bitter peptides and this has also been reported by Chobert *et al*. (1988) that long hydrolysis time had an effect on the taste of the final hydrolysates which are usually bitter after taste. According to Panyam *et al.* (1996), protein hydrolysates with low DH after limited proteolysis had better functional properties than the original proteins and that enhanced functional properties such as water and oil absorption, foaming capacity or emulsifying activity are important food ingredients indicators.

Again, the rate of hydrolysis and final DH were also dependent upon the initial level of protein denaturation. The differences in reaction conditions of each enzyme such as hydrolyzing temperature and pH could have affected the extent of protein unfolding, thus resulting in varying degrees of protein hydrolysis (Chobert *et al*., 1988).

4.4 Effect of limited proteolysis on the protein profile of Bambara groundnut

SDS-PAGE of the hydrolysates from Bambara groundnut proteins for each enzyme gave multiple bands as presented by the electrophoregram in figures 4.2 below.

Figure 4.2: ***Electrophoregram of Bambara goundnut protein hydrolysates. From left to right lane 1 protein marker, lane 2 papain at DH 1.9 %, lane 3 papain at DH 4.4 %, lane 4 pepsin at DH 1.5 %, lane 5 pepsin at DH 4.7 %, lane 6 alcalase at DH 0.7 %, lane 7 alcalase at DH 5.2 %. Each pair is at the 10th and 120th min respectively.***

Enzymatic protein hydrolysates containing short chain peptides with defined amino acid composition and molecular size are preferred for specific formulations (Chobert *et al*., 1988).

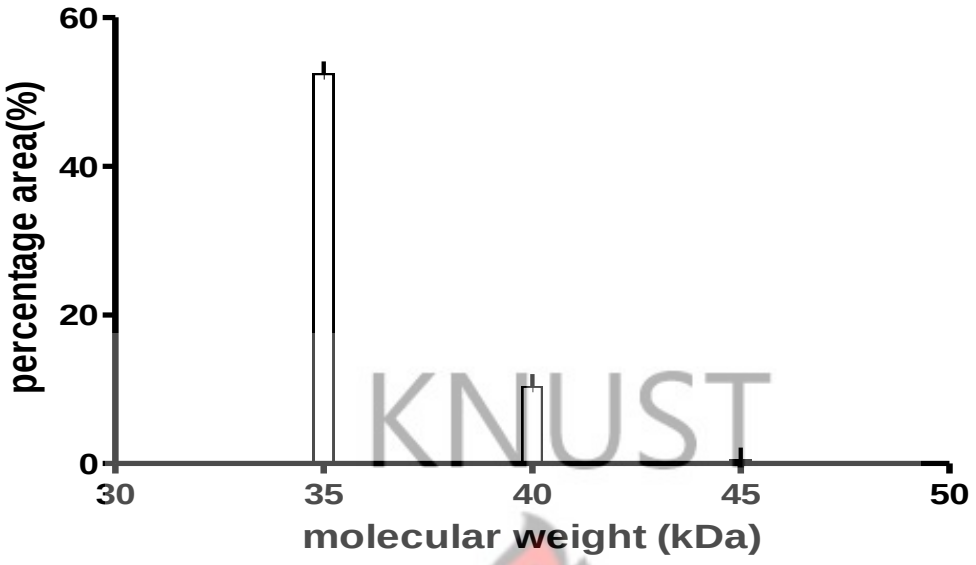

Figure 4.3: *Percentage band area of the molecular weights of papain hydrolysis of Bambara groundnut protein hydrolysate at a DH of 1.9 %.*

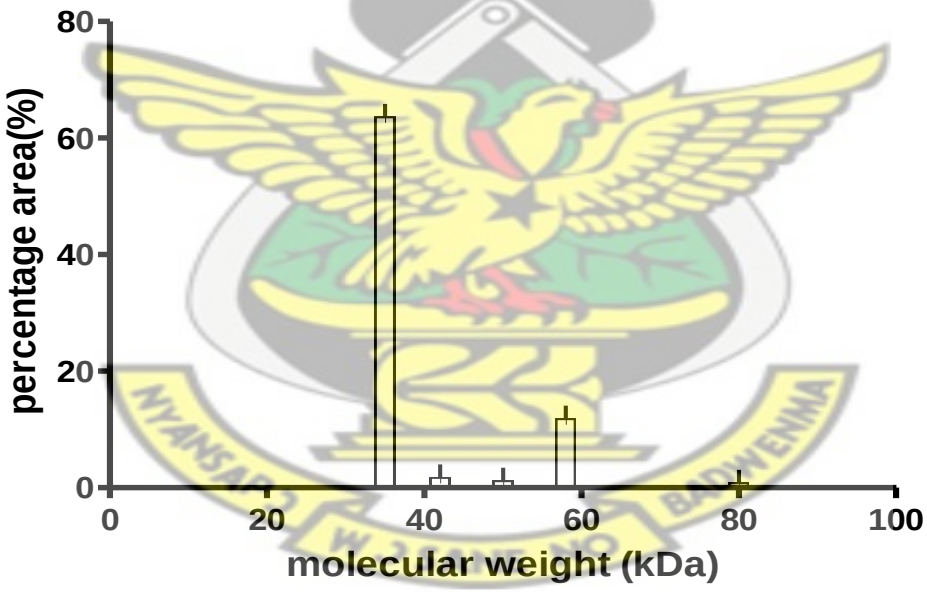

Figure 4.4: *Percentage band area of the molecular weights of papain hydrolysis of Bambara groundnut protein hydrolysate at a DH of 4.4 %.*

Thus protein hydrolysates offer an alternative to intact proteins and elemental formula in the development of special formulations designed to provide nutritional support. Upon limited

proteolysis, proteins were separated into large numbers of fractions resulting in increasing bands as reported by Barać *et al.* (2011).

At degree of hydrolysis of 1.9 % from figure 4.3, three (3) peptides have been hydrolysed with the prominent peak being that of the least molecular eight of 35kDa the highest molecular wieght at this time , thus, 10 min was 45 kDa.

A degree of hydrolysis of 4.4 % from figure 4.4, was however, achieved in the 120th min, five bands appeared, indicating five peptide groups hydrolysed. The prominent peak was 35 kDa and this showed an increase compared to three peaks at the 10th min .

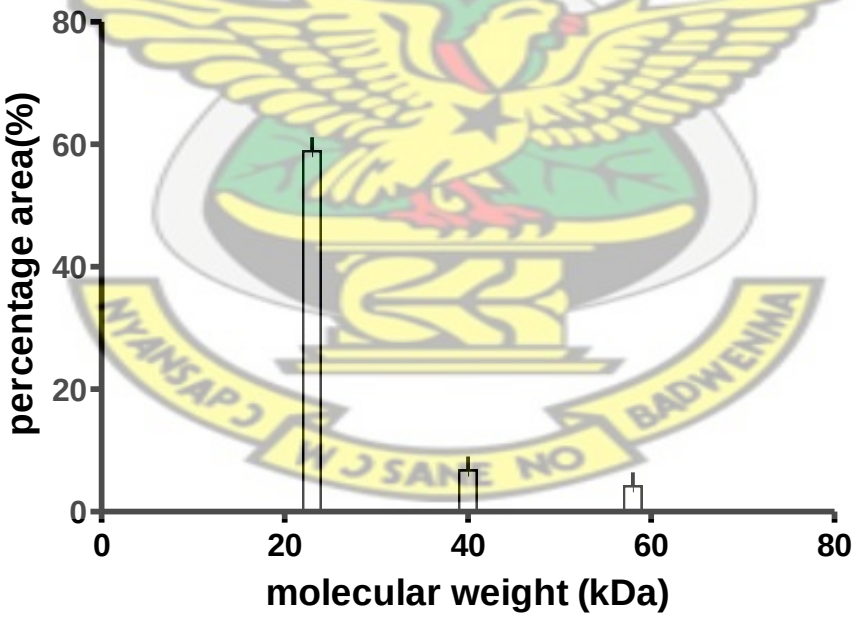

Figure 4.5: Percentage band area of the molecular weights of pepsin hydrolysis of bambara groundnut protein hydrolysate at degree of hydrolysis 1.2 %.

From figure 4.5, a degree of hydrolysis 1.2 % was achieved in the 10th min of hydrolysis time, three (3) bands appeared giving significant peaks ranging from 23 kDa to 58 kDa. The prominent peak was observed at 23 kDa.

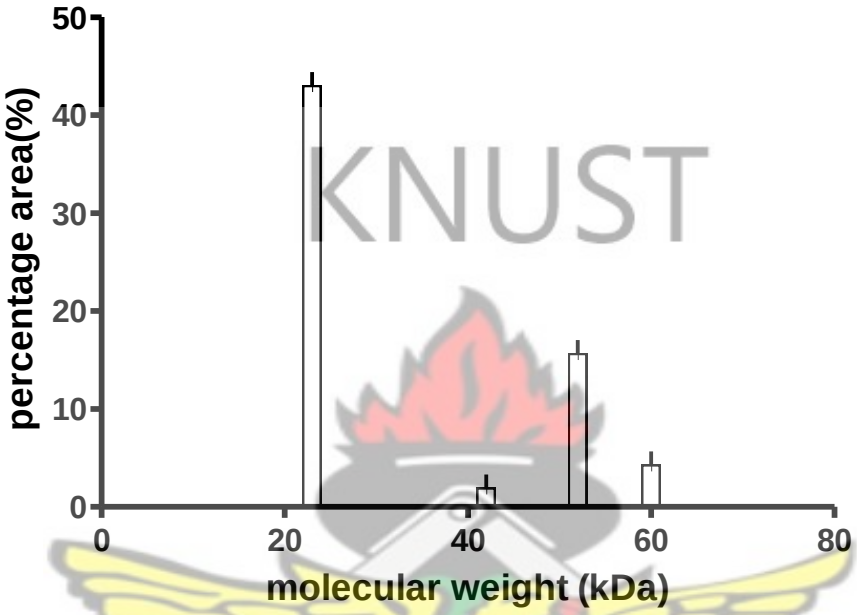

Figure 4.6: Percentage band area of the molecular weights of hydrolysis of bambara groundnut protein hydrolysate for pepsin at a DH of 4.7 %.

At degree of hydrolysis 4.7 % from figure 4.6, four (4) bands appeared giving peaks ranging from 23 kDa to 60 kDa. The prominent peaks were observed at 23 kDa and 52 kDa respectively. There was an increase in the number of peaks as compared to the situtation at DH of 1.2 % . Both had their highest peak at 23 kDa even though there was an introduction of new band peak at a DH of 4.7 %.

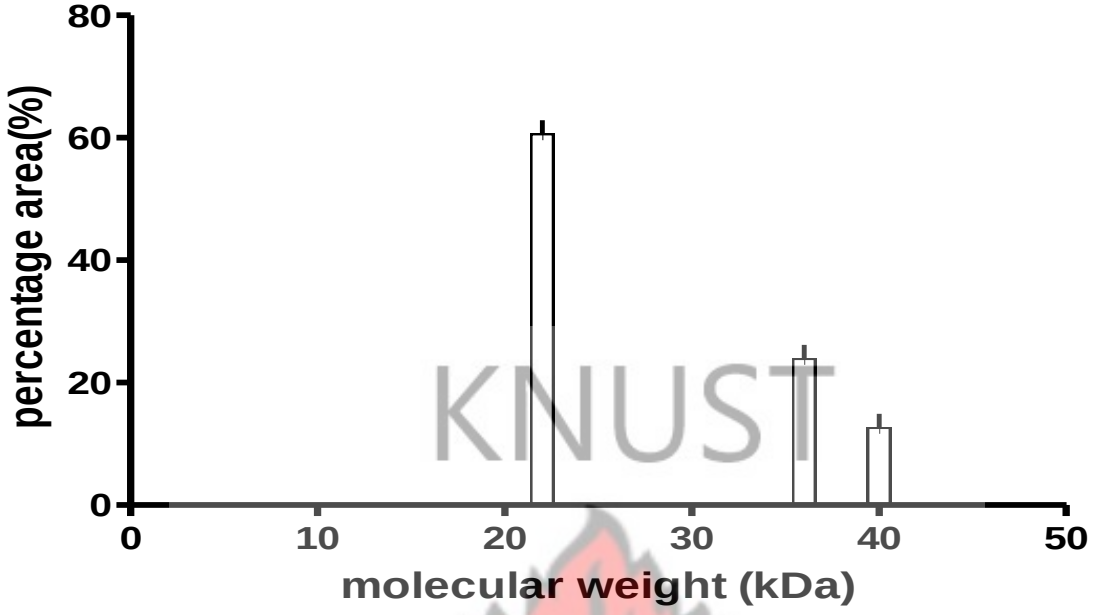

Figure 4.7: Percentage band area of the molecular weights of alcalase hydrolysis of Bambara groundnut protein hydrolysate at a DH of 0.7 %.

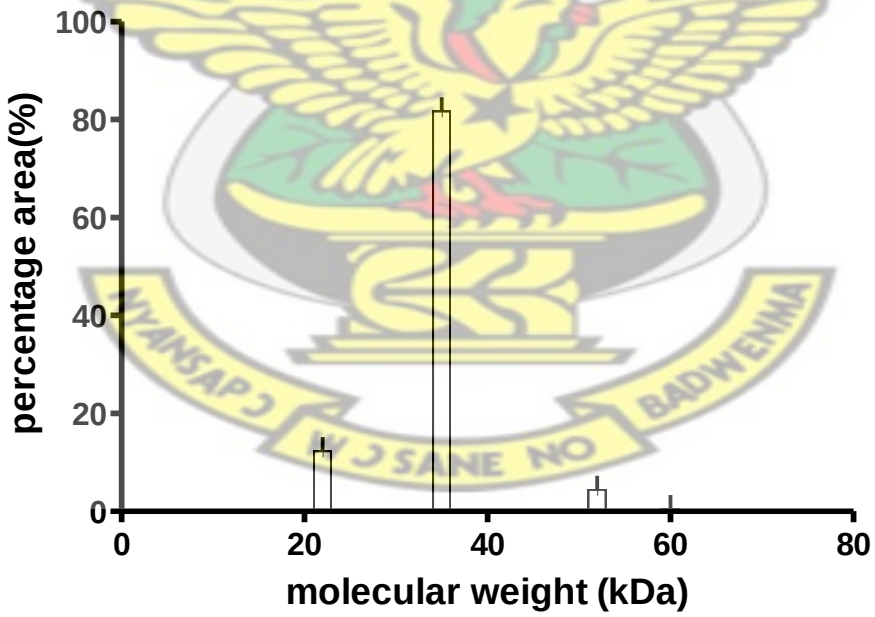

Figure 4.8: ***Percentage band area of the molecular weights of alcalase hydrolysis of bambara groundnut protein hydrolysate at a DH of 5.2 %.***

At degree of hydrolysis 5.2 from figure 4.8, which was achieved in the 120th min, four bands appeared giving significant peaks ranging from 22 kDa to 52 kDa. Prominent peaks were observed at 22 kDa and 36 kDa . There was an increase in the number of peaks as degree of hydrolysis increased for this hydrolysate when compare to that of figure 4.7 where the degree of hydrolysis was 0.7 and three peaks appeared.

4.5 Data analysis of Pegion pea protein hydrolysis

Similarly, after the DH responses were loaded and run for treatment of the Pigeon pea proteins, the sequential model sum of squares table (Table 4.5) was obtained. As in table 4.1, this table also gave the tool to check for the suggested regressional model that would best fit the response.

Table 4.5 ***Sequential model sum of squares table showing the suggested model (*) of the highest order polynomial where the additional terms are significant and the model is not aliased for the treatment of Pigeon pea proteins***

Source	Sum of Squares	df	Mean Square	F Value	p-value prob> F
Mean vs Total	130.83	1	130.83		
Linear vs Mean*	25.63	3	8.54	22.22	0.0001
2FI vs Linear	1.27	2	0.64	1.89	0.1990
Quadratic vs 2FI	0.39	1	0.39	1.17	0.3058
Cubic vs Quadratic	0.59	3	0.20	0.50	0.6918
Residual	2.75	7	0.39		
Total	161.46	17	9.50		

A significant p-value of 0.0001 ($p<0.05$) was obtained for linear *vs* mean model and the design expert process suggested this regressional model for the DH response based on the same fact that it was the model that had the highest order polynomial where the additional terms are significant and the model is not aliased.

Table 4.6 ***Lack of fit test table showing the suggested model (*) of the highest order polynomial with the immediate biggest prob> F value for the treatment of the Pigeon pea proteins***

Source	Sum of Squares	df	Mean Square	F Value	p-value prob> F
Linear*	2.35	8	0.29	0.56	0.78
2FI	1.08	6	0.18	0.34	0.89
Quadratic	0.69	5	0.14	0.26	0.92
Cubic	0.10	2	0.05	0.09	0.91

Table 4.7 ***Model summary statistics table showing the suggested model (*) of the highest order polynomial with the maximized*** **r-squared** ***value the treatment of*** **Pigeon pea** ***proteins***

Source	Std. Dev.	r-squared	adjusted r-squared	Predicted r-squared	PRESS
Linear	0.62	0.84	0.80	0.71	8.95
2FI	0.58	0.88	0.82	0.65	10.63
Quadratic	0.58	0.89	0.83	0.62	11.56
Cubic	0.63	0.91	0.80	0.61	11.95

The model summary statistics as was studied from table 4.7 focused on the model maximizing particularly the "adjusted r-squared" and the "predicted r-squared". It was aimed at selecting model with r^2 value closer to 1. However, the design expert suggested the linear model because the cubic and quadratic models values were too far apart.

Table 4.8 ***Analysis of variance table showing the significance(*) of the suggested model as well as the significance of the factor(s) and their interractions in the regressional model that has been obtained for the treatment of Pigeon Pea proteins***

Source	Sum of Squares	Df	Mean Square	F Value	p-value prob> F
Model*	27.88	9	3.10	7.90	0.0062
A-Time of hydrolysis*	23.51	1	23.51	59.93	0.0001
B-Enzymes	2.16	2	1.08	2.76	0.1309
AB	1.31	2	0.65	1.67	0.2559
A^2	0.39	1	0.39	0.99	0.3525
A^2B	0.56	2	0.28	0.72	0.5204
A^3B	0.04	1	0.04	0.09	0.7689

Table 4.8 above shows that the times of hydrolysis for Pigeon pea protein was significant ($p<0.05$). This time round there were no interactions between any of the factors and the model was not responsive to any interaction of the higher order as well.

4.6 Effect of time on the degree of hydrolysis for the three enzymes on Bambara groundnut

The addition of enzyme is a decisive factor affecting the hydrolysis rate and its magnitude depends, among others, on the kind of hydrolysed protein (Surówka *et al*., 2003). However, from the figure above there was a gradual increase in degree of hydrolysis for all three enzymes with alcalase having the highest degree of hydrolysis at start time of 10 min.

From figure 4.9, on the 10^{th} minute, alcalase had the highest degree of hydrolysis with papain and pepsin starting at similar degree of hydrolysis and ending being the least in degree of hydrolysis. Papain's low performance on Pigeon pea may be because Pigeon pea proteins are low in cysteine amino acid according to Eltayab *et al*. (2008). The results obtained in figure 4.9

are comparable to that obtained by Govindaraju and Srinivas (2006) who obtained hydrolysates with low DH 3-5 %, resulting from treatment by papain, alcalase and fungal protease.

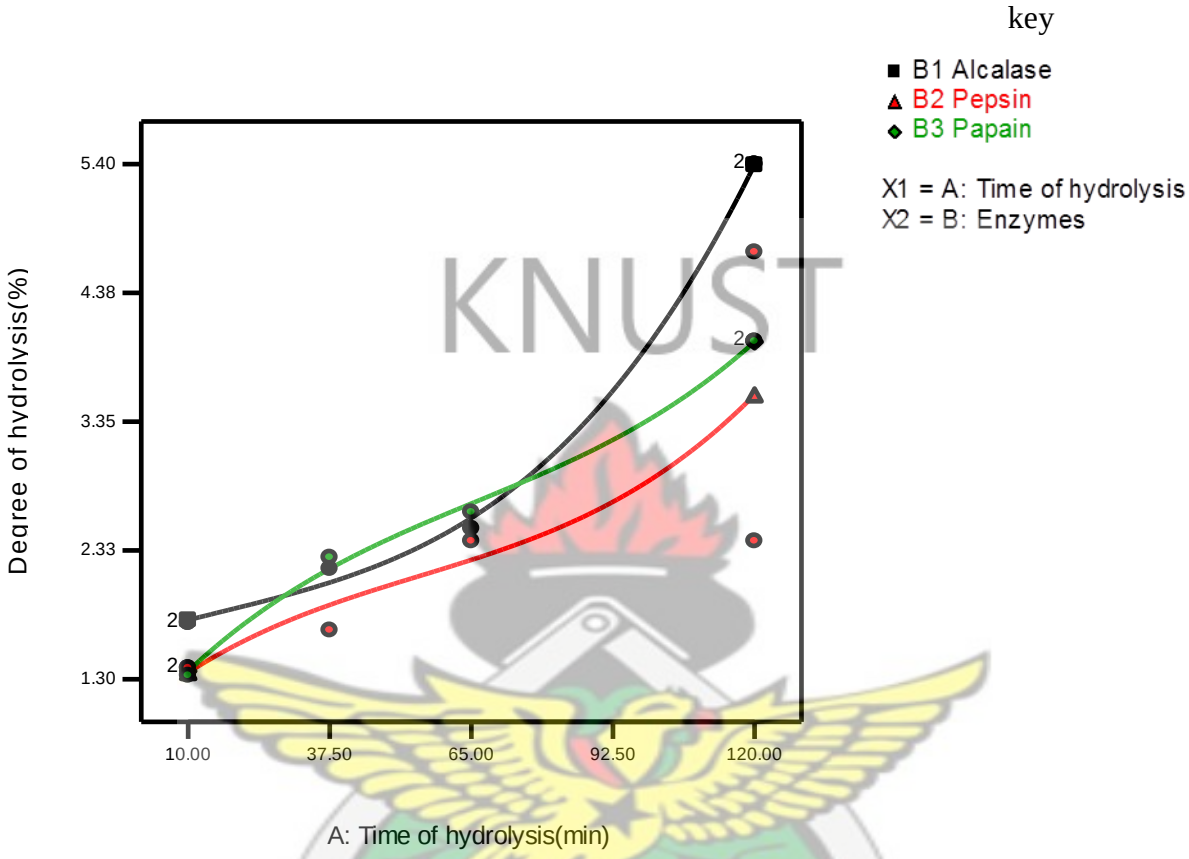

Figure 4.9 ***Regressional plots showing the time of hydrolysis and the degree of hydrolysis of Bambara groundnut protein hydrolysates as a result of proteolysis (■ Alcalase, ▲ Pepsin and ♦ Papain)***

Their results indicated that emulsifying property of hydrolysates was improved with low DH, but extensive hydrolysis resulted in remarkable reduction in emulsification meaning the possibility exist that further determination of the hydrolysates could have recommended surface functional properties. From the figure 4.9 at the end of the hydrolysis, alcalase hydrolysates maintained being the highest in terms of the degree of hydrolysis score. These suggest that for Pigeon pea,

alcalase performance is remarkable and must be monitored closely in order to prevent excessive hydrolysis that would not yield resourceful surface functional properties.

4.7 Effect of limited proteolysis on the protein profile of Pigeon pea

In general, figure 4.10 below show that there has been reduction in the molecular weights of proteins following limited proteolysis with increasing degree of hydrolysis. The degree of hydrolysis increased positively with peaks. In limited hydrolysis, the enzymes are said to breakdown proteins of larger molecular weights to smaller molecular weights.

Figure 4.10*: Lane 1 protein marker, lane 2 papain at DH of 1.6 %, lane 3 papain at DH of 4.0 %, lane 4 alcalase at DH of 2.2 %, lane 5 alcalase at DH of 5.4 %, lane 6 pepsin at DH of 1.7 %, lane 7 pepsin at DH of 4.7 % for Pigeon pea protein hydrolysate, representing 17th min and 100th min respectively.*

The length of peptide chain influences the rate of absorption, and therefore, the nutritional value of food proteins (Silvestre, 1994). The increased number of bands can be attributed to the

polymerization of proteins of larger molecular weights. It was also observed from the two electrophotogram that as the degree of hydrolysis increases the thickness of the bands reduces. Enzymatic hydrolysis separated total Pigeon pea proteins into multiple components with molecular weight (MW) ranging from 97 kDa to 23 kDa. Barac *et al.* (2011) reported that upon hydrolysis, pea protein hydrolysate ranges from 104.8 kDa to 9.8 kDa which originated mainly from vicilin and legumin.

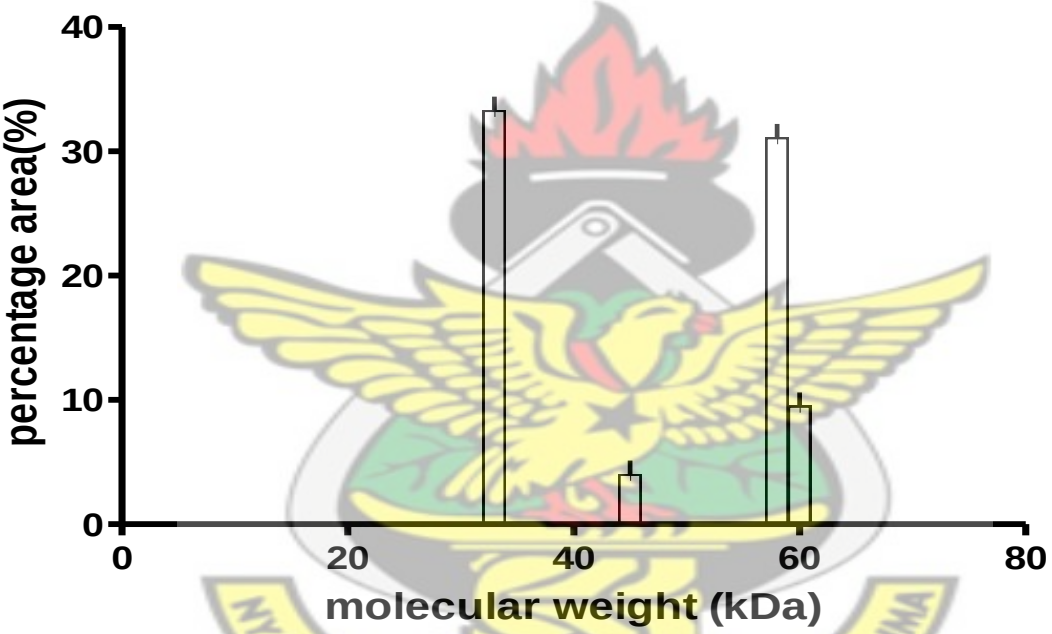

Figure 4.11: Percentage of band area for papain hydrolysis of pigeon pea protein hydrolysate at degree of hydrolysis 1.6 %

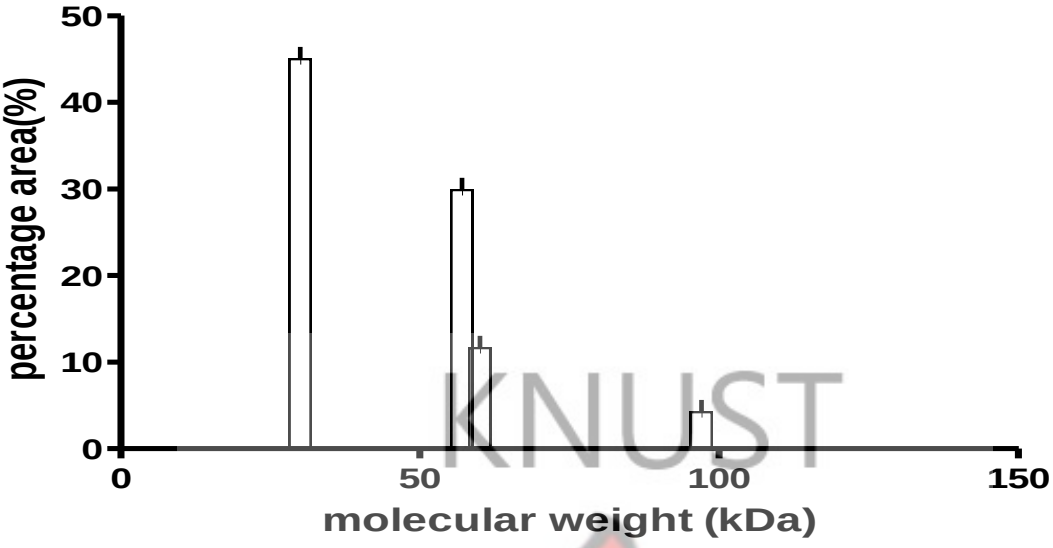

Figure 4.12: Percentage band area of the molecular weights of hydrolysis papain hydrolysis of pigeon pea protein hydrolysate at degree of hydrolysis 4.0 %

Figure 4.11 shows the hydrolysis at degree of hydrolysis 1.6 % which was achieved in the 10th minute, four (4) bands appeared giving significant peaks ranging from 33 kDa to 60 kDa. At degree of hydrolysis 4.0 % in figure 4.12, which was achieved in the 120th min, four bands appeared again giving significant peaks ranging from 30 kDa to 97 kDa. A protein with an apparent MW of about 97 kDa, which was present at the 120th min, has been reported to be lipoxygenase (Crévieu *et al*., 1997).

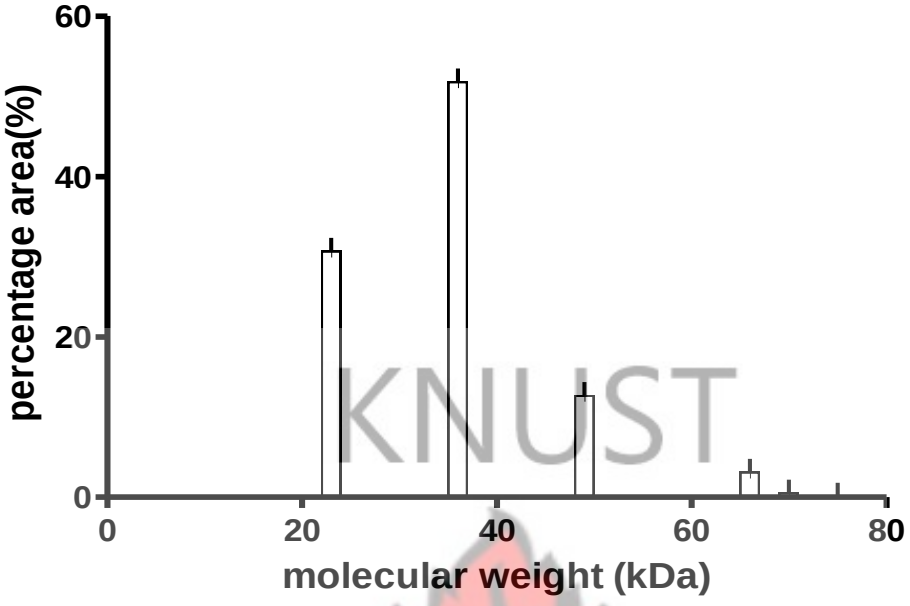

Figure 4.13: Percentage band area of the molecular weights of hydrolysis alcalase hydrolysis of pigeon pea protein hydrolysate at degree of hydrolysis 2.2 %

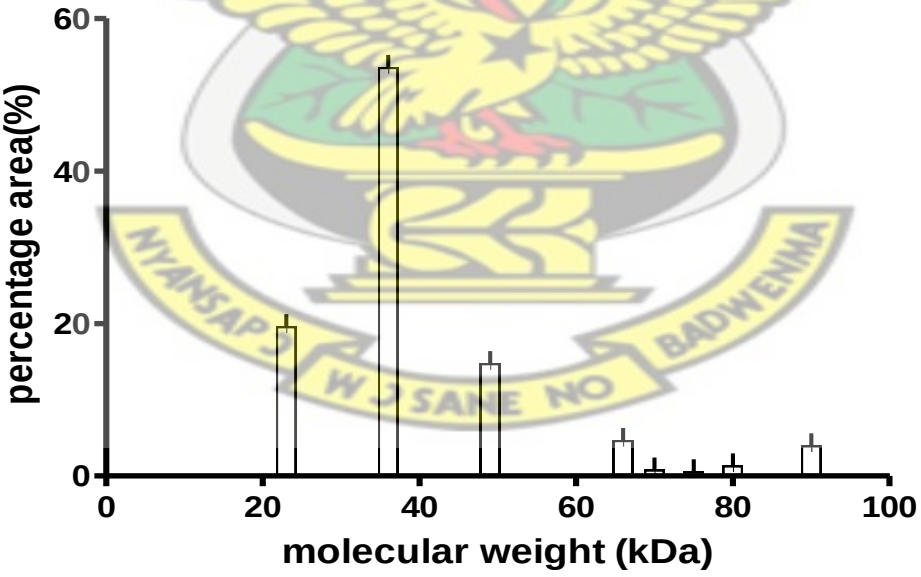

Figure 4.14: Percentage of peak area for alcalase hydrolysis of pigeon pea protein hydrolysate at degree of hydrolysis 5.4 %

A degree of hydrolysis 2.2 % from figure 4.13 was achieved in the 10th minute, six bands appeared giving significant peaks ranging from 22 kDa to 97 kDa. At degree of hydrolysis 5.4% from figure 4.14 which was achieved in the 120th min, eight bands appeared giving significant peaks ranging from 23 kDa to 90 kDa. Prominent peaks were observed at 36 kDa the highest and 23 kDa . this showed high increase in bands and may be beacuase Alcalase had high degree of hydrolysis in limited proteolysis of Pigeon pea compared papain and pepsin.

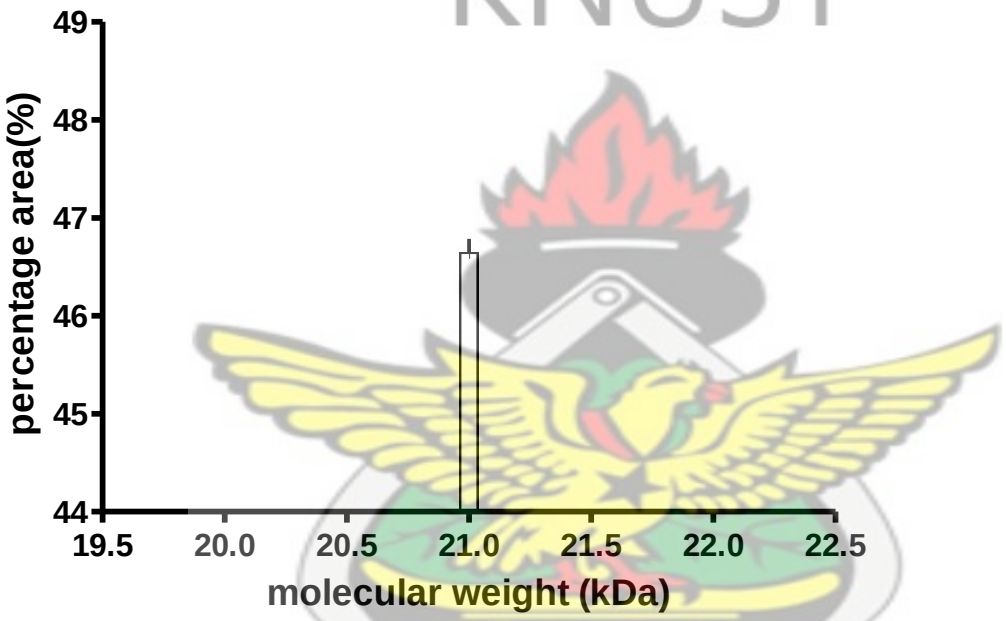

Figure 4.15: Percentage band area of the molecular weights of hydrolysis pepsin hydrolysis of pigeon pea protein hydrolysate at degree of hydrolysis 2.2 %

At degree of hydrolysis 2.2 % which was achieved in the 10th min from figure 4.15, one band which appeared on 21 kDa .there were no bands at the 120 min hydrolysis. Generaly, pepsin's hydrolysate was the least in terms of degree of hydrolysis of Pigeon pea. No band at all was observed from electrophoregram in Figure 4.10 and may be because produced peptides can

interact with unhydrolysed protein via hydrophobic interactions resulting in increase of the insoluble protein fraction as reported by Paraman *et al.* (2006).

The functional properties of the legumes after this hydrolysis are generally going to be altered. Govindaraju and Srinivas (2006) obtained hydrolysates with low DH 3-5 %, which were treated by papain, alcalase and fungal protease. Their results indicated that emulsifying property of hydrolysates was improved with low DH, but extensive hydrolysis resulted in remarkable reduction in emulsification.

From this study alcalase are expected to have high impact on the functional properties of Bambara groundnut and Pigeon pea proteins from Figure 4.1 and 4.9 for Bambara groundnut and Pigeon pea respectively. This is therefore indicative that new food product can be produced from Bambara groundnut and pigeon pea proteins following limited proteolysis.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

The use of alcalase, pepsin and papain from 10 min to 120 min on both Bambara groundnut and Pigeon pea protein isolates was able to produce hydrolyzed proteins with increased peptide size distribution under increasing degree of hydrolysis. SDS-PAGE analysis confirmed that degree of hydrolysis has an influence on the peptide profile of hydrolysates following hydrolysis. Again on the average, alcalase showed the highest degree of hydrolysis in Pigeon pea protein hydrolysate. Papain on the otherhand showed the highest degree of hydrolysis and SDS-PAGE analysis confirmed it produced smaller peptides in its action on Bambara groundnut proteins.

5.2 Recommendations

Further works should be done to relate the degree of hydrolysis and peptide profile on the functional properties of the resultant hydrolysate so as to help in the production of new and improved food products from the two underutilized protein sources.

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APPENDIX ONE

Appendix 1.0: Preparation of reagents

1.1 Preparation of 0.01 M sodium hydroxide

The solution was prepared by dissolving 0.04g of the sodium hydroxide pellets in distilled water in a 1000 mL volumetric flask to produce 1000mL of solution.

1.2 Preparation of 1 M hydrochloric acid

A volume of 86mL of the concentrated acid solution was added to a 1000mL volumetric flask half filled with distilled water a little at a time. The flask stood in cold water with a constant gentle swirling. The volume was then made to the mark with distilled water.

1.3 Preparation of the Bradford reagent

Fifty milligram of Coomassie brilliant blue dye G250 was dissolved in 50 mL methanol. A 100 mL of 85% phosphoric acid was added to the solution and this solution prepared was added to 500mL distilled water and was mixed thoroughly. It was filtered to remove precipitate and additional 350 mL of distilled water was added. It was stored in a refrigerator at 4^0C when not in use.

1.4 Preparation of the Ninhydrin solution

Twenty–five grams of Ninhydrin crystals were dissolved in 100 mL ethanol. Fifty milliliters of glacial acetic acid was added slowly while mixing. The solution was filtered and stored in a dark bottle.

1.5 Preparation of Tris buffer

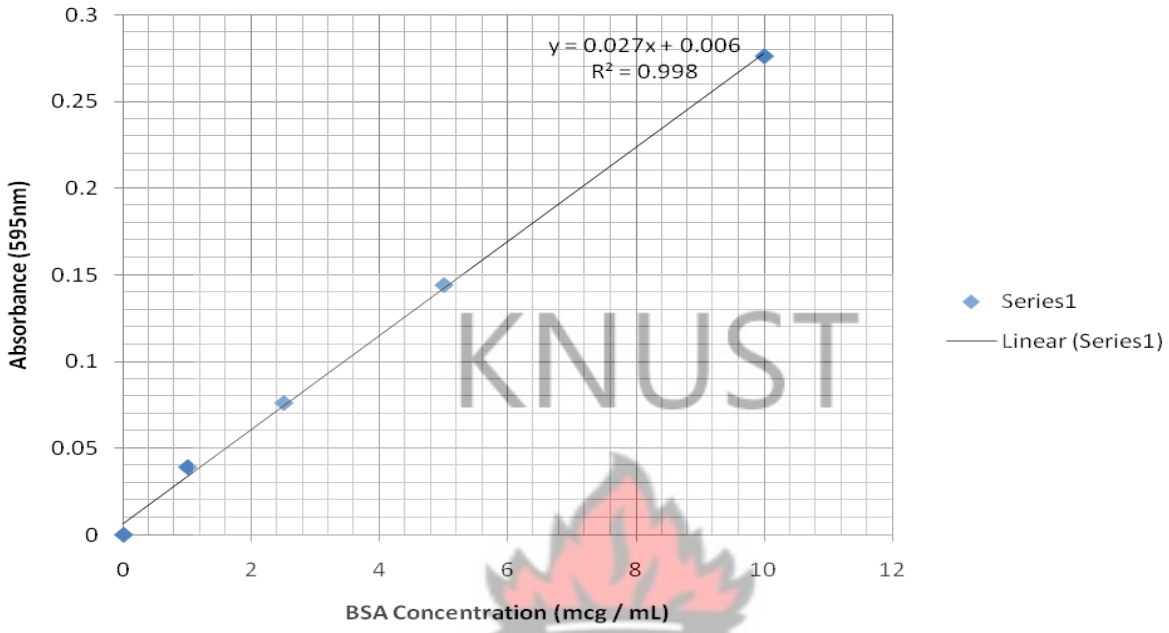
121.14g of tris was dissolved in 800ml of distilled water. The pH was adjusted to 7.0using an appropriate volume of HCl. The final volume was topped to the 1 litter mark. The resultant solution was autoclaved and stored at room temperature.

APPENDIX TWO

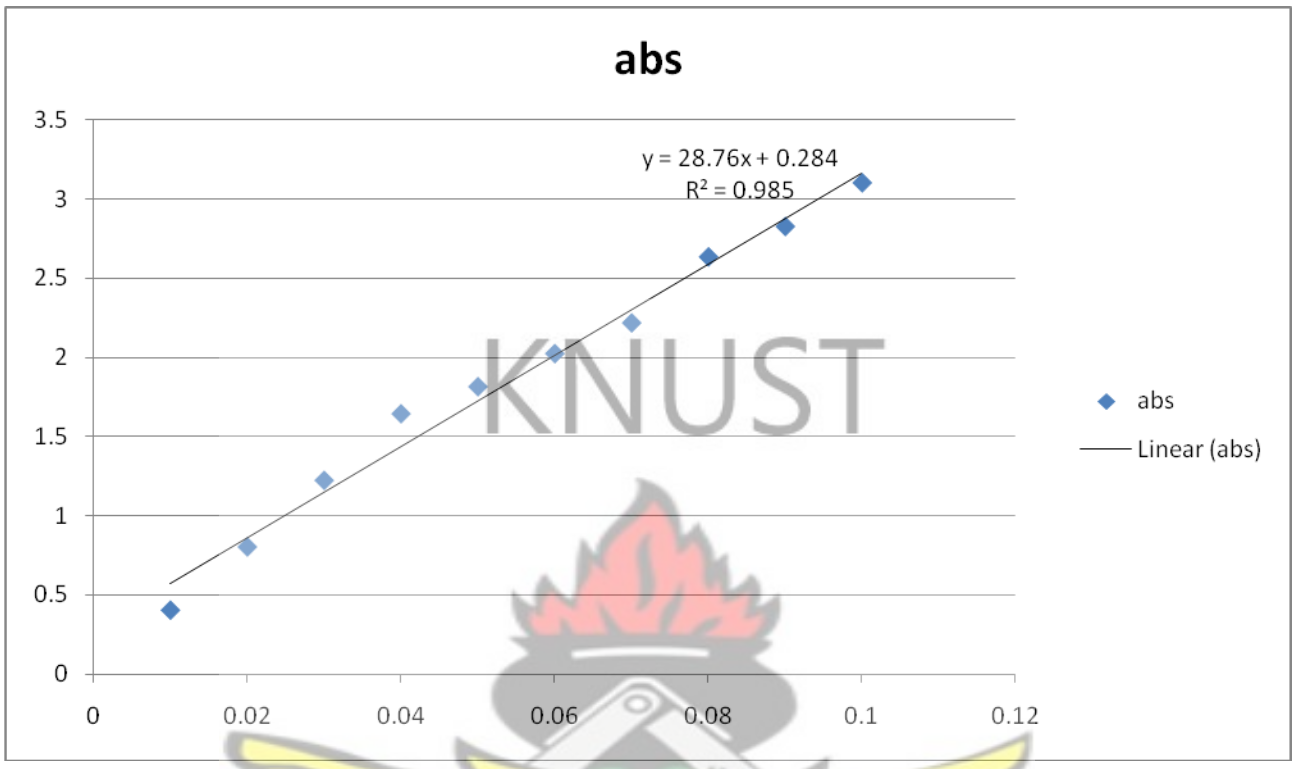
2.1 Table for the preparation of standards for the Bradford BSA assay at pH 8.0

Sample	Volume BSA working solution (0.5 mg/mL)	Volume water/ mcL	Volume Bradford reagent/ m	Absorbance (595 nm)		
Blank	0	800	200	N/A	N/A	N/A
BSA Standard – 1.0 mcg/mL	2	798	200	0.040	0.038	0.039
BSA Standard – 2.5 mcg/mL	5	795	200	0.078	0.074	0.076
BSA Standard – 5.0 mcg/mL	10	790	200	0.144	0.143	0.144
BSA Standard – 10.0 mcg/mL	20	780	200	0.273	0.279	0.276

2.3.3.1 A graph of BSA concentration with absorbance at pH 8.0



2.2 Preparation of leucine standard in ninhydrin reagent



2.3 Calculation of degree of hydrolysis

where A is the absorbance at 570 and b and m are the y intercept and slope of the calibration curve.

The degree of hydrolysis at each time point was then calculated from the following equation:

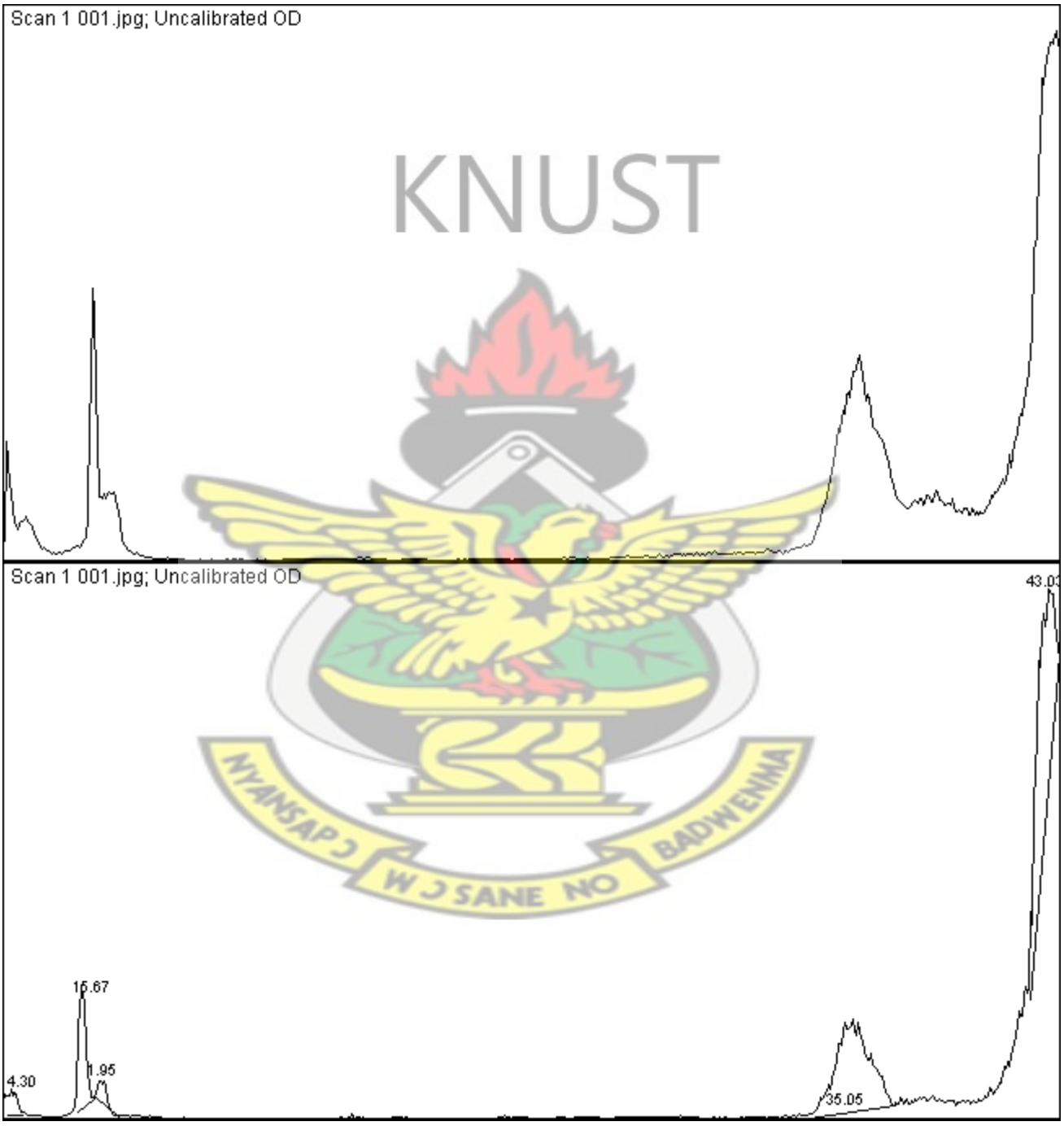
$h = (y\text{-}.0284)/28.76$

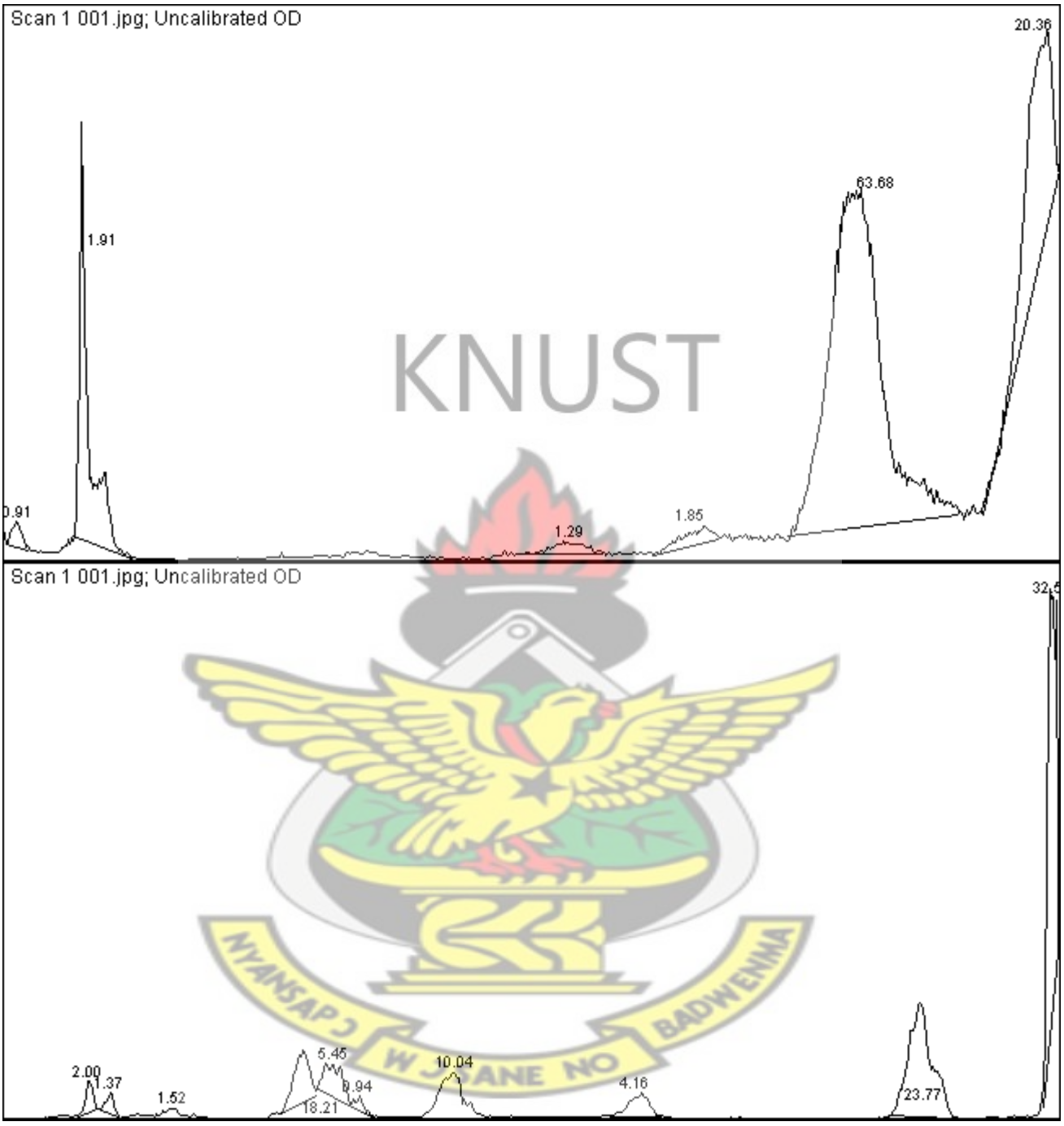
$D_H = h/h_{tot}$ x100 %

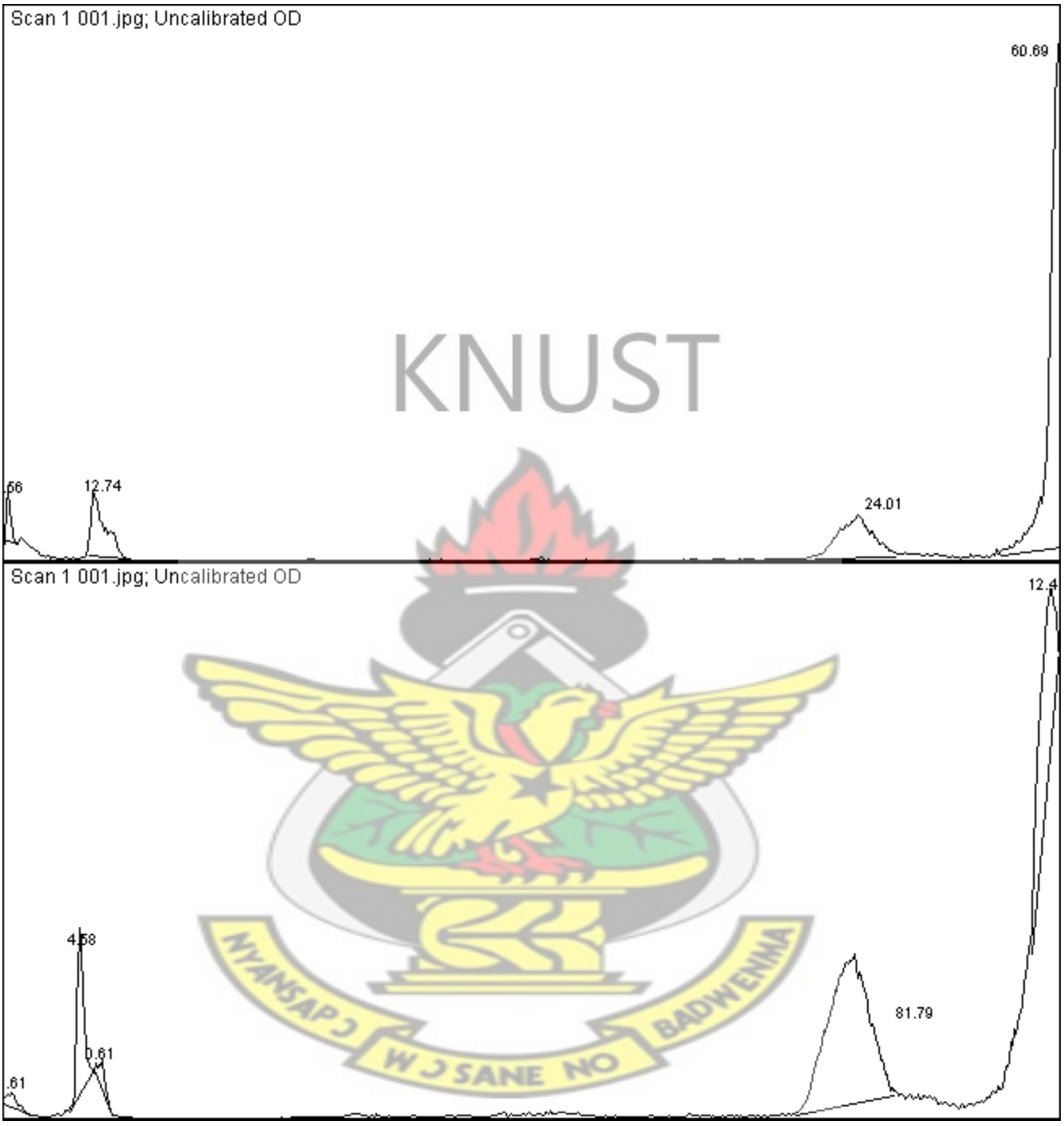
where h_{tot} is the total amount of peptide bonds given as 8 (obtained as the reconstituted 8 % (w/v) in deionized of the sample protein prepared in the sample characterization stage).

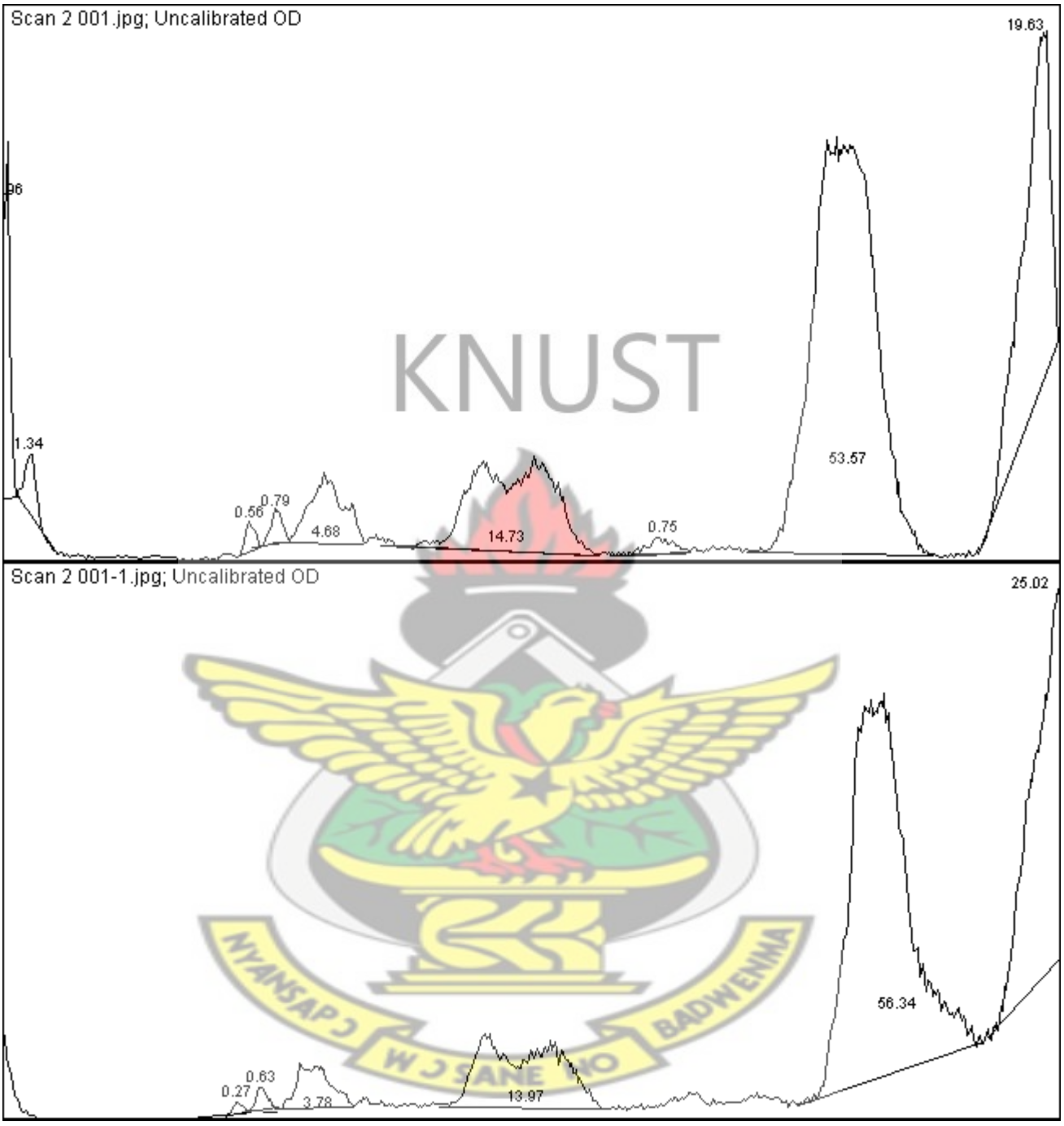
APPENDIX THREE

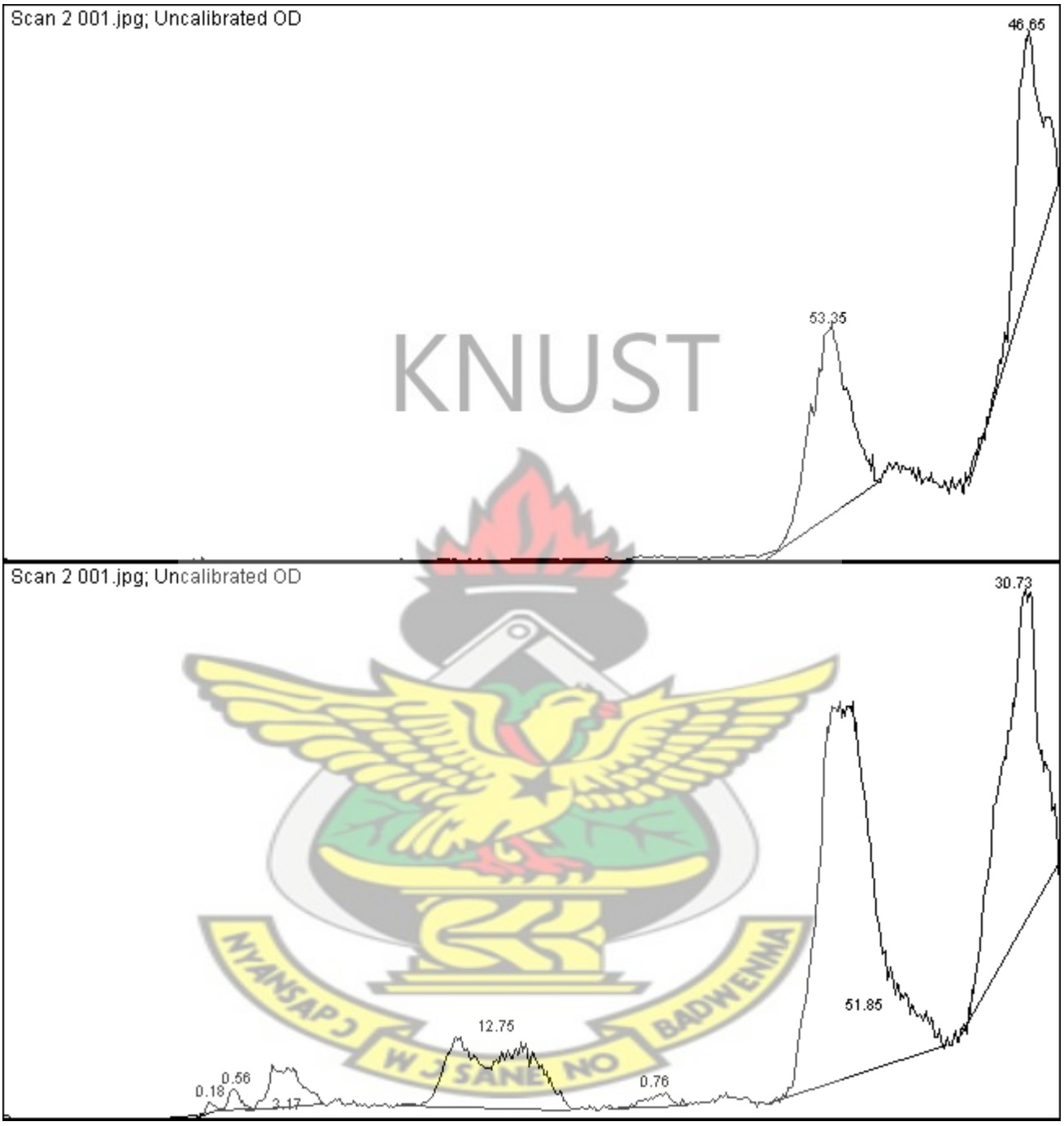
3.1 Peaks of electrophotogram after Image –J analysis




Scan 1 001.jpg; Uncalibrated OD
20.36
63.68
1.91
0.91
1.29
1.85
Scan 1 001.jpg; Uncalibrated OD
2.00
1.37
1.52
5.45
18.21
8.94
10.04
4.16
23.77


Scan 1 001.jpg; Uncalibrated OD
60.69
12.74
24.01
Scan 1 001.jpg; Uncalibrated OD
81.79


Scan 2 001.jpg; Uncalibrated OD
19.63
96
1.34
0.56
0.79
4.68
14.73
0.75
53.57
Scan 2 001-1.jpg; Uncalibrated OD
25.02
0.27
0.63
3.78
13.97
56.34


Scan 2 001.jpg; Uncalibrated OD
46.65
53.35
Scan 2 001.jpg; Uncalibrated OD
30.73
51.85
12.75
0.56
0.18
3.17
0.76