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Student ID number: 03230120

First name: Noriza

Surname: Ahmad

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Chief supervisor: Assc. Prof Dr. Marie Wong

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THE ENGINE OF THE NEW NEW ZEALAND

BEEF HYDROLYSIS BY ZYACTINASE™ ENZYMES

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

at Massey University, Auckland,

New Zealand.

NORIZA BINTI AHMAD

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Abstract

Protein hydrolysis is the term that applies to all possible ways of splitting proteins to produce products with lower molecular weight. There is a continuous search for novel products derived from waste materials. In the developed nations considerable amount of meat off-cuts are discarded each year. Utilizing these leftovers by developing new technology for protein recovery and modification and production of a broad spectrum of food ingredients greatly enhances its final value.

The aim of this research was to partially hydrolyse beef meat protein with a commercial kiwifruit product called Zyactinase^{TM}, which is essentially freeze-dried kiwifruit to determine the effect of various processing conditions that influence the extent of beef meat hydrolysis. Secondly to determine the peptide and amino acid profile of the beef meat sample after hydrolysis. Thirdly to determine the relative reaction of Zyactinase^{TM} on various beef meat protein fractions. This study also aimed to evaluate the rate and the extent of partial enzymic hydrolysis of lean beef using Zyactinase^{TM} enzymes in order to obtain a better understanding of protein hydrolysis reaction.

Lean beef minced was partially hydrolysed using the Zyactinase enzymes for different processing times (up to 360 minutes), temperatures (27°C to 70°C) and varying enzyme concentrations. No pH adjustment on the raw material was carried out except for pH studies. The hydrolysates were collected and analysed for total nitrogen content and degree of hydrolysis. The method used to characterize the extent of protein hydrolysis was SN-TCA index (fraction of nitrogen soluble in trichloroacetic acid) also called non-protein nitrogen NPN. Peptide and amino acid in protein hydrolysates were analysed by HPLC and different protein fractions in the hydrolysates were characterised by SDS-PAGE.

The relationship between the reaction temperature, enzyme concentration and processing time to the total nitrogen and NPN were determined. The total nitrogen content remained relatively constant throughout the hydrolysis process. In addition, the NPN content increased as the temperature, processing time and enzyme concentration increased. The optimum pH range for the enzyme's activity was 4 – 5.6 and optimum temperature was 60°C. Furthermore, most of the higher molecular weight protein bands on SDS- PAGE disappeared after hydrolysis and lower molecular weight protein

bands increased in intensity. Zyactinase was also found to digest protein in the myobrilla and sarcoplasmic meat fractions at similar rates as whole beef meat.

The results provide basic understanding of the kiwifruit enzymes action toward protein that may lead to improved methods for recovering meat protein or developing new food materials.

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List of abbreviations

The following table describes the significance of various abbreviations and acronyms used throughout the thesis.

Abbreviation	Meaning
-COOH	Carboxyl group
-NH2	Amino group

ADP	Adenosine diphosphate
APS	Ammonium persulfate
ARI	Allegenicity reduction index
ATP	Adenosine triphosphate
C	Weight percentage of cross linker
Ca^{2+}	Calcium
Da	Dalton
DH	Degree of hydrolysis
DM	Dry matter
DTNB	Ellman's Reagent (5,5'-dithio-bis-[2-nitrobenzoic acid])
DTT	Dithiothreitol
EC	Emulsification capacity
F-actin	Filament actin
G- actin	Globular actin
HMM	Heavy meromyosin
HPLC	High performance liquid chromatography
IgE	Immunoglobulin E
k_{cat}	Turnover number
k_m	Michaelis constant
LMM	Light meromyosin
N	Nitrogen
NPN	Non-protein nitrogen

OPA	o-phthalaldehyde
pI	Isoelectric point
SDS	Sodium dodecylsulfate
SDS - PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH	Sulfhydryl group
T	Total monomer concentration
t	Time
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TN-C	Troponin C
TN-I	Troponin I
TN-T	Troponin T
T_S	Shrink temperature
v/v	Volume over volume
V_0	Initial velocity
V_{max}	Maximum velocity
w/v	Weight per volume

Chapter 1
Introduction

Many humans consume meat, as a source of protein, as part of their daily diet. The Australia - New Zealand Food Standard Code (2000) defines meat as the whole or part of the carcass of any buffalo, camel, cattle, deer, goat, hare, pig, poultry, rabbit or sheep, slaughtered other than in wild state, but does not include –

(a) the whole or part of the carcass of any other animal unless permitted for human consumption under the law of a State, Territory or New Zealand; or

(b) avian eggs, or foetuses or part of foetuses.The definition is expanded to include meat flesh, offal (such as kidney liver and brains) and other edible tissues (Food Standards Australia New Zealand, 2000).

Over the course of history, religious, cultural factors, moral, ethical belief and lifestyle have been a deciding factors for meat consumption in different populations (Dinyal and Dinyal, 2003, Font-I-Furnols & Guerrero, 2014). Religious and cultural factors have had considerable influence in defining what meat is considered acceptable, or unacceptable for consumption. For example, Muslims forbid the consumption of any part of pork or their derivative and regulate the way other Halal animals are slaughtered, whereas Hindu mandates the avoidance of beef.

The shortage of protein in some parts of the world contributes to the need for new methods that will improve the recovery and quality of the protein produced. In New Zealand the export meat industry was worth NZ$ 6.1 billion in the 12 months to the end of June 2012. This was approximately 26% of the country's total exports and made up the second largest food export after dairy. By-products and co-products from meat contribute NZ$1.2 billion, or 22% of the overall meat industry's total export receipt (Meat Industry Association of New Zealand (Inc), 2013). In a Western country such as New Zealand meat selection is based on a whole cut and there are many other edible parts not utilized. The 2011 Red Meat Sector Strategy Report (Meat Industry Association of New Zealand (Inc), 2011) identified farming for meat production was struggling and this was the main driver for conversion of sheep and beef farms to dairy production. New products from meat will generate new value to the beef carcasses.

Around 60 to 70 % of a beef carcass is by–product, of which 40% is edible and 20% remains inedible. Currently, the by-product is underutilized, being used for high price delicacies (cheek meat, oxtail) and lower grade protein material is being used for animal feed, fertilizer, or fuel. Annual meat production is projected to increase from 218 million tonnes in 1997-1999 to 376 million tonnes by 2030 (Meat Industry Association of New Zealand (Inc), 2011)

Proteolytic modification of food protein is an ancient technology by which mankind has been able to improve the palatability and storage stability of the available protein resources (Kristinsson & Rasco, 2000). Functional properties such as solubility, dispersibility, foaming and emulsification can be improved by the limited proteolysis of protein (Fennema, Parkin, & Damodaran, 2008; Quaglia & Orban, 1990).

Protein hydrolysate produced from proteolysis of proteins is an alternative to intact proteins. They have been used as an ingredient in the development of special food formulations designed to provide support to patients with special nutritional needs. The hydrolysis process influences the molecular size, hydrophobicity and polar groups of the hydrolysate (Adler-Nissen, 1986; Kristinsson & Rasco, 2000). The characteristics of the hydrolysate directly affects the functional properties and ultimate end use as a food ingredient (Kristinsson & Rasco, 2000).

The effect of proteolytic enzymes found in kiwifruit on gelatine and haemoglobin have been known for some time (Arcus, 1959). Kiwifruit protease affects muscle tenderness and structure (Lewis & Luh, 1988a; Samejima et. al., 1991; Wada, Hosaka, Nakazawa, Kobayashi, & Hasegawa, 2004; Wada et. al., 2002). Actinidin, the key protease in kiwifruit, has been considered a better meat tenderizer than papain because it does not over-tenderize the meat surface or result in a ‘slimy’ texture (Lewis & Luh, 1988). By regulating the pH , the enzymic activity of Actinidin can be controlled, in contrast to papain and bromelain enzyme activity which non-selectively hydrolyzes all myofibrillar protein over a wide pH range (Nishiyama, 2001).

The main aim of this research was to characterise and model Zyactinase^{TM} hydrolysis of beef.

Hence the individual objectives were:

1. To determine the extent to which Zyactinase™ enzyme complex can hydrolyse beef meat under various conditions including pH, temperature, substrate and enzyme concentration. The extent will be assessed by degree of solubilisation of hydrolysed protein and monitoring the peptides and amino acids formed.
2. To determine the extent to which Zyactinase™ enzyme complex can hydrolyse specific protein fractions from beef meat.
3. To mathematically model for the reaction mechanism for the hydrolysis of beef meat with Zyactinase™ enzyme complex.

Zyactinase™ enzyme a semi-purified complex of enzymes extracted from kiwifruit has not previously been use to hydrolyse whole beef in natural state/ form. Though there have been publications on hydrolysis of other meat proteins with kiwifruit, crude kiwifruit extracts and pure actinidin none have looked at this semi-purified complex which may provide more specific activity than a crude extract but have advantages over a pure actinidin enzyme.

Also other protease enzymes have been investigated but it is unknown how Zyctinase™ will hydrolyse natural state of beef meet and what conditions provide the desired end product suitable for further application. Application of enzymes to hydrolyse beef meat with a view of applying this technology to waste meat fractions. In this research good quality fractions of beef meat were used to determine the action of the Zyactinase™ enzyme complex on the different fractions present in beef meat to ensure the interference of complex materials of difference fraction of beef meat were eliminated. Separate chapter discussing each fraction of beef protein was allocated to see the effect of zyactinase toward beef protein fraction in relation to whole beef protein.

Chapter 2

Literature Review

In order to understand the process of meat hydrolysis with enzymes, in this chapter firstly, muscle structure and composition will be described briefly. Subsequently, the methods of protein modification (hydrolysis) and their possible effects on meat hydrolysis will be discussed. This will be followed by a summary of previous research focused on meat hydrolysis using different enzymes. Finally, the use of kiwifruit and kiwifruit protease enzyme for meat tenderization and the enzyme's effect on meat and other substrates will be reviewed.

2.1 Muscle structure

On average muscle tissue contains 70 - 75% water, 22% nitrogenous compounds (predominantly protein), 1 - 5% fat, 1% ash (primarily represented by the elements of potassium, phosphorus, sodium, chloride, magnesium, calcium and iron) and 1% carbohydrate (primarily glycogen antemortem and lactic acid postmortem) (Lawrie & Ledward, 2006). These values vary from species to species as shown in Table 2.1.

Table 2.1: Proximate composition of meat from various sources expressed as a percentage of weight of edible portion (Fennema et al., 2008)

	Red Meat (% w/w)			Poultry (% w/w)		Fish (% w/w)	
	Beef	Pork	Lamb	Chicken	Turkey	Cod	Tuna
Water	70.62	72.34	73.42	74.76	74.12	81.22	68.09
Protein	20.78	21.07	20.29	23.09	24.60	17.81	23.33
Lipid	6.16	5.88	5.25	1.24	0.65	0.67	4.90
Ash	1.02	1.04	1.06	1.02	1.02	1.16	1.18

Muscle cells (or fibres) are long cylindrical cells, often several millimetres long, and surrounded by a connective tissue (shealth), called endomysium (Figure 2.1). Bundles of fibres are further enclosed by another connective sheath, called perimysium. Multiple bundles of fibres are then held together to create a muscle. This muscle is surrounded by a third sheath of connective tissue, called epimysium, which not only creates a unique and separate muscle, but also attaches the muscle via tendons to the bone (Winger, 1979).

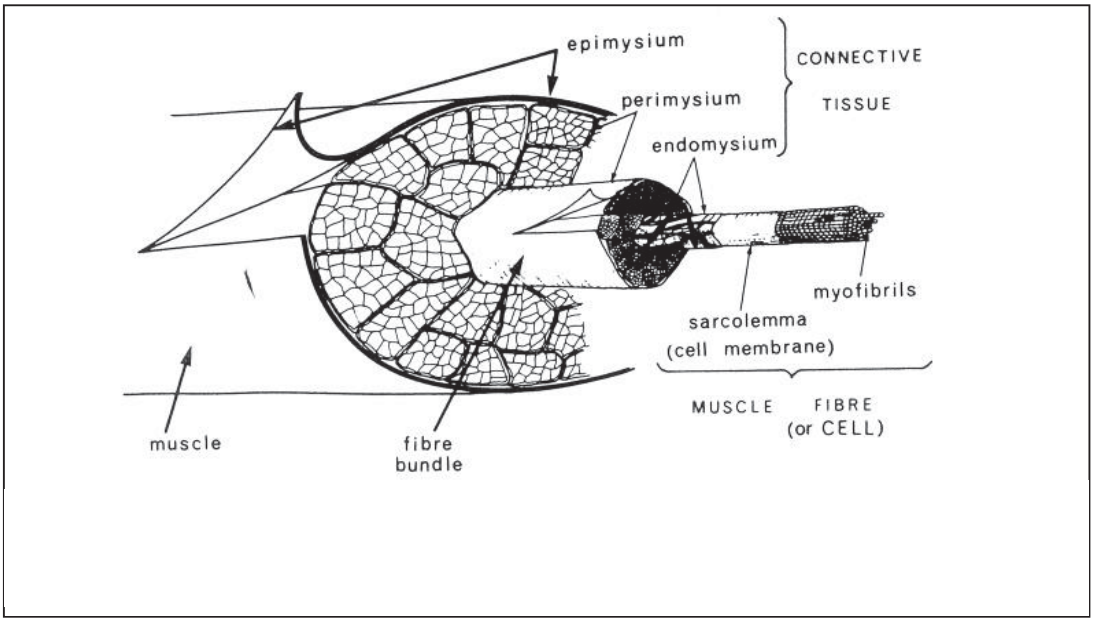

Figure 2.1: Diagrammatic representation of striated muscle structure (Winger, 1979)

Underneath the endomysium of muscle fibre is another layer known as sarcolemma, which is a plasma cell membrane and has a net-like structure. Each muscle fibre contains many parallel elements called myofibrils (e.g. one thousand myofibrils) with each about 1 - 2 μm in diameter which run the length of the fibre (Lawrie & Ledward, 2006). Each myofibril also contains a number of smaller subunits, called filaments (myofilaments), which are made up primarily of two major muscles proteins, actin and myosin. The actin and myosin proteins form thin and thick filaments, respectively, and these two filaments are aligned across the myofibril with alternating thin (actin) and thick (myosin) filaments and their overlap in certain regions. The interaction between actin and myosin filaments is responsible for muscle contraction and relaxation, which is unique to animals (Lawrie & Ledward, 2006). About 75 - 80% of the myofibrils

consist of alternating thin (actin) and thick (myosin) filaments. Each myofibril is also surrounded by a fluid membrane called sarcoplasm (cytoplasm), which is the intracellular substance in a muscle fibre and consists of around 80% water and other components such as proteins, enzymes, lipids, carbohydrates, inorganic salts, and some metabolic by-products (Lawrie & Ledward, 2006).

2.2 Muscle proteins

Most muscle tissue contains around 22% nitrogenous compounds (Lawrie & Ledward, 2006). Of the 22% nitrogenous compounds, 19% is protein and the remainder includes non-protein nitrogenous components such as vitamins, creatine, nucleotides, ammonia, methylamine, free amino acids, carnosine and anserine (Kauffman, 2012). The proteins in skeletal muscle can be divided into three major types: sarcoplasmic proteins, myofibrillar proteins and stromal (connective tissue) proteins, according to their solubility (Lawrie & Ledward, 2006). Sarcoplasmic proteins are soluble in water and hence are called water-soluble proteins, these also include enzymes, myoglobin and haemoglobin. Myofibrillar proteins are proteins that are insoluble in water, but soluble in high ionic strength salt solutions (typically 0.15M KCl or higher) and are called salt-soluble proteins. Examples of myofibrillar proteins are contractile protein (myosin and actin) and regulatory proteins (troponin and tropomyosin). The third group of proteins are classified as stromal proteins and they are insoluble in both concentrated salt solutions or water. These are known as connective tissue proteins and include collagen and elastin. Myofibrillar proteins constitute 60% of the total protein in the muscle, whereas sarcoplasmic proteins represent 29%, connective tissue (stromal) proteins make up 6% of the total protein and the remaining 5% of protein are called granular proteins related to non-structural cellular organelles, such as mitochondria (Kauffman, 2001).

Myofibrillar proteins are responsible generally for muscle contraction and structural support of the animal. Sarcoplasmic proteins are responsible for the metabolism in an animal cell, which is primarily represented by enzymes and myoglobin as indicated above. Albumins and globulins are the main sarcoplasmic proteins and around 90 different proteins belong to the group of sarcoplasmic proteins. Albumins are soluble in water or weak salt solutions whilst globulins are soluble in weak salt solutions only but insoluble in water. Myoglobin (red colour of meat) and haemoglobin (colour of blood) are the most important types of globulin proteins.

Connective tissue proteins are designed to transmit force and hold muscles together. The main representative in the group of connective tissue proteins is collagen (40 – 60%), tropocollagen, and elastin (around 10%) is present as well. Hence, the insoluble protein become the major part of connective tissue, around 30%.

2.2.1 Myofibrillar Proteins

Muscle myofibrils have a long cylinder shape, 1 - 2 μm in diameter, and extend the entire length of the muscle fibre. The major myofibrillar proteins are myosin, actin, tropomysin, troponin, actinins, C-protein, M-protein, F-protein, I-protein, myo-mesin, the filament protein desmin, Z-protein and titin (Price & Schweigert, 1987). Myofibrillar proteins undergo unfolding and aggregation before they can form a three–dimensional protein network (Lantto et al., 2005).

Myofibrillar proteins can be subdivided into three categories according to their physiological and structural roles in muscles of a live animal. Firstly, the contractile proteins myosin and actin are responsible for muscle contraction and building-up myofibrillar structure. Secondly, the regulatory proteins, which include tropomyosin and troponin, control and regulate binding of actin to myosin for muscle contraction. Thirdly, the cytoskeletal proteins responsible for the intracellular structure, including titin, nebulin, desmin, and other small proteins that act as supporting structure of muscle fibre sarcomere (Lawrie & Ledward, 2006).

The main myofibrillar proteins, adding up to 55 – 60% of the total myofibrillar protein, are myosin (around 42%) and actin (around 16%) as well as tropomyosin, troponin and actinin. Actin and myosin are also known as thin and thick myofilaments, respectively, and are responsible for muscle contraction and relaxation (Kauffman, 2000).

As mentioned above, myofibrils comprise approximately 75 – 80% thick and thin filaments. The thick and thin filaments of the myofibril, which are referred to as A band and I band, consist of myosin and actin as the main components, respectively. The overlap of the myosin and actin filaments accounts for the banding or striated appearance of the muscle myofibril (Forrest et al., 1975). The smallest functional unit of a myofibril is called a sarcomere and is normally defined as the repeating structural unit contained between two Z-lines (Figure 2.2). Thin filaments (mainly actin) are connected

to the Z-lines and extend into the sarcomere to overlap with the thick filaments (mainly myosin), shown in Figure 2.2 by the two dark areas either side of the H-zone. Thin filaments do not stretch into the H-zone in relaxed muscle. The thick filaments are not anchored in the sarcomere, but reside in the A-band in the middle of the sarcomere at rest. The overlap of myosin and actin gives the fibre a striated appearance of the myofibril (Lawrie & Ledward, 2006).

The interaction of myosin and actin allows muscles to contract causing movement due to the globular heads of myosin in the thick filament forming cross-bridges that interact with the actin in the thin filaments. Two other large filamentous proteins, titin and nebulin, also play important roles in myofibril assembly (Labeit & Kolmerer, 1995) and regulation of the actin-myosin interactions (Taylor et al., 1995).

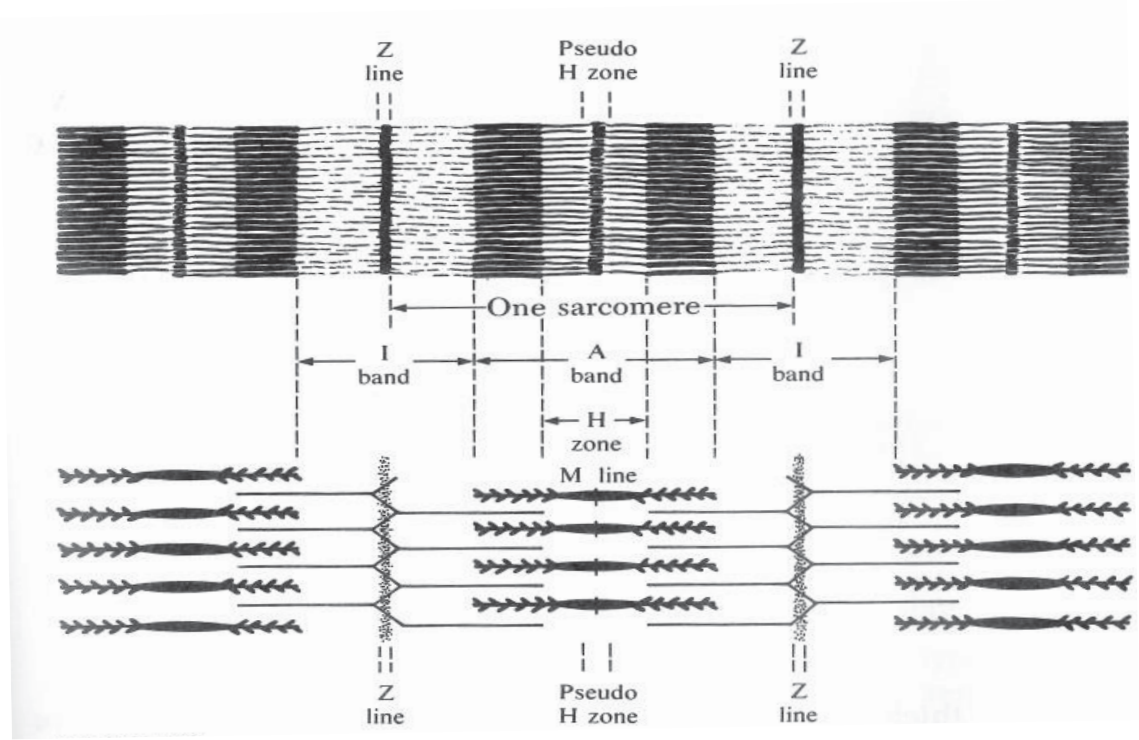

Figure 2.2: Portion of two myofibrils and a sarcomere (Forrest et al., 1975)

2.2.1.1 Myosin

Myosin is the major myofibrillar protein. About 26% of the muscle proteins are myosin (Kauffman, 2012). It also known as the 'thick' filament made from around 280 molecules of myosin. The molecular weight of myosin is around 490,000 ~ 500,000 Da. Each myosin molecule consists of two large polypeptides, each called a 'heavy chain', and four small subunits, each called a 'light chain'. Each of the heavy chains has a molecular weight of about 200,000 Da and each light chain has a molecular weight range of 16,000 to 30,000 (Price and Schweigert, 1987). Myosin is soluble in a high ionic strength salt solutions and is insoluble at low ionic strength. This unique solubility property makes it easy to isolate myosin in a pure form (Price and Schweigert, 1987).

Each myosin molecule exhibits a long tail and two pear-shaped heads in which two heavy chains form a long helical tail structure by winding around each other and at one end the two heavy chains are folded into two separate globular heads, each with two light chains (Rayment et al.,1993). The molecule is relatively highly charged due to high contents of acidic (glutamic and aspartic acids) and basic (lysine and arginine) amino acids. It has thus some affinity for calcium and magnesium ions (Lawrie & Ledward, 2006).

Myosin can be extracted with KCl solution of concentrations higher than 0.15 mol/L from the myofibrillar muscle structure after the removal of water–soluble proteins (Kijowski, 2001). Myosin molecules under the effect of sodium dodecylsulfate (SDS) dissociates into its subunits of high and low molecular weights.

There are two classes of light chain that are present in skeletal muscle myosin: an alkali light chain and a DTNB light chain. The alkali light chain can be removed from the myosin molecule under alkaline condition while the DTNB light chain can be removed by the sulfhydryl reagent, 5,5'-dithiobis-2–nitrobenzoic acid (DTNB) (Weed & Lowey 1971). Each myosin heavy chain is associated with one alkali light chain and one DTNB light chain. The two heavy chains join together and are folded into an α-helical confirmation for most of their length and at the NH_2-terminus forms a globular structure referred to as 'myosin head'. The myosin light chains are associated with these head portions.

Each thick filament in the muscle fibre contains approximately 280 myosin molecules, which are arranged in a staggered array with the heads protruding from the surface of the filaments (see Figure 2.2). The filaments also exhibit bipolar symmetry around the central axis so that the left and right halves of the filament are mirror images of one another (Weeds & Lowey, 1971). The myosin molecule contains regions in its polypetide chains, which are susceptible to mild proteolytic cleavage.

Proteolytic fragments of myosin are known as heavy meromyosin (HMM) and light meromyosin (LMM). LMM contains parts of both heavy chains and HMM comprises parts of heavy chains and the four light chains. Heavy meromyosin, which contains all the ATPase and actin-combining properties of myosin, is sited on the periphery of the myosin filaments. The properties depend upon the free SH group of the molecule (Lawrie & Ledward, 2006) . There are three properties of myosin that are (Freeman, 2000):

1. It is an enzyme with ATPase activity
2. It has the ability to bind with actin and activate the myosin ATPase.
3. It can aggregate with itself to form filament.

The isoelectric point (pI) of myosin, where negative and positive charges are of the same number within the protein, is at a pH value of 5.0, which means it has negative charge at normal meat pH value which is typically in the range of 5.4 to 6.2 (Sikorski, 2001). The tail of the myosin molecule is rich in acidic amino acids as well as alkaline amino acids and the COO^- as well as the NH_3^+ groups are attracted to each other inside the tail. Those charged ions hold the thick filament together and are also responsible for the limited solubility of myosin in water. Myosin also carries the enzyme myosin ATPase activity (where ATP is adenosine triphosphate), which plays a major in role in the movement of muscle fibres during the contraction and relaxation of a muscle (Freeman, 2000).

Myosin forms an irreversible gel when heated. This gel exhibits a high water-binding capacity and very strong elastic properties. Gelation of myosin optimally occurs at temperatures between 60 - 70°C at a pH of 6.0 and an ionic strength of 0.6 M (Yasui et al., 1979; Yasui et al.,1980)

2.2.1.2 Actin

The thin filament comprises mainly actin, which with myosin forms the contractile components of muscle. Actin represents 13% of total muscle protein and has an isoelectric point at pH 4.7 (American Meat Institute Foundation, 1960). Actin is a globular protein made up a single polypeptide chain which binds one molecule of nucleotide (ATP or ADP) and divalent cation (Ca^{2+} or Mg^{2+}) per monomer (Price & Schweigert, 1987). It can exist in two forms, G–actin (Globular actin) and F-actin (Filament actin). G-actin is a small single globular monomer with the molecular weight of about 43,000 Da to 48,000 Da and is made up of 350 amino acids (Silverthorn, 2004). G-actin polymerizes into F-actin, which is part of the polymeric thin filaments, in the presence of salts and small amounts of ATP, commonly found in the sarcoplasm (Lawrie & Ledward, 2006). Two strings of F-actin molecules wind around each other in a double helix, like a chain of pearls, curled around the string-like tropomyosin, which itself is also made out of two curled strings. After every seventh actin molecule of the thin filament, a troponin complex made out of 3 types of troponin polypeptides, denoted as troponin I, C and T, is attached to the actin. Troponin complex has a high affinity for Ca^{2+} ions.

F-actin combines with myosin to produce mechanical energy for muscle movement. Troponin and tropomyosin have a controlling or regulating impact on the contraction and relaxation of the muscle fibres but are not directly involved in the contraction and relaxation. Denaturation of these contractile fibres (myosin and actin) starts at 55 – 60°C.

Actin can be found in most cells throughout the eukaryotic cell which require mobile function (Polland & Weihing 1974, Bhadriraju et. al., 2007). Skeletal and cardiac muscle contains α-actin, whereas non muscle tissue of higher vertebrate contains β-actin and γ-actin species (Price & Schweigert, 1987).

2.2.1.3 Tropomyosin

Tropomyosin is an α-helical molecule approximately 41 nm long with similar amino acid composition to myosin (Vetterkind & Morgan, 2012). It consists of 2 subunit chains (α and β), each with molecular weight 33,000 Da. (Lawrie & Ledward, 2006) These subunits can be separated by SDS-gel electrophoresis or by ion exchange chromatography. Their functions are to bind stoichiometrically with actin (1:7 molecules) and with troponin (Hill, Mehegan, Butters & Tobacmans, 1992).

2.2.1.4 Troponin

Troponin has a molecular weight of 80,000 Da which confers Ca^{2+} sensitivity to the actomyosin ATPase and has been shown to have a high affinity for calcium ions above threshold concentrations of 10^{-6} M (Price & Schweigert, 1987). Troponin is located in the thin filament at a periodicity of 40nm and is bound to tropomysin. Troponin can be divided into 3 units as mentioned above with different function. Troponin C (TN-C) has molecular weight of 18,000 Da and binds with calcium ion. The second one is troponin I (TN-I) with molecular weight of 23,000 Da and inhibits the interaction of myosin and actin. The third component binds strongly to tropomyosin known as Troponon T (TN-T) with molecular weight of 38,000 Da.

2.2.1.5 Actinin

Actinin plays a role in regulating the physical state of physical movement. The four classes of actinins are α-actinin, β-acyinin, γ-actinin and euactinin. α-Actinins is the major actinin found in skeletal muscle. It is an acidic protein of molecular weight 95,000 Da and has the role of gluing the thin filament at the Z-line. Mild treatment of myofibrils with proteases results in a marked decrease in the density of Z-lines together with the release of intact α-actinins. β-Actinin is a minor component of myofibrils around 0.4% of actin. It consists of two subunits 37,000 Da and 34,000 Da molecular weights. β-Actinin is located at the free end of actin filaments in each sarcomere and thus preventing actin from forming networks rather than filaments. γ-Actinin is a protein of molecular weight of 35,000 Da. This protein is rich in serine and glycine residues, which inhibits the polymerization of actin at the nucleation step. Euactinin is a protein of molecular weight of 42,000 Da and has a similar amino acid composition as actin except for higher proline content.

2.2.1.6 Filament protein

Filament proteins represent protein that plays a structural role in architecture of the myofibril and the muscle cell. It provides a mechanical continuity along the length of the myofibril, which ultimately gives rise to the elasticity of the myofibril. The major filament proteins (or scaffold proteins as they are sometimes called) are connectin (titin), desmin, and Z-protein. Connectin or titin forms a three-dimensional net of a very thin filament and linking neighbouring Z-lines. Desmin has a molecular weight of 55,000 Da and forms classical intermediate (10 nm) filaments. There are two forms of desmin: α and β with isoelectric points of pH 5.65 and pH 5.7, respectively. Desmin is soluble with at low pH (1.0 N acetic acid) and when dialysed against water, polymerizes to form a network of 10 nm filaments. Z- protein has the same molecular weight as desmin but it not able to react with an antidesmin antibody.

2.2.1.7 Minor regulatory proteins

Minor proteins such as M-protein, C-protein, F–protein and I-protein play a role in regulating the filamentous nature of myofilament. M-protein has a molecular weight of 165,000 Da and is found at the M-line of the thick filaments. The protein consists of a single peptide, which is about 13% α- helix and 35% β-structure (Lawrie and Ledward 2006).

C-protein comprises 2% of the myofibrils protein and has a molecular weight of 135,000 Da and a relatively high proline content (71/1000 residues). C-protein copolymerizes with myosin filaments with a specific periodicity but does not appear to be bound to the myosin rod along the length of the thick filament.

F-protein has molecular weight of 121,000 Da and has an amino acid composition that is very different from other myosin associated proteins. F-protein will bind to myosin filament but C–protein can inhibit this binding.

I–protein has a molecular weight of 50,000 Da and is high in aspartic and glutamic acid content. It is located at the A band except for the central region. In the absence of Ca^{2+}, I-protein inhibits actomyosin ATPase activity. This leads to the suggestion that I–protein inhibits the unnecessary splitting of ATP in relaxed muscle.

2.2.2 Sarcoplasmic proteins

Sarcoplasmic proteins are water-soluble proteins and account for 29 % of the total protein in muscle. These proteins are normally extracted with low ionic strength potassium phosphate buffer solutions of ~0.06 mol/L (Keeton & Eddy, 2004) or with allow salt concentrations less than 0.1 M KCl (Xiong, 1997). Sarcoplasmic proteins are a mixture of several hundred molecular species the complexity of which has been shown by modern proteomic techniques, such as two dimensional electrophoresis (Bendixen, 2005). Sarcoplasmic protein contains hundreds of different proteins, most in small quantities. Most of sarcoplasmic proteins are part of the energy production system. It includes all the remaining soluble muscle cell proteins that are not associated with the contractile apparatus either functionally or mechanically. These include many soluble enzymes involved in anaerobic metabolism, as well as the proteins of the mitochondria comprising the enzymes of the tricarboxylic acid cycle and those of the electron transport system. Several sarcoplasmic proteins are enzymes of the glycolytic pathway and may be present in more than one form (isozymes) (Lawrie & Ledward, 2006). Sarcoplasmic proteins share common physicochemical properties such as low molecular weight, high isoelectric pH and have globular or rod shape structure (Xiong, 1997).

Sarcoplasmic proteins can be divided into four different components based on their sedimentation velocity in differential centrifugation: 1) pellet after 1000 *g* is nuclear; 2) pellet after 10,000 *g* is mitochondrial; 3) pellet after 100,000 *g* is microsomal, and; 4) supernatant after 100,000 *g* is cytoplasmic (Xiong, 1997).

Sarcoplasmic proteins are soluble at low ionic strength and the glycolytic enzymes (which contribute the major proportion of the sarcoplasmic proteins) are bound to the myofibrillar protein in vivo, assists the orientation and control of these enzyme reactions in the muscle (Lawrie & Ledward, 2006)

2.2.2.1 Myoglobin

Myoglobin a sarcoplasmic protein is the protein pigment responsible for the red colour in meat. Myoglobin acts as oxygen storage, which is responsible for maintaining oxygen supply in muscles and facilitates diffusion of molecular oxygen. Oxygen

transported to the muscle by haemoglobin diffuses from the capillaries to the muscle fibre where it is bound by myoglobin for subsequent use in aerobic metabolism.

Myoglobin is an important component of these proteins. Myoglobin is a globular protein with 153 amino acids (Young & West, 2001) and a molecular weight of 16,800 Da. However the molecular weight differs among the species (Xiong, 1997). In addition, the concentration of myoglobin differs according to species, breed, age and individual muscle cuts (Young & West, 2001). Cattle usually have 2-5 mg/g myoglobin in muscle (Hunt & Hedrick, 1977).

Meat colour lightens and eventually turns to a brown-grey hue during cooking (Young & West, 2001). The lightening is due to an increased reflection of light arising from light scattering by denatured myofibrils protein (actin and myosin) as they bind less water, thus creating a gap between myofibrils. In turn, this creates a step change in refractive indices resulting in light scattering and increased reflectivity (Offer et al., 1989). Changes in meat colour during cooking are caused by loss of chroma and change in hue. The brown grey hue in cooked meat is caused by myoglobin denaturation (about 75°C) which results in chemical changes forming globin hemichrome in which iron is in Fe^{2+} state (Young & West, 2001).

The various known complexes of myoglobin can be grouped into two major classes of ionic and covalent bond types, each containing iron the ferrous(2^{+}) or ferric (3^{+}) ion state. The ferrous complex bound to oxygen is a red in colour and gives sharp peaks at 535 – 545 mμ and 575 - 585 mμ. Myoglobin without oxygen forms a purple pigment that can combine with water, known as deoxymyoglobin, which will give a diffuse absorption band with a maximum at 555 mμ. Oxidation of the iron compound to the ferric complex also binds with water but not with oxygen and is called metmyoglobin, with absorption peak at 505 mμ and weaker peak at 627 mμ.

2.2.2.2 Haemoglobin

Haemoglobin is the oxygen–binding respiratory protein found in red blood cells whose main function is to transport oxygen to all the cells of the body and the removal of carbon dioxide to the lungs that results from aerobic metabolism. Haemoglobin has a molecular weight of 68,000 Da and consists of four protein subunits each folded around

a central heme. There is relatively little haemoglobin inside meat as the blood is lost during slaughter and little remains inside the muscle tissue. Blood in muscle tends to look black, so it is an undesirable component of meat as food.

2.2.3 Connective tissue proteins

Connective tissue, also known as stroma protein, is protein that is insoluble in 0.15 M KCl solution. Connective tissue proteins hold and support the muscle through the component tendons, epimysium, perimysium and endomysium. Connective tissue consists of various fibres embedded in an amorphous ground substance rich in proteoglycan and glycoprotein. The structural stability imparted by the connective tissue is largely determined by the properties of collagen fibres and elastin, which are the main proteins of connective tissue. Collagen has a significant influence on meat tenderness. Elastin is a protein with rubber like properties. The relative proportion of connective tissue and muscle fibres differ among various muscles and as such, it contributes to the relative difference of meat tenderness (Lawrie, 1998). Stromal protein also includes the insoluble proteins that constitutes of membranes in many intracellular organelles that are present in a small amount.

2.2.3.1 Collagen

Collagen is the main component of connective tissue and is found in ligaments, tendons and muscle tissue. Collagen can be divided into three major components based on their macromolecular structure: 1) striated fibrous collagen (Type I, II and III collagen); 2) nonfibrous collagen (Type IV or basement membrane collagen, and; 3) microfibrillar collagen (Type VI, VII (matrix microfibril), type V, IX and X (pericellular collagen) (Xiong, 1997).

The building blocks for collagen are units of tropocollagen, which is a triple-helical structure, made out of three intertwined polypeptide α-chains. The triple helical structure is stabilised by hydrogen bonds. The α-helix has an abundance of proline and hydroxyproline. Each collagen contains about 33% glycine, 12% proline, 11% hydroxyproline and lacks any tryptophan (Xiong, 1997). The central triple – helical region of a collagen molecule is composed of segments of tripeptides with repeating Gly – X - Y sequence where X and Y are often proline and hydroxyproline (Varnam & Sutherland, 1995). The molecular weight of tropocolagen is 300,000 Da with each α-

chain being 100,000 Da. Numerous molecules of tropocollagen align themselves next to each other to form collagen. The structure is a consequence of the amino acid content and sequence of the component α-chains. Cross-links on the side chain of the amino acids lysine and histidine, as well as hydrogen bonds stabilize the helical structure of tropocollagen within collagen. Such cross-links are unusual in proteins and only occur in collagen and elastin.

Native collagen has an isoelectric point in the range pH 7 to 8 (American Meat Institute Foundation, 1960). Collagen is insoluble in water and in salt and/or phosphate solutions. In contrast, procollagen is water-soluble. The triple helix swells if exposed to acid for a prolonged period of time and 'absorbs', or holds, water during this process of swelling. The amount of collagen in meat is important because it is considered to be of low biological value because of deficiencies in lysine, tryptophan and sulphur amino acids (Young & Pellett, 1984). Due to its structure, collagen is resistant to the action of most proteases. Collagenases are the only enzymes that are able to cleave peptide bonds in the triple–helical regions of collagens.

The strength of collagen increases with the increasing biological age of the animal and the solubility of collagen, in old animals, is reduced as a higher number of cross-links are formed within the collagen molecule (Varnam & Sutherland, 1995).

The method used to estimate collagen involves quantifying one of the major components, the amino acid hydroxyproline, which is present at about 12.5 % (Vazquez-Ortiz & Gonzalez Mendez, 1996). The quantity of hydroxyproline is multiplied by a factor of eight to covert to collagen.

Heating these fibres in water to temperatures in the range 60- 70°C results in collagen shrinking to about one–third to a quarter of its original length. This is known as the shrink temperature (Ts) and is a key characteristic of the collagen because the temperature-induced shrinkage is quite sharp over a narrow temperature range. Collagen from different sources may have different values for Ts. Increasing the temperature to 80° C causes the transformation and solubilisation of collagen to water-soluble gelatine.

2.2.3.2 Elastin

Elastin is a minor component present within connective tissue at around 4% of the amount of collagen and around 0.8 % of the total meat protein. Elastin has a molecular weight of 70,000 Da, with a similar amino acid composition to collagen. Each elastin molecule contains about 33% glycine, 10-13 % proline and 40% hydrophobic amino acids (Pearson & Young, 1989).The amino acid hydroxyproline is found within elastin at 1%. Elastin is yellowish in colour, almost insoluble in water and salt, it is also resistant towards diluted acids. Elastine has low levels of histidine and lacks hydroxylysine. The molecular structure is a random coil. Elastin fibres are composed of a glycoprotein–rich myofibrillar components surrounded by an amorphous protein fraction.

2.3 Amino acids

Amino acids are the building blocks of proteins. The amino acid composition of proteins is often used to define their nutritional quality. Humans require 20 different amino acids to synthesize all necessary proteins. All these 20 amino acids are alpha-amino acids, given that both functional groups, the 'acid' carboxyl group (–COOH), as well as the 'alkaline' amino group (–NH2), are attached to the same carbon atom, the α-carbon atom. This α-carbon atom is also referred to as the 'chiral centre'. Glycine, the simplest amino acid, is the only non-chiral amino acid. Eight of those 20 amino acids are essential, which means that the human body cannot synthesize these eight essential amino acids. The eight amino acids are: Isoleucine, Threonine, Leucine, Valine, Lysine, Tryptophan, Methionine and Phenylalanine are obtained from the food. Some nonessential amino acids can be classified as conditionally essential where the amino acids may become temporarily essential when it become insufficient due to rapid growth or critical illness. Protein-containing food is digested into individual amino acids, from which the required body proteins are synthesized. Meat is a good source of essential amino acids as shown in Table 2.2. Meat proteins contain amino acids not usually found in plant proteins, such as methyl histidine hydroxymethyllysine (Friedman, 1996).

Beef has higher content of Leucine, Lysine and Valine than pork or lamb and a lower content of threonine. Glutamine was the most abundant free amino acid (4.0-5.7 mg/g dry weight) followed by taurine, alanine, glutamate, and β-alanine found in beef (Wu et al.,2016). The amino acid contents may be affected by processing and storage but

unless processing conditions are both severe and prolonged, such destruction is minimal. Rather more importantly is the possibility that certain amino acids activity may lost or reduce by being bound to other substances in the meat or food. (Hurrel, 1980).

In protein, amino acids link to each other through a peptide bond. Peptide bonds occur when the carboxyl group (COOH) of one molecule reacts with the amino group (NH_2) of another. A peptide is a compound consisting of two or more amino acids. Oligopeptides have 10 or fewer amino acids. Polypeptides and proteins are chains of 10 or more amino acids but peptides consisting more than 50 amino acids are classified as protein (Fatih, 2009). The process that breaks the peptide links is known as hydrolysis.

Table 2.2: Amino acid composition in fresh meat

Amino acid	Category	Beef (% crude protein)	Pork (% crude protein)	Lamb (% crude protein)
Isoleucine	Essential	5.1	4.9	4.8
Leucine	Essential	8.4	7.5	7.4
Lysine	Essential	8.4	7.8	7.6
Methionine	Essential	2.3	2.5	2.3
Cysteine	Essential[a]	1.4	1.3	1.3
Phenylalanine	Essential	4.0	4.1	3.9
Threonine	Essential	4.0	5.1	4.9
Tryptophan	Essential	1.1	1.4	1.3
Valine	Essential	5.7	5.0	5.0
Arginine	Essential for infants	6.6	6.4	6.9
	Non -essential			
Histidine		2.9	3.2	2.7
	Non -essential			
Alanine	Non -essential	6.4	6.3	6.3
Aspartic acid	Non -essential	8.8	8.9	8.5
Glutamic acid	Non -essential	14.4	14.5	14.4
Glycine	Non -essential	7.1	6.1	6.7
Proline		5.4	4.6	4.8
Serine		3.8	4.0	3.9
Tyrosine		3.2	3.0	3.2

[a]conditionally essential amino acid

(Source: Lawrie & Ledward, 2006)

All amino acids contain at least one nitrogen atom and this is normally used to quantify the amino acid (and protein) content of food. Crude protein analysis is conventionally based on multiplying the total nitrogen (N) by a factor of 6.25 (Jones, Munsey, & Walker, 1942). The value of 6.25 is based on the assumption that protein contains 16 % of nitrogen. A factor of 6.25 is used for determining total protein from total N. Different factors originally determined by Jones et al. (1942), are currently used to calculate proximate protein amounts based on nitrogen content in different foods. These factors range from 6.37 for human milk, to as low as 5.55 for gelatine and 5.18 for almonds. The nitrogen factor for meat and fish is 6.25 (Greenfield & Southgate, 2003).

2.4 Functional properties of protein

Protein functionality can be defined as any physicochemical properties of protein that allow protein molecules to interact among themselves and their environment to produce or improve the quality and stability of the final product (Xiong, 1997). In processed food products, the functional properties of protein such as solubility, emulsifying capacity, emulsion stability, water binding, gelation and cohesion are inter-related (Kinsella, 1976). The functional properties can be determined by the quantity, composition, conformation and physical properties of the muscle proteins present in the muscle food system (Kinsella, 1976). Protein functionality can be improved to provide greater formulation flexibility, ensure reliable ingredient uniformity, and minimize processing losses (Brekke & Eisele, 1981).

2.4.1 Protein solubility

The solubility of protein is important for manufactured muscle food. Protein solubility is related to other functional properties such as providing good emulsion, foams, gelatine and whipping properties. By definition, protein solubility is the protein concentration in the solvent in a single, or two phase system (protein solution in either a liquid, or in a liquid-liquid phase) at the equilibrium state (Hall, 1996). Solubility of protein relates to surface hydrophobic (protein-protein) and hydrophilic (protein –solvent) interactions with water. The relative proportions of surface hydrophilic and hydrophobic groups dictate the degree of interaction with the solute (solvation) by water. Muscle solubility is

defined as the amount of the total muscle protein (%) that goes into solution under specified conditions, which remains in solution after a specified centrifugal force (Xiong, 1997).

Most sarcoplasmic proteins are globular proteins that have relatively low molecular weights and are naturally soluble within muscle. The structure and the distribution of sarcoplasmic proteins allow them to interact freely with surrounding water (Xiong, 1997).

Myofibrillar proteins generally require relatively high ionic strength to dissolve in water and myofibrillar proteins are highly organized, integral structural units. The isoelectric points of myofibrillar proteins are relatively low between pH 5 – 6. Protein solubility can be affected by several factors such as pH, temperature, ionic strength, the presence of other components, protein concentration and physical parameters. Protein solubility is important for rapid diffusion to the interface. Intermolecular bonds affect the rate and extent of protein unfolding. Electrostatic, hydrophobic, and steric factors can both facilitate and inhibit molecular arrangement and retard coalescence of proteins. Solubility between post rigor fast- twitch (white) and slow- twitch (red) muscles are different. Protein solubility of both white and red myofibrils increases, but at different rates, indicating that dissolution of the myofibrils in the different fibre types follows different time– dependent processes (Xiong, 1997).

Enzymatic modification with protease may alter the properties of the original protein molecules, forming smaller protein fragments with different solubilities, intramolecular bond arrangements and changing the proportion of charge groups (Smith & Brekke, 1984).

2.4.2 Effect of pH on protein solubility

Solution pH has significant influence on protein solubility. The degree of protein extraction in muscle is affected by divalent cations present in muscle. Solubility of myofibrillar protein of white muscle is more sensitive to pH and divalent cation than protein from red muscle (Xiong, 1997).

Several authors stated that a protein usually has the least solubility at the isoeletric point (pI) where protein-protein interaction increases because the electrostatic forces of

the molecules are at a minimum and less water interacts with the protein molecules. At a pH value below or above their isoelectric point, proteins carry a net positive or net negative charge, respectively. Electrostatic repulsion and hydration of charged residues promotes solubilisation of protein. When solubility is plotted against pH, most food proteins exhibit a U shaped curve, with minimum solubility occurring at the isoelectric pH of the protein. The occurrence of minimum solubility near the isoelectric point is due to the lack of electrostatic repulsion which promotes aggregation and precipitation via hydrophobic interaction.

The change in pH of meat during heating may be caused by changes in charge, or hydrogen bonding, or both, within the myofibrillar proteins (Hamm1966), or by splitting of hydrogen bonds, which releases additional positive charges. The heating may also cause the formation of new hydrogen bonds around the isoelectric point of actomyosin. During heat-coagulation of tissue myofibrils or actomyosin, the number of dye-binding acidic groups of the proteins decreased. Between 30 and 40°C a mild denaturation occurred (Hamm & Deatherage, 1960) which resulted in unfolding of protein chains and formation of new salt and/or hydration bonds. A stronger denaturation started at 40°C and continued to 50°C. The changes in this temperature range were mainly determined by the disappearances of acidic groups in the muscle proteins. A decrease in acidic groups also occurred between 50 and 55°C. In the range 55 - 80°C new cross-linkages may have been formed and the loss of acidic groups was observed (Hamm & Deatherage, 1960).

2.4.3 Effect of ionic strength on protein solubility

Protein solubility depends on the ionic strength of the extraction buffer. Solubility begins at an ionic strength close to 0.5 and reaches max at 1.0. In the meat industry addition of salt concentration above 0.5 M (2 % salt) is widely used allowing solubilisation and extraction of myofibrillar proteins necessary for producing desirable meat product functionalities (Xiong, 1997). The ionic strength of salt solution is given by Equation 2.1:

$$\mu = 0.5\sum C_i Z_i^2 \qquad \text{Equation 2.1}$$

Where C_i is the concentration of an ion and Z_i is its valence. At low ionic strength (<0.5), ions neutralize charges at the surface of protein. The charge screening affects solubility in two different ways, depending on the characteristic of the protein surface. Solubility decreases for those proteins that contain high incidence of nonpolar patches. The decreased solubility is caused by enhanced hydrophobic interaction. In contrast, increased solubility is caused by a decrease in the ionic activity of the protein macromolecule.

2.4.4 Effect of temperature on solubility

In contrast to pH and ionic strength, the solubility of most protein generally increases with temperatures between 40 -50. Exceptions occur with highly hydrophobic proteins such as β-casein and some cereal proteins that exhibit a negative relationship with temperature. Above 50°C, the increase in thermal kinetic energy causes protein unfolding (denaturation), exposure of nonpolar groups, aggregation and precipitation that also decreases solubility (Fennema et al., 2008). Proteins are denatured by the effect of temperature on the non-covalent bonds involved is stabilization of secondary and tertiary structure; for example, hydrogen, hydrophobic and electrostatic bonds (Wong et al, 1996). When the secondary and tertiary structures of a protein are unfolded, the hydrophobic groups (i.e, the sulfidril groups SH -, initially inside the protein molecules) interact and reduce water binding. Any hydrophobic interactions lead to aggregation, followed by coagulation and precipitation. Therefore Denaturation decreases protein solubility compared to native protein, and leads to aggregation and difficulty of reversal upon cooling (Mine, 1995, Kim, 1998, Langendorff et al., 1999, Pelegrine and Gasperetto, 2005).

2.4.5 Emulsification capacity (EC)

Emulsification capacity is the most widely used test for meat protein functionality (Smith & Brekke, 1984). During the preparation of a protein-based emulsion, the formation of an interfacial film occurs in three stages: Diffusion of proteins to the interface, protein unfolding, and then re-arrangement of the denatured protein molecules to their lowest free energy state to form a film. Solubility and EC usually have positive correlations but other properties of proteins may affect emulsification (Smith & Brekke, 1984).

2.4.6 Viscosity

Viscosity of the aqueous protein phase can influence texture and stability of processed meat batters. Meat protein is mainly a charged polymer capable of binding water and causing swelling by the uptake of water and loosening of the polypeptide matrix. As a consequence of swelling, a protein increases its effective hydrodynamic volume, thereby increasing resistant to shear. The flow behaviour of a fluid can be characterized by the stress response of the solution to an imposed shear rate. Dynamic viscosity is defined as the ratio of shear stress to shear rate. In the Newtonian flow, there is a relationship between shear stress and shear rate. Proteins generally exhibit non-Newtonian psuedoplastic characteristics, which depend on the intrinsic factors such as molecular size, volume, shape, surface charge and ease of deformation, as well as extrinsic factors such as pH, temperature, ionic strength, ion type and shear rate.

Myosin is highly viscous in salt solution due to its structure i.e. large length to diameter ratio of the rod portion. Myosin is the major contributor to the viscosity and thickness of an aqueous extract in salted meat and meat emulsions.

2.5 Protein modification via hydrolysis

Modification refers to the intentional alteration of the physicochemical properties of proteins by chemical, enzymatic or physical agents to improve functional properties (Franzen, 1977). A combination of chemical and enzymatic techniques to modify muscle protein may improve functional properties. Chemical modification prior to enzymatic treatment promotes an unfolding and opening of the protein structure, making proteins more susceptible to enzyme action. Conversely, enzyme hydrolysis prior to chemical modification may cause more reactive groups on the protein to become more available to the chemical modifier (Brekke & Eisele, 1981).

Protein hydrolysis is the term that applies to all possible ways of splitting proteins to produce products with lower molecular weight. Basically there are three ways to hydrolyse protein: chemical hydrolysis by strong acid or base; microbial fermentation, or; enzyme hydrolysis (Lieske & Konrad, 1994). Protein hydrolysates may be used as flavour enhancers, functional ingredients, or simply as nutritional additives to food of low protein quality. Protein hydrolysates are currently used to help individuals who

cannot digest whole protein. Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides (Thiansilakul, 2006). The functional properties of protein are those physicochemical properties that govern their performance and behaviour in food system during preparation, processing, storage and consumption (Panyam & Kilara, 1996). The functional properties are affected by peptide hydrolysis and reduction in the molecular weight during hydrolysis (Adler-Nielssen,1976; Turgeon et al.,1992).

Chemical modification involves acetylation or succinylation of protein with acid anhydrides, phosphorylation, lipohilization, glycosylation, thiolation, reductive alkylation and covalent attachment of amino acids to change the net charge on the protein (Panyam & Kilara, 1996). Acylation reaction, involving the direct addition of a chemical group to a functional group of an amino acid side chain via a substitution reaction, offers a great opportunity for modifying proteins (Brekke & Eisele, 1981). Acetylation results in the conversion of a cationic amino group to an electrically neutral residue, for a gain of a net negative charge. Meanwhile, succinylation converts the cationic amino group to an anionic residue, for a gain of two negative charges (Brekke & Eisele, 1981). Chemical modification can also alter digestibility. Alkaline hydrolysis of protein is not preferable because of the low reaction rates and the presence of fat in the material can cause problems associated with soap formation such as emulsification and loss of fat (Criswell, Litchfield, Vely & Sachsel, 1964). It can also cause destruction of amino acids and the resulting material is difficult to neutralise (Lahl & Grindstaff, 1989). Acid hydrolysis produces higher solubilisation than enzyme hydrolysis (Criswell et al., 1964) but destroys some amino acids such as tryptophan, reducing the nutritional value of the hydrolysate (Criswell et al., 1964).

Enzymatic hydrolysis has many advantages. The reaction is specific, the rate was reported to be reasonably fast at a moderate temperature and the resulting hydrolysate can produce flavour, taste or some unique physical properties that can only be achieved through specific enzyme activity (Chen, 2005). The amino acid composition of the hydrolysed product is unchanged by the process (Criswell et al., 1964).

2.6 Enzymatic modification of protein

The constituents of an enzymatic hydrolysate of protein can be divided into six fraction of protein according to their: metaprotein; protease; peptone; subpeptones, and; amino

acids (Wasteneys & Borsook, 1924). Endopeptidases cleave the peptide linkage between two adjacent amino acid residues in the primary sequence of a protein, yielding two peptides (Panyam & Kilara, 1996). Enzymatic modification has been used extensively to tailor the functionality of proteins to meet specific needs. Three distinct effects accompany enzymatic hydrolysis of proteins:

1. A decrease in molecular weight
2. An increase in the number of ionizable groups
3. The exposure of hydrophobic groups

Enzymatic hydrolysis processes can produce different products compared to chemical reactions such as acid and alkali hydrolysis. Undesirable side reactions that may inadvertently alter flavour, texture, nutrition or food safety, are minimised. Until recently, the main use of enzymes to modify food protein has been more or less uncontrolled protein hydrolysis catalysed by protease. In the past, major advances in the preparation of the protein hydrolysates have included the development of better control over the specificity and degree of protein hydrolysis. Factors affecting the enzymatic hydrolysis of protein include: enzymes specificity, extent of protein denaturation, substrate and enzymes concentration, pH, ionic strength, temperature and absence or presence of inhibitory substances (Panyam & Kilara, 1996). The specificity of enzymes is the key factor determining the number and location of peptide linkages that are hydrolysed (Panyam & Kilara, 1996). Partial hydrolysis of proteins with protease enzymes is used to modify the protein structure (Brekke & Eisele, 1981).

2.6.1 The extent of enzymatic hydrolysis process

In order to control the extent of hydrolysis and the properties of the final product, understanding the specificity and activity properties of the enzyme is important. Hydrolysis processes can be controlled by a variety of techniques that include termination of the reaction at a specific time, degree of hydrolysis, choice of enzymes, reaction milieu, denaturation condition of the protein prior to adding enzymes and the combination of non-enzymic modification with enzymic modification (Haard, 2001). The hydrolysis process can also be controlled by the molecular size, hydrophobicity and polar groups of the hydrolysate (Adler-Nissen, 1986; Kristinsson & Rasco, 2000). The final product of hydrolysis (hydrolysate) formed by controlled conditions may yield desirable functional and pharmacological properties, high nutritional value and desirable organoleptic qualities. The characteristics of the hydrolysate directly affect

the functional properties and the uses as a food ingredient. Extensive hydrolysis is normally used to produce hypoallergenic hydrolysates with the biggest peptides < 5,000 Da and almost 90% of them < 500 Da, whereas the hydrolysates used for protein supplements may undergo less extensive hydrolysis (Panyam & Kilara, 1996).

The functional properties of protein hydrolysates depend on the types of enzymes used in their preparation. This is because each enzyme has a unique functionality and reactivity within a protein structure and these properties contribute to different polypeptides being released during hydrolysis. Improvement in protein functionality would provide greater formulation flexibility, ensure reliable ingredient uniformity, and minimize processing losses (Brekke & Eisele, 1981).

Endopeptidase enzymes are often very specific and cleave peptides bond inside the protein, usually leading to relatively small peptides but only a small amount of free amino acids. Generally, less specific enzymes will produce smaller peptides. The exopeptidase enzymes cleave an amino acid from the end of the protein polypeptide chain resulting in a mixture of free amino acids and larger peptide fractions, depending on the reaction time.

It is imperative to have a good understanding of a specificity and activity properties of the enzymes available for protein hydrolysis in order to control the extent of hydrolysis and properties of the end product. The functional properties of protein hydrolysates depend on the types of enzymes used in their preparation. This is because each enzyme has different size and physicochemical properties which contribute to different polypeptides released during hydrolysis. In general, solubility of most proteins improves after hydrolysis regardless of the enzymes. The greater the degree of hydrolysis (DH), the higher is the solubility; however the net increase depends on the types of enzymes used. Higher protein solubility is particularly important in acidic protein drinks in which precipitation and sedimentation is undesirable.

2.7 Mechanism of protein hydrlysis

The first hypothesis by Gizelius & Ericson-Quensol in 1939 suggested that enzymatic hydrolysis of protein occurs by an ‘all or none’ principle (Nekluydov et al., 2000). That is, part of the protein molecule is rapidly hydrolysed to end products, while other parts of the protein remain intact. So the resulting hydrolysate will have only two products:

the non-cleaved molecules of the initial protein and the end products of the reaction. No intermediate products would be found in the hydrolysate. This hypothesis is described by the classic Michaelis –Menten equation (Gonzalez & Camacho , 2004).

At the beginning of hydrolysis (stationary phase), the Michaelis constant was negative. However, it has since been shown that reactions formed intermediate products in addition to the initial and end-products. Thus, the digestion of proteins by proteolytic enzymes may follow one of the following courses (Mihalyi & Szent-Gyorgyi, 1952):

1. Gradual degrading, with continuous splitting off of low molecular weight products from the parent molecules;
2. Explosion-like disintegration of the protein molecule into smaller fragments and different sized polypeptides;
3. Formation of a well-defined high molecular weight intermediate, which is further, hydrolysed into low molecular weight substances.

Partial proteolysis occurs when a protease selectively cleaves off one or several peptide fragments, leaving a high molecular weight proteinaceous fragment. Hydrolysis depends on the rate of individual stages. If the initial protein was cleaved at a higher rate than the intermediate products, the process would display a gradual and progressive increase in rate. If the intermediate products were cleaved at a higher rate than the initial protein, the process would look like a burst degradation of protein, apparently yielding the end-product in one step as hypothesised by Gizelius & Erikson–Quensol.

Inhibition of proteolysis can occur if the end-products interfere with proteolysis. As a result, the rate of hydrolysis would decrease over time and the kinetic curves would resemble a complex S–shape of function.

It is also possible that peptide reformation could occur during the hydrolysis process. However, in aqueous solutions or suspensions of protein, the equilibrium lies so far to the right that degradation and not synthesis is thermodynamically favoured (Zhang, 1983). Thus synthesis is normally neglected and the kinetic scheme describing the action of endopeptidases on peptide bond hydrolysis can be simplified as shown in Equation 2.2:

$$E + S \xrightarrow{k+1} ES \xrightarrow{k+2} EP + H - P' \xrightarrow{k+3} E + P - OH + H - P' \qquad \text{Equation 2.2}$$

Where S is substrate, E is the enzymes and P and P' are the resulting peptides.

The enzymic hydrolysis of peptide bonds can be described by the Michaelis-Menten equation and has been found to follow pseudo-first order kinetics (Richard, 1955; Ottesen,1956; Biszku et al., 1973; Solti et al., 1975). The Michaelis-Menten equation shows how the initial rate of this reaction, V, depends on the substrate concentration, [S].

$$v = \frac{[S]V_{max}}{K_m + [S]}$$

A well-defined final peptide profile, of critical importance for effective digestion and absorption, can be achieved using mild conditions and enzymatic proteolysis (Lahl & Braun,1994).

2.7.1 Degree of hydrolysis (DH)

DH is defined by Adler-Nissen (1986) as the percentage of cleaved peptide bonds (Equation 2.3):

$$DH = \frac{h}{h_{tot}} * 100\% \qquad \text{Equation 2.3}$$

Where h_{tot} is the total number of peptide bonds per protein equivalent and h is the number of hydrolyzed bonds. h_{tot} is dependent on the amino acid composition of the raw material (Nielsen, Petersen, & Dambmann, 2001).

There are three essential methods for determining DH: Firstly, determination of the amount of non-protein nitrogen released during hydrolysis by precipitating using a strong acid; secondly, determination of free α-amino groups, and thirdly, using titration of the released protons (Silvestre, 1997).

An hydrolysate with a high DH value often possesses a bitter taste (Shahidi, Synowiecki, & Balejko, 1994). Partial hydrolysis of protein can greatly affect, and usually improve solubility. Solubility is also important for foaming and emulsifying

properties which are generally improved by partial hydrolysis. The foaming and emulsifying properties improved up to DH < 10% and then decreased above 10%. The stability of foams and emulsions made with protein hydrolysates are generally lower than that of intact protein. Short polypeptides are unable to form a cohesive viscoelastic film at the air-water and oil-water interface.

Hydrolysates possess lower allergenicity compared to proteins. The antigenic sites (epitopes) that bind to immunoglobulin E (IgE) exist in the intact protein, whereas in protein hydrolysates the epitopes are destroyed by proteolytic cleavage. A higher DH value results in a greater reduction of allergenicity. ARI (allergenicity reduction index) is used to calculate the efficiency of protease to reducing the allergenicity. ARI can be defined as the ratio of % reduction in allergenicity to % DH(Mahmoud et al., 1992)

Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides (Thiansilakul, Benjakul, & Shahidi, 2007). The hydrolysis procedure also effectively defats the product, the soluble hydrolysates having protein to fat ratios two to three times greater than those found in the original material (Webster, Ledward, & Lawrie, 1982).

The reduction of pH to close to the isoelectric points and the unfolding and exposure of hydrophobic areas promotes the formation of aggregates of low molecular weight (Lahl & Braun, 1994). The transfer of the reaction equilibrium to the opposite direction and the accumulation of soluble peptides also facilitate the plastein reaction (Cheftel et al., 1989; Sgarbieri, 1996). Proteases can degrade myofibrillar proteins over a range of temperature, but collagen must be denatured above 50°C before it can be solubilised by papain, with an optimum rate of breakdown occurring at 65°C (Pearson & Dutson, 1994).

2.7.2 Conversion of nitrogen to protein

This use of a single factor for protein content of 6.25 as discussed earlier, is confounded by two considerations. First, not all nitrogen in foods is found in proteins: it is also contained in variable quantities of other compounds, such as free amino acids, nucleotides, creatine and choline, where it is referred to as non-protein nitrogen (NPN). Only a small part of NPN is available for the synthesis of (non-essential) amino acids.

Second, the nitrogen content of specific amino acids (as a percentage of weight) varies according to the molecular weight of the amino acid and the number of nitrogen atoms it contains (from one to four, depending on the amino acid in question). Based on these facts, and the different amino acid compositions of various proteins, the nitrogen content of proteins actually varies from about 13 to 19 percent. This would equate to nitrogen conversion factors ranging from 5.26 (1/0.19) to 7.69 (1/0.13). In response to these considerations, Jones (1942) suggested that N x 6.25 be abandoned and replaced by N x a factor specific for the food in question. These specific factors, now referred to as “Jones factors”, have been widely adopted. Jones factors for the most commonly eaten foods range from 5.18 (nuts, seeds) to 6.38 (milk). It turns out, however, that most foods with a high proportion of nitrogen as NPN contain relatively small amounts of total N (Merrill & Watt, 1955; and 1973).

As a result, the range of Jones factors for major sources of protein in the diet is narrower. Jones factors for animal proteins such as meat, milk and eggs are between 6.25 and 6.38; for the vegetable proteins that supply substantial quantities of protein in cereal/legume-based diets are generally in the range of 5.7 to 6.25. Use of the high-end factor (6.38) relative to 6.25 increases apparent protein content by two percent. Use of a specific factor of 5.7 (Sosulski & Imafidon, 1990) rather than the general factor of 6.25 decreases the apparent protein content by nine percent for specific foods. In practical terms, the range of differences between the general factor of 6.25 and Jones factors is narrower than it at first appears (about one percent), especially for mixed diets

2.7.3 Bitter Peptides

One of the most undesirable properties of protein hydrolysates is their bitter flavour. Extensive hydrolysis contributes to formation of bitterness due to accumulation of low molecular weight peptides containing hydrophobic amino acids (Panyam & Kilara, 1996). The bitterness originates from certain peptides released during hydrolysis. In general bitterness is caused by the presence of peptides of too short chain length and is related to hydrophobicity. Ney (1971) formulated the Q-rules where peptides with a mean residue hydrophobicity of <1.3 kcal/mol are not bitter. On the other hand, peptides with a mean residue hydrophobicity of the peptides of >1.4 kcal/mol are bitter. Mean residue of hydrophobicity can be calculated using free energies of transfer of amino acid residue from ethanol to water. Formation of bitter peptides in protein

hydrolysates depends on the amino acid composition, sequence and the types of enzymes used. The bitterness can be reduced or eliminated by using a mixture of endo and exopeptidases, which further breakdown into fragments that have < 1.3 kcal/mol mean residue hydrophobicity (Fennema, Parkin & Damodaran, 2008). It can also be reduced by selecting non-bitter proteases or by additional use of specific peptidases that are capable of debittering the hydrolysates (Panyam & Kilara, 1996).

Five variables that can influence bitterness (Adler-Nissen, 1986):

1. Hydrophobicity (HΦ) of the substrate, since a high value of HΦ, generally results in relatively larger amounts of highly hydrophobic peptides
2. DH, which influences both the concentration of soluble hydrophobic peptides and their chain length
3. Enzymes, which in combination with DH influence the peptides hydrophobicity
4. Any separation step included in the hydrolysis process
5. Masking effect from components other than peptides.

2.8 Previous studies on meat hydrolysis and tenderness

The difference between meat tenderizing and meat hydrolysis is the extent of degradation of protein (Adler Nielsan, 1986. The most widely used enzymes for tenderization are the plant enzymes papain, bromelain and ficin (Dransfield & Etherington, 1981). The use of these enzymes is limited due to regulatory controls and inability to control the activities of currently used enzymes (Ashie, Sorensen, & Nielsen, 2002). At the same time the enzymes have broad specificities and therefore indiscriminately breakdown the major proteins that are myofibrilla, sarcopalsmic and connective tissue (Ashie et al., 2002). Enzymic hydrolysis of meat protein generally increases solubilisation of free amino acid groups and hydroxyproline, which may result in loss of muscle integrity and reduce shear force or increase tenderness (Fogle et al. 1982). Ficin was effective in improving protein solubility and emulsification capacity (Smith & Brekke, 1984). Proteolysis was effective in improving protein functionality. The most important protein functional properties in processed meat products are solubility, emulsifying capacity, gelation, and water binding (Kinsella, 1982). Proteolysis alters these properties by changing the molecular size, conformation, solubility and strength of inter- and intramolecular bonds of the protein molecules (Kinsella, 1976).

Enzymatic modification can increase protein solubility and has potential as partial substitution for salt in processed meat products (Brekke & Eisele, 1981). Partial hydrolysis with protease is the most common method of enzymatic modification (Brekke & Eisele, 1981). A limited hydrolysis may improve the emulsifying capacity of meat proteins (Du Bois et al., 1972; Smith & Brekke, 1985). Limited hydrolysis not only improved model functional properties but also actual performance in frankfurters, however, the adhesiveness of meat (chicken) decreased after a limited hydrolysis (Schnell et al., 1973). Partial proteolysis of mechanically deboned fowl myofibrillar protein improved protein solubility, emulsifying capacity and gelation (Smith & Brekke, 1985). The extent of proteolysis needed for optimum improvement varied according to the functional properties examined (Smith & Brekke, 1985).

Meaty flavoured hydrolysate can be produced from bones, offals (Moss & Tautman, 1972) and bone residues after mechanical deboning (Behnke et al., 1984). A kinetic expression which regarded the substrate as a composite of both soluble and insoluble protein was proposed for the reaction. It gave a good fit to the reaction curves although not significantly better than a simpler model based on soluble substrate only (O'Meara & Munro, 1985). Denaturation of myofibrillar protein occurred at 30 - 70°C, this was accompanied by fibre shrinkage and a more compact protein than in native tissue (O'Meara & Munro, 1985). Denatured myofibrillar is resistant to hydrolysis so that the peptide fragments formed are not readily released from the compact fibrous structure or are susceptible to aggregation, contributing to low solubilisation at given DH (O'Meara & Munro, 1985).

There are two primary structural features of muscle that influence tenderness which are integrity of the myofibrils (termed the actomyosin effect) and the connective tissue contribution (termed a background effect). Fat also plays a minor role. There are six characteristics of perceived tenderness (Cover *et al*. (1962). Three characteristics relate to the myofibrillar portion, two relate to product adhesion, and the remaining characteristic relates to connective tissue components. Myofibrillar proteins are located intracellularly while connective tissue proteins (primarily collagen) are located extracellularly. This difference in distribution of key proteins within the muscle increases the challenge of improving tenderness.

Exogenous enzymes added to meat to enhance tenderness react differently with the myofibrillar and connective tissue portions of the meat. Currently, just five of the many exogenous enzymes that have been studied have been classified as 'Generally Recognized as Safe' (GRAS) by USDA's Food Safety Inspection Service (FSIS) and come from varying plant, bacterial and fungal sources (papain, bromelain, ficin, *Bacilus* protease and aspartic protease (Bekhit et al., 2014, Barret et al., 2004). Papain, bromelain and ficin are cysteine protease are usually used for meat tenderization (Barret et al., 2004). However, actinidin was not in the GRAS list in as recently but shows potential on digesting in meat protein (Bekhit et al., 2014; FDA 1997). However kiwiberry extract had received GRAS notice in 2006 (FDA, 1997). There are 6 possible actinidin protease in which have a similar molecular weight of 23.5 kDa. There is growing interest in this protease has it has less of a tenderizing effect compare to traditional proteases (Bekhit et al., 2007; Christensen et al., 2009; Han et al., 2009; Lewis and Luh, 1988; Toohey et al., 2011; Wada et al., 2002).

All plant proteases generally have the ability to hydrolyse myofibrilla protein and connective tissue. However the enzyme does not hydrolyse native collagen in raw form but is able to do so in its denatured form (Wada et al., 2002).

There are a few factors available to control the enzyme activity. Temperature is probably the most controllable factor influencing enzyme activity. Most of the exogenous enzymes used to tenderize meat have an optimal activity in the 50-70°C temperature range. Most of the enzymes have an optimal pH in the normal range of meat, but some function best at a more acidic or alkaline pH for optimal protein degradation. Many of the enzymes do not penetrate meat, causing the method of application to play an important role in achieving uniform distribution into the meat product. The proper identification of enzyme, time, temperature and pH will be dependant upon the processing system and desired final product.

2.8.1 Previous studies with kiwifruit and meat hydrolysis

Chinese gooseberry or kiwifruit, botanically known as *Actinidia chinensis Planch,* is native to Southern China and commercially developed in New Zealand from seeds of *Actinidia deliciosa* in 1904 (Ferguson, 2004). It was launched in the world market in 1960 by the name kiwifruit. The kiwifruit industry has progressed rapidly as kiwifruit can be obtained in most supermarkets throughout the world. In the market, kiwifruit is in the

form of fresh fruit, semi processed ingredient in other food products such as in ice-cream, yoghurt, cakes, jams, juices and also wine. It can be considered as a highly nutritional product because it contains many health promoting and therapeutic compounds such as vitamins, minerals and also shows strong pro-oxidant and antioxidant capacities (Cassano, Figoli, Tagarelli, Sindona, & Drioli, 2005; Motohashi et al., 2002; Wang, Cao, & Prior, 1996).

2.8.2 Proteolytic enzymes (Actinidin)

Kiwifruit contains endogenous proteases. The search for kiwifruit protease activity began in an extract of ripe Chinese gooseberry from New Zealand commercial strains. Its activity against gelatine and haemoglobin were studied by Arcus (1959). The given named of the protease is actinidin (Arcus,1959) and officially known as actinidin (EC 3.4.22.14) by the International Union of Biochemistry and Molecular Biology (IUBMB) Enzymes Nomenclature List (*Enzyme Nomenclature* 1992). The enzyme was subsequently purified in a crystalline form and shown to resemble papain in its action (McDowall, 1970). The protease activity was shown to be enhanced by cysteine, Na_2S, KCN and EDTA (Arcus, 1959; Yamaguchi, Yamashita, Takeda, & Kiso, 1982). Different varieties of kiwifruit show different strengths of proteolytic activity (Nishiyama, 2000).

The enzymes is found at high level in developing kiwifruit and can constitute up to 50% of soluble protein in mature fruit at harvest (Praekelt, McKee & Smith, 1988). The proteolytic activity of kiwifruit is in the sarcocarp representing 70% of total fruit weight and found to have the highest level of actinidin activity (Boyes, Strubi & Marsh, 1997; Yamaguchi et al., 1982).

Concentrations of actinidin were reported to change during ripening. Immature fruit contains small amounts of actinidin and their proteolytic activity doubles or triples when the fruit is ripe (Lewis & Luh, 1988).

2.8.3 The structure of actinidin

The actinidin molecule consist of 220 amino acids, which are folded into two domains and consist of three disulphide bridges between amino acids 22-65, 56-98 and 156-206 (Baker, 1977; Baker, 1980). Domain one consists principally of residues from the first half of the amino acid chain (residues 19-115 and 214-218) and contains several α-

helices whereas domain two (residues 1-18 and 116-213) contains one stretch of α-helix but made-up mostly of β-sheet regions (Baker, 1977 ; Baker, 1980).

Actinidin shows a close similarity to the 3-dimensional structure of papain (EC 3.4.22.2), with 50% similarities in the amino acid residue (Baker, 1977; Carne & Moore, 1978). It also has similarities in kinetic behaviour and specificity (Baker, Boland, Calder, & Hardman, 1980). They also have substantial similarities in their chemical and physical properties, similar amino acid sequences about the active site cysteine and histidine residues have been demonstrated for papain, ficin and stem bromelain (Baker, 1976; Carne & Moore, 1978). The sulfylhydryl protease are closely related to the serine protease (Carotti, Hansch, Mueller, & Blaney, 1984). However, variations are found in the amino acid compositions, molecular weights, isoelectric points and carbohydrate contents (Carne & Moore, 1978).

McDowall (1970) found actinidin to have a molecular weight of 12,800±700 Da, and was partially resolved from inactive protein with an apparent molecular weight of 15,400±800 Da using Sephadex gel filtration. Boland & Hardman (1972) rectified the actinidin molecular weight with 26,000±50 Da obtained using a calculated partial specific volume of 0.720 and from amino acid sequence. The molecular weight of actinidin is 23,500 Da found by Carne & Moore (1978). Actinidin appears to show lower preference for an aromatic acylamido function compared to papain (Boland & Hardman, 1972).

Actinidin contains two types of protease which are A1 and A2 (McDowall, 1973). Another study showed actinidin had six proteases, that can be divided into two groups by the effect of DL-Dithiothreitol (DTT) and zinc (Zn^{2+}) on the activity (Sugiyama, Ohtsuki, Sato & Kawabata, 1996).

2.8.4 Properties of actinidin

In the earliest studies actinidin was reported to have optimum activity at pH 5 - 7 with significant persistent activity at pH 3.2 and pH 10.1, it had a minimum solubility near pH 3.1 and is readily soluble at pH 2.8 when pI is about 3 (McDowall, 1970). With a combination of temperature and pH, highest proteolytic activity in kiwifruit was found at pH 7.3 - 7.6 and temperature 58 - 62°C by Yamaguchi et al (1982). Further findings

show that actinidin had different effects on different proteins/substrates as the optimum condition differs for different substrates. An example, when the substrate was gelatine in citrate–phosphate buffer, kiwifruit actinidin had the optimum activity at pH 4.0 to 4.3 (Arcus, 1959), optimum activity was at pH 5 to 7 was seen with N-benzoyl-L-arginine ethyl ester as substrate (McDowall, 1970), optimum actinidin activity at pH 5.8 to 6.4 when N-α-CBS-Lysine –p-nitrophenlyl ester was used as substrate (Boyes et al., 1997) and for degradation of α-lactalbumin and β-lactoglobulin the optimum condition was at pH 4 and 41.6°C (Vázquez-Lara, Tello-Solís, Gómez-Ruiz, García-Garibay & Rodríguez-Serrano, 2003).

2.8.5 Actinidin activity

Two proteins of actinidin resolved on gel chromatography have the same molecular weight and charge, it is thought the inactive protein may be enzymes in which the active thiol has been oxidized to a suphonate (Boland & Hardman, 1972) and this suggested that actinidin contained two types of protease that are A1 and A2 (McDowall, 1973). Another study showed actinidin had six types of protease, two mentioned earlier and another four unknown proteases that can be divided into two groups by the effect of DTT and Zn^{2+} on the activity (Sugiyama et al., 1996). A broad range of pH optimum from pH 5.0 to pH 6.5 is observed for the k_{cat}(rate of catalyst with a particular substrate), k_m (The Michaelis constant) and k_{cat} / k_m (k_{cat} / k_m =Catalytic efficiency) The k_{cat} value found in actinidin was 29 $\pm$ 2 sec^{-1} and more or less similar to papain and ficin but the k_m value was 22 $\pm$ 2 μM which was considerably higher than papain and ficin (Boland & Hardman, 1972).

For most substrates the K_m values are higher for actinidin than for papain (ratio typically 5:1 to 30:1) and this shows the difference between actinidin or papain catalysed hydrolysis lies primarily in the strength of binding (Baker et al., 1980). For all substrates with an aromatic acylamido group including Bz-Arg-pNA, k_{cat}/k_m was considerably less for actinidin compared to papain by a factor of between 10 and 110 (Baker et al., 1980). The major difference between actinidin and papain lies, therefore, in the interaction between aromatic N-substituents and their binding sites (Baker et al., 1980). In actinidin, trp 69 is replaced by thr. Other changes include pro 68 becomes Ile 70, phe 207 become ser 213 and at the end of S_2 subsite in papain becomes Met 211, making the pocket notably shorter (Baker, 1977). Actinidin Met 211 projects into the

hydrophobic pocket into which the un-substituted phenyl ring of I binds (Carotti et al., 1984).

2.9 Kiwifruit enzymes and their effects on food proteins

The effect of kiwifruit on proteins have long been studied, starting with its effect on gelatine and haemoglobin (Arcus, 1959). The proteolytic enzymes from plant origin have proven to have proteolytic effect on myofibrillar protein and/or collagen and their crude extract and slices have been practically used as meat tenderizer (Sugiyama et al., 2005).

Kiwifruit extracts have an effect on muscle tenderness and structure (Lewis & Luh, 1988; Samejima, Choei, Isioroshi, & Hayakawa, 1991; Wada, Hosaka, Nakazawa, Kobayashi, & Hasegawa, 2004; Wada, Suzuki, Yaguti, & Hasegawa, 2002). Crude actinidin was reported to hydrolyze myosin, actin and collagen molecules (Samejima et al., 1991). It disorganises actin and myosin filaments (Wada et al., 2004). In the hydrolysis of native myosin heavy chain using actinidin, the myosin heavy chain decreased with concurrent increase of C-protein and α- actinin with time (Lewis & Luh, 1988). Previous studies, when meat pieces were immersed in crude actinidin solution, the cutting strength for raw and cooked meats decreased gradually with time of soaking and increased the tenderness (Kawashima, Tanaka, & Aoyagi, 1994; Samejima et al., 1991; Tsutsumi et al., 1994; Wada et al., 2002). The pre-treatment with kiwifruit juice significantly decreased the shear force required to cut connective tissue but increased the liberation of collagen-related peptides (Sugiyama et al., 2005, Liu et al. 2011). Kaur et 2010b observed beef muscle protein bands decrease intensity above 25 kDa which indicate actinidin enhance the digestion of beef muscle protein under gastric condition. Actinidin significantly ($P < 0.05$) increased water-soluble proteins and water holding capacity which resulted in a more tenderized beef and produce more stable emulsion those impreve the texture of sausage (Aminlari, Shekarforoush et al. 2009)

Actinidin can be considered as a better meat tenderizer compared to papain because it does not produce off flavours and does not over tenderize the meat surface for the same degree of overall tenderness (Lewis & Luh, 1988). However, actinidin did not hydrolyze as much as papain (Lewis & Luh, 1988). Over tenderization of meat surface using actinidin can be controlled to some extent, by regulating the pH condition in contrast with papain and bromelain which non-selectively hydrolyzes all myofibrillar

protein over a wide pH range (Nishiyama, 2001). Proteolytic effect of actinidin on myofibrillar protein can be control by varying pH as described earlier. At pH 3 - 4 actinidin thoroughly hydrolyzed all the myofibrillar protein whereas at pH 5.5 - 8 actinidin only hydrolyzed myofibrillar protein not actin (Nishiyama, 2001). Kiwifruit extract does not appear to greatly enhance protein digestion after gastric plus intestinal digestion" (Kaur et al. 2010).

Actinidin showed complete degradation of α- lactalbumin and 65.3% degradation for β-lactoglobulin when used as pure protein. Whereas in commercial dairy whey a degradation of 89.1% and 84.6 %, respectively, was observed (Vázquez-Lara et al., 2003). Actinidin had relatively low proteolytic activity toward Atlantic cod viscera (Aspmo, Horn, & Eijsink, 2005). Aspmo et al (2005) also found actinidin showed low solubilisation yields compared to other commercial protease such as alcalase, bromelein, neutrase, papain, protamex^{TM} and supermix. Actinidin was found to hydrolyze α-casein and β-casein in milk (Nishiyama & Oota, 2002; Yamaguchi et al., 1982). The occurance of a bitter taste in mixtures of milk proteins and raw kiwifruit which was attributed to a caseolytic protease in kiwifruit splitting casein into bitter peptides (Bachmann & Farah, 1982).

Kiwifruit proteases not only show proteolytic activities in globular proteins but also fibrous proteins such collagen that raises questions on the collagenases properties of this enzyme.

2.10 Collagenase properties in kiwifruit enzymes

Several authors had reported that plant extracts apparently have a collagenase properties but that was measured at temperatures above 50°C in which collagen was converted to gelatin (Sugiyama et al., 2005). Collagen contributes to one third or more protein from animals making it important to degrade collagen into smaller units as a source of protein. Collagenase activity in the tissue around the kiwifruit seed was higher than that in the core and the optimum was at pH 5.0 and 60°C and has found to have a molecular weight 52,000 Da by gel filtration and 60,000 Da by SDS–Page (Tsutsumi, Nagahiro, Tanaka, Nakajima, & Yoshinaka, 1998).

Current studies show that actinidin was unable to hydrolyze the triple helical structure of collagen (Morimoto, Kunii, Hamano, & Tonomura, 2004; Sugiyama et al., 2005; Sugiyama, Ohtsuki, Sato, & Kawabata, 1997, Kaur et al., 2010) but it can degrade denatured collagen (Sugiyama et al., 2005). For example, actinidin was able to hydrolyze aterocolagen (pepsin-hydrolyzed collagen) from yellowfin tuna at specific sites on the inside of the inter-strand cross-linking peptides at acidic pH (Morimoto et al., 2004). But actinidin had no cleaving activity toward collagen at neutral pH (Morimoto et al., 2004). Kaur et al 2010b observe the effect of actinidin toward gelatin under stimulated gastric condition which is pH 1.9.In other studies, actinidin could degrade the insoluble collagen in the cattle achilles tendon into collagen subunit chains, $\propto$-chain and β-chain at neutral and acidic pH (pH 3.3) but not at pH 2.7-2.9 (Wada et al., 2004). At acidic pH, the cross-linked subunits of acid-soluble collagen were converted to monomeric subunits, suggesting that the globular domains, in which cross-links preferentially occur, can be degraded by kiwifruit juice (Sugiyama et al., 2005). The connective tissue treated with kiwifruit protease had soluble α-chain collagen compare to untreated samples where α-chain collagen was not obtained (Wada et al., 2002). Collagen and gelatin are said to be difficult to digest due high level of proline and hydroxyproline in which it prevent the flexibility of protein backbone that facilitate enzyme binding to active site (Kaur et al, 2010b)

However in recent study, Mostafaie et al. (2008) demonstrated collagenase properties of actinidin where it was able to hydrolyze collagen type I and type II at neutral and alkaline buffer. This protease has a potential for isolation of different cell populations from various solid tissue (Mostafaie et al., 2008)

2.11 Conclusions

Meat muscle can be divided into three major types according to their solubility. Myofibrill proteins are proteins that are soluble in high ionic strength or concentrated salt solution and are also called salt-soluble protein. Muscle proteins that are soluble in water are called water-soluble proteins or sarcoplasmic. The third group are insoluble in either concentrated salt solution or water and are known as connective tissue. Protein hydrolysis can be obtained by chemical, microbial fermentation and enzymes hydrolysis. The extent of the hydrolysis determined using degree of hydrolysis is correlated to the number of peptide bonds cleaved. Nitrogen content with conversion

factor of 6.25 will be used for calculation of protein content in meat. Kiwifruit enzyme, actinidin, has been shown to provide a mild hydrolysis reaction compared to other proteases such as papain. A number of authors have published research on the use of kiwifruit, crude kiwifruit or actinidin for tenderization of various meat proteins. There is no published literature on the use of kiwifruit enzymes for the hydrolysis of beef protein.

Chapter 3
Materials and Methods

This chapter describes the methodology and experimental design used for the meat hydrolysis experiments. It will cover the chemicals and equipment used in all assays, preparation of beef mince as a raw material, the beef hydrolysis process and the chemical analyses used on raw materials and hydrolysates.

3.1 Chemical preparations

Unless describes, the chemical were prepared according to supplier's instruction.

3.1.1 Acrylamide/Bis solution for SDS PAGE

The following solutions, 30%T (total monomer concentration), 2.67%C (weight percentage of cross- linker) were purchased from Bio-Rad Laboratories, Inc. (USA). The % T and % C is defined in Equations 3.1 and 3.2.

%C is the weight percentage of cross-linker:

$$\%C\ (w/w)=\frac{\text{gram bis-acrylamide}}{\text{gram bis-acrylamide+gram acrylamide}}\times 10 \qquad \text{Equation 3.1}$$

%T is total monomer concentration:

$$\%T\ (w/v)=\frac{\text{gram acrylamide+gram bis}}{\text{total volume (ml)}}\times 100 \qquad \text{Equation 3.2}$$

The pore size of acrylamide gels can be varied by regulating the % T and % C. The selected acrylamide/bis solution used was 30 % w/v of 30% T, 2.67% C contains 29.2 g acrylamide and 0.8 g N'N'-bis-methylene-acrylamide made up to 100 ml in a volumetric flask with distilled water (Bio-Rad manual). This solution was stored in 4°C.

3.1.2 Ammonium persulfate (APS) Solution (10% w/v) for SDS PAGE

This solution was prepared immediately before use by dissolving 100 mg ammonium persulfate (reagent grade, Bio-Rad Laboratories, Inc., USA) in 1.0 ml distilled water.

3.1.3 Boric acid solution, 4 % w/v Kjeldahl analysis

Approximately 200 g of boric acid (analytical grade, Labserv, Thermo Fisher Scientific, Australia) was accurately weighed and dissolved in 3.0 l of hot distilled water. Then 50 ml of 0.1% bromocresol green (analytical grade, Acros Organic, Thermo Fisher

Scientific, Australia) and 35 ml of 0.1 % methyl red (analytical grade, Acros Organic, Thermo Fisher Scientific, Australia) were added to the solution. The solution was cooled and made up to 5.0 l with distilled water in a volumetric flask.

3.1.4 Bradford reagent

The Bradford reagent was composed of 0.01% w/v Coomassie blue G-250, 4.7% w/v ethanol and 8.5% w/v phosphoric acid. About 100 mg of Coomassie blue G 250 (microscopy grade, Sigma-Aldrich, USA) was dissolved in 50 ml of 95% ethanol (high grade anhydrous alcohol, Anchor Ethanol, NZ). The solution was then mixed with 100 ml of 85% phosphoric acid (analytical grade, Fisher Scientific, UK) then diluted with distilled water in a one litre volumetric flask. The solution was then filtered twice through Whatman No.1 filter paper, then stored at room temperature and used within one month.

3.1.5 Destaining Solution for SDS PAGE

The destaining solution used for SDS-PAGE was 2.5% methanol (99.9%, analytical grade, Fisher Scientific, UK) and 0.5% acetic acid (analytical grade, Fisher Scientific, UK). The destaining solution was made by mixing 250 ml of methanol and 50 ml of acetic acid then diluted to one litre using distilled water.

3.1.6 Electrophoresis Running Buffer, pH 8.3 for SDS PAGE

Approximately 30.3 g of Tris-hydroxymethyl-aminomethane base (electrophoresis purity reagent, Bio-Rad Laboratories Inc., USA), 144 g glycine (reagent grade 99%, Sigma) and 10.0 g sodium dodecyl sulphate (SDS) (electrophoresis powder bioreagent, Fisher Scientific, UK).

SDS was dissolved in distilled water and made up to one litre in a volumetric flask. The stock solution (10x concentration) was then stored at 4 ± 1°C. One hundred millilitres of the stock solution was diluted with distilled water in a volumetric flask, made up to one litre, for each electrophoresis run.

3.1.7 Iodoacetate–KCl solution

A solution containing 5 mM sodium iodoacetate and 0.15 M KCl was prepared for pH determination. About 0.93 g sodium iodoacetate (Sodium salt 99%, Arcos Organic,

Thermo Fisher Scienctific Ltd, Australia) and 11.18 g KCl (analytical grade, Labserv, Thermo Fisher Scientific Ltd, Australia)) were dissolved in one litre of distilled water in a volumetric flask.

3.1.8 Kjeldahl Catalyst Tablets

The tablets each contained 1.5 g potassium sulphate (K_2SO_4) and 0.15 g copper sulphate ($CuSO_4.5\ H_2O$) (Foss, Denmark).

3.1.9 OPA reagent

The OPA reagent was prepared as follows: 7.620 g disodium tetraborate decahydrate and 200 mg sodium-dodecyl-sulfate (electrophoresis powder bioreagent, Fisher Scientific, UK) were dissolved completely in 150 ml distilled water. This was prepared fresh on a daily basis. One hundred and sixty milligrams of ortho-Phthalaldehyde 97% (analytical grade, Alfa Aesar, USA) was dissolved in 4 ml ethanol (high grade anhydrous alcohol, Anchor Ethanol, NZ). This OPA solution was then transferred quantitatively to the previously prepared tetraborate decahydrate /SDS solution rinsing with distilled water. One hundred and seventy-six milligrams of dithiothreitol 99% DTT) (grade, supplier, country) was added to the OPA solution. The solution was made up to 200 ml with distilled water in a volumetric flask.

3.1.10 Phosphate Buffer (0.03 M) for SDS PAGE

The phosphate buffer was 0.03 M phosphate, pH 7.4. Approximately 4.06 g of potassium dihydrogen phosphate (KH_2PO_4) (analytical grade, Labserv, Thermo Fisher Scientific, Australia) was dissolved in 900 ml water and the pH was adjusted to 7.4 using 1M potassium hydroxide (KOH) (analytical grade, Labserv, Thermo Fisher Scientific, Australia). The solution was made up to one litre with distilled water in a volumetric flask.

3.1.11 Phenolphthalein Indicator Solution

The phenolphthalein solution was prepared by dissolving 1 g phenolphthalein (analytical grade, Sigma Aldrich, USA) in 100ml of 60% ethanol (high grade anhydrous alcohol, Anchor Ethanol, NZ).

3.1.12 Derivatization reagent for SDS PAGE

The derivatization reagent consisted of methanol, TEA (HPLC grade, Fisher Scientific, UK), distilled water, phenylisothiocyanate (PITC) at a ratio of 7:1:1:1, respectively. The reagent was made fresh daily.

3.1.13 Protein Electrophoresis Size Standard for SDS PAGE

The standard consisted of mixture of eight blue-stained recombinant proteins (10 - 250 kD) with two pink reference bands at 25 and 75 kD (Precision plus protein™ Dual, Biorad Laboratories Inc., USA).

3.1.14 Phosphate Buffer (0.1 M) for SDS PAGE

This phosphate buffer was 0.1 M phosphate, 1.1 M KCl, pH 7.4. Approximately 13.6 g of postassium dihydrogen phosphate ((analytical grade, Labserv, Thermo Fisher Scientific, Australia), 5.0 g KOH (analytical grade, Labserv, Thermo Fisher Scientific, Australia) and 82.0 g potassium chloride (analytical grade, Labserv, Thermo Fisher Scientific, Australia) were dissolved in 900 ml distilled water. The pH was adjusted to 7.4 using 1 M KOH (analytical grade, Labserv, Thermo Fisher Scientific, Australia) and the solution was made up to one litre with distilled water in a volumetric flask.

3.1.15 Resolving Buffer (1.5 tris-HCl, pH 8.8) for SDS PAGE

Exactly 18.15 g of Tris- base (electrophoresis purity reagent, Bio-Rad Laboratories Inc., USA) were dissolved in 60 ml distilled water. The pH of the solution was then adjusted to pH 8.8 using 6 M HCl and made up to 100 ml in a volumetric flask with distilled water. The solution then stored at 4 ± 1°C.

3.1.16 Sodium Hydroxide (NaOH), 40% w/v for Kjeldhal analysis

The NaOH standard was prepared by dissolving 400.0 g of NaOH (analytical grade, Fisher Scientific, UK) in one litre of distilled water in a volumetric flask.

3.1.17 Sample Buffer (0.0625 M tris-HCl, pH 6.8) for SDS PAGE

The sample buffer included 2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol and 0.01 % bromophenol blue.

A solution containing 12.5 ml of 0.5 M tris-HCl at pH 6.8, 25 ml glycerol (analytical grade, Labserv, Thermo Fisher Scientific, Australia), 20ml 10% SDS (electrophoresis powder bioreagent, Fisher Scientific, UK) and 2 ml of 0.5% w/v bromophenol blue (electrophoresis purity reagent, Bio-Rad Laboratories Inc., USA) was diluted to approximately 99 ml with distilled water in a volumetric flask. To this solution was added 500 µl of β-mercaptoethanol (electrophoresis purity reagent, Bio-Rad Laboratories Inc., USA). The final solution was diluted to 100 ml, immediately before use.

3.1.18 Sodium dodecyl sulphate (SDS), 10 % w/v for SDS PAGE

Approximately, 10 g of Sodium dodecyl sulphate (SDS) (electrophoresis powder bioreagent, Fisher Scientific, UK) was dissolved in 90 ml water, the solution was made up to 100 ml with distilled water in a volumetric flask and stored at room temperature for up to one month.

3.1.19 Stacking Gel (0.5 M tris- HCl, pH 6.8) for SDS PAGE

Approximately, 6.0 g of Tris-base (electrophoresis purity reagent, Bio-Rad Laboratories Inc., USA) was dissolved in 60 ml distilled water. The pH of the solution was then adjusted to pH 6.8 using 6 N HCl and made up to 100ml with distilled water in a volumetric flask. The solution was then stored at 4 ± 2 °C.

3.1.20 Trifluoroacetic acid (TFA) (0.1 % v/v) for HPLC analysis

About 1.0 ml of TFA (optima LC/MS, Fisher Scientific, UK) was added to a one litre volumetric flask and made up to volume with distilled water. Fresh solutions were prepared for each batch of samples analysed on the HPLC.

3.1.21 Trichloroacetic Acid (TCA) Solution (15% w/v) for precipitation of protein

Dilute solutions of TCA (are unstable and decompose to form toxic gases. Therefore, a stable stock solution of 30% TCA in water was prepared by adding 30.0 g of TCA (analytical grade, Labserv, Thermo Fisher Scientific, Australia) into a 100ml volumetric flask and made up to volume with distilled water and stored at 4 ± 2 °C. When required, the stock solution was diluted with distilled water to give a 15 % (w/v) working solution.

3.2 Raw materials

3.2.1 Preparation of minced lean beef

Beef meat was purchased from a local butchery (Maxi Meat Mart, Auckland, N.Z). This lean beef was selected because it contains a relatively high proportion of meat muscle and little connective tissue or fat to reduce variability in hydrolysis reaction to zyactinaseTM. In subsequent experiment other meat protein such as collagen, sarcoplasmic and myobrillar were analysed separated to determine if Zyactinase can hydrolyse the proteins All visible fat and connective tissue were removed with a knife (Figure 3.1). The lean beef meat was then cut into small pieces and then placed in the meat grinder (Dimock) (Figure 3.2). The meat was minced twice using a 3 mm plate (Figure 3.3). This process was carried out to make sure the meat sample was homogeneous for the hydrolysis experiments.

One large batch of beef meat was purchased, minced and stored frozen to ensure one batch of meat was used for all experiments. A total of 40 kg of minced lean beef was allocated for the experiments, with the 40 kg divided into 120 ± 5 g individual portions. These individual potions were vacuum-packed into small foil pouches (140 x 200 mm) for easy handling and stored at -20 ± 2°C until required.

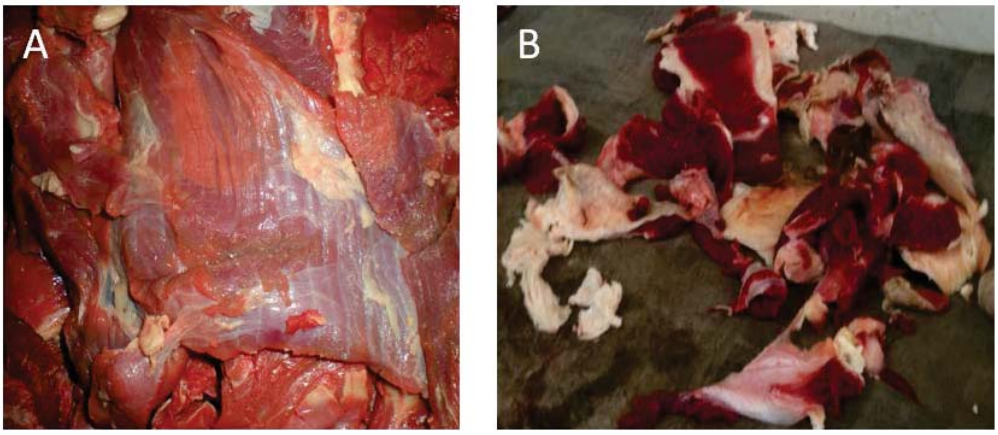

Figure 3.1: (A) Lean beef meat, (B) Visible connective tissue and fat remove from the beef meat.

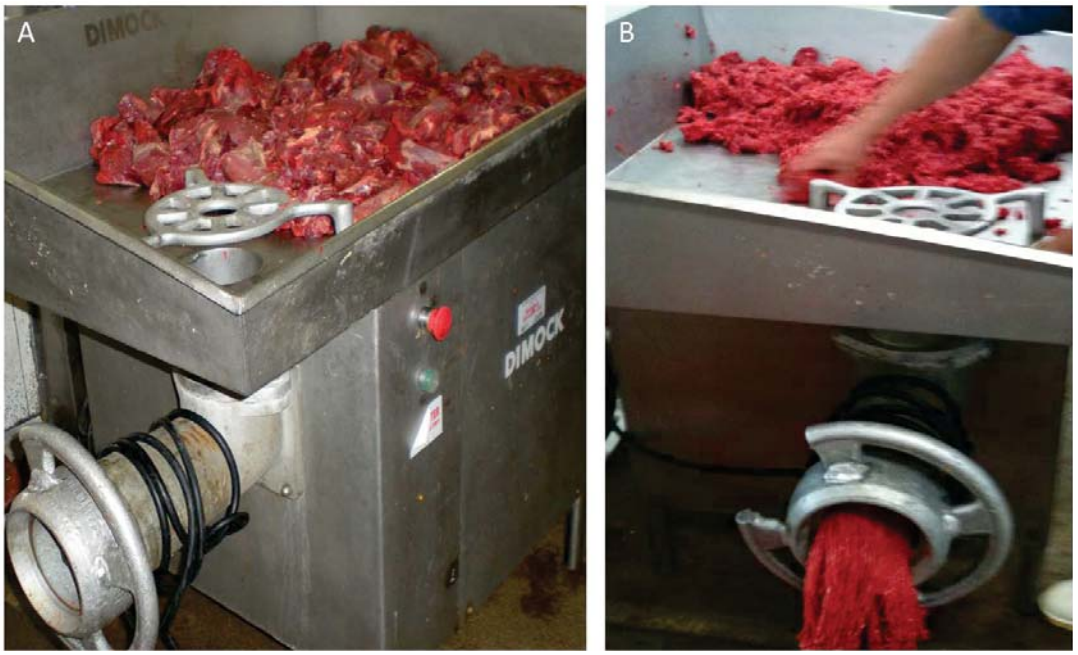

Figure 3.2: (A) Dimock meat grinder; (B) Mixed minced beef going through the meat grinder for the second time.

Figure 3.3: Grinder plate with hole size of 3 mm attached to meat grinder.

3.2.2 Sarcoplasmic protein (water soluble protein)

Sarcoplasmic protein was separated from a sample of lean minced beef. The protein extraction technique reported by Helander (1957) was used with minor modification. Ten grams of thawed minced beef wa weighed to the nearest 0.01g. About 190 ml of cold (3 ± 2°C) 0.03 M phosphate buffer was added to the minced beef and the mixture was homogenized in a Waring blender (Waring, model HGBTWTS3 8010es, USA) for

15 seconds at full speed, followed by 15 seconds at low speed. The homogenate was then transferred to a 250 ml centrifuge tube and stored in ice until all samples were prepared. The homogenate was centrifuged (Heraeus, Multifuge 1S-R, Germany) at 1,400 x *g* for 20 minutes at 4 ± 1°C. The supernatant liquid from the centrifuged homogenate was decanted through a double layer of woven cheesecloth and stored at 4 ± 2°C. The sediment pellet from the centrifugation step was rinsed from the centrifuge tube into a 250 ml beaker using 90 to 100 ml phosphate buffer. The mixture was then homogenised for a further 30 seconds using the Waring blender. The Waring blender was washed with approximately 50 ml of 0.03 M phosphate buffer and the liquid from the washings were added to the original homogenate. The homogenate was then stirred and stored on ice until all samples were prepared. The final homogenate was re-centrifuged (IECB -22M, Model 3495/3496, USA) at 5,000 x *g* for 30 minutes at 4 ± 1°C. All the supernatant liquid obtained from the procedure above was designated as water soluble protein or sarcoplasmic protein and stored at -20 ± 2°C.

3.2.3 Myofibrilla protein (salt-soluble protein)

The pellet from Section 3.2.2 was re-suspended with 200 ml of 0.1 M phosphate buffer containing 1.1 M KCl. The suspension was homogenised using a Waring blender (Waring, model HGBTWTS3 8010es, USA) for 15 seconds at high speed, followed by 15 seconds at low speed and then centrifuged at high speed (IECB -22M, Model 3495/3496, USA), at 10,000 x *g* for 30 minutes at 4 ± 1°C. The suspension was passed through woven cheesecloth twice. The procedure was repeated two more times to dissolve all the myofibrilla protein from the sample. All supernatant liquid obtained from these procedures were combined and designated as salt-soluble protein or myofibrilla protein and stored at -20 ± 2°C.

3.2.4 Connective tissue

Connective tissue substrate was prepared using two methods. Small amounts of tissue were obtained from the preparation of myofibrilla protein fraction (Section 3.2.3). The tissue retained on the cheesecloth was washed with 0.1M phosphate buffer containing 1.1M KCl to remove contaminating myofibrillar protein and was stored at -20 ± 2°C as a connective tissue fraction.

The second method used to obtain connective tissue, was prepared using chuck beef, because this cut contained higher amounts of connective tissue than the other meat used in 3.2.1. The chuck beef was cut into small pieces and minced for 15 seconds in a Waring blender (Waring, model HGBTWTS3 8010es, USA). The connective tissue entwined itself around the blender blade. The connective tissue strands were collected and washed with 0.1M phosphate buffer, then centrifuged (IECB -22M, Model 3495/3496, USA) at 10,000 x *g* for 30 minutes to ensure of removal of both myofibrilla and sarcoplasmic proteins. The resulting washed strands of connective tissues were then stored at -20 ± 2°C (O'Meara and Munro 1985)

3.3 Enzymes used for hydrolysis reactions

The enzyme complex used in this study was a commercial product made by Vital Foods Limited, Auckland, New Zealand. The complex was a freeze-dried kiwifruit extract, called Zyactinase™. The extract was prepared from kiwifruit variety Hayward (*Actinidia deliciosa)* by molecular differentiation. The most prominent enzyme in Zyactinase™ is actinidin, a thiol cysteine protease. Zyactinase™ is designed to be used as digestive enhancer. It has activity between 180 - 210 U/mg. The Zyactinase™ is a commercial enzyme complex and due to confidentiality issues no other information can be provided on the enzyme manufacture or separation.

3.4 Beef hydrolysis

3.4.1 Hydrolysis process setup

Protein hydrolysis experiments were conducted in 500 ml glass beakers in a controlled temperature water bath (Grant Instruments, GD120, UK). The setup of the equipment is shown in Figure 3.4. Agitation at 200 rpm was achieved by using a gate paddle impeller as shown in Figure 3.5 attached to an overhead stirrer (IKA®, RW20 digital, Germany). Frozen vacuum-packed minced beef was thawed overnight at 4 ± 2 °C. The thawed minced beef was weighed accurately, placed into the glass beaker and the beaker was placed in the controlled water bath (Grant Instruments, GD120, UK) at the required temperature (Figure 3.4). The minced beef was stirred occasionally until it reached the water bath temperature. The time required varied according to the hydrolysis temperature. For example, hydrolysis at 50°C took approximately 20 minutes for the meat to warm from 4 to 50°C.

The enzyme powder (amount based on concentration required % w/w)was first mixed in 8 ml of warm reverse osmosis water to form a slurry. All of the enzyme slurry was then added only after the minced lean beef reached the required temperature (40°C, 50°C, 60°C). The hydrolysis reaction started (t = 0) when the enzyme slurry was added to the heated minced beef. During the first 15 seconds, the mixture of beef and enzyme were stirred constantly using a fork, because using the stirrer resulted in the beef rolling into a ball (Figure 3.6) which stopped the stirrer. Note: Within one minute of addition of the enzyme slurry, the stirrer could function normally. The hydrolysis process was continued with constant stirring (200 rpm) for the desired hydrolysis time.

At the completion of the hydrolysis process, the enzyme reaction was terminated by placing the beaker containing the hydrolysate in a shaking water bath (Grant Instruments, GLS 400, UK) at 95 ± 1°C for 10 minutes with occasional stirring with a fork. Previous preliminary trials on deactivation of the enzymes determined there was no further enzymes activity at 95°C. The time required for the slurry to reach 95°C was approximately 5 minutes. The hydrolysate was then cooled in an ice-water bath (0 ± 2°C) until the hydrolysate reached 18 ± 2°C. It was then packaged into a plastic container, frozen and stored at -20 ± 2°C until analysis.

Each hydrolysis reaction was carried out in duplicate with separate meat samples. When investigating the effect of substrate concentration and pH on the hydrolysis process, distilled water was added to the beef meat samples, as necessary, to adjust the substrate concentration. Each duplicate sample was analysed at the various points in the hydrolysis reaction in triplicate.

Figure 3.6 shows the visual observations during the beef hydrolysis process. At the end of the hydrolysis process, minced beef was converted to thick brown slurry. The visual observations changed depending on the hydrolysis temperature or enzyme concentration.

Figure 3.4: Experimental set up for hydrolysis process

Figure 3.5: Gate stirrer used to mix beef samples during hydrolysis experiments.

Hydrolysis time (minutes)	Observations	
t = initial	At the beginning of the experiment, minced beef was placed in a controlled temperature water bath.	
t = 0	On adding enzyme, the minced beef formed a ball-like structure, clinging to the stirrer and causing the stirrer blade to stop. Hand mixing using a fork facilitated the mixing.	

t = 2

As hydrolysis began, the meat surface developed a glossy appearance. The stirrer was able to stir the beef although there were still large beef particulates within the slurry.

t = 3

The mixture started to liquidize forming a very thick grainy appearance. Beef particles still clumped together in the mixture.

t = 5

The beef mixture changed to a brown-pinkish slurry. Some beef particles could be seen still attached to the stirrer.

t = 11	The mixture became light brown in colour and the particulates disappeared to produce a uniform liquid slurry.	
t = 30	Browning of the beef slurry occurred at the edge of the beaker. The viscosity of the slurry increased.	
t = 120	The beef slurry was a dark brown colour. The slurry was observed to increase in viscosity.	

Figure 3.6: Qualitative observations during hydrolysis of beef: Hydrolysis conditions; 60°C, 2% w/w enzyme concentration.

3.5 General chemical analyses

3.5.1 Moisture content

The moisture content of the meat and hydrolysates was determined by the air oven drying method (Method 950.46, AOAC, 1991). Two grams of minced meat or hydrolysate were weighed accurately (Mettler Toledo, ML204, USA) to four decimal places and spread evenly into a pre-weighed moisture dish. The samples were then dried at 105 ± 1°C in air oven (Contherm, Digital series 2300, NZ) for 16 hours or until the weight was constant. Once the samples were cooled to room temperature inside desicator, the moisture dish containing the dried sample was weighed to the nearest ± 0.1 mg. The moisture content was determined by measuring the mass of the minced beef and meat hydrolysate before and after the water was evaporated in the oven as in Equation 3.3

$$\% \text{ Moisture} = \frac{M_{Initial} - M_{Dried}}{M_{Initial}} \times 100 \qquad \text{Equation 3.3}$$

Where:

$M_{initial}$ = initial weight of sample

M_{Dried} = weight of sample after drying to constant weight.

Each determination of moisture content was carried out in triplicate. The total solids in minced beef and hydrolysates were calculated based on of the amount of solids remaining after evaporation of all the water, Equation 3.4.

$$\% \text{ Total solids} = 100 - \% \text{ Moisture} \qquad \text{Equation 3.4}$$

3.5.2 Ash content

The ash content of the minced beef and meat hydrolysates was determined by dry ashing following air oven drying (Method 920.153, AOAC, 1991). Ten grams of minced beef meat or meat hydrolysate were weighed accurately (Mettler Toledo, ML204, USA) into pre-weighed dried porcelain crucibles and dried in an air oven (Contherm, Digital

series 2300, NZ) at 105 ± 1°C for 12 hours to remove excess water. The porcelain crucible was then heated over an open gas flame until the sample charred and no smoke was observed due to any burning. The charred sample was placed in muffle furnace (Vulcan®, A550, USA) capable of maintaining a temperature of 550 ± 5°C for 16 hours, until a white-grey ash was formed. The samples were cooled to room temperature and the porcelain crucible containing the ash was weighed (Mettler Toledo, ML204, USA) to the nearest ± 0.1 mg. Each determination of ash content was carried out in triplicate.

The calculation for ash content was determined by measuring the mass of the minced meat or meat hydrolysate before and after the ashing procedure as in Equation 3.5.

$$\text{Ash, \%} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100 \qquad \text{Equation 3.5}$$

3.5.3 Fat content

The fat content in minced beef and meat hydrolysate was determined using the Mojonnier method (Mills et al., 1983). A meat emulsion was prepared by accurately weighing (to 4 decimal places) (Mettler Toledo, ML204, USA) a 20 g sample of thawed minced beef or meat hydrolysate into a 200 ml beaker. To the meat emulsion 100.00 g of 0.1 M NaOH was added and then heated in a water bath at 70 ± 2°C for 5 minutes with occasional stirring. The sample was then homogenised immediately using a homogeniser (Success,Dyna Pasion, Malaysia) at high speed (20000rpm) for four minutes. The homogeniser was washed with 50 ml pre-warmed distilled water to remove any meat sticking to the homogeniser and the meat emulsion weight was made up to 200.00 g using distilled water.

Ten grams of the emulsion (representing 1.00 g of minced beef or meat hydrolysate) was then weighed into a Mojonnier tube for solvent extraction. There were two steps of extractions: The first extraction involved a mixture of 1.5 ml ammonia, 10 ml ethanol, 25 ml ethyl ether, and 25 ml petroleum ether. The second step involved extracting with a mixture of 5 ml ethanol and 25 ml petroleum ether.

After adding the solvent mixture (extraction 1 or 2 above) the Mojonnier tube was mixed by inverting manually for about one minute. The samples were then centrifuged (Funke Gerber, Super Vario-N, Germany) for two minutes. The upper layer of the extraction containing solvent with fat was poured into a pre-weighed beaker leaving the meat or hydrolysate emulsion in the bottom part of the tube. The beaker containing solvent and fat was heated using a hot plate (Stuart, CB302, USA) at minimum heat (approximately 40°C) inside the fume hood, allowing the solvent to evaporate and leaving the fat in the beaker. The second extraction followed the first set of solvents. After mixing with the new solvent, the Mojonnier tube was centrifuged (Funke Gerber, Super Vario-N, Germany) for two minutes and the upper layer was added to the previous beaker and the solvent evaporated. The beaker was reweighed and the amount of fat was measured. Fat content was calculated using Equation 3.6.

$$\% \text{ Fat} = 100 \times \frac{((\text{g beaker} + \text{g fat}) - (\text{g beaker})) - \text{mean blank residue}}{\text{g sample}}$$

Equation 3.6

All samples were analysed in triplicate. A blank was conducted using 10 g of distilled water instead of meat emulsion.

3.5.4 pH measurement

3.5.4.1 pH measurement of minced beef and meat hydrolysate samples

The method recommended by Bendall (1973) was used to measure the pH of the minced beef and meat hydrolysate samples. A 0.5 to 1.0 g sample was added to approximately 10.0 ml iodoacetate-KCl solution and homogenised for 30 seconds using handheld homogeniser (Success, Malaysia). The pH of the homogenate was measured at room temperature (25C) using a standard pH meter with pb20 probe (Sartorius, Basic meter pb11, Germany). Duplicate determinations were completed on each sample.

3.5.4.2 pH measurement during meat hydrolysis process

The pH and temperature profile of the hydrolysates during the hydrolysis reactions were measured every 15 minutes using a hand held pH meter (EC-PCSTestr35, Waterproof, UK) with a spear electrode inserted into the centre of the reaction mixture. The pH meter was rinsed with distilled water after every reading. The pH meter was calibrated using pH 7.0, pH 4 and pH 10 buffers every day before experiments started.

3.5.5 Total nitrogen content

The protein content of the hydrolysates was determined by the Kjeldahl method (Method 981.10, AOAC, 1990) with some modification. Sample sizes of 0.8 to 1.0 g of minced beef and meat hydrolysate were accurately weighed and added to a 250 ml digester tube, along with two Kjeldahl catalyst tablets. Twenty millilitres of concentrated sulphuric acid was added and the mixture digested for 45 minutes at 200°C, then heated to 270°C and held for 45 minutes, then heated to 330°C and held for 30 minutes, then finally heated to 400°C and digested (Foss Tecator, DS 20, Denmark) until the sample became a clear solution (approximately 3.5 hours). After digestion the cooled material was then diluted with 70 ml distilled water.

About 70 ml of 40% NaOH was added to the tube, which was placed into a distillation unit (Foss Tecator, Kjeltec system 1026, Denmark) and the solution distilled using direct steam injection. The condensate from the distillation was collected in 50 ml of 4% w/w boric acid solution. The boric acid solution was then titrated with 0.1 M HCl until a grey-mauve colour end point. The nitrogen content on the sample was calculated using Equation 3.7.

$$N\ per\ sample = \frac{\text{(ml HCl of sample-ml HCl of blank)} \times \text{HCl normality} \times 14.008}{\text{g of sample}} \qquad \text{Equation 3.7}$$

The total nitrogen content in the minced lean beef samples was determined 10 times and all hydrolysate samples collected were analysed in duplicate.

All nitrogen data were converted to protein by multiplying the results by the conversion factors. The average nitrogen (N) content of proteins was found to be about 16 percent, which led to use of the calculation N x 6.25 (1/0.16 = 6.25) to convert nitrogen content into protein content.

3.5.6 Non-protein nitrogen (NPN) content

Non-protein nitrogen was determine in minced beef and meat hydrolysate and used as an indicator of the extent of the hydrolysis reaction. Protein was precipitated by adding 20 ml of 15% w/v tricholoroacetic acid (TCA) to a 10.0 ± 0.01 g of minced beef or meat hydrolysate. The mixture was homogenised with a handheld homogenizer (Success,

D130, Malaysia) for one minute and allowed to stand at room temperature for 30 to 45 minutes. The homogenate was then filtered through Whatman No. 1 filter paper and the nitrogen content on the clear supernatant containing the NPN fraction was determined as described later. All supernatants were stored at 4 ± 1°C until analysis within three days after precipitation with TCA.

For the NPN analysis, 25 ml of clear supernatant was added to each 250 ml block digester tube, along with two Kjeldahl catalyst tablets. Exactly 18 ml of concentrated sulphuric acid was added and the mixture heated for 60 minutes at 200°C, then 270°C for 45 minutes, then 330°C for 30 minutes and finally digested at 400°C until the sample became a clear solution (approximately 3.0 hours). After digestion the samples were diluted with 70 ml distilled water. Changes to the method were made to avoid sample overflow from the tube in the digestion block due to the originally high water content. The digested sample was neutralised with 50 ml of 40% NaOH and distilled as in Section 3.5.5.

The non-protein nitrogen content of the sample was calculated using Equation 3.8.

$$\text{g N per sample} = \frac{(ml\ HCl\ of\ sample - ml\ HCl\ of\ blank) \times HCl\ normality \times 14.008\ x\ final\ volume}{g\ of\ sample\ x\ aliquot\ volume}$$

Equation 3.8

3.5.7 Protein determination by Bradford method

This method was used to determine the protein concentrations in hydrolysate samples and the enzyme slurry before SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Under acidic conditions, the Bradford reagent (Coomassie Brilliant Blue G-250 dye) specifically binds to arginine and phenylalanine residues in protein molecules, forming a blue complex that can be measured at 595 nm (Bradford, 1976). Bradford reagent (5 ml) was added to the sample solution, mixed and the colour was allowed to develop for five minutes at room temperature. The absorbance was read in a UV-Vis spectrophotometer (Shimadzu, Japan) at 595 nm. All determinations were carried out in duplicate.

3.6 The effect of reaction parameters on minced beef hydrolysis

A series of experiments were conducted to examine the effect of varying reaction conditions on the NPN and total nitrogen contents of beef hydrolysates. These experiments were carried out to determine the effect of time, pH, temperature, and enzyme and substrate concentration. Reaction conditions were chosen based on preliminary experiments carried out by Vital Foods Ltd (unpublished).

3.6.1 Effect of pH on the extent of hydrolysis

Based on the meat hydrolysis procedure described in Section 3.4.1, 80 g of minced meat was diluted with 320 ml of reverse osmosis water. Dilution of minced meat was carried out to allow changes in pH to be made in the reaction solution, to avoid gelatinisation of undiluted meat that will occur at low pHs. Immediately before the mixture was placed in the water bath the minced beef and distilled water were homogenised for one minute in a Waring blender (Waring, model HGBTWTS3 8010es, USA). The pH of original sample was measured (Sartorius, pb 20, Germany) and the pH was adjusted using 1 M HCl or 1 M NaOH to pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 ± 0.2, respectively.

3.6.2 Effect of substrate concentration on the extent of hydrolysis

In this experiment, the substrate (beef meat) concentration was varied by addition of distilled water to the minced beef. The quantity of distilled water added to the beef was determined by Equations 3.9 – 3.11 (Adler Nilsen, 1976). The enzyme concentration and processing temperature were fixed at 2% w/w and 60 ± 2°C, respectively. The total mass of reaction mixture was 240 g per batch which contained minced beef, water and Zyactinase™. The % protein in the reaction mixture was based on the initial protein content in the minced lean beef (PR%) which was 22.20 ± 0.06 g protein/100 g meat.

$$MP = M\left(\frac{S\%}{100}\right) \qquad \text{Equation 3.9}$$

$$MR = 1.1MP\left(\frac{100}{PR\%}\right) \qquad \text{Equation 3.10}$$

$$MW = 1.1\left(M - M_{enz}\right) - MR \qquad \text{Equation 3.11}$$

Where:

MP = Mass of utilized protein (g);

M = Total mass of reaction mixture (minced beef, distilled water and enzyme) (g);

S% = Percent protein in reaction mixture;

MR = Mass of minced beef (g);

PR% = Percent protein in minced lean beef;

MW = Mass of distilled water (g);

M_{enz} = Mass of enzyme solution

The substrate concentrations (S %) were varied from 2% protein up to 22.20 ± 0.06% w/w. At the maximum substrate concentration there was no addition of distilled water. The substrate preparation (containing water and meat only) was homogenised for one minute at medium speed in Waring blender (Waring, model HGBTWTS3 8010es, USA) to ensure a homogeneous composition before it was placed in the water bath. Experiments were conducted using the standard meat hydrolysis setup describe in Section 3.4.1.

3.6.3 Effect of reaction time on the extent of hydrolysis

The standard hydrolysis method described in Section 3.4.1 was used for this experiment. Undiluted mince beef was used at a constant temperature and enzyme concentration.

3.6.4 Effect of temperature on the extent of the hydrolysis

From early studies in this current work, the lowest temperature chosen was 40°C. No adjustment to pH was made, but the pH values for the initial raw material and during processing were recorded. The reaction temperatures selected for this trial were 40, 50, 60, 65, 70 and 75 ± 2 °C (Yamaguchi et al., 1982). The meat was heated to the desired temperature before the enzyme slurry was added to the sample as described in Section 3.4.1. The beaker containing the hydrolysis reaction solution was removed from the water bath after 1, 30, 60, 90, 120 and 180 minutes of hydrolysis time. The hydrolysate was then placed in an ice water bath to cool to 18 ± 5°C before being packaged into a plastic container, frozen and stored at -20 ± 2°C until further analysis.

3.6.5 Effect of enzyme concentration on the extent of hydrolysis

This experiment was designed to determine the effect of Zyactinase™ concentration on the extent of hydrolysis. The enzyme concentrations added into the hydrolysis mixture were based upon the original weight of raw minced beef meat. The percentages of

enzyme addition selected were 1%, 2% and 4% w/w. For 2% enzyme concentration, for example, 2.00 ± 0.01g was added to 100 g of meat. In all instances, the enzyme was mixed to a slurry with 8 ml of warm reverse osmosis water just before it was added to minced beef.

3.7 Polyacrylamide gel electrophoresis (SDS- PAGE) analysis

3.7.1 Gel preparation

SDS-PAGE was performed according to the method of Laemmli (1970), using the Bio-Rad Mini-PROTEAN® Tetra Cell multi-casting chamber (Bio-Rad Laboratories Inc., USA) to cast the gel with 1.5 mm thickness. The gel formulation was based on the Bio–Rad Mini Protean(R) 3 cell instruction manual as shown in Table 3.1. The gel mixtures were gently poured in the casting modules. After filling the separating gel to about 2 cm from the top plate, distilled water was gently overlaid to create a flat surface on the separating gel and to protect the gel from atmospheric oxygen. After polymerization (45 to 60 minutes), the distilled water was poured off and replaced with 4% stacking gel up to the height of the short plate. Finally the comb was placed at the top of short plate to form the wells. After polymerization of the stacking gel (30 to 40 minutes), the comb was removed from the gel and wells were created in the gel. The wells were then washed with distilled water and running buffer to remove any air created during the polymerization of the gel. The prepared gel was used within a day of preparation.

3.7.2 Sample preparation for SDS-PAGE

Samples were diluted to a final protein concentration of 2 mg ml^{-1} in a sample buffer. The sample was mixed using a vortex mixer for 1 minute, heated for five minutes at 95°C and centrifuged at 6,000 x *g* for five minutes.

Broad range molecular weight marker mixes were used to estimate the relative molecular weights of the proteins.

3.7.3 Run conditions for SDS-PAGE

Electrophoresis was performed using a constant voltage (Bio-Rad, PowerPac™ Basic, USA) of 150 volts at room temperature (approximately 20ºC). The electrophoresis was performed until the dye reached the bottom of the gel.

Table 3.1: Preparation of resolving and stacking gels for analytical and preparative SDS-PAGE. (Bio-Rad formulation, Bio-Rad Laboratories Inc., USA)

Materials	Stacking gel	Resolving gel	
	4%	12%	X%
Acrylamide/bis (30% T; 2.6% C)	1.32 ml	4.0 ml	0.33*X ml
0.5M Tris – HCl, pH 6.8	2.52 ml		
1.5M Tris-HCl, pH 8.8		2.5 ml	2.5 ml
10% SDS	100 µl	100 µl	100 µl
Distilled water	6 ml	3.35 ml	7.35 – (0.33*X) ml
TEMED	10 µl	5 µl	5 µl
10% APS	50 µl	50 µl	50 µl
Total	10 ml	10 ml	10 ml

*X= Percentage of required gel, Ammonium Persulfate (APS), Tetramethylethylenediamine (TEMED)

3.7.4 Staining and destaining for SDS-PAGE

Immediately after ending electrophoresis, gels were removed from plates and placed in Coomassie brilliant blue R-250 solution from Bio-Rad (Bio-Rad Laboratories Inc., USA) for 30 to 45 minutes. The gels were then washed with distilled water and soaked with destaining solution overnight.

3.7.5 SDS-PAGE imaging

The gels were scanned using a scanner (Canon, CanoScan LiDE 20, USA) and data were analysed by comparing the molecular weight of the SDS-PAGE pattern Precision Plus Protein™ Dual Colour Standards molecular weight standards (Bio-Rad Laboratories Inc., USA).

3.8 Hydrolysis of meat protein fractions (sarcoplasmic, myofibrilla and connective tissue)

The hydrolysis of protein fractions were carried out the same as the meat hydrolysis procedure described in Section 3.4.1, with slight modification. The protein extracts of sarcoplasmic (Section 3.2.2), myofibrilla (Section 3.2.3) or connective tissue (Section 3.3.2) were placed in a 500 ml beaker and heated to the reaction temperature (40°C).

No adjustments of pH were made. The enzyme slurry was added (2% weight enzyme per weight of reaction mixture) and the system stirred to start the reaction. About 10 ml samples were collected from the hydrolysis process at 1, 10, 30, 45 and 60 minutes. Each aliquot was placed in a shaking water bath for 10 minutes at 95°C to inactivate the enzymes. The nitrogen content in the protein fractions was determined before the hydrolysis process in order to have standardized nitrogen content to enzyme ratio for all the protein fractions (Table 3.2).

Table 3.2: Nitrogen content of the starting material for meat protein fraction hydrolysis

Protein fraction	Nitrogen content g /100 (DM)
Meat	11.68
Sarcoplasmic	2.70
Myofibrilla	4.83
Connective tissue	0.95

* DM = dry matter

3.9 Peptide analysis on hydrolysates

3.9.1 Sample preparation

The hydrolysate sample was diluted with 0.1 % trifluoroacetic acid, TFA (optima LC/MS, Fisher Scientific, UK) to achieve a 1:5 dilution of sample. The sample was homogenised using a stomacher for 8 minutes then centrifuged using a high speed centrifuge (IECB- 22M, USA) at 10,000 x *g* for 20 minutes. The supernatant was then filled into a 5 ml plastic syringe and filtered through 0.45 µm and 0.2 µm syringe filters in series, before injection into the high performance liquid chromatograph (HPLC).

3.9.2 Chromatographic conditions for peptide analysis

The separations were performed using Shimadzu HPLC system, consisting of a SCL-10AVP pump with LC 10ATV system controller and SPD M10AVP diode array detector (Shimadzu, Japan). A 10 µl aliquot of filtrate (Section 3.9.1) was injected into the HPLC. Separation of peptides was carried out using a 250 x 4.6 mm, Jupiter 5µ C18 300A column (Phenomenex, USA) at 40 ± 1°C. The mobile phase consisted of solvent A containing 0.1% TFA (optima LC/MS, Fisher Scientific, UK) in distilled water and solvent B containing 60 % acetonitrile and 0.085% TFA (optima LC/MS, Fisher

Scientific, UK) in distilled water. Solvent C containing 60% acetronitrile was used to flush the HPLC system after each run. All mobile phases; A, B and C were filtered through a 0.20 µm nylon filter (Millipore, USA) and degassed prior to each analytical run. The HPLC gradient consisted of an initial isocratic gradient of 1% solvent B for 5 minutes, followed by a linear gradient from 1% to 60% Solvent B in 20 minutes. The system was then flushed with 60% acetonitrile (Solvent C) for 5 minutes at the end of each run and prior to the next injection (Table 3.3). The separation was monitored at a UV wavelength of 214 nm.

Table 3.3: The chromatographic gradient programme used for peptides separation

Time (minutes)	Flow type	Solvent A 0.1 % v/v TFA	Solvent B 60% acetonitrile and 0.085% TFA in water	Solvent C 60% acetronitrile
0 – 5	Isocratic	99 %	1 %	-
5 - 20	Gradient	99 - 40 %	1 – 60 %	-
20 – 25	Isocratic	-	-	100 %

3.9.3 Chromatograph analysis

A synthetic peptide standard mixture (Sigma Aldrich,H2016- 1VL, USA) with different molecular weights was injected into the column to find the best chromatographic conditions to obtain an elution profile for the peptides. The peptide mixture contained 0.5 mg of five different protein peptides; (1) GLY-TYR, molecular weight of 238.2 g mol^{-1}, (2) Methionine Enkephalin Acetate, MW = 573.7 g mol^{-1} for free base (TYR-GLY-GLY-PHE-MET), (3) VAL-TYR-VAL, MW = 379.5 g mol^{-1}, (4) Leucine Enkephalin, MW = 555.6 g mol^{-1} for free base (TYR-GLY-GLY-PHE-LEU) and (5) Angiotensin II Acetate, MW = 1046.2 g mol^{-1} for free base (ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE). The chromatograph for samples was compared before and after the hydrolysis to indicate the degradation of protein during the hydrolysis process.All analysis were done in duplicate.

3.10 Amino acid analysis on hydrolysate

3.10.1 Sample preparation

Minced meat and hydrolysate samples were extracted for individual amino acid analysis by precipitating the proteins with 5% TCA (Mullen et al., 2000). Samples of minced meat and hydrolysates were homogenised with MilliQ reverse osmosis water (dilution 1:2) in a stomacher (IUL instruments, Basic, Spain) for one minute, then 50% TCA was added to achieve a final concentration of 5% TCA in the solution. The mixture was then centrifuged cold (4 ± 1°C) at 3600 x *g* for 20 min (IECB-22M, USA). The supernatant was filtered through Whatman No. 1 filter paper and a 250 µl aliquot was placed in a 2 ml microcentrifuge tube. Next, 250 µl of each sample plus 50 µl of an internal standard solution (5mM neoleucine) were mixed on a vortex mixer for one minute and 20 µl of the supernatant was derivatized to their phenylthiocarbamyl derivatives according to the method of Bidlingmeyer *et al.* (1987).

3.10.2 Sample derivatization for amino acids

The supernatant containing the free amino acid standard or hydrolysed sample was placed in a 2 ml centrifuge tube and dried using the Speed vac (Eppendorf, Germany) at 30°C for 10 minutes. The pellet obtained after drying was then soaked with 20 µl reagent containing methanol: distilled water: triethylamine in a ratio 2:2:1 and dried again using the Speed vac. The sample was now ready for derivatization.

PITC amino acids were formed by adding 20 µl derivatization reagent to dried samples, mixed and left for 20 minutes at room temperature. Reagents were then removed under vacuum using the Speed vac. Once the derivatised sample was dried, 400 µl diluent consisting of 5mM sodium phosphate (pH 7.6) with 5 %v/v acetonitrile was added and the sample was ready for separation using HPLC.

3.10.3 Chromatographic conditions for amino acid analysis

The derivatised amino acids were separated using a Shimadzu HPLC system, consisting of a SCL-10AVP pump with LC 10ATV system controller and SPD M10AVP diode array detector (Shimadzu, Japan). A 10 ul aliquot of derivatised amino acid was injected into the HPLC column, a Gemini® 5 µm C18 110 Å LC 30 x 2 mm column at 40 ± 1°C. Two mobile phase solvents were used for the separation with a flow rate at

0.8 ml/min. Solvent A was 0.14 M sodium acetate trihydrate solution with 0.5ml TEA (HPLC grade, Fisher Scientific, UK) adjusted to pH 6.40 with acetic acid, solvent B was acetonitrile:water (60:40 v/v). The chromotgraphic gradient programme is shown in Table 3.4. Separation was monitored at a UV wavelength of 254 nm.

Table 3.4: The chromatographic gradient programme used for amino acid separation

Time (minute)	Flow type	Solvent A (%)	Solvent B(%)
0	Isocratic	90.0	10.0
0 - 6	Gradient	90.0 - 87.5	10 - 12.5
6 - 38	Gradient	87.5 - 42.0	12.5 - 58.0
38 - 71	Gradient	42.0 - 0	58 - 100
71 - 79	Isocratic	0	100
79 - 81	Gradient	0 – 90.0	100 - 10

3.10.4 Chromatograph analysis.

An amino acid standard mixture was prepared by mixing 95 µl of 250 pmol/µL amino acid standard mixture (Sigma-Aldrich, USA) with 5 µL 10 Mm Norleucine and derivatized using the same procedure described in Section 3.10.2. Individual 10 mM amino acids (Sigma-Aldrich, USA) were also prepared and derivatised using the same procedure as described in Section 3.10.2. The chromatographs obtained for individual amino acids were used to determine the order of amino acid elution compared to the amino acid standard mixture.

3.11 Kinetic studies of meat hydrolysis

All kinetic studies of the hydrolysis reaction were performed at 60°C and without pH adjustment. Two sets of experiments were conducted:

(i) enzyme concentration was fixed and the meat concentration varied,

(ii) meat concentration was fixed and the enzyme concentration varied.

The minced beef (substrate) concentration (S%) was varied by addition of distilled water to produce a 240 g reaction mixture containing minced beef, reverse osmosis water and Zyactinase^{TM} enzyme. The amount of water added to achieve the desired substrate concentrations were determined with Equations 3.9 to 3.11. To ensure a

homogenised substrate was obtained, minced beef with reverse osmosis water was pulverized using a Waring blender for 30 seconds at high speed followed by 30 seconds at low speed. The homogenate of minced beef and reverse osmosis water was then placed in a 500 ml beaker before being placed into the controlled temperature water bath. The reaction mixture was heated to 60 ± 1 °C; it took approximately 15 min for reaction mixture to reach 60 ± 1°C. The selected enzyme concentrations were 2%, 4% and 7.5% w/w based on the total weight of the reaction mixture. The procedure was as described in Section 3.4.1. Initially samples of hydrolysate were taken every minute until five minutes of hydrolysis time, then samples were taken at 15, 30, 45, 60 and 120 minutes.

3.11.1 OPA analysis for amino acids and degree of hydrolysis (DH)

The concentration of α-amino acids was determined using the ortho-Phthalaldehyde (OPA) method describe by Nielsen (2001). Serine was dissolved in distilled water at concentrations ranging from 0.005 to 0.05 M to provide standards. Fifty milligrams of serine was diluted with 500 ml distilled water (0.9516 meq/l) as a reference standard. For the meat and hydrolysates samples, 0.1000 to 1.0000 g was weighed accurately, mixed with distilled water and made up to 100 ml in a volumetric flask. The assays were conducted by adding 400 µl serine standard, or 400µl diluted sample into 3 ml OPA reagent and mixed for five seconds. The mixture was left to stand for exactly two minutes before being read at 340 nm in a UV-Vis spectrophotometer (Shimadzu, Pharma spec, UV -1700, Japan).

The degree of hydrolysis (DH) measures the percentage of peptide bonds hydrolysed during the protein hydrolysis DH was calculated using hydrolysis equivalent (h_{eq}) that corresponded to the number of peptide bonds cleaved during the hydrolysis, expressed as meq/g protein. Therefore, DH = (h_{eq}/h_{total}) x 100. h_{total} is the sum of the millimoles of individual amino acids per gram in the unhydrolysed protein.

The mean values obtained for the standards (three replicate measurements for each concentration) were used for calculations of free α-amino groups as shown in Equation 3.12.

Determination of h_{eq} :

$$\text{Serine-NH}_2 = \frac{\text{Abs sample-Abs blank}}{\text{Abs standard-Abs blank}} \text{ x } 0.9516 \text{ meq/l x } 0.1 \frac{100}{X} P \qquad \text{Equation 3.12}$$

Where,

Serine-NH_2 = meq serine NH_2/g protein,

Abs = Absorbance

X = g sample;

P = protein % in sample;

0.1 is the sample volume in litres (L).

h_{eq} was then calculated as

$$h_{eq} = \frac{Serine\_NH_2 - \beta}{\alpha \frac{meqv}{gprotein}} \qquad \text{Equation 3.13}$$

where

α is 1.00 and β is 0.40 (Adler-Nissen, 1976)

Calculation of DH:

$$DH = \frac{h}{htotal} x10 \qquad \text{Equation 3.14}$$

where,

h_{total} is 7.6 (Adler-Nissen, 1976)

3.11.2 Determination of initial velocity

The initial velocity (V_0) can be defined as the rate of change in concentration of α-amino acid (Serine-NH_2) / (Serine-NH_2/min). This corresponds to the slope of the α-amino acid (Serine-NH_2) versus time graph. The slope was determined from the linear part of the curve (up to five minutes of reaction time) for each substrate concentration.

3.11.3 Determination of K_m and V_{max}

The initial velocity obtained from Section 3.11.2 was then plotted against the substrate concentration (S%). The graph will give the value of V_{max} and K_m. V_{max} represents the maximum activity, whereas K_m is defined as the concentration of substrate that gives half of the maximum enzyme activity in the reaction. The value of V_{max} and K_m were

obtained by linear transformation using the Lineweaver and Burk method or by using non-linear regression of Michaelis–Menten equation.

3.12 Data analysis

All data were statistically analysed by Minitab software (version 15.0, Minitab, USA). The results of protein solubility obtained from all the trials were analysed using the two-way ANOVA to study the interaction between the reaction variables. All the graphs were plotted by Microsoft Excel or Sigma Plot 12.5. The relationship between variables was assessed by a Pearson correlation and significance was calculated using the one-way sample *t* test for a correlation coefficient.

Chapter 4
Effect of hydrolysis conditions on the total nitrogen and non-protein nitrogen contents

The enzymic hydrolysis of beef by Zyactinase™ is potentially affected by pH, temperature, reaction time, and enzyme and substrate concentration. Kiwifruit extract or enzyme has been investigated for a number of different proteins (Arcus, 1959a and b, ,Sugiyama et al. 2005, Kaur, L and Boland, M, 2013, Zochawska-Kujawska et al. 2013). However using Zyactinase™ a patented enzyme complex has not been investigated fully to determine the effect of reaction conditions on the hydrolysis of beef. This chapter investigates the properties of the Zyactinase™ and optimises the reaction conditions for the hydrolysis of beef meat. Zyactinase™ hydrolysis of meat was determined by monitoring the increase in non-protein nitrogen (NPN) released as the protein moieties in the meat were hydrolysed to amino acids or smaller peptides with molecular weights below 800 Daltons. In addition, SDS-PAGE patterns of meat hydrolysates were characterised to follow protein changes.

4.1 Characterisation of Zyactinase™ enzyme complex

Zyactinase™ is a semi-purified freeze-dried extract from kiwifruit. It contains a wide range of components, including proteolytic enzymes, carbohydrates, fibre and many micronutrients that are found naturally in the original fruit. The moisture content of Zyactinase™ was 2.5%. The proteins were evaluated using SDS-PAGE analysis. For SDS-PAGE analysis, 1.0 g of Zyactinase™ powder was dissolved in 1.0 ml of distilled water and the protein content was determined by the Bradford analysis. The Zyactinase™ was then diluted to 1 mL with SDS-PAGE sample buffer to give 1.0 g protein per millilitre of sample buffer. Figure 4.1 shows four clear bands around; 28 kDa (a), 24 kDa (c), 23 kDa (d) and 17 kDa (f) and one less clear band around 27 kDa (b) in the Zyactinase™ sample (Z). The M lane represents standard marker proteins of known molecular weight bands. The Zyactinase™ SDS-PAGE pattern was consistent with the SDS-PAGE pattern

derived from raw kiwifruit extract (Afshar-Mohammadian, Rahimi-Koldeh, & Sajedi, 2011; Tuppo et al., 2008).

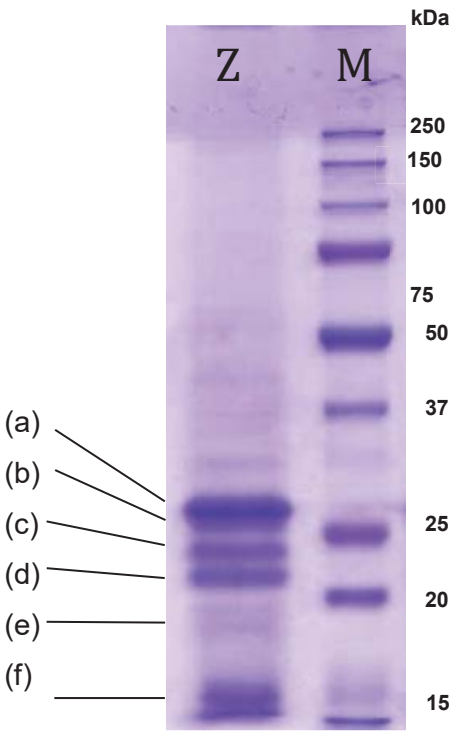

Figure 4.1: SDS-PAGE of the enzyme ZyactinaseTM on 12% gel; Lane Z: 1.0 g protein /ml buffer and Lane M: Molecular weight marker. (a) Actinidin, (b) kiwellin, (c) TLP and (d) KiTH

According to Larocca, Rossano, & Riccio (2010), there are six protein bands in the range 17 – 30 kD found in green kiwifruit extract. The band at 30 kDa was identified as actinidin, band at 26 kDa was kiwellin while bands at 25 and 24 correspondent to thaumatin- like protein (TLP) (Larocca, Rossano & Riccio, 2010). A band at 20 kDa was named KiTH (Tuppo et al., 2008) and the lower band of 17 kDa could correspond to a propeptide of actinidin (Pastorello et al., 1998).

Actinidin is the major kiwifruit protease and it contains a free sulfhydryl group that is required for enzyme activity (McDowall, 1970) and is thus grouped in the class of plant thiol proteases. This enzyme has 220 amino acids with a molecular weight of 23 kDa (Kamphuis, Drenth, & Baker, 1985). From previous studies, Carne & Moore (1978) suggested that kiwifruit of *Actinidia chinesis* contained a single enzyme. In contrast, Bolland & Hardman (1972) found there were two active proteins resolved on a wide

chromatography column on DEAE cellulose with a molecular weight of 26 kDa. Furthermore, Sugiyama et al. (1996) found six acidic isoforms with slightly different isoelectric points (pI) but identical N-terminal sequences. The two kiwifruit proteases, kp4 and kp6, were purified by the ion exchange chromatographic method. Sugiyama et al. (1997) demonstrated that two of these acidic isoforms showed differing substrate specificities and pH optima. Actinidin has several isotopes with the same pI but different molecular masses (Larocca, Rossano & Riccio, 2010)

4.2 Proximate analysis of meat

The original meat protein was analysed for moisture, protein, fat and ash. The results are presented in Table 4.1. The mince beef meat had an average nitrogen content of 3.55%, which was converted to an average protein content of 22.2 % (based on wet weight) by multiplying the nitrogen-to-protein conversion factor of 6.25. These results fall within the average muscle content of protein for lean beef meat (Lawrie & Ledward, 2006).

Table 4.1: Proximate composition of minced beef used for all experiments. Results are mean values of 10 replicates ± standard deviation.

Composition	Minced beef meat	
	Wet basis (wt%)	Dry basis (wt%)
Moisture	70.2 ± 0.3	
Nitrogen	3.55 ± 0.01	
Protein	22.20 ± 0.06	74.4 ± 0.8
Fat	6.5 ± 0.4	21.9 ± 0.5
Ash	1.08 ± 0.04	3.7 ± 0.2

The composition of these components may vary depending on the type of meat muscle (Jeremiah, Dugan, Aalhus, & Gibson, 2003). On average lean muscle contains 1% ash (potassium, phosphorus, sodium, chlorine, magnesium, calcium and iron), 5% lipid, 1% carbohydrate, 15 - 22% nitrogenous compounds and 72% moisture as reported by Lawrie & Ledward (2006). It was reported that muscle showed significant variation of mineral content (ash) among muscles of different animals and within different muscle tissues from the same animal (Doornenbal & Murray, 1982; Marchello, Milne, & Slanger, 1984). Ground beef is reported to contain relatively large quantities of potassium, sodium and phosphorus and smaller amounts of calcium, copper, iron, magnesium and zinc (Marchello et al., 1984).

4.3 Proximate analysis of hydrolysate

4.3.1 Hydrolysis process

The hydrolysis reaction for this trial was carried out by mixing thawed minced beef, pre-heated to the reaction temperature (40°C) and Zyactinase™ powder, wetted with water to form a slurry, as described in Section 3.4.1. Neither the pH nor the meat substrate and enzyme concentrations were adjusted. The reaction was allowed to proceed for 180 minutes before being terminated by destroying the enzyme in a near-boiling water bath. At the end of hydrolysis process the minced meat had been converted to athick brown slurry (refer to Figure 3.6 in Section 3.4.1). The extent of hydrolysis, measured in terms of total nitrogen and NPN contents in the hydrolysate, was assessed using Kjeldahl analysis.

Nitrogen in meat originates from either protein or non-protein sources. NPN is that nitrogen which is not precipitated by 15% trichloracetic acid (TCA) (Erlanger, Kokowsky, & Cohen, 1961). This effectively means nitrogen compounds with molecular weights below 10,000 Daltons (Bell, 1963). NPN contributes a relatively small proportion of the total nitrogen in raw meat and mainly originates from free amino acids, peptides, alkaloids, nitrogenous glucosides, lipids and nucleic acids, ammonium salts, and amides such as urea, hippuric acid, guanidine, creatine and glutathione (Lawrie & Ledward, 2006).

4.3.2 Proximate composition of hydrolysate

A beef mince sample as described above was hydrolysed at 40 °C for 120 minutes, using 2% w/w Zyactinase™ to produce a hydrolysate product. The proximate composition of the hydrolysate product is given in Table 4.2.

Table 4.2: Proximate composition of meat hydrolysate. Results are mean values 3 replicates ± standard deviation.

Composition	*Hydrolysate product*	
	Wet basis (wt%)	Dry basis (wt%)
Moisture	65.3 ± 0.3	-
Nitrogen	4.12 ± 0.01	-
Protein	25.77 ± 0.08	74.3 ± 0.6
Fat	7.6 ±0.2	21.8 ± 0.5
Ash	1.33 ± 0.04	3.9 ± 0.1

In comparison to the original beef sample (Table 4.1) the total solids (dry basis) are identical, showing there was no loss of dry material during the hydrolysis reaction. However, there was a loss of moisture from evaporation, a change in moisture content from 70.2% to 65.3 %.

4.3.3 Moisture loss during the hydrolysis process

Because there was an overall moisture loss during hydrolysis, the rate of change was measured and the results are shown in Table 4.3. Results were triplicate measurements obtained from three completely different experimental runs. As a result of this moisture loss, a cover was placed on the beaker to minimise moisture losses for future work. The

cover was made from plastic container with the hole in the middle of the cover to allow the mixer to be placed.

The moisture content for each reaction was determined after inactivation of the enzyme at 95°C. This step further increased the loss of moisture because of the high temperature.

Table 4.3: Moisture content (g water per 100 g sample) during hydrolysis at 40°C (Results are mean ± standard deviation, n = 3)

Time (Minute)	Enzyme concentration (%w/w)			
	Control 0%	1%	2%	4%
0	72.68 ± 1.04			
1	72.91 ± 1.62	72.51 ± 0.84	72.31 ± 0.53	71.28 ± 0.69
30	69.51 ± 0.50	71.31 ± 0.45	71.83 ± 0.35	70.58 ± 0.53
60	66.94 ± 1.19	70.50 ± 0.49	69.07 ± 0.44	69.17 ± 0.76
90	64.75 ± 0.96	68.72 ± 0.30	68.76 ± 0.92	70.02 ± 0.80
120	65.83 ± 2.03	67.98 ± 0.49	67.78 ± 0.90	69.39 ± 0.57
180	65.33 ± 0.41	67.02 ± 0.51	67.02 ± 0.55	68.47 ± 1.04

The loss of moisture was found to be significantly greater ($p<0.05$) for the control sample (without enzyme addition) compared to the hydrolysates processed with added enzyme at 40°C. There were no significant ($p<0.05$) correlations among the various enzyme concentrations and the moisture loss. There was no significant difference in the moisture contents between 1% and 2% enzyme concentration for all reaction times. The observed low moisture content for the control samples was most likely caused by the relatively non-homogenous nature of samples and possibly some moisture-binding compounds in the Zyactinase. The majority of water was held within the muscle structure, between the muscle cells and within the muscle cell (Offer & Cousins, 1992), the addition of enzyme initiates degradation of the protein structure in the meat that leads to the release of trapped water from the muscle tissue. The greater the enzyme concentration, the faster protein degradation occurred. Unhydrolysed meat will have a larger surface area as the particles are not uniform and do not create a single surface as happens when the Zyactinase creates a slurry.

The moisture loss was taken into account in calculations of the total nitrogen content in hydrolysate samples by calculating all results on a dry weight basis. Figure 4.3 shows a comparison of total nitrogen results at 40°C and 2% enzyme concentrations for 180 minutes reaction time, based on a wet or dry weight basis. Similar results found at 50 and 60°C are presented in Appendix A1.1. For both graphs in Figure 4.3 a sudden drop in the total nitrogen content was observed at the beginning, one minute into the reaction. This feature will be discussed further in Section 4.4.4 which discusses the effect of addition of enzyme.

4.3.4 Changes in pH during hydrolysis process

The hydrolysis process was carried out without changing the pH or adding water at the start of the process. The operational pH was recorded before the addition of enzyme (0 minute), after the addition of enzyme (one minute) and every 20 minutes during the hydrolysis process (up to180 minutes).

The results are shown in Figure 4.4. The raw beef, processed without any addition of Zyactinase™ (control), did not change in pH at any stage throughout the process. However for the sample with Zyactinase™ added, there was a drop of pH at the beginning of hydrolysis process, clearly related to the inherent pH of the Zyactinase™ itself. This pH then increased slightly during hydrolysis, but the final hydrolysate had a lower pH than the original minced meat.

A decline in pH is most likely caused by the acidic components in the enzyme complex, as Zyactinase™ is pH 3.1. A decline in pH may also be caused by the freeing of amino acid carboxyl groups from the protein chain by the proteolytic enzyme (Hsu et al., 1977). However, as the major drop in pH occurs on addition of the Zyactinase, before any significant hydrolysis occurs, this is not a likely cause. The pH change may be caused by a structural change in the meat proteins caused by the low Zyactinase pH exposing amino acids that were initially buried inside a 3-D protein structure in the unhydrolysed protein (Mozersky and Panettieri, 1983). The stabilisation of the pH after the initial drop possibly reflects the strong buffering capacity of the animal muscle. These buffering constituents

are believed to be inorganic phosphate, nucleotides, organic acids, taurine and protein bound histidine residues (Okuma & Abe,1992). Most meat muscle has good buffering capacity in the pH range of 6.5 to 7.5 (Okuma & Abe, 1992). Generally red fibre muscle has lower buffering capacity than white muscle under acidic conditions (Rao & Gault, 1981).

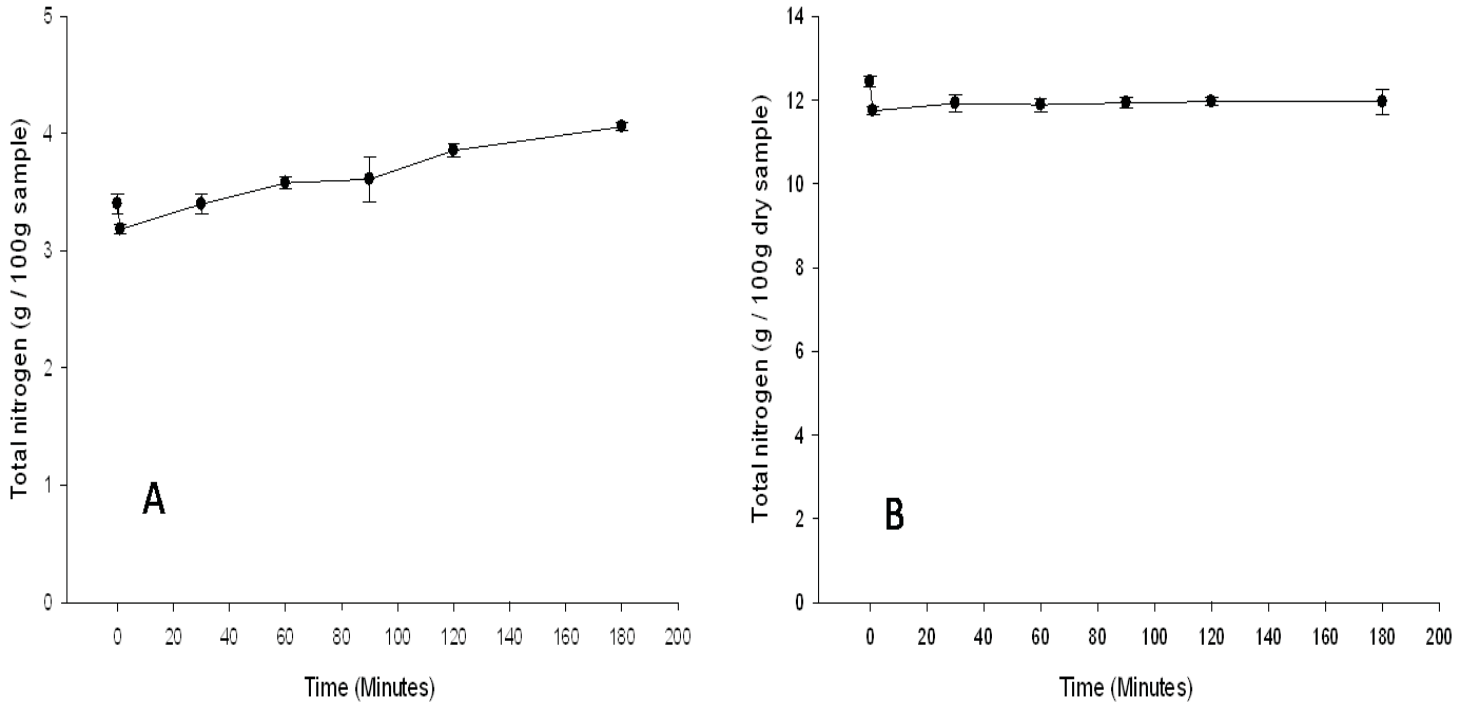

Figure 4.3: Total nitrogen content in hydrolysate reactions at 40°C and 2% enzyme concentration: (A) wet weight basis, (B) dry weight basis. Mean value ± standard deviation, for n=6 trials.

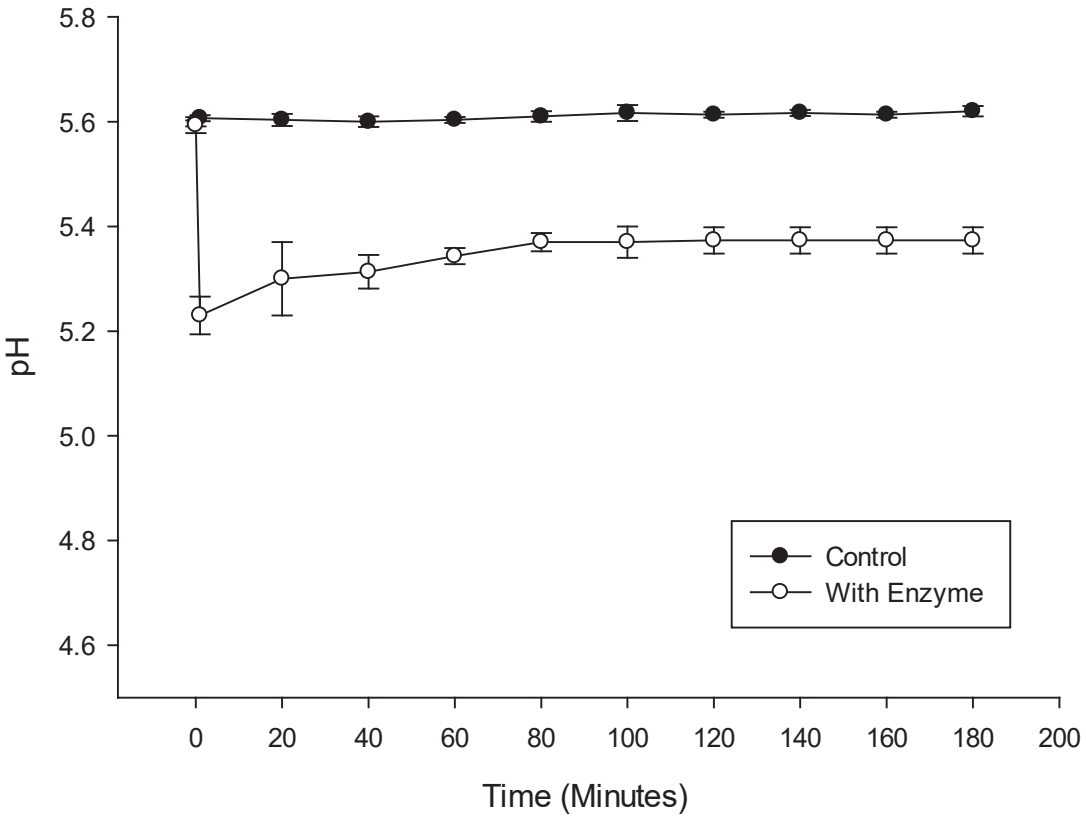

Figure 4.4: Changes of pH during hydrolysis at hydrolysis process at 40°C and 2% enzyme concentration.

The pH can affect the substrate or enzyme by causing their protein structures to unfold, which may occur at the pI of the protein molecule, thereby exposing the hydrophobic amino acid residues buried inside the molecule. The open structure leads to further cleavage of the protein molecules and this can contribute to an increase in the formation of hydrolysis products (Sun, Luo, Shen & Hu, 2011). The pH changes can also induce conformational changes and affect enzyme activity (Duarte, Arroyo, Beristain, Argaiz & Garcia, 1988).

4.4 Effect of hydrolysis conditions on the extent of the hydrolysis process.

Various hydrolysis conditions, such as time, temperature, pH and substrate and enzyme concentrations, were altered to assess their importance on the extent of the hydrolysis process. The intention of this research was to optimize the hydrolysis process for minced beef with Zyactinase™.

4.4.1 The rate of hydrolysis

The rate of the release of NPN was studied at 60°C and 2% enzyme for up to 360 minutes. Results are shown in Figure 4.5. The control was minced beef without any enzyme addition. The control sample showed no significant changes over the 360 minutes. However, the rate of enzymic reaction increased dramatically and constantly throughout the entire reaction time.

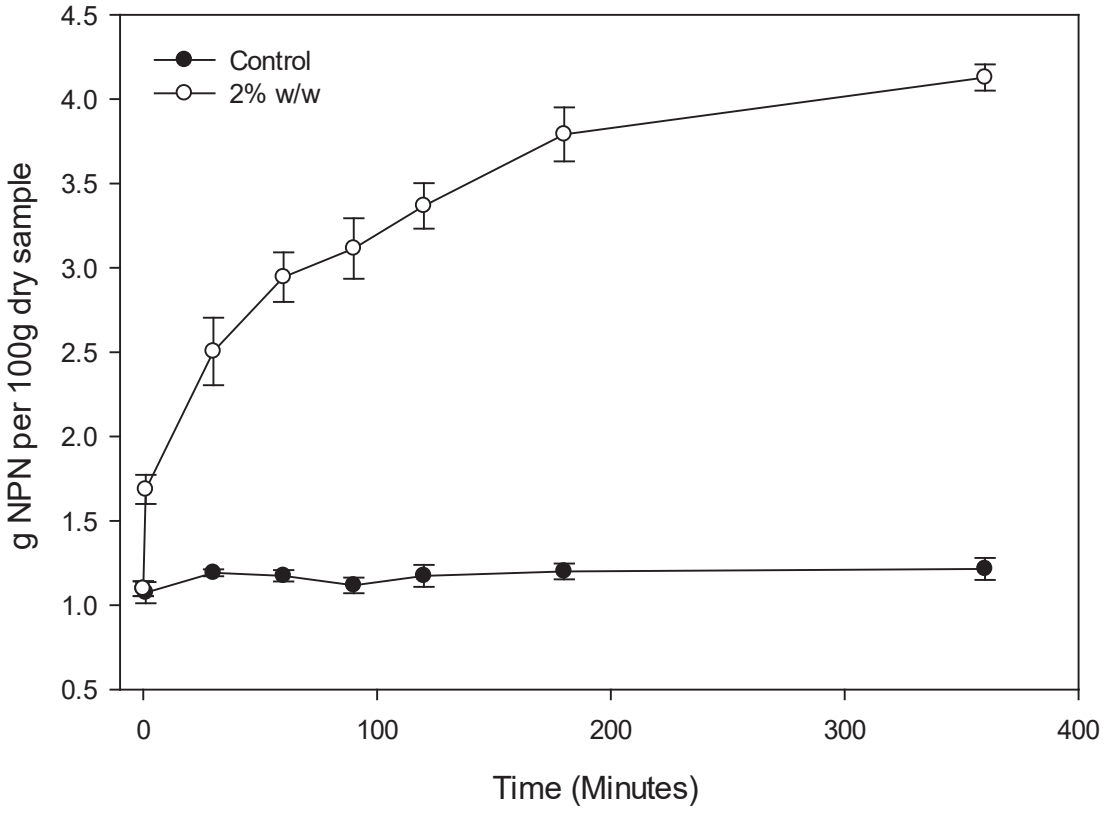

Figure 4.5: NPN content (g/100 g dry sample) of hydrolysates at 60°C without enzyme addition (control ●) and 2% enzyme concentration (○). Data points are mean values ± standard deviation, n = 6.

Similar curves for the formation of NPN have been observed during the solubilisation of bovine rumen (Clark, Cutler, O'Meara, & Munro, 1987), solubilisation of beef (O'Meara & Munro, 1984) and enzymic hydrolysis of fish protein (Archer, Ragnarsson, Tannenbaum & Wang, 1973).

In the first 90 minutes, the observed rapid increase in NPN could be because the enzyme interacts rapidly with the soluble protein particles and the polypeptide chains that are loosely bound to the surface of the insoluble meat proteins. The more compacted core proteins are cleaved more slowly (Benjakul, Seymour, Morrissey & An, 1997). Denatured protein which had reduced solubility, has been found to be less susceptible to hydrolysis by added exogenous enzymes (Klompong, Benjakul, Kantachote & Shahidi, 2007). Endogenous enzymes such as cathepsin in raw fish muscle provide additional proteolytic activity in addition to commercial exogenous enzymes during hydrolysis reactions (Mackie, 1982). However, the control value in this experiment clearly shows little, if any, endogenous hydrolysis.

Figure 4.6 shows the changes of total nitrogen content as a function of time at different enzyme concentrations. In contrast to NPN, the total nitrogen content remained constant throughout the hydrolysis process. There was no significant difference ($p<0.05$) in the total nitrogen content throughout the processing time (0 to 180 minutes) in the control experiment (without enzyme addition). There was also no significant difference ($p<0.05$) in the total nitrogen content in reactions with added enzymes after the sudden drop at 1 minute.

However, there was a significant drop ($p<0.05$) at the beginning of the experiment when the enzyme was first added to the meat. Clearly, the greater the amount of added enzyme, the greater the drop in total nitrogen at the beginning of hydrolysis. Whereas no significant ($p<0.05$) drop was observed for the control sample.

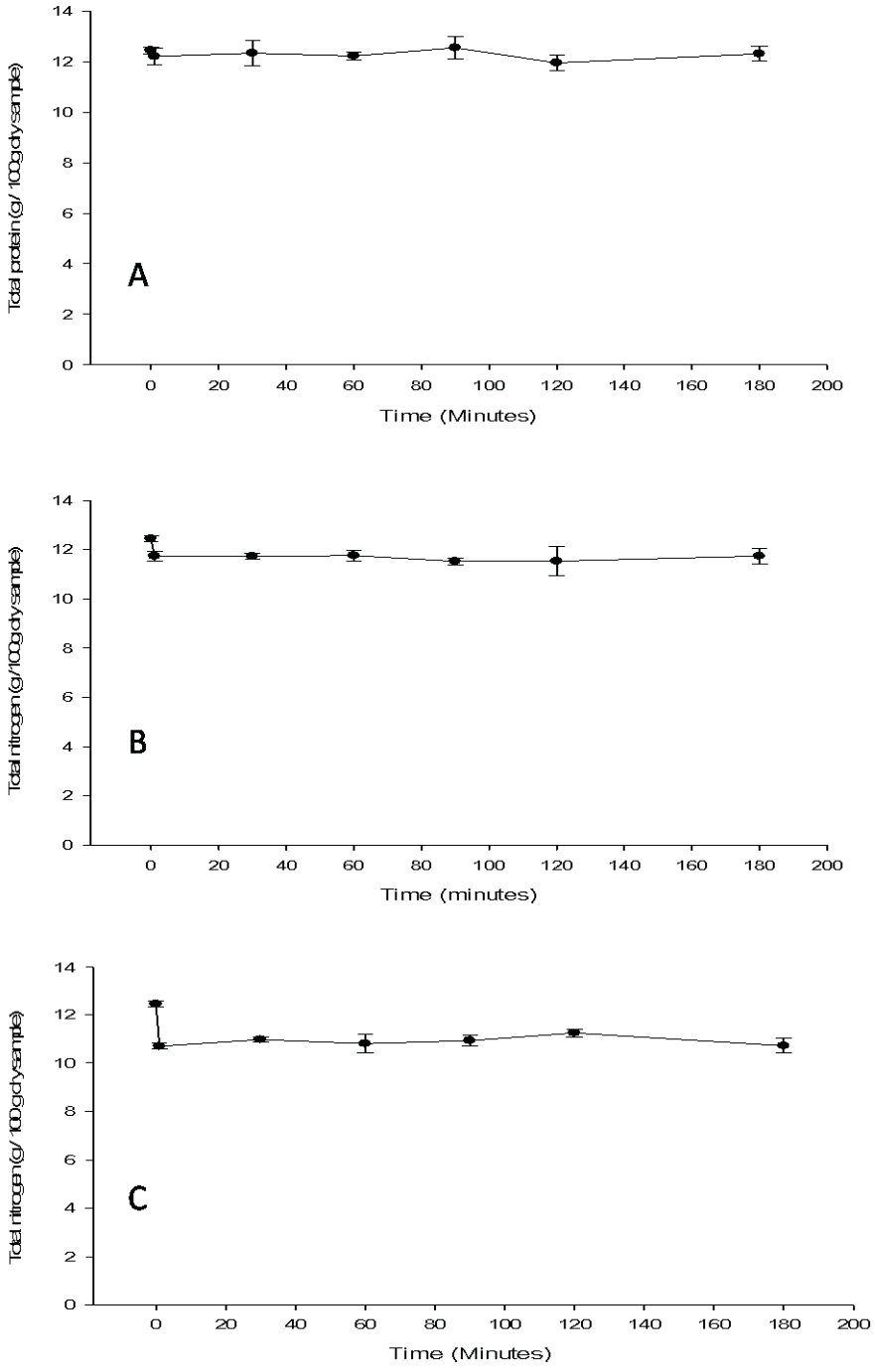

Figure 4.6: Total nitrogen content for hydrolysate at 40°C (A) without enzyme addition (B) 1% enzyme concentration (C) 4% enzyme concentration. Data points are mean values ± standard deviation, n = 6.

The same results were obtained for the hydrolysis reactions at 40 °C and 50 °C (shown in Appendix A1.2). This can be explained by the dilution effect of a low-nitrogen containing Zyactinase™ (compared to the meat).

4.4.2 Effect of pH on the hydrolysis at 50°C

In this set of experiments, the reaction conditions were kept constant for temperature (50°C), time (180 minutes) and enzyme concentration (2 g Zyactinase™ per 100 g meat). To facilitate pH adjustment, the meat was mixed with distilled water to create a slurry (see Section 3.4.1). The original pH of the starting material (lean beef mince) was pH 5.6 ± 0.2. The pH of meat slurry was adjusted by the addition of either 1M NaOH or 1M HCl. Once adjusted, the meat slurry was heated to 50°C before the enzyme was added. The pH of the reaction mixture was also monitored regularly throughout the hydrolysis. The results are presented in Figure 4.7. There was a noticeable change in pH immediately after the enzyme was added to the meat slurry (first minute). The direction and extent of those changes depended upon the starting meat pH.

Following the initial change in pH, the system pH then stabilised and remained constant until the end of the process. The pH of the Zyactinase™ enzyme was 3.10 ± 0.5. Where the initial meat slurry possessed a pH value below the Zyactinase™, the addition of enzyme increased the pH slightly. Whereas, when initial meat slurries had pHs higher than Zyactinase™, the addition of enzyme decreased the pH of the reaction mixture. The higher the starting pH, the greater the extent of pH drop, indicating that Zyactinase™ had a very powerful buffering system and a lot of acidic components.

Figure 4.9 shows the results of the rate of hydrolysis for the various pH systems used in this experiment. The control sample (no added enzyme) is shown at the bottom of Figure 4.9 and it does not change during the time scale of the trial. There is evidence that the optimum pH for the hydrolysis reaction with Zyactinase™ was in the range pH 4 to 5.5 and the rate slowed significantly in more acid or alkaline conditions ($p<0.05$). In order to see the optimum pH more clearly, the NPN changes found at 60 and 120 minutes is shown in Figure 4.10.

The degree of pH change following the addition of Zyactinase™ is shown in Figure 4.8.

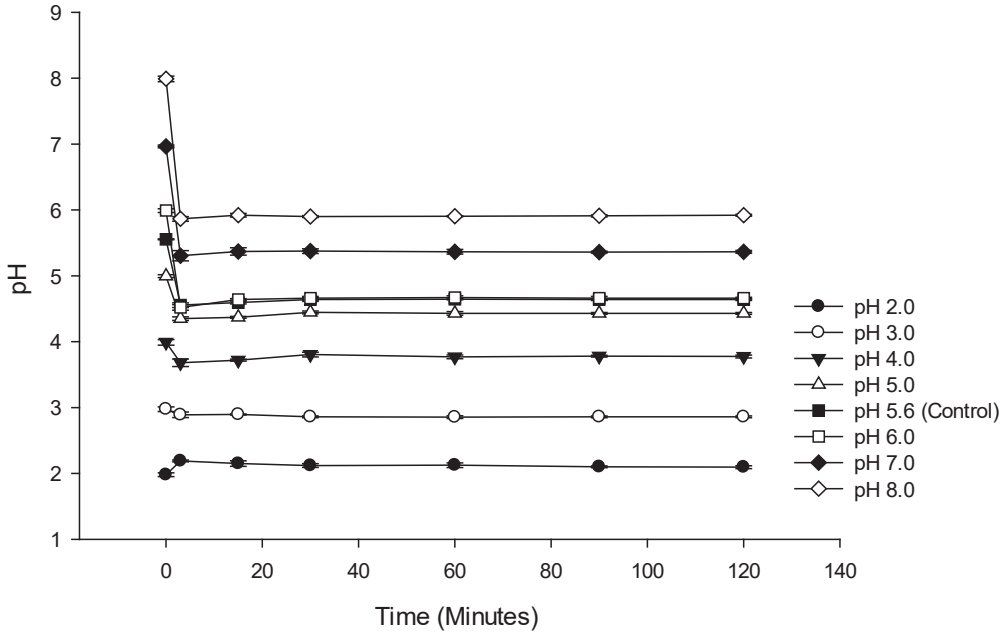

Figure 4.7: Changes in pH value during the hydrolysis process at 50°C and 2% enzyme concentration. Data points are mean values ± standard deviation n = 3

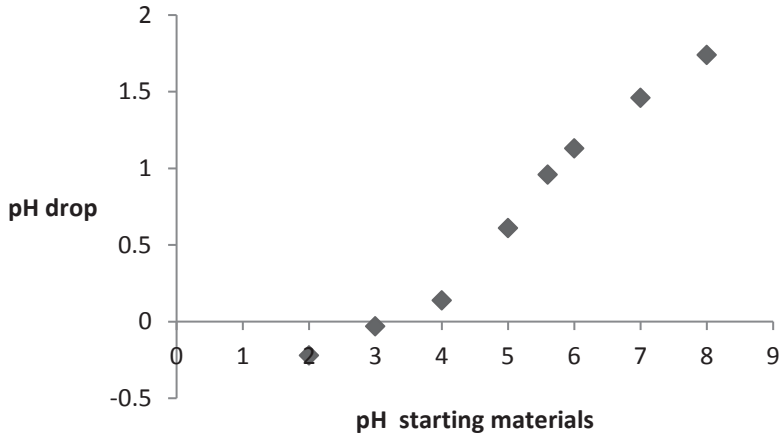

Figure 4.8: The change in pH after addition of enzyme in the mixture (pH was measured at 90 seconds reaction time). Data points are mean values ± standard deviation n = 3

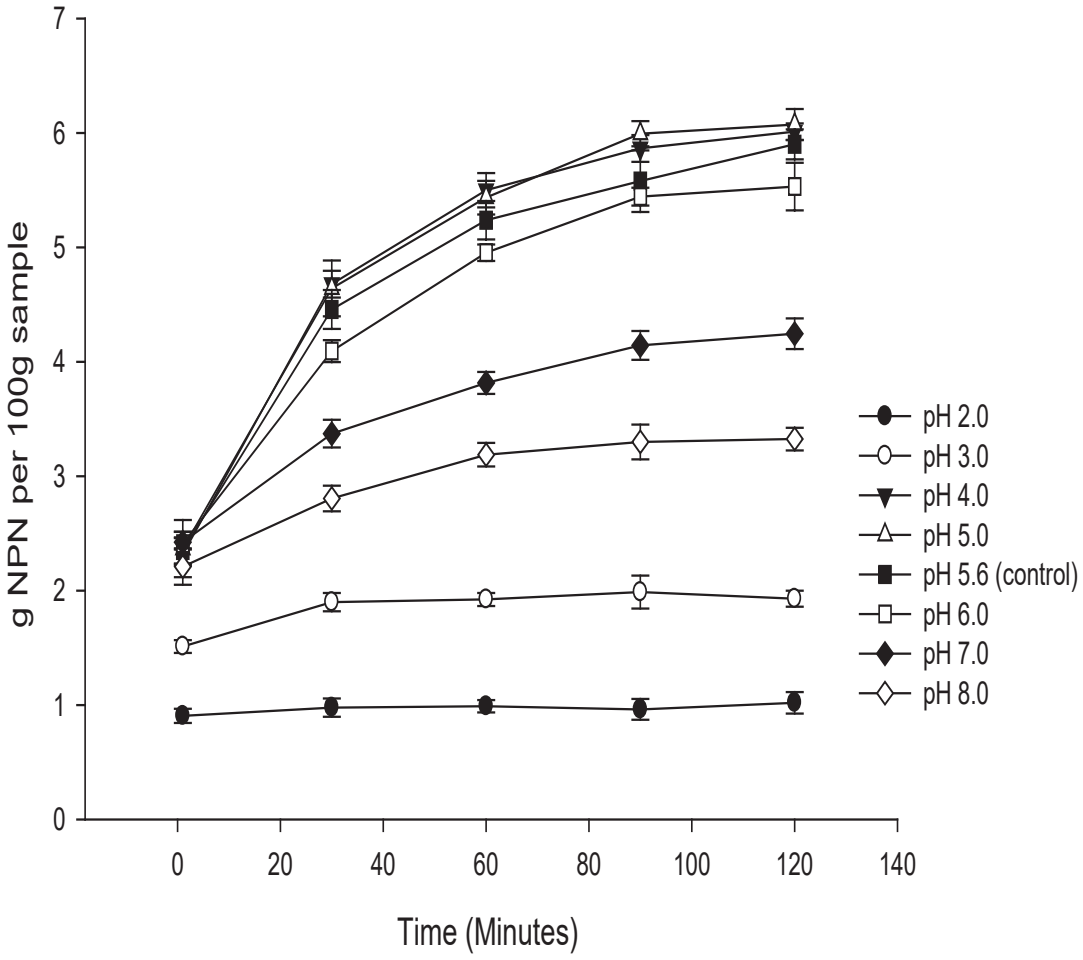

Figure 4.9: NPN content of the hydrolysates for different initial pHs of diluted meat, at 50°C, 2% enzyme addition. Data points are mean values ± standard deviation. n = 6.

The rate of hydrolysis measured by NPN was not significantly different between pH 4 and 5.5 and this was the optimal pH range. Hence, it would be possible to carry out the hydrolysis process at the natural pH of the mince beef, without a pH adjustment.

Extremely acidic environments (pH 2 and 3) appeared to retard the hydrolysis process. Kiwifruit enzymes have been found to have high activity in the acidic pH region (pH 4 - 5.5) due to a negatively charged group on the kiwifruit protease and low isolectric point (pI) values of pI 3.45 and pI 3.33 (Sugiyama, Ohtsuki, Sato, & Kawabata, 1997). In earlier

studies actinidin was reported to have the optimum activity at pH 5 - 7 with significant persistent activity at pH 3.2 and pH 10.1. At acidic pH (4 - 5.5), the enzyme remains soluble due to the net negative charge (electrostatic repulsion) (Sugiyama, Ohtsuki, Sato, & Kawabata, 1997).

Actinidin activity is pH dependent and it has been reported that its maximum activity is between pH 3 to 4 for myofibrilla protein (Nishiyama, 2001) which is what was also seen in Figure 4.10 that maximum hydrolysis occurred between pH 4 to 5.6. Hence there is considerable variability in the pH optima for actinidin, which depends on the substrate and hydrolysis conditions.

As meat pH drops below its isoelectric point (approximately pH 5.6) there is an increase in the net positive charge of the protein side group which can cause a decrease in electrostatic attraction forces between adjacent protein chains. This reduction in attraction leads to an increase in the diameter of the myofibrillar elements and a weakening of the connective tissue due to hydration. The maximum swelling observed may double in volume in cut beef at pH 3.55 after an immersion time of 24 hours at 4°C (Gault 1985). As the pH of the sample decreases below the pI, there is also an increase in the diameter and length of the sarcomere. Swelling observed in meat at low pH was due to myofibrillar swelling across the muscle fiber axis. Muscle fibers started swelling at pH 4.75 reaching a maximum around pH 3. Collagen fibres start to swell at pH 4.2 reaching maximum around pH 2.5 (Guvtavson, 1956). This could explain why hydrolysis reactions rates were greater at approximately pH 4, when the myofibrillar elements are maximally swollen, allowing the enzyme easier access to the reaction sites. At the same time, kiwifruit enzymes have been reported to selectively hydrolyse the myofibrillar protein over a wide range of pHs. For example, actinidin hydrolysed all the myofibrillar protein at pH 3 – 4, whereas myosin, but not actin was hydrolysed at pH 5.5 – pH 8 (Nishiyama, 2001).

Based on these current results, the optimum pH for Zyactinase^{TM} hydrolysis is the natural pH of beef. Therefore, all subsequent hydrolysis reactions were carried out without the need of pH adjustment.

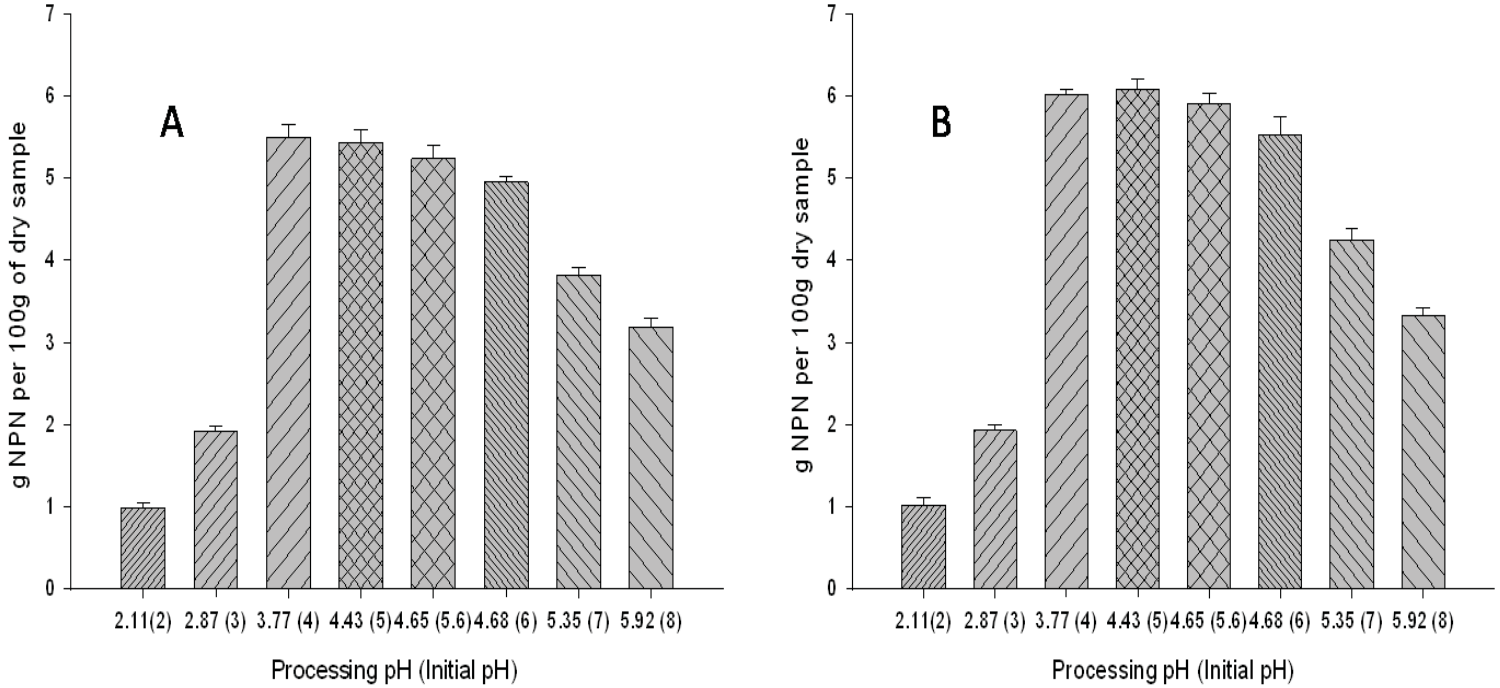

Figure 4.10: NPN concentration at different pHs after (A) 60 minutes and (B) 120 minutes of hydrolysis time. Mean value ± standard deviation for n=6 trials.

4.4.3 Effect of temperature on the hydrolysis process

The optimum temperature for hydrolysis reaction will be a compromise between reaction rate and enzyme inactivation. Ideally, the enzyme needs to be stable throughout the hydrolysis process, while the reaction process must be sufficiently fast to be effective in commercial processes. For this experiment, the reaction conditions were 2% w/w enzyme concentration used on pure meat for up to 180 minutes of hydrolysis.

4.4.3.1 Impact of temperature on NPN

The results are shown in Figure 4.11. An increase in hydrolysis temperature resulted in an increase in NPN and therefore the extent of reaction up to 65°C. At 75°C the extent of reaction decreased and the actual hydrolysis stopped at around 30 minutes into the hydrolysis. Similarly, at 70ºC the hydrolysis reaction appeared to cease after about 60 minutes, as there was no significant difference between the NPN content at 60 and 180 minutes. It appears that hydrolysis temperatures above 65ºC lead to inactivation of Zyactinase™ enzymes.

It was observed that during the hydrolysis at 60°C, white stringy material (collagen) collected on the stirrer at the beginning of the hydrolysis (after 1minute) but was not present at 180 minutes. It is proposed that the connective tissue started to gelatinise and may have been more exposed to proteolytic enzyme digestion, resulting in this stringy material being dissolved. This has been observed by Ishioroshi, Samejima, & Yasui (1979) and Jones, Carroll, & Cavanaugh (1977). At 40°C and 50°C the white stringy material remained present during the entire hydrolysis reaction and it remained attached to the stirrer even after 3 hours of hydrolysis. This observation was also reported by O'Meara & Munro (1984).

The optimum temperature for the maximum production of NPN by Zyactinase™ was determined by plotting the NPN contents at 2 hours of hydrolysis (Figure 4.12). The optimum temperature for kiwifruit hydrolysis of blood, egg and gluten was reported to be between 58 - 62°C (Yamaguchi, Yamashita, Takeda, & Kiso, 1982). However, the optimum for degradation of milk whey proteins, α-lactalbumin and β-lactoglobulin, was found to be 41.6°C at pH 4 (Vázquez-Lara et al., 2003).

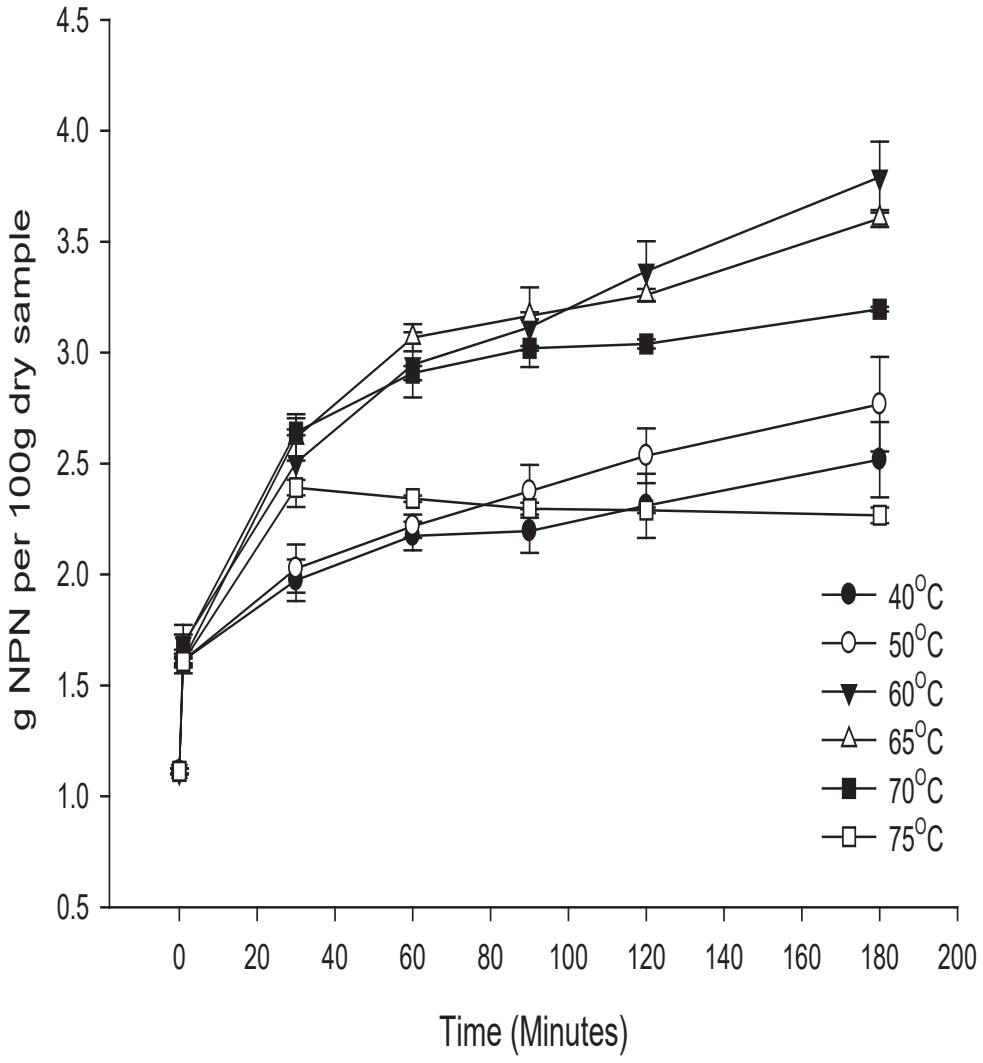

Figure 4.11: NPN content (g/100 g dry sample) of hydrolysate at different processing temperatures and 2% enzyme concentration. Data points are mean values ± standard deviation, n = 6.

The optimum hydrolysis reaction temperature of 60°C probably caused the unfolding of the protein molecules allowing the enzyme to cleave the peptide bonds (Hamm, 1966), while the endomysial collagen was congealing and becaming non-fibrous at 60°C (Jones et al., 1977). The temperature of heat-induced gelation of myosin occurs at 60 – 70 °C and pH 6 (Ishioroshi et al., 1979). However, salt soluble protein starts to unfold at 30 - 32°C followed by protein-protein association at 36 - 40°C and gelation occurred at 45 - 50°C (Xiong & Brekke, 1990a, 1990b). Most sarcoplasmic proteins aggregated between 40 and 60°C, but this could be extended to 90°C (Davey & Gilbert, 1974).

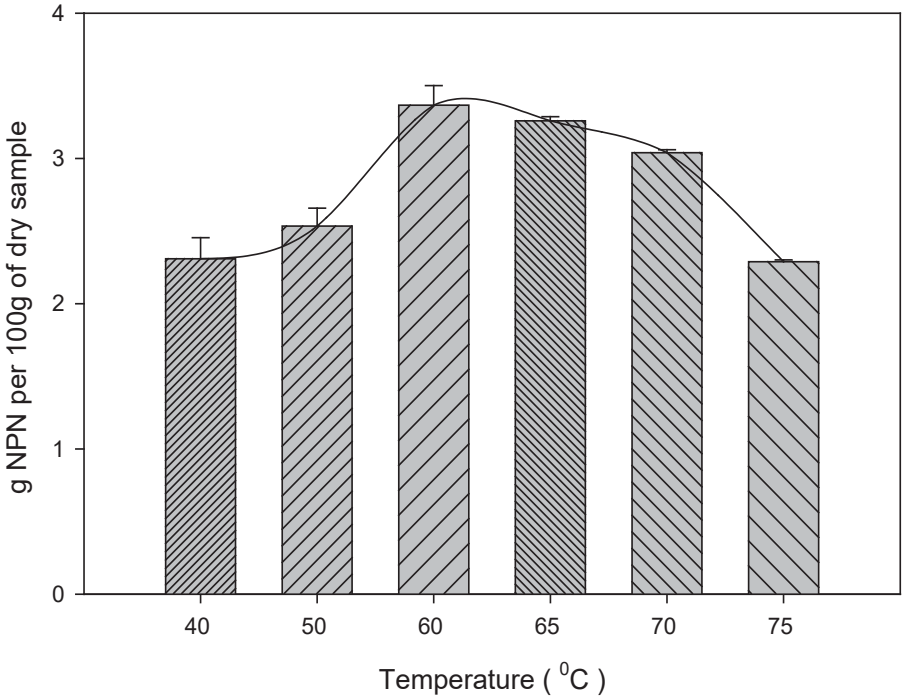

Figure 4.12: NPN concentration (g/100 g dry sample) in hydrolysates after 120 minutes at different reaction temperatures, with 2% enzyme addition. Data points are mean values ± standard deviation, n = 3.

4.4.3.2 Impact of temperature on SDS-PAGE results

The hydrolysates were assessed by SDS-PAGE. Figure 4.13 shows the electrophoretic pattern of hydrolysates from different temperatures and at different times during the hydrolysis.

Beef muscle contained many proteins ranging in molecular weight from approximately 17 kDa to a size greater than that able to enter the gel. Specific proteins identified by their molecular weight included myosin heavy chain (220 kDa), β-actinin (130 kDa), α-actinin (95 kDa), desmin (75k Da), actin (42 kDa), tropomysin β-chain (39 kDa), troponin T (35 kDa) tropomysin α- chain (34 kDa), myosin light chain 1 (23 kDa), troponin I (21 kDa) , troponin C (18 kDa) and myosin light chain 2 (17 kDa).

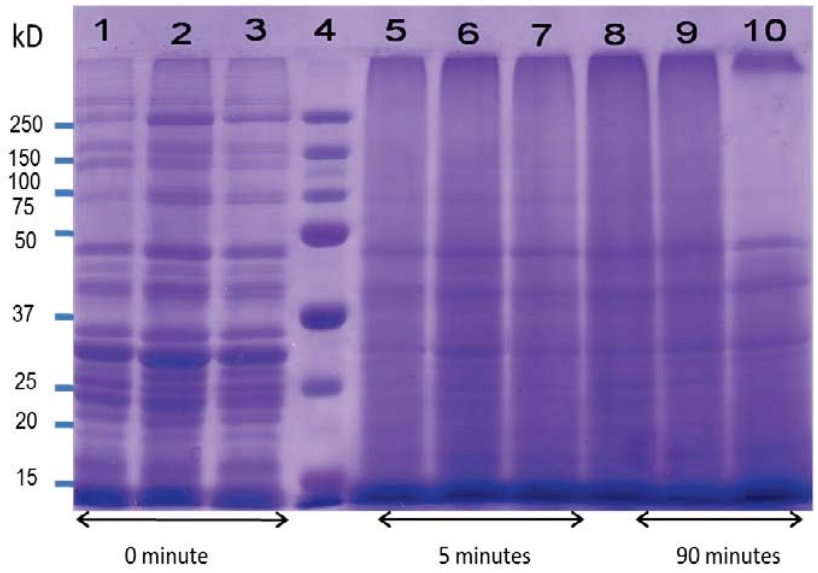

Figure 4.13: SDS –PAGE patterns of beef muscle proteins after hydrolysis with 2% w/w ZyactinaseTM at different temperatures for different reaction times. Lanes 1, 2 and 3: T = 40°C, 50 °C and 60 °C , respectively, 0 minutes, without enzyme; lane 4 : molecular weight marker; Lane 5: T= 40°C , t= 5 minutes; Lane 6: T = 50°C , t = 5 minutes; Lane 7: T = 60°C , t = 5 minutes; Lane 8: T = 40°C , t = 90 minutes; Lane 9: T = 50°C , t = 90 minutes; Lane 10: T = 60°C , t = 90 minutes. (T = temperature, t = time)

With no enzyme addition the protein bands showed similar electrophoretic patterns for 40°C, 50°C and 60°C, as would be expected. The SDS- PAGE patterns of the meat digested for 5 minutes and 90 minutes were different and had changed significantly from the original meat samples. The protein bands corresponding to high molecular weight proteins, such as myosin heavy chain (220 kDa), β-actinin (130 kDa), α-actinin (95 kDa); bands above 45 kDa were observed either to decrease in intensity or to disappear completely. The results are consistent with Lewis and Luh (1988), who reported that actinidin caused significant tenderization of broiled steak by hydrolysing the myofibrillar proteins.

As a control, minced beef was processed at 60°C, but no Zyactinase^{TM} was added. From Figure 4.14 it appears there was no reaction, or significant breakdown of the proteins as all the protein bands remained unchanged.

Heating beef muscle has been found to increase troponin-I and decrease the titin concentration due to the heat instability of high molecular weight titin, whereas, other myofibrillar proteins did not change significantly (Claeys et.al.,1995).

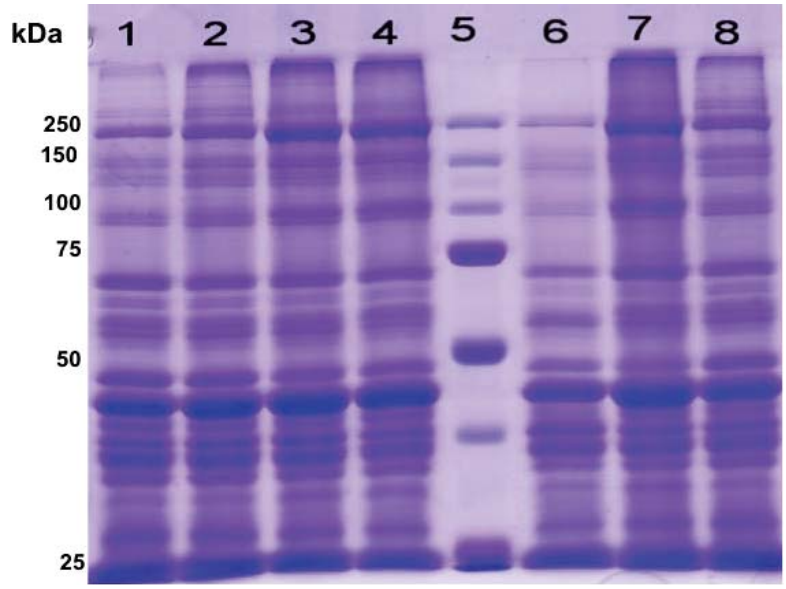

Figure 4.14: SDS –PAGE of beef muscle after reaction with no enzyme at 60 °C for different times (0, 30, 60 and 90 minutes). Lane 1,2,3,4, 6,7 and 8 : t= 1 minute, 30 minutes, 60 minutes, 90 minutes, 120 minutes , 180 minutes and 0 minute, respectively. Lane 5: molecular weight marker.

Attempts were also made to assess the rate and extent of protein destruction using SDS-PAGE analysis at three temperatures (40, 50, 60 °C) over the entire hydrolysis process. The results are shown in Figure 4.15. It appears the changes in electrophoretic patterns happened almost immediately the enzyme reaction began, then the pattern qualitatively remained the same for the remainder of the hydrolysis time. There were dark blue bands at the bottom of gels which were lower molecular weight protein bands that could not be separated on the gel system chosen. There appeared to be little difference in the SDS page results for the three temperatures used. As the electrophoretic analysis involved the use of SDS, which tends to dissociate proteins into discrete globular molecules, these results suggest the enzyme quickly destroys key peptide bonds that hold large molecules together and the SDS splits them apart.

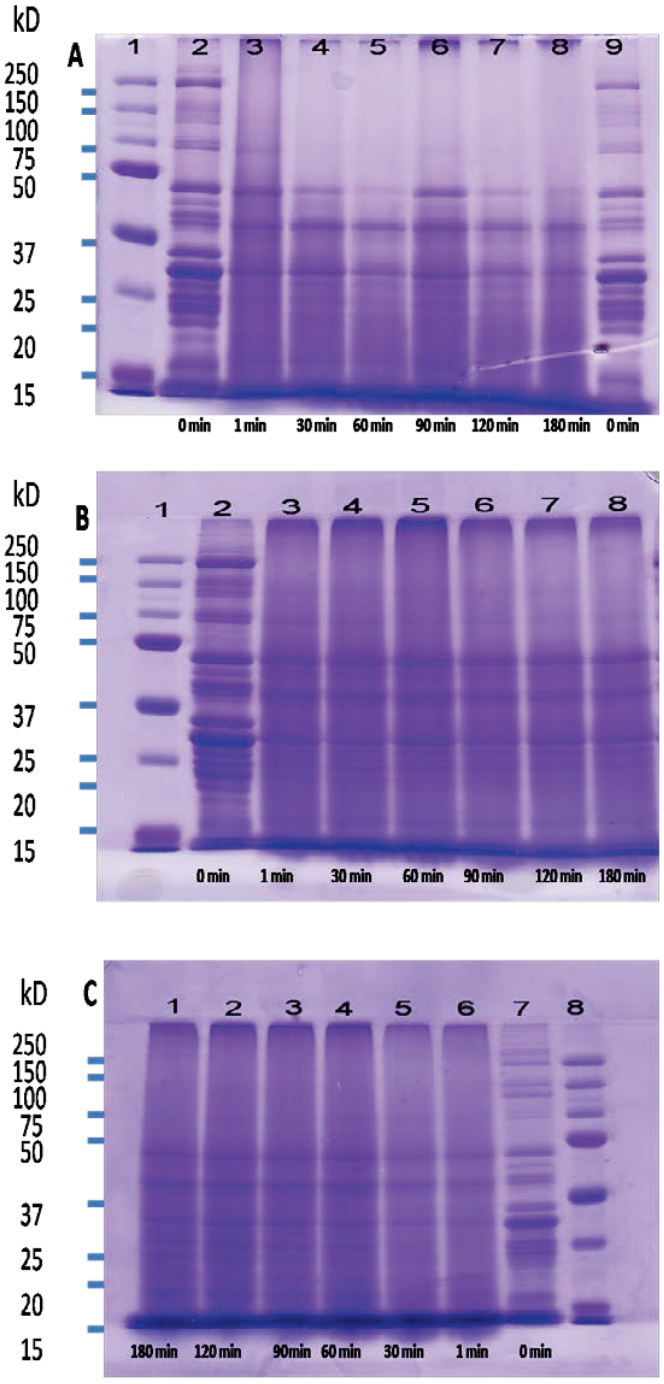

Figure 4.15: SDS -PAGE of beef muscle hydrolysate with 2% enzyme concentration for different hydrolysis times and temperatures: A: 60°C, B: 50°C, C: 40°C.

4.4.4 Enzyme concentration and reaction parameters

The rate and extent of hydrolysis were determined at various enzyme concentrations. Figure 4.16 shows the NPN content released at different enzyme concentrations and at different temperatures. At the low temperature, 40°C, the rate of production of NPN was slower than at both 50°C and 60ºC. At higher temperatures the rate of hydrolysis was faster in the first 90 minutes then the rate decreased. At all temperatures, the rate of hydrolysis at high enzyme concentrations (4%) starts to level off after 90 minutes. At 60ºC, addition of 4% enzyme resulted in a 288% increase in NPN after 180 minutes compared to 118% increase in NPN for 1% enzyme addition.

Quantitatively, not all the protein in the meat was converted to NPN. The total nitrogen content of the meat was 11.1 ± 0.9 g per 100 g dry sample, whereas the highest NPN concentration was 4.3± 0.2 g per 100 g dry sample after 180 minutes hydrolysis at 60°C and 4% enzyme concentration. There was an initial drop in the pH of the reaction medium when Zyactinase™ was added to the meat. This can be seen in Figure 4.17 and was reported earlier in Section 4.4.2. As the enzyme concentration was increased the initial pH drop became larger. As Zyactinase™ has a natural pH of 3.1, this pH decline was expected. There was no significant difference in the pH drop between the control and 1% enzyme concentration ($p>0.05$). However there was a significant difference in the pH drop with 2 % and 4% enzyme concentrations. In all instances, there was no significant change in pH after that initial drop, until the end of the hydrolysis at 180 minutes. Addition of 4% enzyme resulted in 17% increase in NPN after 180 minutes compared to 1% enzyme.

The SDS-PAGE electrophoretic patterns of the hydrolysates produced using different enzyme concentrations are presented in Figure 4.18. As before, the high molecular proteins, such as myosin heavy chain (220 kD), disappeared within one minute after the enzyme addition as can be seen in Lanes 3, 6 and 8 in Figure 4.18. As the time progressed, other high molecular weight proteins (β-actinin, α-actinin and desmin (>75kDa) started to disappear and the intensity of the bands between 75 and 25 kDa decreased (Lanes 4, 7 and 9, respectively). The control samples shown in Lanes 1 and 2, without the addition of any enzyme, did not show any protein fraction changes.

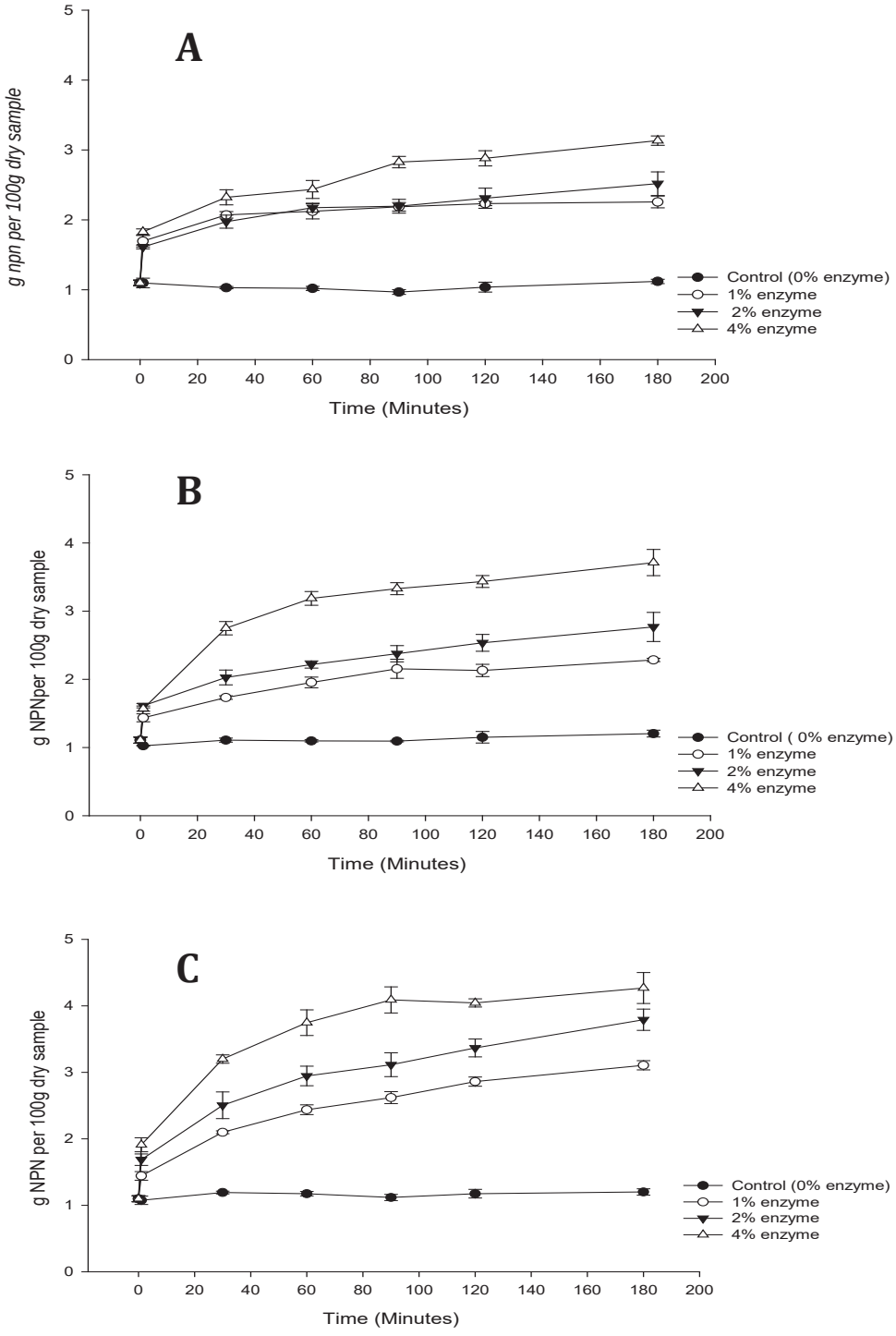

Figure 4.16: NPN content (g/100 g dry sample) at different enzyme concentrations and different processing temperatures. A: 40 °C, B: 50 °C, C: 60 °C. Data points are mean values ± standard deviation, n = 4 to 6

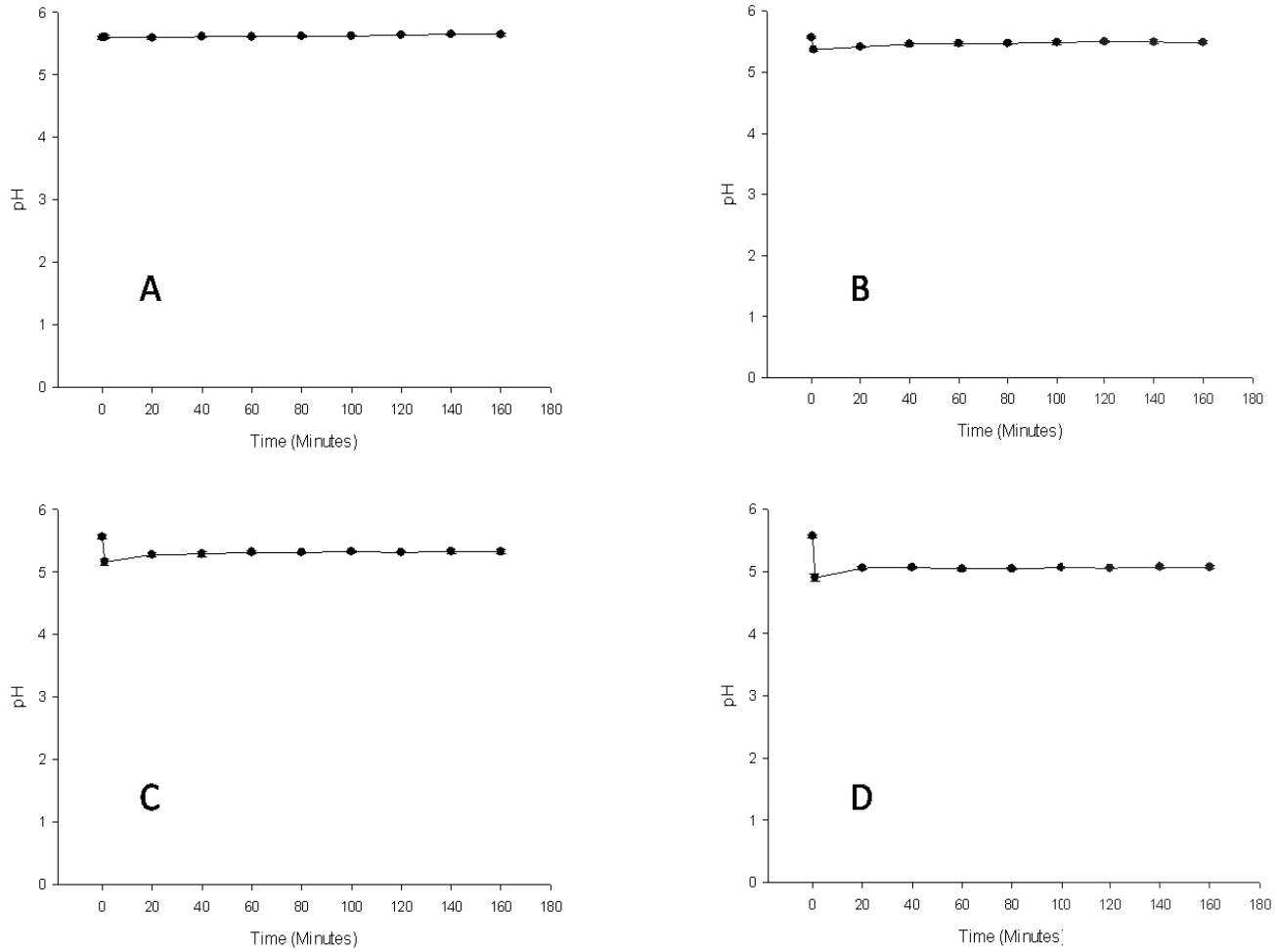

Figure 4.17: Changes in pH during hydrolysis at difference enzyme concentrations: (A) no enzyme addition, (B) 1% enzyme concentration, (C) 2% enzyme concentration, (D) 4% enzyme concentration. Data points are mean values ± standard deviation, n = 2

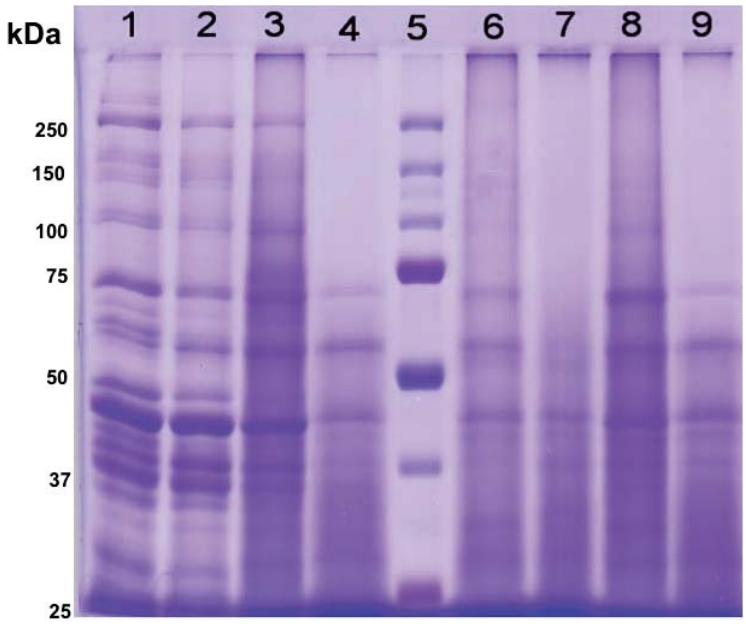

Figure 4.18: SDS-PAGE: Beef muscle after hydrolysis with ZyactinaseTM enzyme at different enzyme concentrations for 0 & 120 minutes (1 mg/ml protein per load). Lane 1: 60°C, 0 minute, without enzyme; Lane 2: 60°C,120 minutes, without enzyme; Lane 3: 60°C, 1 minute, 1%w/w enzyme; Lane 4: 60°C,120 minutes, 1%w/w enzyme; Lane 5: Molecular weight marker; Lane 6: 60°C,1 minute, 4%w/w enzyme; Lane 7: 60°C,120 minutes, 4%w/w enzyme; Lane 8: 60°C, 1 minute, 2%w/w enzyme; Lane 9: 60°C, 120 minutes, 2%w/w enzyme.

There appeared to be a greater degree of disappearance of protein bands with increasing enzyme concentration. For example, after one minute of the hydrolysis reaction with 1% enzyme concentration, myosin heavy chain and α-actinin bands decreased in intensity, whereas they were not visible for 2% and 4% enzyme concentrations (Lane 3 (1% w/w), Lane 6 (4%w/w) and Lane 8 (2% w/w)) (Figure 4.18). Similarly, after 120 minutes hydrolysis, actin (42 kDa) disappeared in the 4% enzyme concentration, but it was still present at 1% and 2% enzyme concentrations (Lanes 4, 7 and 9).

It has been reported that the enzyme concentration generally has a greater effect on reducing the hydrolysis time compared to a temperature increment (James et al, 2005) and the enzyme concentration has a strong impact on the catalytic process (Straathof, 2003).

4.4.5 Effect of substrate concentration

In this current experiment, substrate concentration is the weight percent of protein in the reaction mixture. A fixed enzyme concentration and temperature were used (2% w/w enzyme concentration at 60°C). Substrate concentration was varied by diluting the

meat with distilled water, thereby altering the dry weight of meat taken for the reaction system. Results are shown in Figure 4.19 (A).

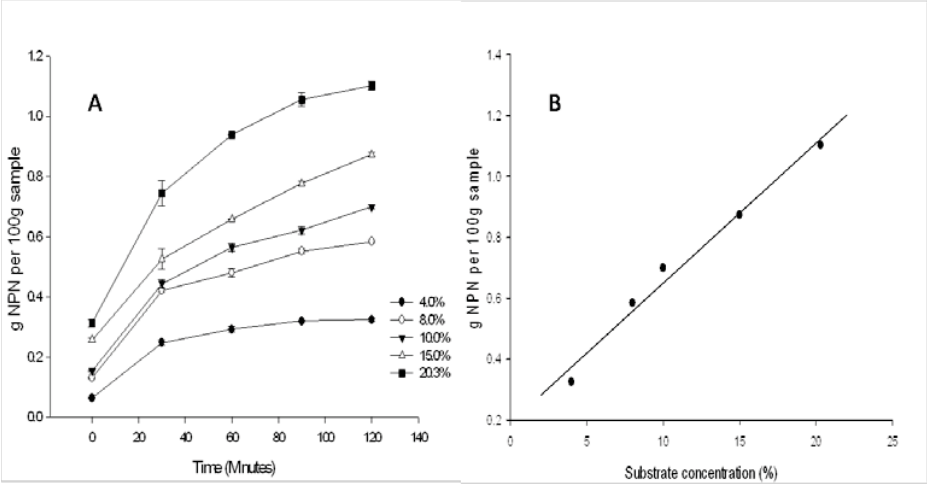

Figure 4.19: Progress curve for NPN content (g/100 g dry sample) at (A) different substrate concentrations and (B) at 120 minutes (r^2 = 0.98). Data points are mean values ± standard deviation, n = 3

The total quantity on NPN produced varied directly with the concentration of substrate: the higher the substrate concentration, the more NPN produced. A comparison of the amount produced in 120 minutes (Figure 4.19B) shows a linear relationship. This is in contrast with ground rumen where the increase of substrate concentration decreased the solubilisation and hydrolysis of substrate, due to the product inhibition of the enzyme and the hydrophobic aggregation of reaction products (Clark et al., 1987). However the hydrolysis of beef by alcalase only showed small changes in the degree of hydrolysis as the meat concentration was increased and decreased solubilisation was observed with increasing meat concentration (O'Meara & Munro, 1984).

4.5 Summary

This chapter showed that the optimum pH for hydrolysis was between 4 and 5.6 and pH 5.6 was the natural pH of the meat. Hence, pH adjustment was not required to optimise hydrolysis. The optimum temperature for Zyactinase™ was found to be 60 ºC**.** Increasing enzyme concentrations up to 4% w/w resulted in 18 % increase in production of NPN. An increase in substrate concentration resulted in a linearly correlated increase in production of NPN. The hydrolysis process was stopped at at 180 minutes to make sure partial hydrolysis is achieved. The hydrolysis relatively slow down after 180 minutes as the reaction curve become platue. With additional enzyme and substrate.

Chapter 5
Peptide and Amino Acid Analysis of Hydrolysates

Proteolysis of meat causes the degradation of major proteins such as myosin and actin into peptides and further proteolysis will convert peptides into their constituent amino acids. The characteristic of peptide and amino acid form by the digestion of Zyactinase were analysis using HPLC. The analysis of the various proteolytic end-products resulting from the Zyactinase^{TM} hydrolysis of meat can have an impact on the final composition of the hydrolysate.

5.1 Peptide analysis

Minced beef was hydrolysed with 1% w/w Zyactinase using 60°C reaction temperature for 0- 180 minutes. The resulting hydrolysate was reacted with 0.1% TCA to precipitate proteins and the slurry was centrifuged to remove the solid precipitate. The supernatant was further processed. The hydrolysate sample was then diluted with the mobile phase 0.1 % trifloroacetic acid (TFA) (1: 5 dilution). The sample was homogenized using a stomacher for 8 minutes then centrifuged at 10000 x g for 20 minutes. The supernatant was then filtered before the injection into the high performance liquid chromatograph (HPLC) and describe in Section 3.9.1. The extraction was than further separated and analyzed as describe in Section 3.9.2.

The HPLC profile of the hydrolysate processed at 60°C and 1% enzyme concentration sampled at different time intervals up to 180 minutes is shown in Figure 5.2. Similar, chromatograms were also observed at other temperatures and enzyme concentrations. A synthetic peptide standard mixture (Sigma Aldrich, USA) with different molecular weights was injected into the column to find the best chromatographic conditions to obtain an elution profile for the peptides as in Section 3.9.3. The column used was Jupiter 300 C18 columns in which depend on hydrophobicity. It allows one to separate proteins with only slight differences in hydrophobicity.

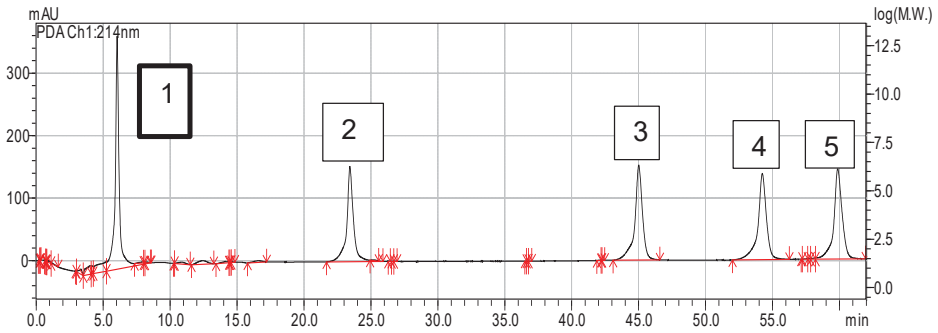

Figure 5.1: The peptide mixture contained 0.5 mg of five different protein peptides; (1) GLY-TYR, molecular weight of 238.2 g mol-1, (2) Methionine Enkephalin Acetate, MW = 573.7 g mol-1 for free base (TYR-GLY-GLY-PHE-MET), (3) VAL-TYR-VAL, MW = 379.5 g mol-1, (4) Leucine Enkephalin, MW = 555.6 g mol-1 for free base (TYR-GLY-GLY-PHE-LEU) and (5) Angiotensin II Acetate, MW = 1046.2 g mol-1 for free base (ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE)

The formation of peptides formed during hydrolysis at different time intervals was monitored with HPLC (Figure 5.2). After one minute hydrolysis, a major peak eluted at 4.6 minutes. The peptides began to elute at retention times of 10, 14, 17, 19 and 24 minutes. A comparison of the peak fractions in Figure 5.2 reveals a marked increase in the overall concentration of peptides as the hydrolysis time and enzyme concentration increased. After 90 minutes of hydrolysis, the increases of concentration of peptides containing components were stabilized. After 90 minutes, there was no significant increase in the peptide peak area and no formation of new peptides.

As hydrolysis progressed the HPLC profile changed considerably. A complex series of peaks eluted from 12 - 30 minutes. These reflected the formation of peptides from the protein and show the complexity of the mixture. In addition, the peaks at 3-5 minutes increased in intensity, reflecting a significant increase in these materials. Hydrolysis to peptides (peaks eluting between 12 – 30 minutes) appeared to reach a stable maximum within 90 minutes and did not appear to change significantly after that time. However, the formation of low molecular weight materials (eluting at 3–10 minutes) appeared to increase constantly throughout the entire hydrolysis process.

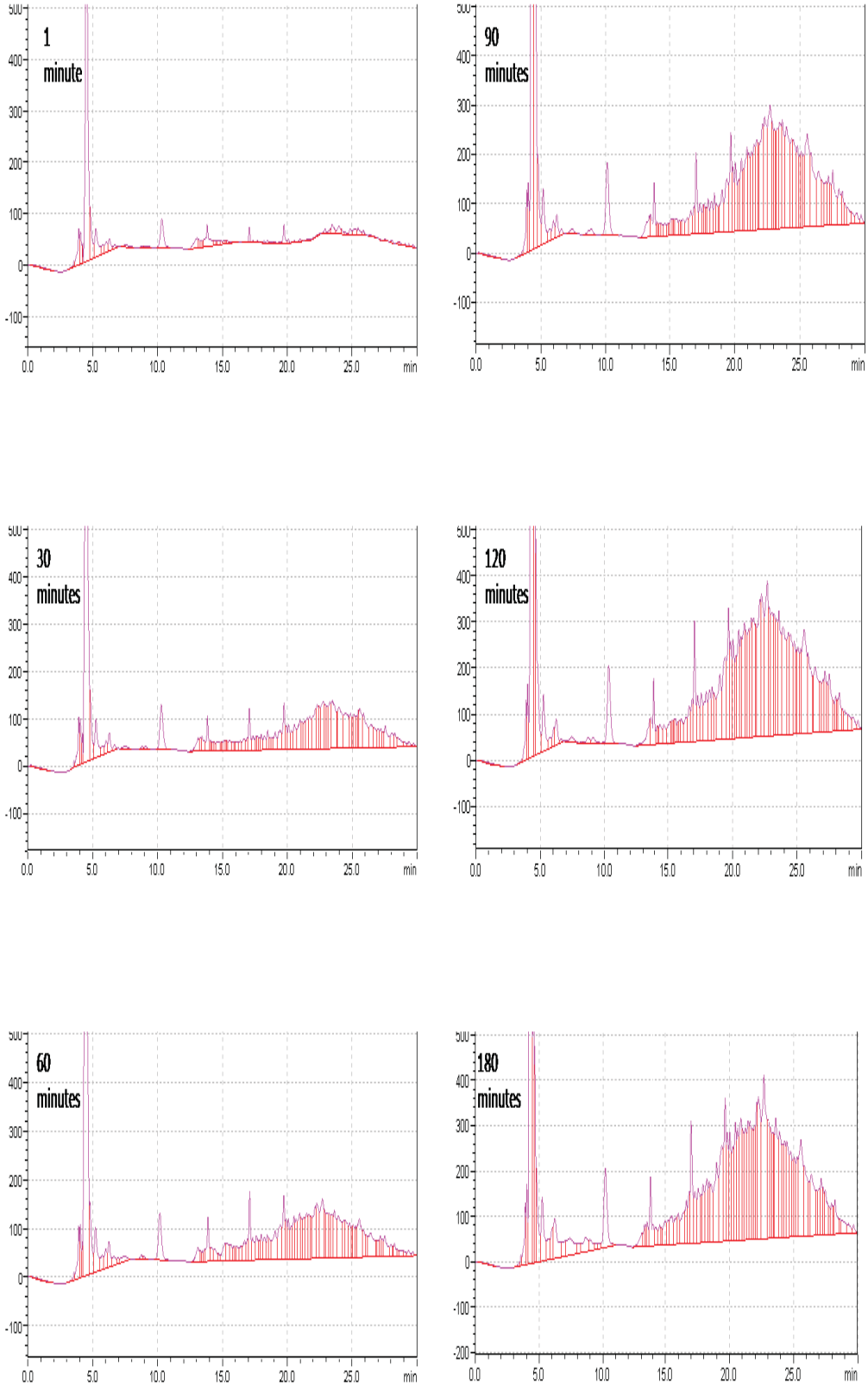

Figure 5.2: HPLC chromatograph of peptides in the hydrolysates processed at 60°C and 1% enzyme concentration after 1, 30, 60, 90, 120 and 180 minutes hydrolysis.

Fadda et al., (1999) with similar HPLC condition as in 3.9.1 and 3.9.2 divided the peptides based on their retention times. Peptides that emerged between 10 and 15 minutes retention time were considered to be hydrophilic, whereas peptides eluting between 15 and 25 minutes were considered to be hydrophobic in nature. Based on these criteria, this current research shows only two major hydrophilic peptides (eluting at 11 and 14 minutes) were derived from the meat hydrolysates and the others were hydrophobic peptides. The significant increase in the hydrophobic nature of the peptides suggests they will contribute to an undesirable (bitter) hydrolysate taste.

According to Ney (1971), bitterness is related to an average hydrophobicity of a peptide (Q value). The Q value was defined as the sum of free energies of transfer of the amino acid side chains from ethanol to water, divided by the number of amino acid residues in the peptide. A peptide was considered bitter if its Q value exceeded 1400 cal/mol and its molecular weight was less than 6000 kDa (Fukui et al., 1983). In addition, Ishibashi et al. (1987a, 1987b), reported that a bitter taste from peptides is more intense when the content of amino acids leucine, phenylalnine and tyrosine are high.The bitter tastes have more instense when hydrophobic acids with l configuration were located at C – terminus of the peptides (Isibashi et, al.,1987a, Isibashi et, al.,1987b, Shinoda et, al., 1986) Adler–Nissen (1988) proposed that the molar concentration and chain length of most hydrophobic peptides were also important properties responsible for bitterness. The bitterness limits the utilization of enzyme hydrolysates in the food industry. Peptides that contain a hydrophobic residue, especially with a long chain or aromatic amino acid, give bitter tastes (Kanehisa *et al,* 1984). Bitterness in meat hydrolysate is asscociated with low molecular weight peptides such as 5 kDa of hydrophobic peptides derives from bovine haemoglobin hydrolysates (Aubes –Dufau et al..,1995)

Food protein hydrolysates have known to have various application in food, cosmetic and pharmaceutical company. The use and characteristic of protein hydrolysates is based on their molecular size (Gauthier et al. 1986). Digestion of food protein released peptide that may exhibit a diverse range of bioactivities (Rutherfurd- Makwick and Moughan, 2005, Kaur et al, 2010a). Large molecular weight peptides (more than 20 amino acid residues) have improvement in the functional properties wheras low molecular weight peptides (di- and tripeptides, and amino acid) have high nutritional and therapeutic values such as preventing disease of hypertension (Bautista et al. 2000; Clemente 2000, Escudero et, al. 2012 ,Vijayalakshmi et al. 1986; Williams 1995).

5.2 Free amino acid formation from Zyactinase™ hydrolysis of meat

The same hydrolysis supernatant from the peptide analysis was used to measure the amino acid content. In this instance, the hydrolysis for 1 minute was compared to that for 120 minutes only. The samples were analysed as described in Section 3.10. Results are shown in Figure 5.3.

The total concentration of amino acids in the hydrolysate doubled from 1 to 120 minutes of hydrolysis. Figure 5.3 shows the quantitative changes in the amounts of various amino acids, comparing the initial and final hydrolysis results for 60 °C and 2% enzyme concentration.

Following hydrolysis, there was a marked increase in the concentrations of proline, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine. However, the concentrations of some of these amino acids were still quite low, less than 1.0 mg/g. Many L- amino acids (arginine, proline, leucine, phenylalanine, trytophan and isoleucine) taste bitter, whereas L- alanine, L –serine and most D-amino acids give a sweet taste. Valine and lysine have both sweet and bitter tastes. Glutamic acid gives a umami taste. Leucine is a significant precursor of volatile aroma compounds such as 2-methyl propanal and 3-metylbutanal. The increase in lysine, arginine and leucine levels is due to the proteolytic activity on myofibrillar proteins, whereas sarcoplasmic protein hydrolysis increases the alanine content (Fadda et al., 1999). Hydrophobic amino acid residues in peptides such as leucine, isoleucine, valine. The presence of phenylalanine, tyrosine and threonine in peptides may also link to antioxidant activity (Sun et al., 2011).

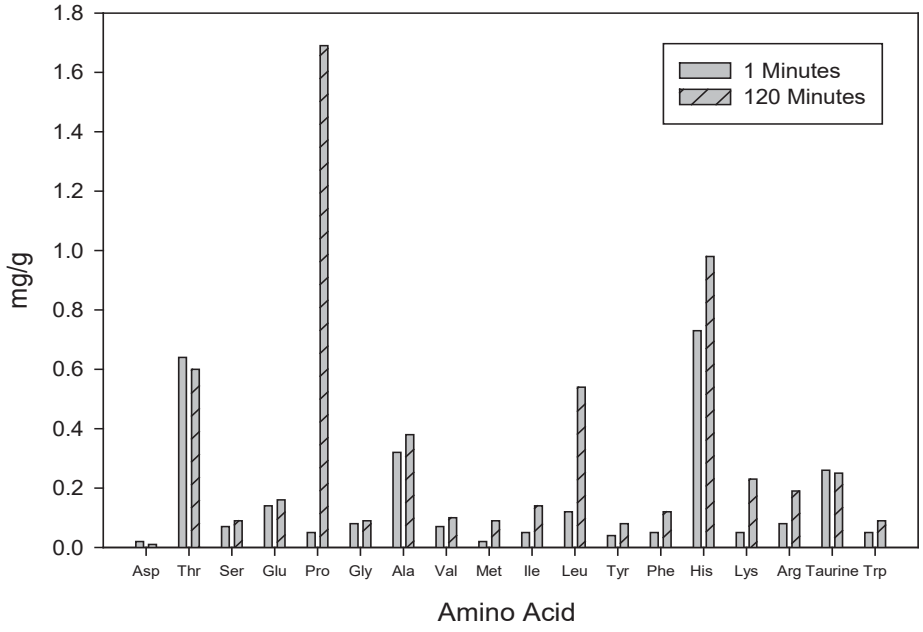

Figure 5.3: Changes of amino acid content after 1 minute and 120 minutes processing time Amino acid concentration in hydrolysates after 1 and 120 minutes, at 60°C and 1%. w/w enzyme concentration.(*Asp= aspartic acid, Thr= threonine, Ser=serine, Glu=glutamic acid, Pro=proline, Gly=glycine, Ala=alanine, Val=valine, Met=methionine, Ile=isoleucine, Leu=leucine, Tyr=tyrosine, Phe=phenylalanine, His=histidine, Lys= lysine, Arg=arginine, Trp= tryptophan)

Some amino acids appeared to decrease after hydrolysis (aspartic acid, threonine and taurine). The predominant free amino acids in fresh muscle are α-alanine, glycine, glutamic acid and histidine (Tallon et al., 1954). Free amino acids such as taurine and anserine are important for taste (Nishimura et al, 1988). An increase of free amino acid has shown to improve the taste of the meat (Nishimura et al, 1988). Comparing with hydrolysis of pig muscle by *Lactobacillus plantarum*, the myofibrillar protein extract give significant increase in alanine (Fadda et al.,1999). Chicken head hydrolysate has been reported to had high glycine content in which contribute to a better product taste (Surowka & Fik,1992).

5.3 Summary

In conclusion, as the time of hydrolysis increased the more hydrophobic peptides were produced in the hydrolysates. The increase in peptides corresponded to NPN results as discussed chapter 4. Higher temperatures and enzyme concentrations led to faster increase in release of peptide residues. The majority of free amino acids present in the hydrolysates increase between 5% and 324 % with proline increasing the most followed by 0.05 mg/g to 1.69 mg/g.

Chapter 6
Hydrolysis of different meat protein fractions

The separation into three crude protein fractions allows a more detailed understanding of the impact of Zyactinase™ on different protein components of meat. Zyactinase™ was used to digest each of the meat fractions for one hour at 40 °C and 2% w/w enzyme concentration. The amount of NPN released was measured to determine the extent of protein hydrolysis of different protein fractions. The various protein degradation reactions were followed using SDS–PAGE analysis.

6.1 Protein separation

Meat proteins were separated according to their solubility. Those proteins soluble in water are called sarcoplasmic proteins. The proteins insoluble in water, but soluble in 0.15M KCl are called myofibrillar proteins and the insoluble residue left after KCl extraction is called connective tissue. The raw beef material used for this trial was prepared as described in Section 3.2. The protein extraction technique reported by Helander (1957) was used with minor modification. The extraction of the sarcoplasmic fraction was described in Section 3.2.2, myofibrillar fraction in Section 3.2.3 and the connective tissue fraction in Section 3.2.4.

6.1.2 Hydrolysis of meat protein fractions

The nitrogen content of the protein fractions was determined before the hydrolysis process and the protein fractions were diluted with distilled water to ensure a uniform nitrogen concentration across all samples. Total nitrogen content and NPN content for all the protein fractions were determine as described in Section 3.5.5.

The pH of all protein extracts was adjusted to pH 5.6, to replicate the pH of the beef before enzyme extraction. The hydrolysis of the protein fractions was conducted as described for whole meat hydrolysis experiments in Section 3.4.1, with any modifications described in Section 3.8. The appropriate liquid protein fraction (sarcoplasmic or myofibrillar proteins) were placed in a 500 ml beaker, heated to the reaction temperature (40°C) before the addition of 2% w/w Zyactinase™ enzyme. The pH was adjusted to pH 5.6 prior to the hydrolysis process. Ten millilitre aliquots of the reaction mixture were collected at 1, 10, 30, 45 and 60 min. The hydrolysate aliquot collected was placed in a test-tube in a shaking water bath for 10 min at 95°C to

inactivate the enzymes. The sample was then placed in ice water until it reached 5 ± 2°C, it was then frozen and stored at -20 ± 2 °C.

6.2 Composition of various hydrolysis solutions for each meat fraction

The nitrogen content of the whole meat and for each fraction (sarcoplasmic, myofibrillar, connective tissue) present in meat was presented earlier in Table 3.2. Following the fractionation of 1kg of meat, the yield of connective tissue was only 0.5g (0.05%), as shown in Table 6.1.

Table 6.1: Mass of fractions recovered after separation of beef meat into different fractions.

Sample	Nitrogen content (g /100 g dried sample)	Recovered mass (g)
Whole Meat	11.68	100
Sarcoplasmic	2.70	ND
Myofibrillar	4.83	740
Connective tissue	0.95	0.5

ND Not determine – Sarcoplasmic is dissolved in dilute KCl aqueous solution during extraction.

Because of the low yield of connective tissue, a further sample of chuck beef (containing large amount of connective tissue) was extracted, as described in Section 3.2.4, to obtain sufficient connective tissue for the hydrolysis experiment. As mentioned above, the whole meat and the various meat fractions were diluted to ensure the same protein concentration before hydrolysis. The nitrogen analysis (total nitrogen and NPN) of each diluted hydrolysis solution, for each meat fraction, before the enzyme addition is given in Table 6.2.

The myofibrillar and connective tissue protein solutions had very low NPN content compared to the whole meat and sarcoplasmic proteins extract. The sarcoplasmic proteins had the highest NPN content, which represented 21% of the total nitrogen content in the sarcoplasmic protein extract. This was to be expected, as the initial water extract would have removed all the low molecular weight, water soluble meat NPN material.

Table 6.2: Composition of diluted hydrolysis solutions for each meat fraction. Results are mean values of 3 replicates ± standard deviation.

Protein fraction (diluted for hydrolysis)	Total nitrogen (g nitrogen/100 g dry sample)	NPN (g nitrogen/100g dry sample)
Whole meat	2.33[a] ± 0.22	0.31 ± 0.04
Myofibrillar	2.39 ± 0.24	0.01 ± 0.02
Sarcoplasmic	2.38 ± 0.47	0.50 ± 0.11
Connective tissue	2.31 ± 0.43	0.01 ± 0.01

[a] protein concentration was diluted to ensure approximately equivalent protein content for each fraction hydrolysed. Whole meat protein content reported in Section 3

6.3 Whole meat and meat protein fractions: extent of hydrolysis and NPN released.

The amount of NPN released from the whole meat and meat protein fractions during hydrolysis is shown in Figure 6.1. Using the same concentration of protein, results show myofibrillar and sarcoplasmic proteins were both significantly hydrolysed by Zyactinase™. While the connective tissue was not hydrolysed to the same extent as the other protein fractions, there was a small but significant increase in NPN during hydrolysis. After 60 min of hydrolysis, 78% of the myofibrillar total nitrogen had been converted to NPN, whereas only 38% of the total sarcoplasmic nitrogen had been converted to NPN. Connective tissue had a 1% conversion to NPN after 60 min. The conversion of connective tissue to NPN was relatively low as Zyactinase™ was not able to hydrolyse the connective tissue as extensively as the other protein fractions.

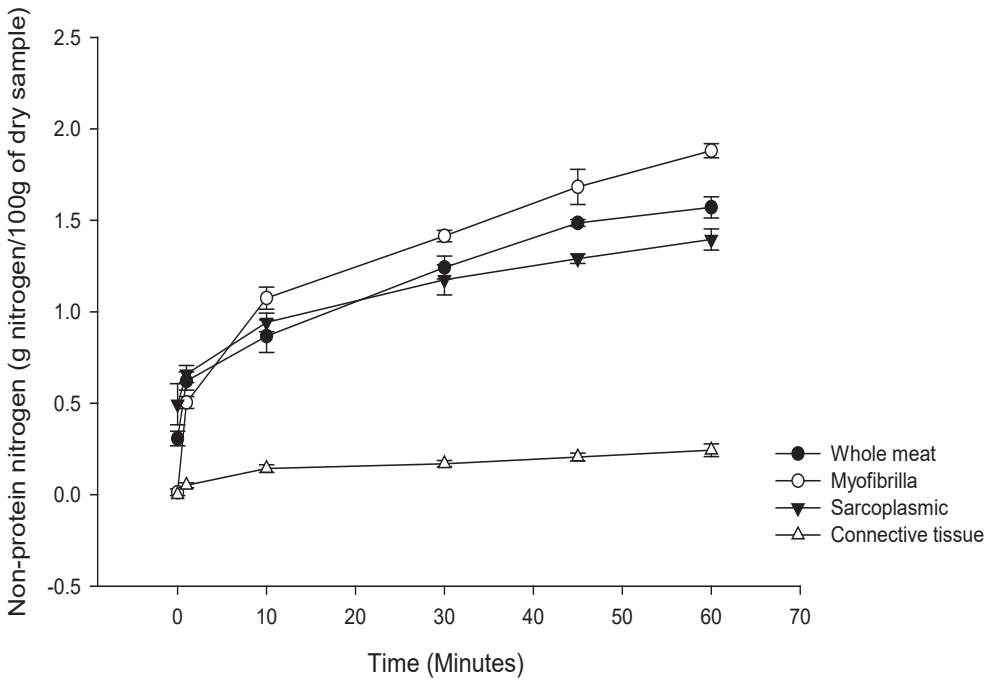

Figure 6.1: Formation of NPN during hydrolysis reactions for whole meat and different protein fractions (myofibrillar, sarcoplasmic, connective tissue). Data points are mean values of 3 replicates ± standard errors.

6.4 SDS-PAGE analysis of protein fractions after hydrolysis

SDS-PAGE provides a sensitive and useful means of observing the effect of proteolysis on protein in the whole meat and its individual protein fractions. The comparison gel, using the entire meat sample, is shown in Figure 6.2.

6.4.1 Whole meat hydrolysis

The control whole meat sample involved hydrolysis with Zyactinase™ at 60°C.An additional 1% of enzyme was added on top of the usual 2% to ensure an excess of enzyme Once the enzyme was added to the meat, the particles started to solubilise and change from a red to a brown colour. An excess concentration of enzyme was added to ensure that this did not become a limiting factor for the hydrolysis reaction. After one hour of hydrolysis, a brown slurry had formed, containing only a small fraction of meat particles.

The results of SDS-PAGE analysis is shown in Figure 6.2. For whole meat hydrolysis, high molecular proteins such as myosin and β-actinin reduced in intensity after 10 min of hydrolysis and completely disappeared after one hour of hydrolysis. Alpha-actinin's protein band showed a slight decrease in intensity once the Zyactinase™ was added to

the reaction mixture, then remained the same for one hour of hydrolysis. Protein bands between 50 to 75 kDa decreased in intensity but still remained after one hour of hydrolysis. Actin (42 kD) was unaffected by the hydrolysis. Protein band troponin T (~30 kD) was slightly reduced in intensity whereas the bands at 20 to 25 kDa disappeared after the addition of Zyactinase™. New protein bands appeared at 18 kDa.

6.4.2 Myofibrillar proteins hydrolysis

The myofibrillar proteins fraction was colourless and had a very “sticky” texture This solution changed to a greenish colour and lost its “sticky”, “tacky” texture upon the addition of the Zyactinase™ enzyme (at time = 1 min). During the hydrolysis process, white particulates appeared at the edge of the beaker after 10 min of hydrolysis, but these disappeared again after 30 min of hydrolysis.

The SDS-PAGE gel showing the progress of hydrolysis of myofibrillar proteins is shown in Figure 6.2. The protein bands in the untreated myofibrillar proteins fraction clearly show the standard moieties found in meat, as shown for whole meat (Figure 6.2). The myosin heavy chain (220 kDa) was clearly lost within 1 min of adding the Zyactinase™ to the sample. In addition, the protein bands representing β-actinin (130 kDa), α-actinin (95 kDa) and troponin-I faded within 1 min and had disappeared after 10 min of hydrolysis time. No changes were observed on the protein bands at 55 kDa (desmin) and 42 kDa (actin) throughout the one hour of hydrolysis reaction. A double protein band at 34 - 36 kDa (tropomyosin α-β-chain) decreased in intensity as the hydrolysis proceeded but remained visible until 30 min of hydrolysis. Troponin–C decreased in intensity but remained visible until 45 min of hydrolysis. A new band appeared at 26 kDa which was assumed to be the band for protein in Zyactinase™ enzyme (Section 4.1). A new protein band at about 15 kDa appeared within one and again at 10min of hydrolysis, but it was not observed at 30 or 60 min.

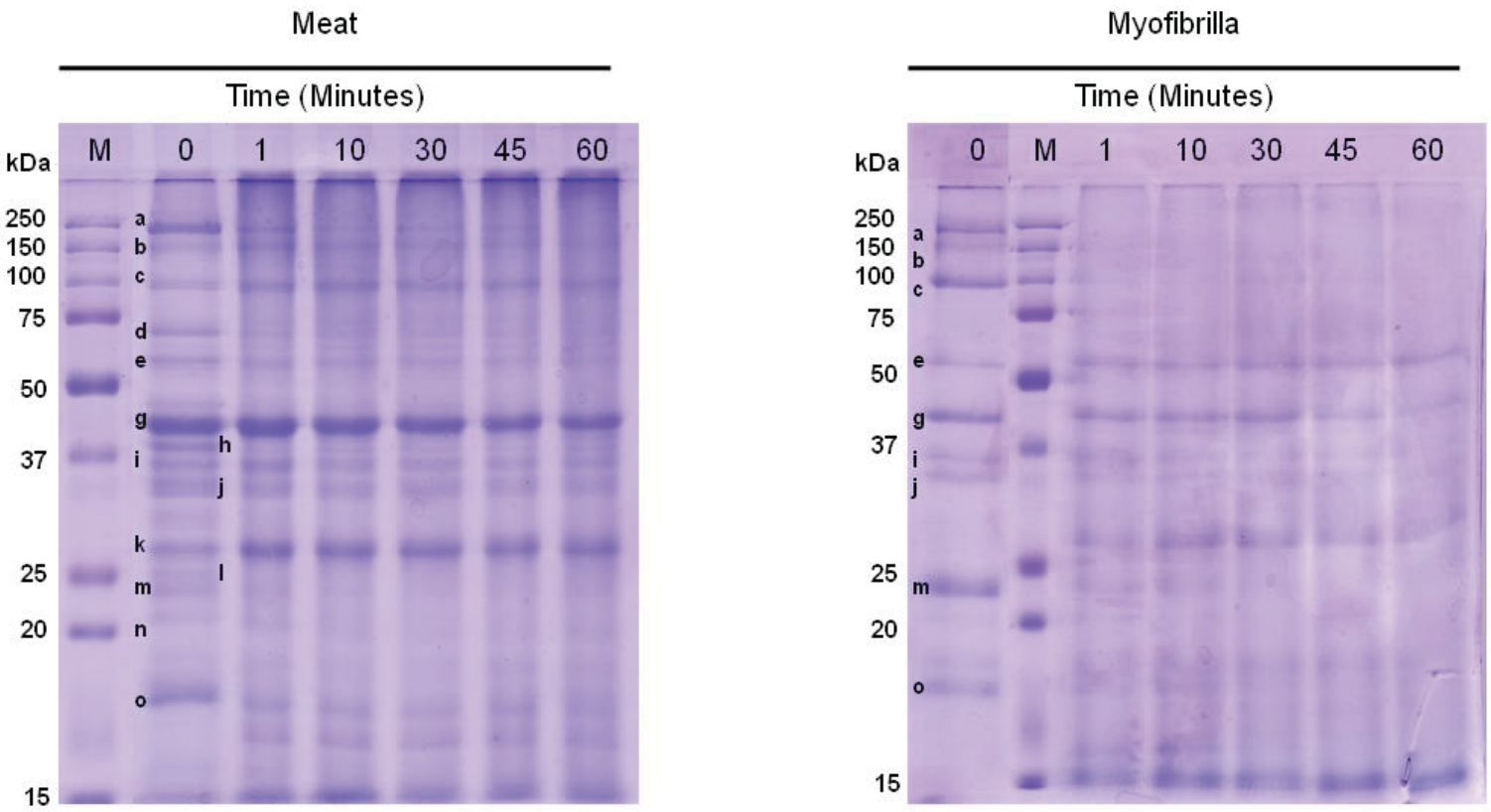

Figure 6.2: SDS-PAGE analysis of whole meat protein and myofibrillar protein fraction hydrolysis. M = molecular markers, 0 – 60 min = processing time. a) Myosin heavy chain (MHC), b) β-actinin, c) α--actinin (95 kDa), d) unknown (75 kDa) e) desmin (55kDa), g) actin (42 kDa), h) unknown (38 kDa), i) tropomyosin β-chain (36 kDa), j) tropomyosin α-chain (34 kDa), k) troponin-T (35 kDa),l) unknown(25kDa), m) unknown (23kDa), n) troponin--I (21 kDa), o) troponin-C (18 kDa).

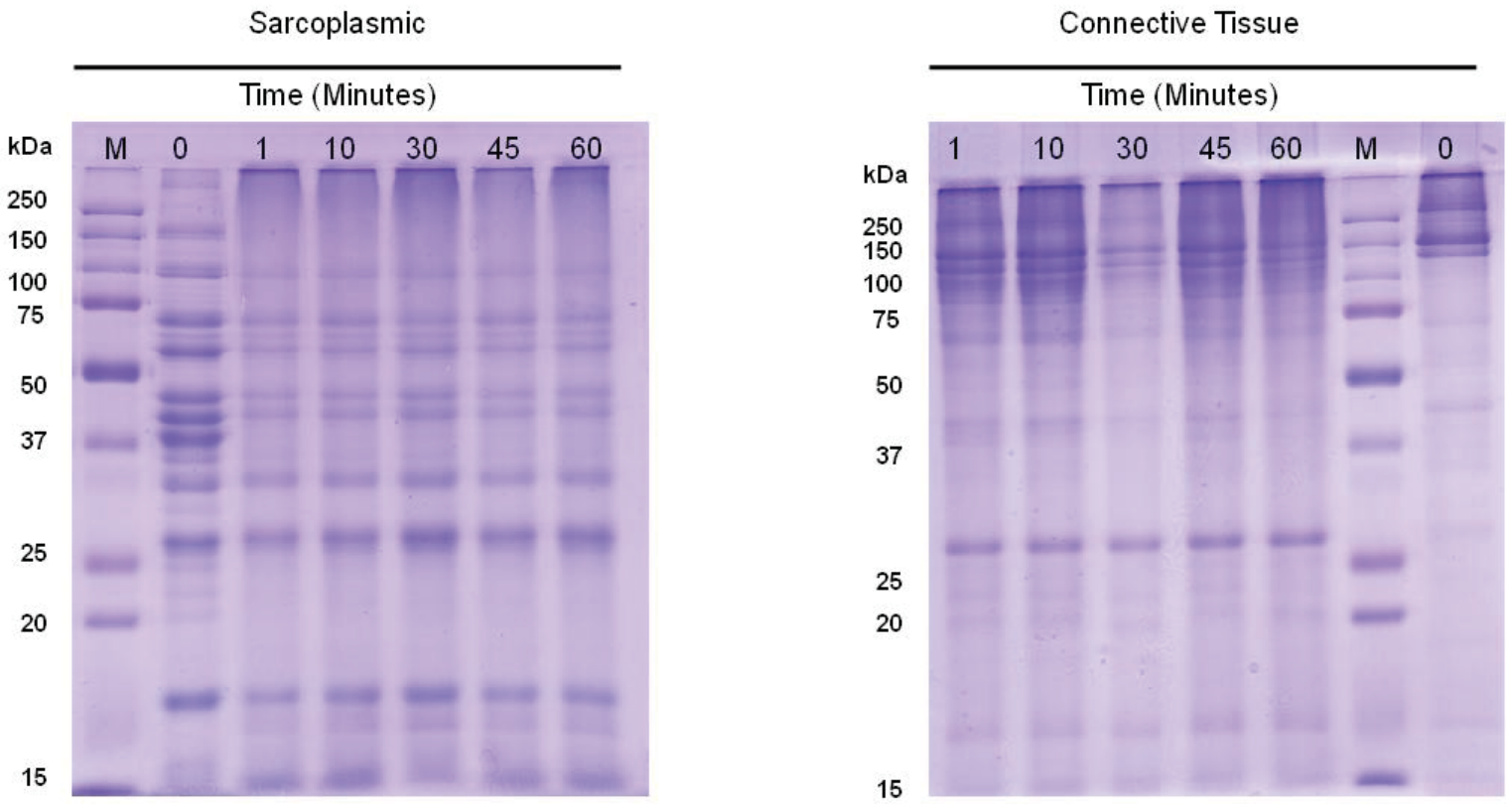

Figure 6.3: SDS-PAGE analysis on sarcoplasmic and connective tissue hydrolysis using ZyactinaseTM. M = molecular markers, 0 – 60 min processing time. Sarcoplasmic: 20 – 15 kDarepresent the formation of smaller proteins/peptides. Connective tissue: B and in Lane 0 represents a) Collagen type 1 (300 kDa), b) β band (200 kDa) and c) α band (100 kDa).

6.4.3 Sarcoplasmic proteins fraction

The sarcoplasmic protein fraction was a red solution. As the sarcoplasmic protein fraction was heated to 40°C, insoluble brown particulates appeared on the reaction mixture and the red solution faded. This is most likely the denaturation and precipitation of myoglobin. The insoluble particulates still remained after addition of Zyactinase™ enzyme (1 minute) and the solution changed to a brown-greenish colour. Even after one hour of hydrolysis some of the insoluble particulates remained. These insoluble particulates remained unchanged, even after the reaction mixture was heated to 95°C for enzyme inactivation.

SDS-PAGE results for the sarcoplasmic proteins extracted are presented in Figure 6.3. The original fraction extracted clearly shows a large number of unique protein fractions (0 channel). This sample was taken after the solution was heated to 40°C, but before Zyactinase™ was added. Thus the brown particulates had formed. Protein bands appeared at approximately 176, 93, 63, 60, 54, 42, 40, 36, 31, 25 and 18 kDa. When the enzyme was added to the reaction mixture (1 min elapsed time) the protein bands at 176, 37 and 34 and 25 to 10 kDa disappeared. However, the protein bands at about 18 kDa and 26 kDa were not affected by the enzyme and remained for the entire hydrolysis process. As mentioned earlier the band at 26 kDa was from the Zyactinase™ enzyme. New protein bands between 15 and 17 kDa were seen to form during hydrolysis. Protein bands at aproximately 94, 63, 60, 54, 43, 40 and 31 kDa decreased in intensity after 1 min, then the bands remained unchanged for the remainder of the experiment.

6.4.4 Connective tissue fraction

The connective tissue fraction was homogenised with distilled water for 24 h to achieve similar nitrogen content as the other meat protein fractions before the hydrolysis process. The connective tissue homogenate consisted of small particles of “stringy” connective tissue dispersed throughout the slurry. There were no changes in the appearance of connective tissue slurry after heating to 40°C. The ‘stringy’ material, seen in the initial slurry, remained visible throughout the entire hydrolysis process. However, this material dissolved slightly upon heating to 95°C, during the inactivation of the Zyactinase™ enzyme.

The SDS-PAGE results are shown in Figure 6.3. The original connective tissue (time 0) showed three major protein bands at approximately 120, 150 and 260 kDa and four

faint bands at approximately 18, 40, 50 and 70 kDa The protein band with apparent molecular weight of 250 kDa significantly decreased in intensity as the hydrolysis proceeded and finally disappeared at 60 min of hydrolysis. Protein bands at approximately 150 kDa and 120 kDa also decreased in intensity, but the bands around 70 and 18 kDa increased in intensity. A number of new protein bands appeared around 80 to 120 kDa. A new, prominent protein band appeared at 26 kDa representing the Zyactinase™ enzyme.

6.5 Enzyme hydrolysis of different protein fractions

Typically, the protein fractions of meat are 11.5% myofibrillar protein, 5.5% sarcoplasmic protein and 2.0% connective tissue protein (Lawrie & Ledward, 2006).
The myofibrillar protein extract used for hydrolysis contained a mixture of denatured and native proteins as thermal denaturation of myofibrillar protein occurs between 30 and 70°C (Forrest et al., 1975). Tornberg (2005) reported that some post-mortem myofibrillar proteins in solution started to unfold at 30 to 32°C, followed by protein–protein association at 36 to 40°C and subsequent gelation at 45 to 50°C. Denaturation and partial denaturation exposed some part of the hydrophobic core, allowing intermolecular bonding or network formation to occur (Oakenfull et al., 1997). Unfolding of the protein structure potentially exposed more peptide bonds for hydrolysis.

The release of NPN during hydrolysis followed a consistent pattern for the whole meat and various fractions, rapidly increasing initially, then slowing down during the later stages of hydrolysis (Figure 6.1). This reflects susceptible peptide bonds being cleaved rapidly and the less susceptible bonds more slowly near the end of the hydrolysis time (Clark et al., 1987).

High molecular weight myofibrillar protein such as myosin (~220 kDa), α-and β-actinin were degraded by Zyactinase™ rapidly during the hydrolysis process, whereas actin and desmin (58 kDa) were resistant to proteolytic attack from Zyactinase™. The lack of proteolysis by actinidin on actin is supported by others ; (Han et al., 2009; Mohr, 1980 Nishiyama, 2001;). Troponin was also degraded early in the hydrolysis process of the whole meat and myofibrillar extract. The loss of troponins and myosin light chains were reported to be responsible for the tenderisation of beef steak in post mortem muscle.

A new protein band appeared at ~20 kDa during whole meat hydrolysis. Han et al (2009) reported a new peptide appeared at approximately 20 kDa when myofibrillar

protein fractions were subjected kiwifruit juices. Protein bands at 28 kDa became more prominent as the hydrolysis time proceeded, consistent with the observations of Han (2009).

There are two contradictory reports on the susceptibility of sarcoplasmic proteins toward enzymatic degradation. Mohr (1980) stated that heat denatured sarcoplasmic proteins were resistant to proteolytic attack whereas O'Meara & Munro (1985) reported the proteins were easily solubilised. In general, denatured globular proteins are relatively easy to hydrolyse compared to native sarcoplasmic protein (Adler-Nissen, 1976). Zyactinase™ was able to degrade the denatured sarcoplasmic protein extract but there were still insoluble particulates present at the end of the hydrolyisis process. When compared to ficin which induced major changes in physiochemical properties of myofibrillar proteins (Ramezani et al., 2003) including aggregation and precipitation, Zyactinase™ did not show the same extent of hydrolysis as ficin. Actinidin was found to significantly increase water soluble protein and water holding capacity which resulted in tenderized beef (Aminlari et al., 2009). The pattern of protein bands for the sarcoplasmic protein fraction was similar to uncooked beef soup stock which contained mainly sarcoplasmic protein (Tajima et al., 2001). Sarcoplasmic protein fraction usually contains globular proteins with relatively low molecular weights ranging from 17 kDa (myoglobin) to 92.5 kDa (Tornberg, 2005). Sarcoplasmic proteins in cooked meat forms a gel between myofibrillar elements, thereby giving a consistency similar to cooked meat (Davey & Gilbert, 1974)

Zyactinase™ did not cause a major degradation of the connective tissue proteins, in comparison to myofibrillar and sarcoplasmic protein fractions (Figure 6.1). However, changes were observed using SDS-PAGE, particularly after one hour hydrolysis time. A major constituent of intramuscular connective tissue is collagen (Type 1). Collagen has its molecules assembled in fibrils and they are immobilized by cross-links that are preferentially formed between the triple-helix and globular domain (Lawrie, 1988). Thus enzymatic degradation is difficult. Collagen and gelatine are extremely resistant to hydrolysis by proteases because of their high levels of proline and hydroxyproline. These amino acids prevent the flexibility of the protein backbone that facilitates binding to the active site of the enzyme (Kaur et al., 2010a). Collagen was reported to be more resistant to protease enzymes than the other myofibre proteins due to its unique triple helix structure (Cheftel, 1977; Swan & Torley,1991; Laser-Reuterswärd et al.,1982; Lieske & Konrad, 1994). Collagen can be extracted using strong acid and alkaline solution or can be digested with pepsin and collagenase. Some authors reported that

actinidin did not digest collagen whereas another researcher reported collagen digestion by actinidin. Kiwifruit juice can degrade the globular domain of the denatured collagen but it cannot cleave the triple–helical domain of collagen, which gives collagen the rigid super molecular structure in the pH range of 3 - 7 (Sugiyama et al., 2005). Wada et al. (2002) reported kiwifruit protease able to slightly digest the α- chain collagen. Wada et al. (2004) also suggested that kiwifruit protease could degrade the insoluble collagen of cattle Achilles tendons into collagen subunit chains (β-and α-chains), at pH 3.3 and 6.0 but not at pH 2.7 - 2.9. Actinidin can digest these two types of collagen at pH 7 and 8.5 but no effect was observed at acidic pH (Mostafaie et al., 2008). Collagen's nutritive value is low due to lack of essential amino acids such as tryptophan and tyrosine (Swan & Torley, 1991). Collagen is high in glycine, proline and hydroxyproline (Etherington & Sims, 1981).

The connective tissue appeared to be solubilised when heated to 95°C for enzyme inactivation and this was probably due to thermal denaturation of proteolysis-weakened proteins within the connective tissue. Collagen when heated in the presence of water at 60 – 70°C, leads to the unfolding of the protein structure. This unfolding is due to the breakage of non-covalent bonds, covalent (disulphide) intermolecular and intra-molecular bonds and a few peptide bonds, resulting in the collapse of the triple helical polypeptide unit which causes gelatinisation (Keeton & Eddy, 2004). However the collagen was not fully transformed to gelatine in this present study because of the very short heating time. Elastin was reported to be very resistant to solubilisation, cooking or enzymatic digestion due to a high content of non-polar amino acids and desmosine cross links (Keeton & Eddy, 2004). This will further explain the lower amount of Zyactinase™ digestion of the connective tissue.

6.6 Summary

The enzymatic hydrolysis with three meat protein fraction extracts has been investigated. Zyactinase™ was found to digest protein in myofibrillar and sarcoplasmic meat fractions at similar rates to that observed in whole meat. Large molecular weight proteins such as myosin and β and α-actinin were quickly digested while actin (42 kDa) was left seemingly untouched. During both myofilbrillar and sarcoplasmic hydrolysis low molecular weight proteins with molecular weight < 20kDa were produced. Connective tissue was hydrolysed by Zyactinase™ but to a much lesser extent than the myrofibrillar and sarcoplasmic fractions. The large molecular weight collagen protein moiety was found to decrease while smaller molecular weight proteins appeared. The

collagen protein structure contributed to its resistance to hydrolysis. These findings are important if Zyactinase™ will be used for hydrolysis of poor quality cuts of beef which contain high percentages of connective tissue.

Chapter 7

Reaction kinetic of meat hydrolysis using Zyactinase™

The effect of various parameters on Zyactinase™ enzyme activity was investigated using a systematic study of the enzyme reaction mechanism. This chapter presents the progress curve for the meat hydrolysis reactions, the relationship between degree of hydrolysis (DH) and non-protein protein nitrogen (NPN) during hydrolysis and the agreement of the hydrolysis reaction to the Michaelis-Menten equation. The effect of initial and added enzyme and substrate concentrations on meat hydrolysis progress was also investigated.

7.1 Correlation between the non-protein nitrogen concentrations (NPN) with degree of hydrolysis (DH) to determine the extent of hydrolysis.

The determination of the extent of hydrolysis using soluble nitrogen content, with Kjeldahl was time consuming and labour intensive. The need for a simple method for the kinetic analysis was required. Amino acids produced as a result of hydrolysis were measured based on the ortho-phthalaldehyde (OPA) method (Nielsen, Petersen, & Dambmann, 2001). OPA methods are based on the specific reaction between OPA and a primary amino group, in the presence of a thiol to form 1- alkylthio-2-alkyl-substituted isoindoles. The OPA method was chosen because of its speed and simplicity compared to the trinitrobenzenesulphonic acid (TNBS) method. The response time for the OPA reaction was two minutes compared to one hour for TNBS. Furthermore OPA reagents are less toxic than TNBS reagents.

The OPA method determined the number of free α-amino groups present which could then be used to determine the degree of hydrolysis (expressed as % DH) as described in Section 3.10. %DH estimates the percentage of peptide bonds which have been cleaved during hydrolysis (Adler-Nissen, 1976).

Figure 7.1 shows a comparison between the NPN value and the %DH, both monitored during the hydrolysis of beef mince with Zyactinase™ at 60°C for 120 minutes. The correlation between %DH and NPN shown in Figure 7.1 had a Pearson correlation coefficient (r) value of 0.937 ($p < 0.05$). This good correlation ($r > 0.9$) gives confidence in moving to the OPA method to monitor the hydrolysis reaction progress.

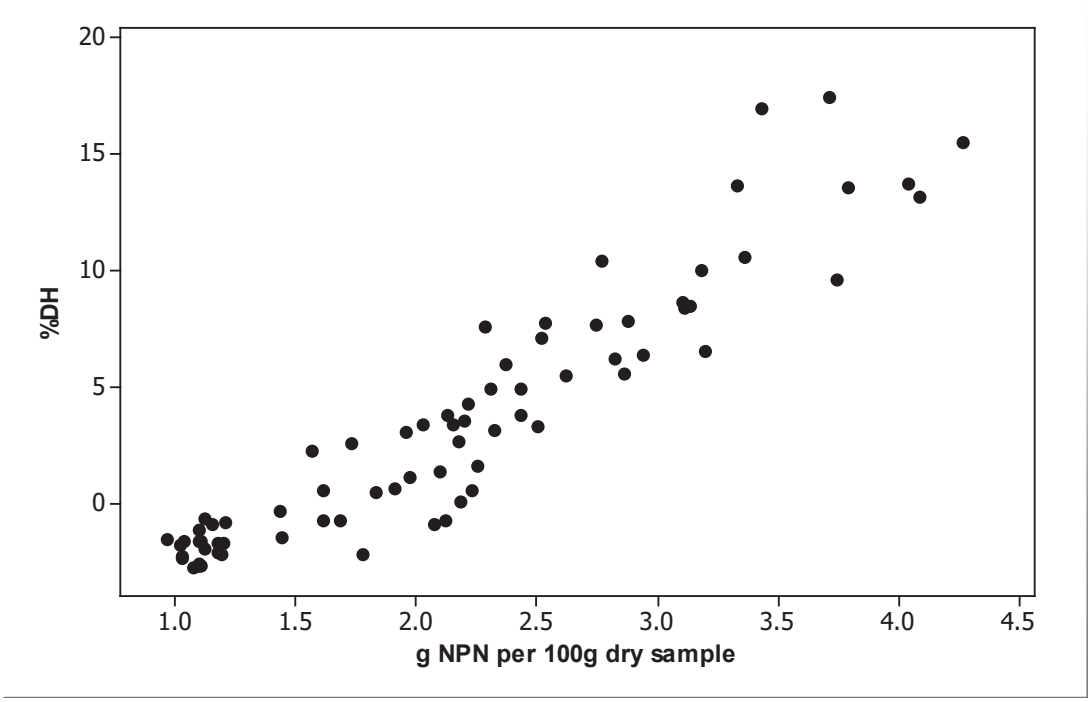

Figure 7.1: Correlation analysis between % DH (by OPA) and NPN (g NPN per 100g sample) or hydrolysis of beef mince at 60°C for 120 minutes.

7.2 Meat hydrolysis progress curve

Enzymatic hydrolysis is complicated due to the following factors: 1) Diversity of reaction components, as peptide fragments produced are both products and reactants. 2) Peptide bonds can be cleaved simultaneously. 3) There can be substrate inhibition, product diversity and enzyme inactivation. 4) Multiple factors influence the hydrolysis reaction and its rate, including pH, temperature, ionic strength and pressure (Qi & He; 2006).

Meat protein consists of peptide bonds and links to different amino acids. During enzyme hydrolysis reactions, these bonds are broken to form low molecular weight protein, peptides or individual amino acids. The smaller protein or peptide chains that are formed may be further hydrolysed to smaller peptides and amino acids by the same reaction mechanism until all the susceptible bonds have been cleaved. A proposed reaction scheme for the meat protein hydrolysis reactions is presented in Figure 7.2. The reaction for hydrolysis of the peptide bond can be expressed by Equation 7.1.

$$\text{Substrate} + \text{Enzyme} \leftrightharpoons \text{ES} \leftrightharpoons \text{Enzyme} + \text{Product} \qquad \text{Equation 7.1}$$

The enzyme binds with the peptide bond in the peptide chain (substrate) forming an enzyme substrate complex (ES). The enzyme catalyses the splitting of the peptide bond thus, forming smaller peptide chains (product). The peptide chain lengths are determined by which peptide bond is broken. However, ES binding can also lead to no result, the enzyme may split from the substrate and the peptide bond remains intact.

Figure 7.2 shows the different possible pathways that could lead to the hydrolysis and breakdown of the meat protein. The meat protein starting material can be hydrolysed and broken down via pathways A, B or F to produce lower molecular weight proteins, peptides and amino acids. Lower molecular weight protein may or may not breakdown further to peptides and/or amino acids. Peptides can also become the substrate which can then breakdown further to amino acids. The hydrolysis process may involve competition between the substrates (meat and low molecular weight proteins and the products (low molecular weight proteins and peptides).

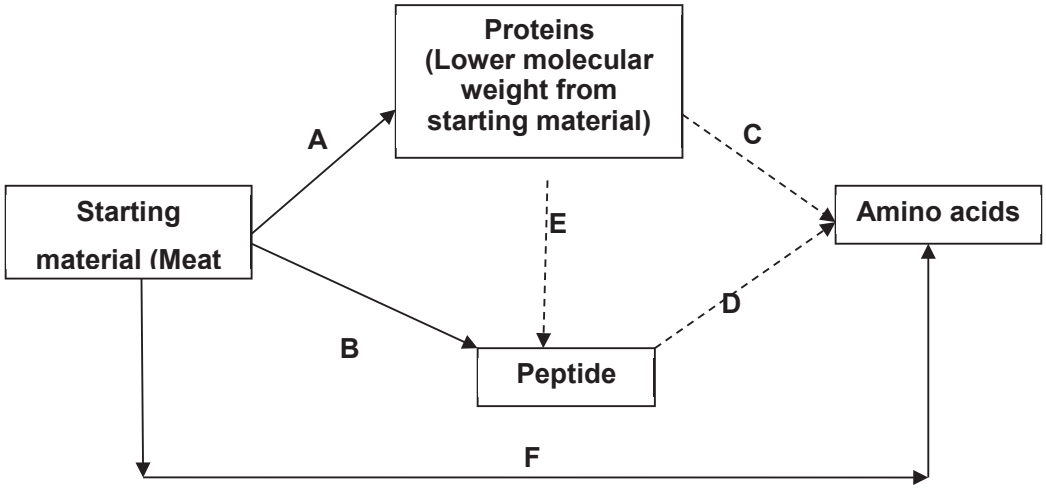

Figure 7.2: Possible reaction pathway for protein hydrolysis

The progress of the hydrolysis reaction was monitored for 180 minutes at 0, 1, 2, 3 and 4% Zyactinase™ enzyme concentrations and at 60°C. The hydrolysis progress curves, monitored using both OPA and SN-TCA (soluble nitrogen by trichloroacetic acid precipitation) methods to give % DH and NPN, are shown in Figure 7.3.

Both Figures 7.3 A and B show similar shaped curves where there is initially rapid hydrolysis on addition of the Zyactinase™ and proportional to the amount of enzyme

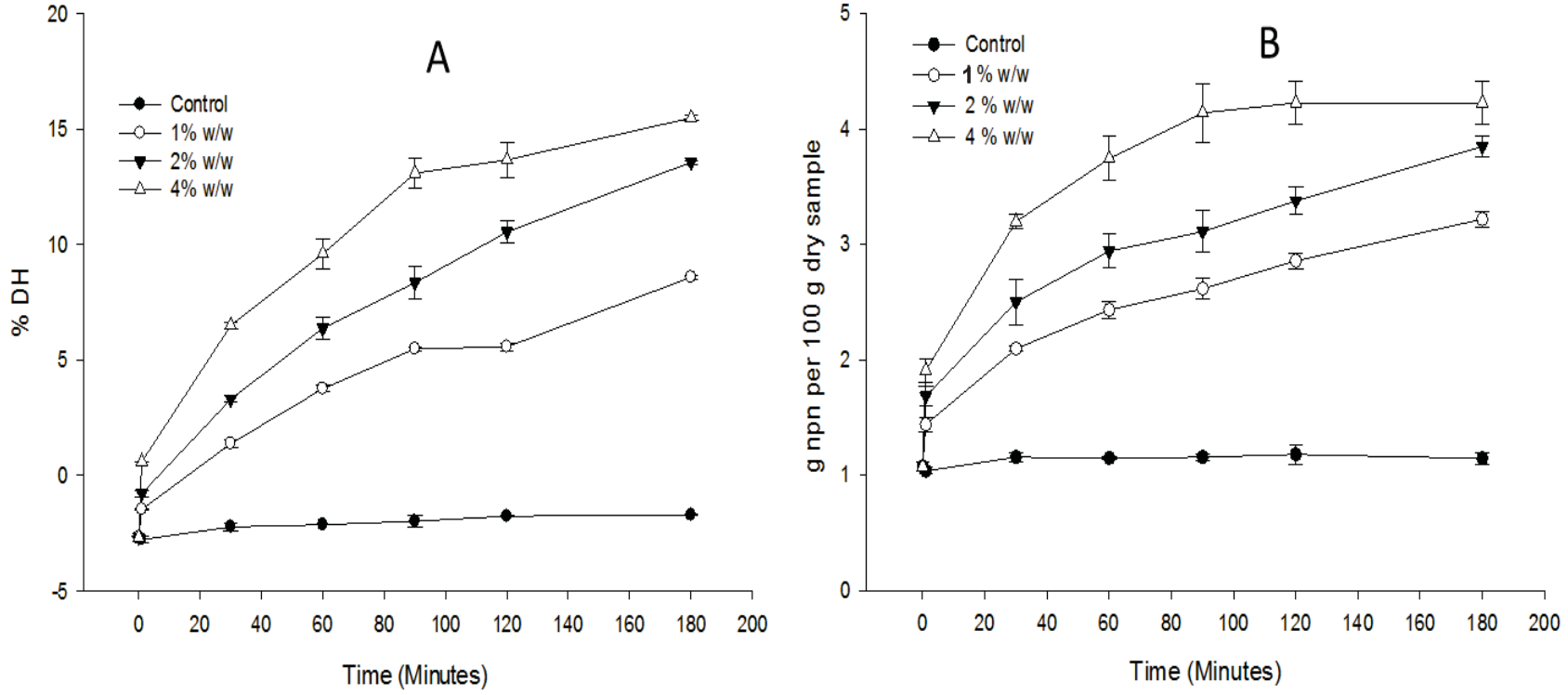

Figure 7.3: Progress curves for hydrolysis at 60°C at different enzyme concentrations (%w/w) monitored by A: OPA method (%DH) and B: SN-TCA method (NPN). Data points are mean values ± standard deviation, n = 6.

7.3 Study of reaction kinetic

The meat hydrolysis process involved at least two steps. In the first step the enzyme molecules bind to the meat resulting in the release of soluble peptides and amino acids (Mohr, 1980). Addition of the enzyme results in an initial rapid hydrolysis phase during which a large number of peptide bonds are cleaved per unit time and a large amount of soluble nitrogen materials were released into solution (Mohr,1980). This is consistent to the results seen in Figure 7.3, where there is initially a rapid hydrolysis reaction rate followed by a slight reduction, followed by a levelling off of the reaction rate as time progresses.

A similar curve was observed for the enzymatic hydrolysis of capelin (Shahidi et al., 1995), sardine (Quaglia & Orban, 1987), and veal bone (Linder et al., 1996). Generally, the enzyme absorbs rapidly onto the insoluble protein particles, then the polypeptide chains that are loosely bound to the surface are cleaved. The more compacted core proteins are hydrolysed more slowly. The rate of enzymatic cleavage of peptide bonds controls the overall rate of hydrolysis (Archer et al., 1973). However, available substrate decreases as reaction time increases (Benjakul & Morrissey, 1997). At the end of the hydrolysis process any reduction in reaction rate could be attributed to the depletion of available substrate, decrease of enzyme activity, or product inhibition (Guerard, Dufosse, De La Broise, & Binet, 2001)

7.4 The effect of addition of new substrate and enzyme during hydrolysis

In attempt to understand the Zyactinase^{TM} enzyme hydrolysis process further and whether the reduction in reaction rate shown in Figure 7.3 was due to substrate depletion, fresh substrate was added to the mixture after 179 minutes of hydrolysis reaction. The time of 179 minutes was chosen because the hydrolysis curve reached a plateau with the addition of 2% enzyme as shown in Figure 7.5. The %DH values were used to monitor the extent of the hydrolysis. Addition of another 50% (by weight) of original mass of substrate (original mass of the substrate = 120 g) was added to hydrolysis reaction after 179 minutes of hydrolysis.

If the reaction was limited by the substrate concentration then a sharp increase in the degree of hydrolysis should be observed in the hydrolysis progress curve at this point in time (Guerad et, al., 2001). Figure 7.4 shows that after 179 minutes the addition of more substrate caused a sharp increase in % DH and the hydrolysis reaction continued afresh. This implies that the substrate was almost depleted at about 179 minutes, but there was still ample enzyme activity available. At higher % DH product inhibition may

occur and it may be due to substrate limitation (Adler-Nielsen, 1986).The small dip in the % DH at the beginning of substrate addition was likely due to a reduction in the enzyme catalysed reaction as the result of a sudden reaction dilution, (Bailey and Ollis,1986). The small dip may also be caused by substrate inhibition, as there was a sudden increase of substrate concentration.

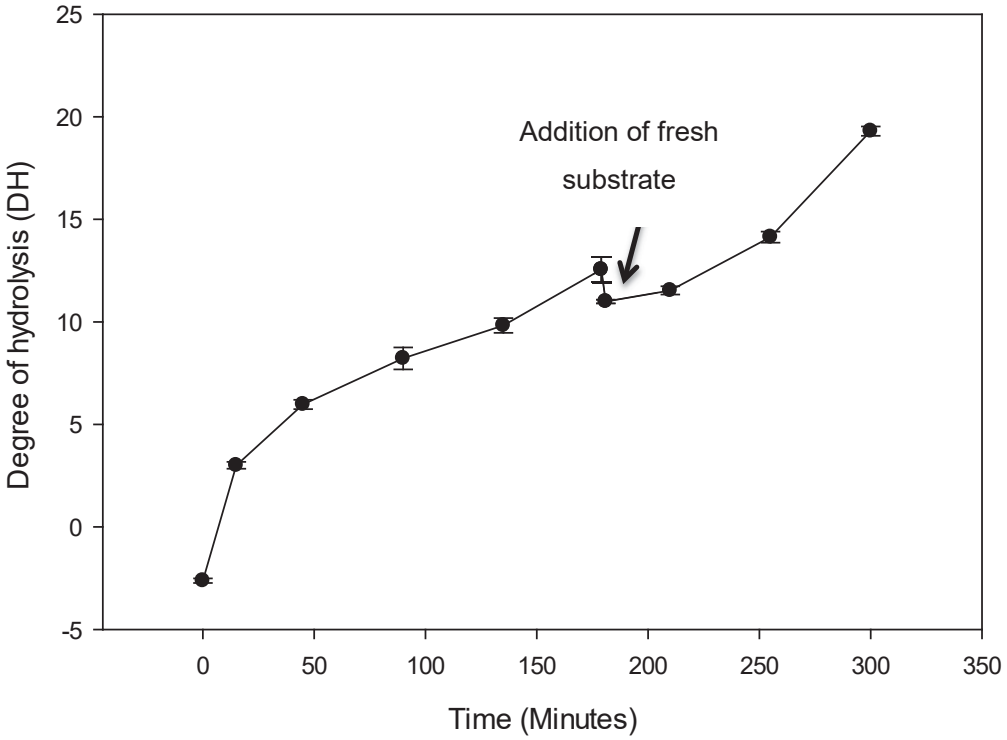

Figure 7.4: Effect of addition of fresh enzyme during hydrolysis. Data points are mean values ± standard deviation, n=4. Hydrolysis at 60°C and 2% w/w enzyme concentration.

Substrate inhibition can occur when high concentrations of substrate are present in the enzymatic process (Bailey & Ollis, 1986). One form of substrate inhibition from meat hydrolysis would be the unproductive binding of the enzyme to non-susceptible peptide bonds. If substrate inhibition occurs, the effect is more prominent as the substrate concentration is increased. This should be noticeable on the plot of initial rate versus substrate concentration; this will be discussed later in the chapter.

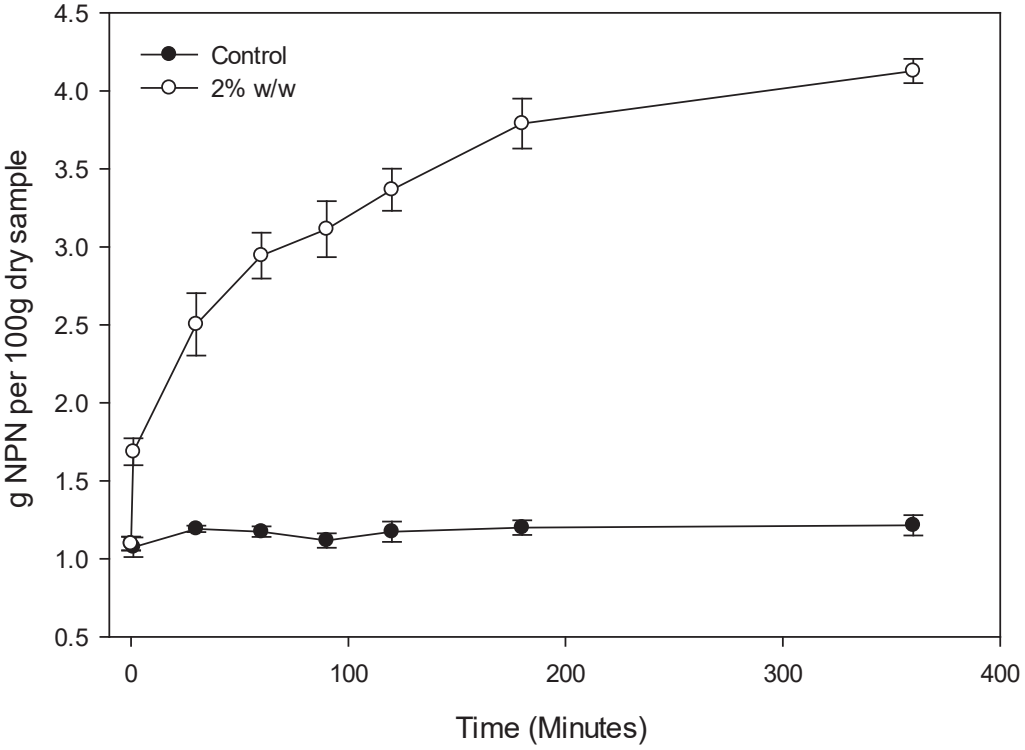

Figure 7.5: Hydrolysis curve of meat hydrolysis using 2% w/w Zyactinase at 60°C and 360 minutes.

Figure 7.5 also shows the majority of peptide bonds in the meat substrate appear to be susceptible to hydrolysis by Zyactinase™ as a steep curve is observed at the beginning of hydrolysis. However, some peptide bonds may be resistant to hydrolysis. This resistance may be caused by steric hindrance from amino acids around the peptide bond, which denies the enzyme access to the peptide bond. (O'Meara and Munro 1984). Resistance to hydrolysis could also be due to lack of suitable adsorption sites around the peptide bond, hence the enzyme was unable to position itself properly on the peptide bond.

To test the existence of enzyme deactivation during the hydrolysis an experiment was carried out with the addition of fresh enzyme after 179 minutes of hydrolysis. Figure 7.6 shows a sharp decreased of % DH on the hydrolysis progress curve after the addition of fresh enzyme. The sudden drop of the % DH after the addition of enzyme was likely due to dilution of the reaction mixture. However, the hydrolysis reaction began rapidly after the addition and enzymatic deactivation did not occur. The typical rapid increase in % DH seen when fresh enzyme was added to fresh substrate (e.g. Fig 7.3) was not

apparent in Fig. 7.6, suggesting that there may have been insufficient substrate or suitable peptide bonds available as no additional substrate was added.

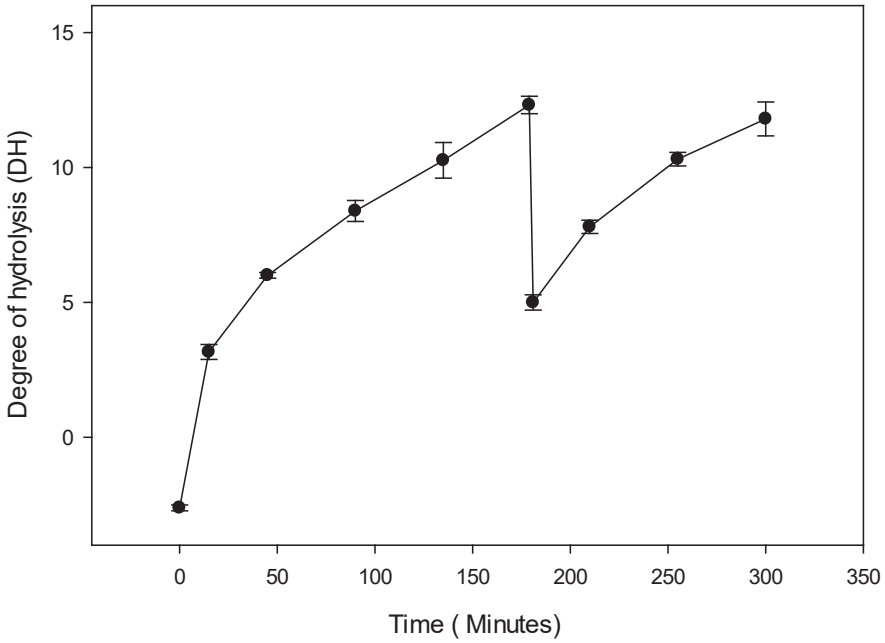

Figure 7.6: Effect of addition of fresh enzyme during hydrolysis. Data points are mean values ± standard deviation, n=4 at Figure 7.4 : Hydrolysis curve at 60°C ,2% w/w and up to 300 minutes hydrolysis time.

The curve in Figure 7.6 started to reach the same level prior to enzyme addition as the hydrolysis process progresses and seemed to reach a plateau around 300 minutes.

7. 5 Reaction rate equation

The Michaelis-Menten equation was used to obtain the rate equations for the meat hydrolysis process. The Michaelis – menten equation was used due to its simplicity. The reaction curve from different % enzyme and time show the same characteristic as meichales menten equation where the initial velocity , start subscript will increase rapidly at low substrate concentrations, then level off to a flat plateau at high substrate concentrations.

Equation 7.2 is the equation for the steady state reaction rate, v.

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad \text{Equation 7.2}$$

Where [S] is the substrate concentration, V_{max} is the limiting or maximum reaction rate and K_m is the Michaelis constant. This equation implies that the reaction rate increases with increasing substrate concentration as an asymptotic curve until it reaches $V_{max.}$ K_m is the substrate concentration when the reaction rate is half of V_{max} and indicates the enzymes affinity towards the substrate, with a small K_m indicating a high affinity. The constants are found after determining the initial linear reaction velocity for various substrate concentrations as shown in Figure 7.7. There are three main zones in the reaction rate (v) versus substrate concentration [S] plot. At very low substrate concentration, the initial velocity (V_0) is proportional to substrate concentration [S]. This zone if linear corresponds to first order kinetics for the enzyme reaction. However, at very high substrate concentration, the velocity is independent of substrate concentration. This zone is known as the zone of zero order kinetics

7.5.1 Initial velocity (V_0) for protein hydrolysis

The hydrolysis reactions were performed at 60°C as it was found in earlier experiments (Chapter 4) that this temperature was optimal for Zyactinase and the meat substrate. The substrate concentrations were varied from 2% to 22.2% protein by addition of distilled water, according to Equations 3.3 to 3.5, to produce 240 g of meat mixture. . The maximum concentration (22.2%) corresponded to the original protein content in the meat, hence no water was added to this sample. The amino acids released were monitored using the OPA method.

The hydrolysis progress curve shown in Figure 7.7 shows the release of an amino acid (serine) as a function of time at 60°C, with 2% w/w enzyme and 22.2% substrate concentration.

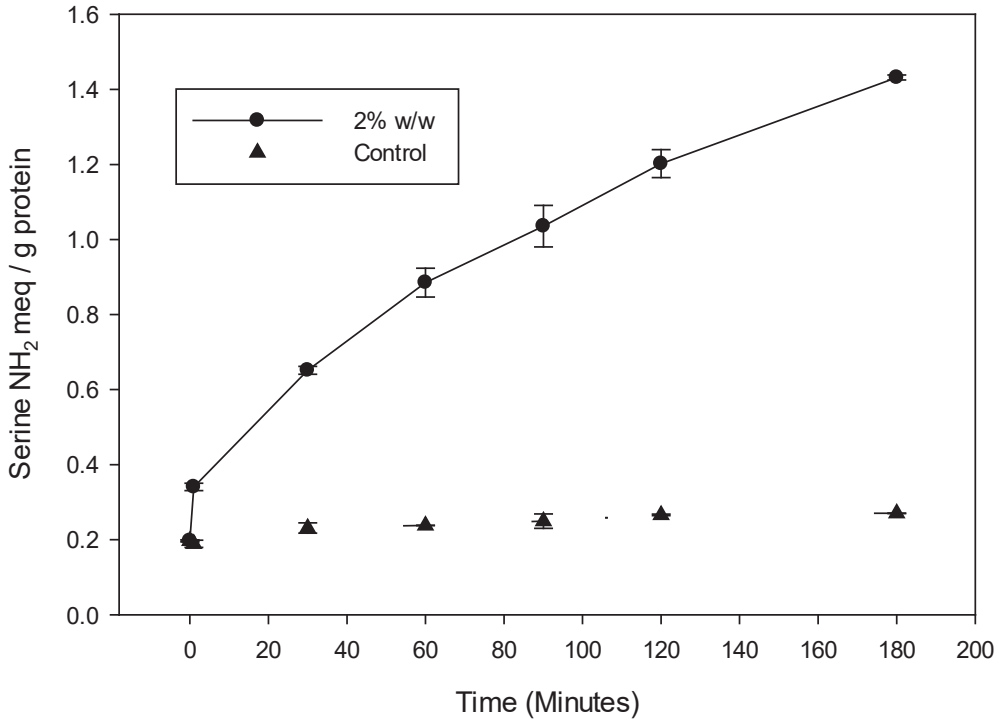

Figure 7.7: Progress curve for amino acid released during hydrolysis reactions at 60°C and substrate concentration 22.2% protein for 180 minutes. Control – no enzyme addition and 2%w/w enzyme added. Data points are mean values ± standard deviation, n=2 to 4.

Figure 7.7 shows that concentration of the amino acid released increased as the reaction time proceeded. The initial velocity (V_0) was determined over the initial portion of the reaction curve. This allowed the estimation of some kinetic parameters at the early stages of the reaction. In this initial part of the reaction, enzyme inactivation would have been negligible and at t = 0 product inhibition can also be ignored. The initial reaction rate velocities (V_0) for each protein concentration were determined as the slope of the amino acid concentration versus time plots over the first five minutes of the hydrolysis reaction.

Figures 7.8 to 7.10 show the concentrations of amino acid released (serine) after five minutes of reaction time for different substrate concentrations and enzyme concentrations (2, 4 and 7.5 w/w).

The initial velocity measurements are only valid if the substrate depletion is between 0 to 10% of the total initial substrate concentration (Copeland, 2000). This is to ensure

that product inhibition will not occur at this stage, as this can contribute to significant error in the estimation of initial rates.

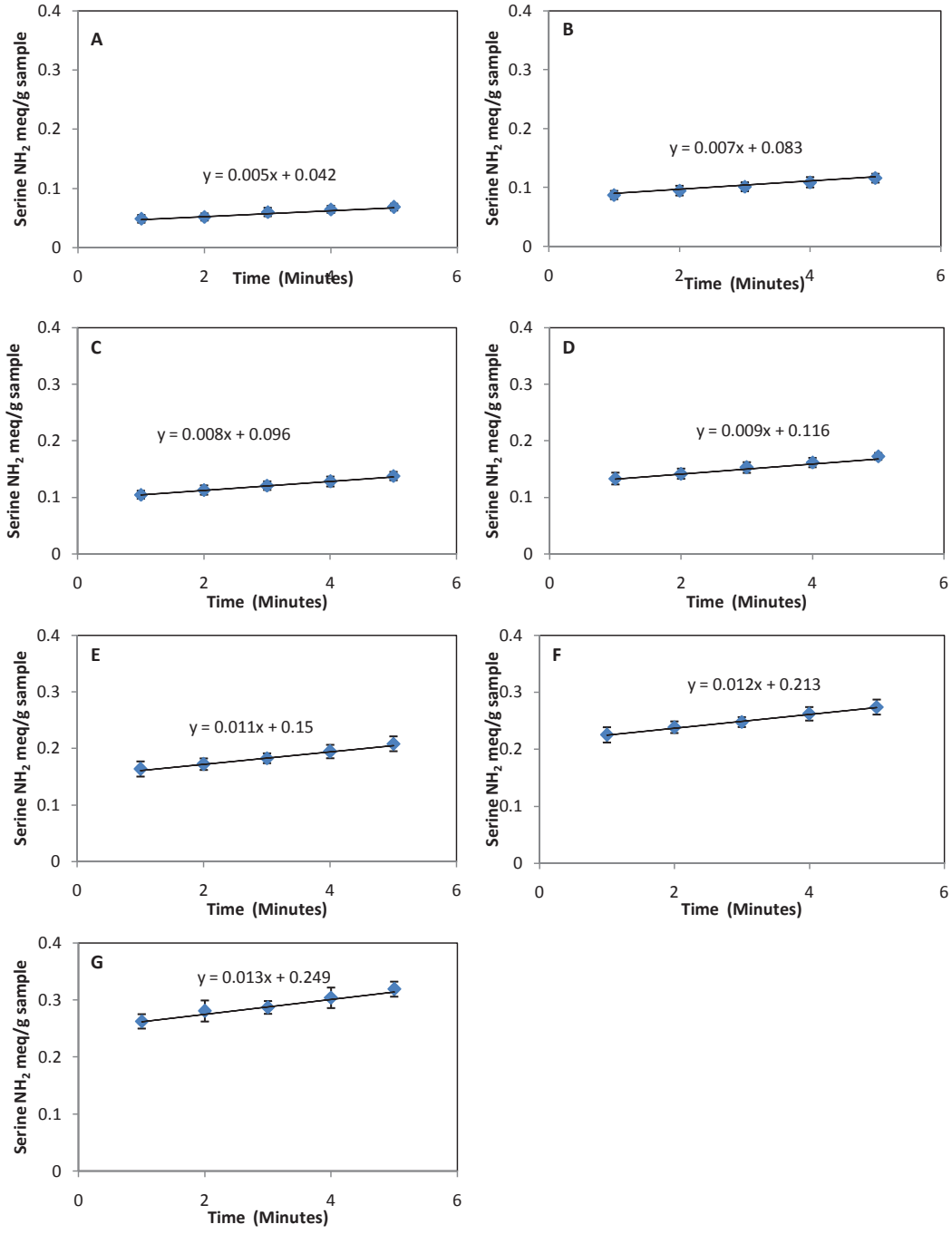

Figure 7.8: Determination of initial velocity (V_0) for the linear part of the hydrolysis progress curve at different substrate concentrations and 2% w/w enzyme concentration. Substrate concentration % w/w protein: A= 2.0%, B= 4.0%, C=6.0%, D=8.0%, E=10.0%, F= 15.0% and G = 22.2%. Data points are mean values ± standard deviation, n = 4.

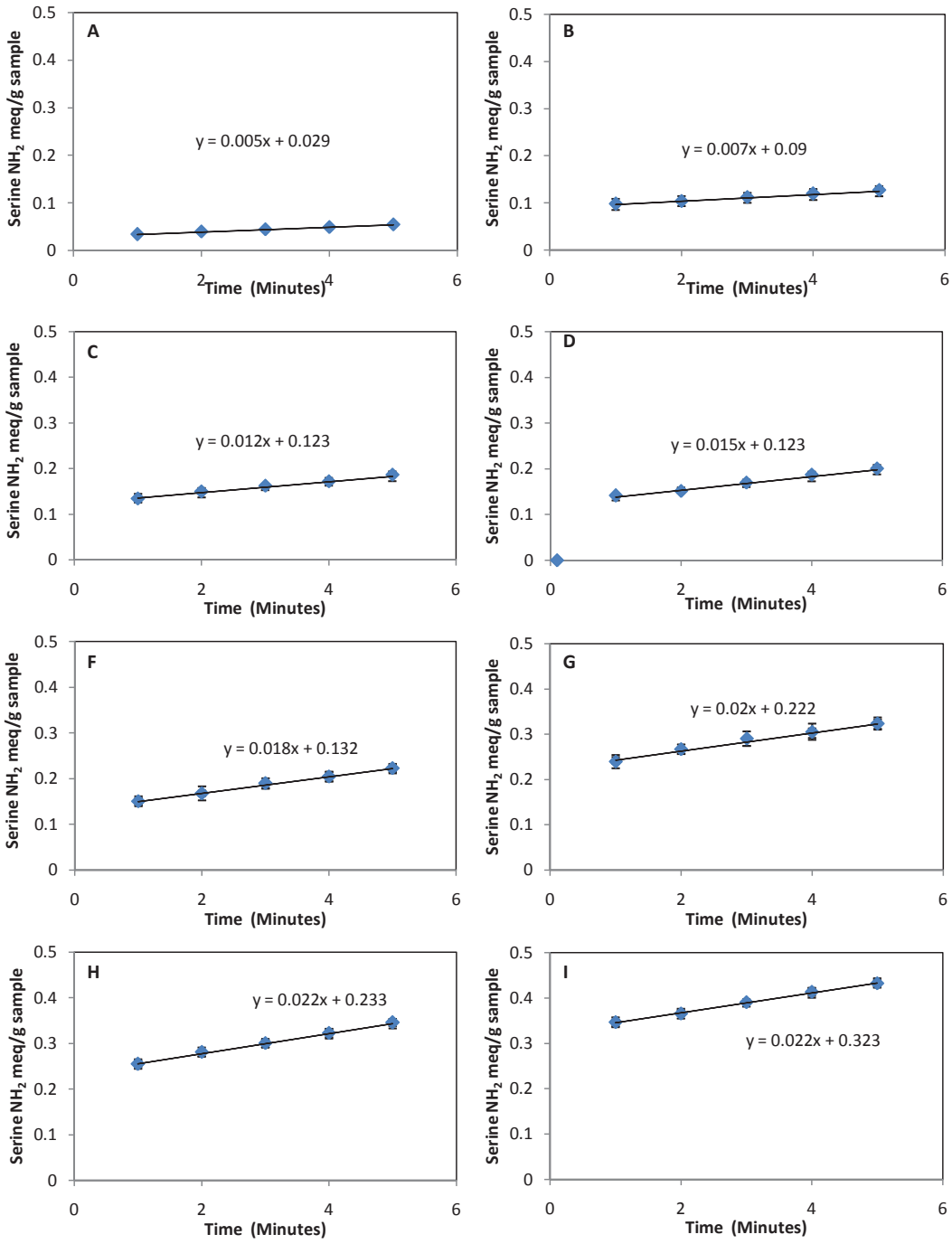

Figure 7.9: Determination of initial velocity (V_0) for the linear part of the hydrolysis progress curve at different substrate concentrations and 4% w/w enzyme concentration. Substrate concentration % w/w protein: A= 2.0%, B=4.0%, C=6.0%, D=8.0%, F=10.0%, G= 12.0%, H = 15.0%, I=22.2% . Data points are mean values ± standard deviation, n = 4.

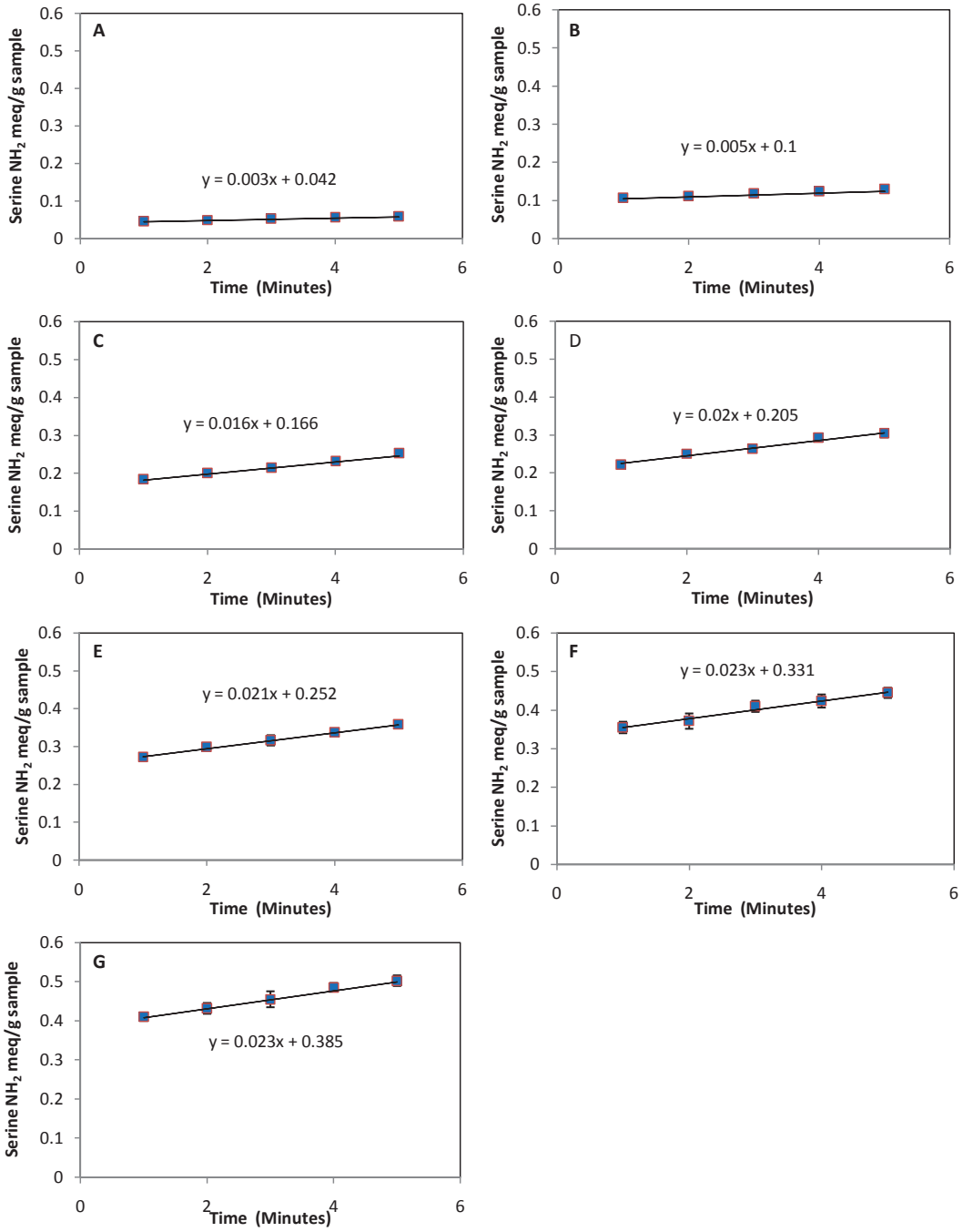

Figure 7.10: Determination of initial velocity (V_0) for the linear part of the hydrolysis progress curve at different substrate concentration and 7.5% w/w enzyme concentration. Substrate concentration % w/w protein: A= 2.0%, B= 4.0%, C=6.0%, D=8.0%, E=10.0%, F= 15.0% and G = 22.2%.Data points are mean values ± standard deviation, n = 4.

7.5.2 Fitting data to classical Michaelis-Menten Kinetics

The plot of initial velocity versus substrate concentration illustrates a property of the enzyme catalysed reaction. Saturation of enzyme with substrate results in the maximum obtainable velocity (V_{max}). The Michaelis-Menten equation predicts that the initial reaction velocity (V_0 (serine NH_2 meq g^{-1} min^{-1})) will vary with respect to substrate concentration [S]. In Figure 7.11 the initial reaction velocity versus substrate concentration curves for 2 %w/w and 4% w/w enzyme shows a hyperbolic pattern whereas the curve for 7.5% w/w enzyme concentration shows a slightly sigmoid shape, where the initial velocities are low at low substrate concentration (2% and 4% w/w protein).

Although the data in Figure 7.10 appears to show a typical Michaelis-Menten relationship, a simple test was carried out with the observed data to determine if it could be described by that model. The data were analysed to determine their fit to an hyperbolic function as shown in Equation 7.3.

$$y = \frac{ax}{b+x} \qquad \text{Equation 7.3}$$

Where y is the velocity, x is the substrate concentration; a represents V_{max} and b represents K_m.

The hyperbolic fit to 2%, 4% and 7.5% w/w enzyme concentration is shown in in Figure 7.11 The R^2 correlation coefficient for each enzyme concentration was found to be 0.977, 0.947 and 0.834, respectively. As more complex enzymatic reactions may be involved with multiple substrates or inhibitory products, the use of a non-linear regression relationship (Equation 7.4) can be used.

The curves in Figure 7.12 can be transformed into linear plots using the double reciprocal (Lineweaver-Burk) plot as shown in Equation 7.4.The double reciprocal (Lineweaver–Burk) plot is the most common linear transformation (Fukuwaka et al., 1985).

$$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \qquad \text{Equation 7.4}$$

The Lineweaver-Burk plot of 1/v versus 1/[S] gives a straight line which has a slope of K_m/V_{max}. K_m (Michaelis constant) and V_{max} were determined using the equation found in

each linear transformation. The transformation plot for 2% and 4% w/w enzyme concentrations gave straight lines with R^2 of 0.969 and 0.968, respectively. Transformation for 7.5% w/w enzyme concentration was found to slightly deviate from a linear relationship, especially at lower substrate concentrations of 2% and 4% w/w protein. K_m and V_{max} were calculated for 2, 4 and 7.5% enzyme concentrations and presented in Table 7.1.

Fitting the Michaelis-Menten equation to the progress curves allowed the determination of V_{max} and K_m values. There was no evidence of interactions or interference from other materials in the substrate that may hinder the reaction. The K_m and V_{max} for different enzyme concentrations did not present any recognizable pattern (Table 7.1). Higher enzyme concentration resulted in higher V_{max} values.

The hydrolysis reaction required at least 5.27 % (w/w protein) (Table 7.1) substrate concentration in order to achieve half the maximum reaction rate for the production of the amino acid (serine). The K_m was low which indicated that there was a strong affinity of the enzyme Zyactinase™ towards beef meat protein as a substrate.

Table 7.1: Michaelis-Menten K_m and V_{max} values for meat hydrolysis at different Zyactinase™ enzyme concentrations – from Lineweaver-Burk transformations.

Enzyme concentration (%w/w)	K_m (%)		V_{max} (meq g^{-1} min^{-1})	
	Non-linear fitting (Figure 7.11)	Lineweaver-Burk transformation (Eq 7.4)	Non-linear fitting	Lineweaver-Burk transformation
2 %	5.27	3.74	0.016	0.015
4%	10.83	15.06	0.036	0.041
7.5%	10.28	NIL	0.037	NIL

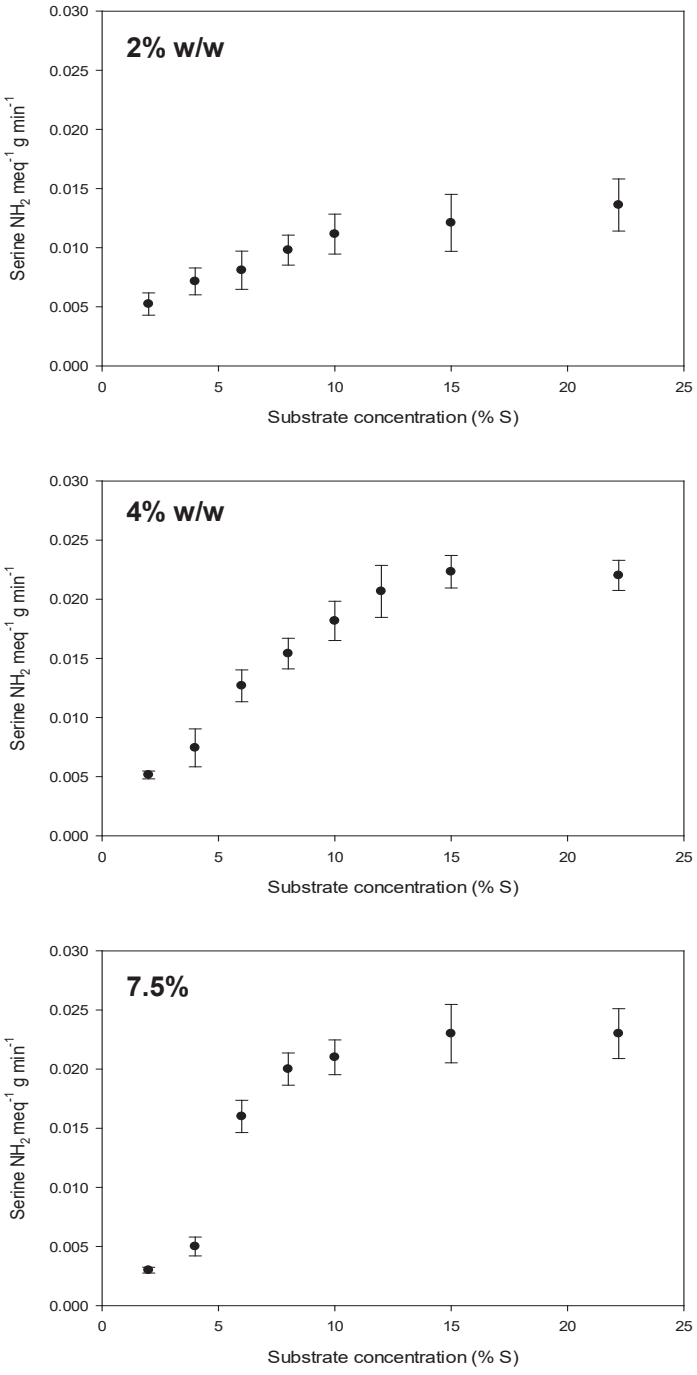

Figure 7.11: Initial velocity (Serine NH_2 (meq g^{-1} min^{-1})) from at different substrate concentrations (Enzyme concentration = 2, 4 and 7.5 % w/w protein). Data points are mean values ± standard deviation, n= 4.

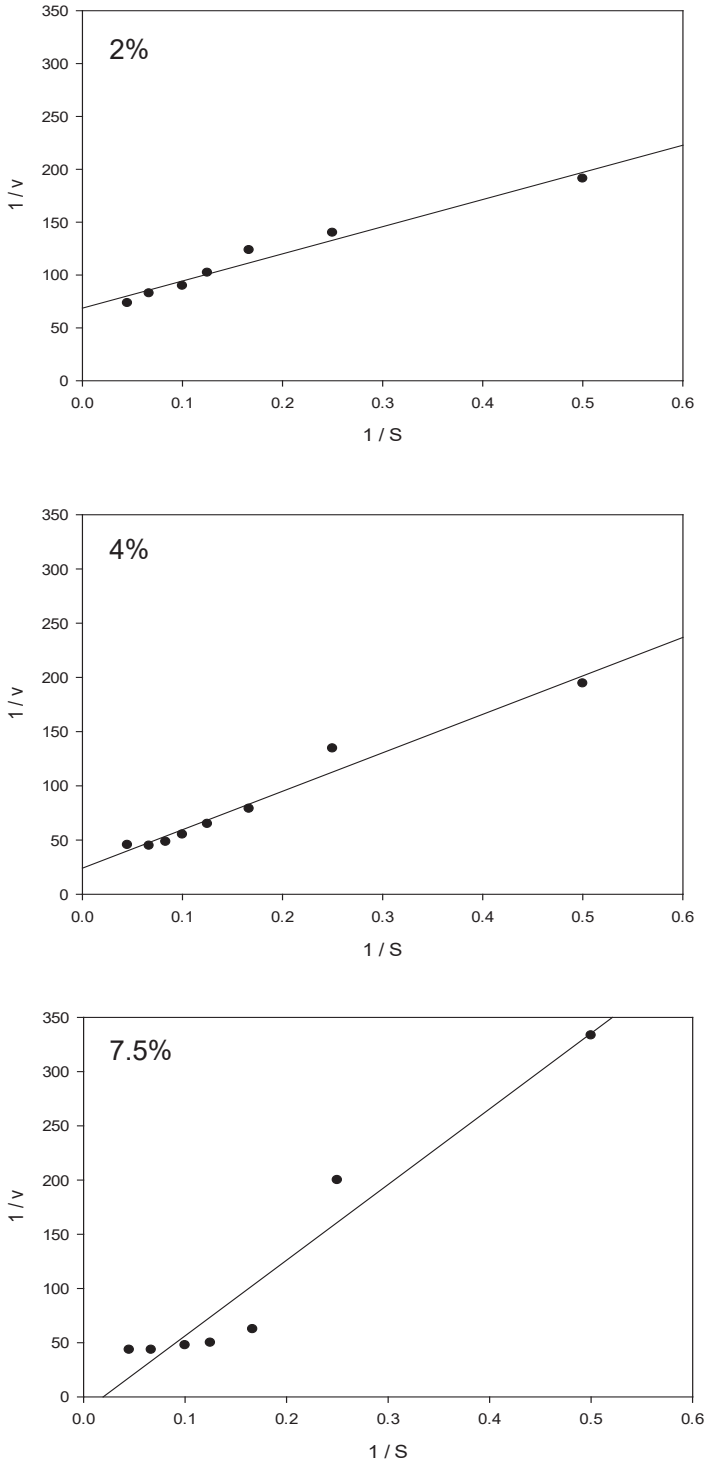

Figure 7.12: Lineweaver-Burk transformation of the progress curve at different substrate concentrations (1/S where S = %S or [S], 60°C. Enzyme concentrations 2, 4 and 7.5% w/w. Data points are mean values ± standard deviation, n= 4.

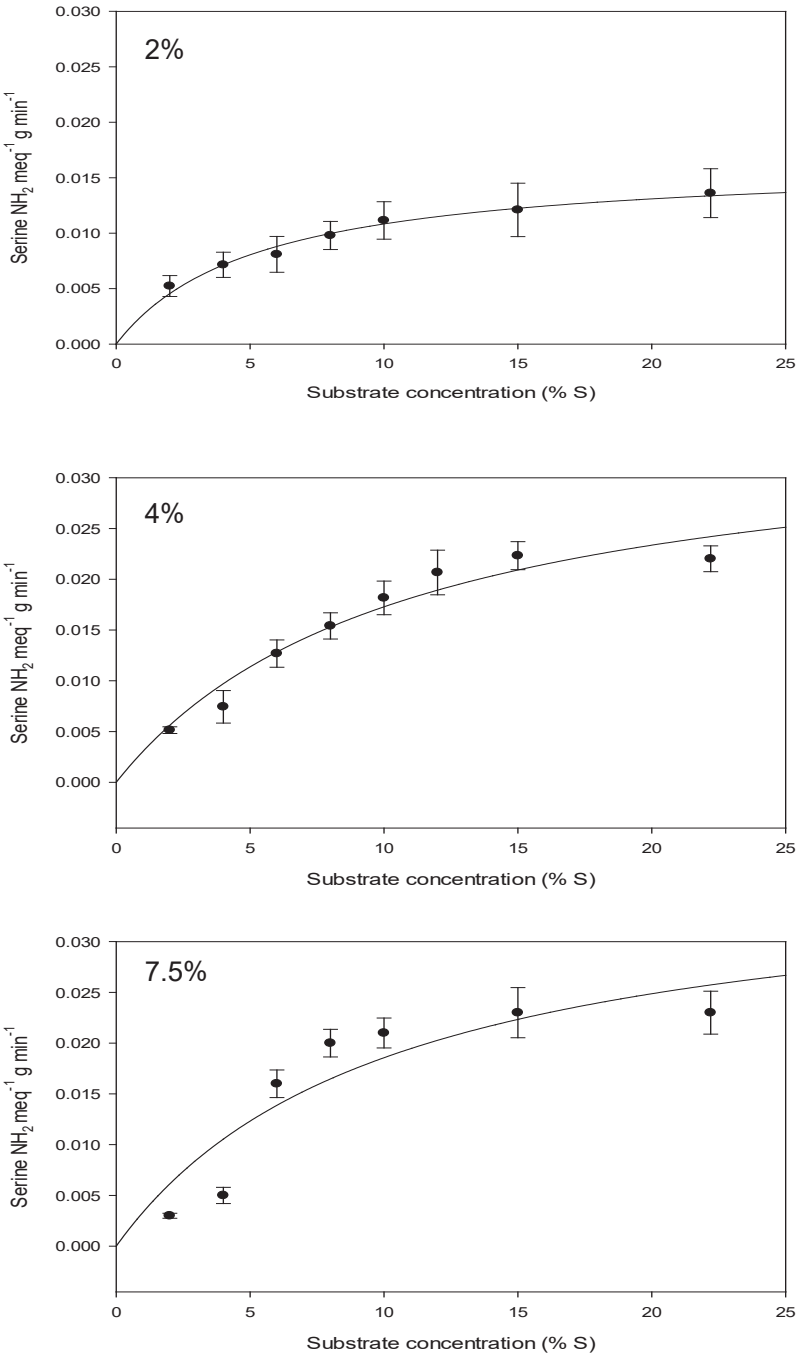

Figure 7.13: Enzyme rate with predicted hyperbolic kinetic pattern on progress curve at different substrate concentrations (1/S where S =% S or [S], 60°C. Enzyme concentrations 2, 4 and 7.5% w/w. Data points are mean values ± standard deviation, n= 4.

7.6 Enzyme Concentration

In order to see the effect of enzyme concentration on the initial rate, the protein hydrolysis was carried out at high substrate concentration where substrate saturation was accomplished. Figure 7.14 shows the rate of amino acid release at 10% (w/w protein) substrate concentration and for different enzyme concentrations. The slope of the linear part of the progress curves for amino acid released at different enzyme concentrations (Figures 7.8 – 7.10) was calculated to obtain an initial velocity value at different enzyme concentrations to construct Figure 7.14.

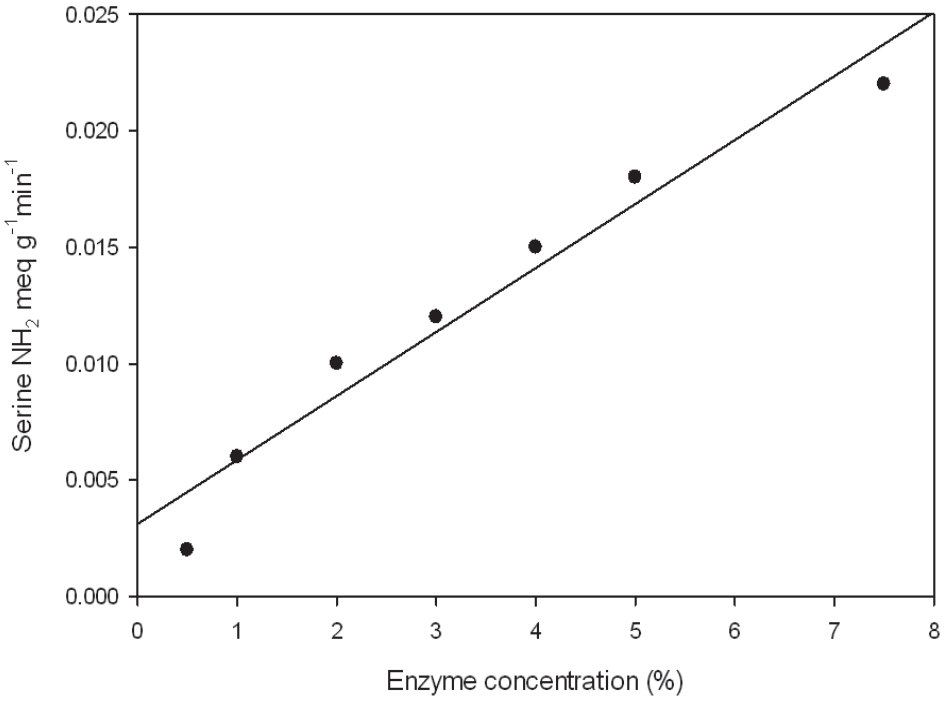

Figure 7.14: Initial rate of amino acid released at different enzyme concentrations (%w/w) and 10% (w/w protein) substrate concentration.

The free amino acids released showed a linear increase with increasing enzyme concentration. A plot of reaction velocity against enzyme concentration (Figure 7.14) gave a linear relationship with R =0.951. Deviations from the straight line were observed for the lowest (0.5%w/w) and the highest (7.5% w/w) enzyme concentrations. The straight line from 1 to 5% enzyme concentrations indicates that the enzyme reaction is first order and progresses faster to the right of Equation 7.1, where many end-products are formed. Substrate inhibition may have occurred at the lowest enzyme concentration because of the relatively high substrate:enzyme ratio, or the enzyme only reacted with the most susceptible peptide bonds that could be easily reached leaving the less susceptible bonds undigested (Clark, Cutler, O'Meara & Munro, 1987).

The deviation at the high enzyme concentration probably reflects insufficient substrate available for maximum reaction rate.

7.7 Summary

This chapter showed the meat hydrolysis curve using Zyactinase™ enzyme had initially a rapid hydrolysis reaction rate followed by a slight reduction in reaction rate, followed by a levelling off of the reaction rate as time progressed. There was substrate inhibition occurring during the hydrolysis process and there was no appearance enzyme inactivation occurring during the hydrolysis process by Zyactinase™. The meat hydrolysis using Zyactinase™ enzyme showed a deviations from the straight line for the lowest (0.5%w/w) and the highest (7.5% w/w) enzyme concentrations. The meat hydrolyis by Zyactinase™ at 1% w/w to 5 % w/w enzyme concentration seem to fit the Michaelis-Menten equation.

Chapter 8
Overall Discussion

The objectives of this research were to:

1. To determine the extent to which Zyactinase™ enzyme complex can hydrolyse beef meat under various conditions including pH, temperature, substrate and enzyme concentration. The extent will be assessed by degree of solubilisation of hydrolysed protein and monitoring the peptides and amino acids formed.
2. To determine the extent to which Zyactinase™ enzyme complex can hydrolyse specific protein fractions from beef meat.
3. To mathematically model for the reaction mechanism for the hydrolysis of beef meat with Zyactinase™ enzyme complex.

All the data from the studies show that the enzyme partially hydrolyses the whole beef protein in such a way it help the understanding the overall reaction of enzyme toward beef protein fraction.

The enzyme Zyactinase™ activates different reactions in different meat protein fractions (Chapter 6). In this study the whole beef meat was separated into three major meat protein fractions; myofibrillar tissue, connective tissue and a liquid portion containing the sarcoplasmic proteins. The meat protein fractions and diluted lean beef meat were then subjected to hydrolysis with Zyactinase™ at different processing temperatures, hydrolysis times and concentrations of enzyme. The myofibrillar protein fraction was found to be mostly hydrolysed and solubilised by the Zyactinase™, followed by hydrolysis of the sarcoplasmic fraction and lastly connective tissue. There were two contradictory reports on the susceptibility of sarcoplasmic proteins toward enzymatic degradation. Mohr (1980) stated that heat denatured beef sarcoplasmic protein was resistant to proteolytic attack whereas O'Meara & Munro (1985) found sarcoplasmic protein from beef was easily solubilised with proteolytic enzymes. In general, the denatured globular protein is relatively easy to hydrolyse compared to native sarcoplasmic protein (Adler-Nissen, 1976). Zyactinase ™ was able to degrade the denatured sarcoplasmic protein extract but there were still insoluble particulates present at the end of the hydrolysis reaction. This implied that Zyactinase™ did not hydrolyse all the proteins as reported for other proteases used in the reported literature. Ficin induced major changes in physiochemical properties of myofibrillar

proteins (Ramezani et al., 2003) including aggregation and precipitation. Zyactinase™ gave similar results to ficin or kiwifruit but did not show the same extent of hydrolysis when compared with papain (Lewis & Luh,1988).

The Zyactinase™ enzyme was found to have enzyme activity that follows the Michaelis-Menten equation within the range of 1% to 5% $^{w}/_{w}$ enzyme concentration. Above 5% enzyme concentration a slight deviation from Michaelis-Menten behaviour occurred especially at lower substrate concentrations (2% and 4%$^{w}/_{w}$) (Chapter 7). The progress curves for meat hydrolysis using Zyactinase™ showed initially a rapid hydrolysis reaction rate then a slight reduction in reaction rate, followed by a levelling off of the reaction rate as time progressed. A similar curve was reported for the enzymatic hydrolysis of crayfish by-product (Baek & Cadwallader, 1995), capelin (Shahidi et al., 1995), sardine (Quaglia & Orban, 1987), and veal bone (Linder et al., 1996). The levelling off of the reaction rate progress curves indicated the depletion of specific substrates, as adding more substrate caused the reaction to accelerate again. Hence Zyactinase™ must only work on specific substrates.

The enzymic hydrolysis of meat proteins by Zyactinase™ was affected by the solution pH, temperature and substrate concentration (Chapter 4). The optimum pH for hydrolysis was between pH 4 to pH 5.6 which is the natural pH of the meat. Hence, pH adjustment was not required to optimise hydrolysis. The optimum temperature for Zyactinase™ was found to be 55°C to 65°C. Increasing enzyme concentrations up to 4% w/w resulted in 25% increase in production of NPN. An increase in substrate concentration resulted in a linear increase in production of NPN. The results from SDS PAGE from the whole meat also indicated that some of the protein bands disappeared, giving lower intensities on the gels. Whereas some of the major protein bands remained unchanged and there were new protein bands that appeared at lower molecular weights. These results indicated that the Zyactinase™ enzyme may have different hydrolytic activity towards different fractions of meat protein, again supporting the hypothesis that Zyactinase™ was very selective for specific protein fractions (Chapter 6). Different reaction temperatures and enzyme concentrations used also affected the peptide and amino acid profile of the hydrolysate produced.

The study of meat hydrolysis by Zyactinase™ enzyme provided a good overview of the enzyme's ability to hydrolyse beef meat protein fractions. Previous studies with kiwifruit enzymes have shown mild reaction compared to the other protease enzymes such as

papain and ficin. The reaction or activity of Zyactinase^{TM} depended upon the substrate type. For example, myofibrillar proteins, the major structural elements of the muscle, were readily and quickly digested, whereas the water-soluble sarcoplasmic proteins were less comprehensively digested and the connective tissue proteins were poorly digested. This allowed the production of a final product which could have specific functional properties for specific applications. The reaction time was very important to ensure bitterness was minimised by controlling the quantity of bitter peptides produced. However there was no sensory analysis completed to evaluate the flavour of the hydrolysates produced.

Chapter 9
Conclusion and Recommendations

9.1 Conclusions

Taken together, the result of NPN from different parameters and SDS–PAGE suggested that Zyactinase™ enzyme was able to partially hydrolysis beef meat. The protein materials were digested to smaller proteins, peptides and amino acid. The optimum pH for hydrolysis was between 4 and 5.6 and pH 5.6 was the natural pH of the meat. Hence, pH adjustment was not required to optimise hydrolysis. The optimum temperature for meat hydrolysis using Zyactinase™ enzyme was found to be 60 ºC**.** Increasing enzyme concentrations do increase the hydrolysis rate such as the use of 4% w/w enzyme concentration resulted in 18% increase in production of NPN compared to 2% w/w enzyme concentration. An increase in substrate concentration is linearly correlated with the production of NPN. The increase of peptides also corresponding linearly to the amount NPN produced. Higher temperatures and enzyme concentrations led to faster increase in release of peptide residues. More hydrophobic peptides were produced in the hydrolysates as the time of hydrolysis progressed. Higher temperatures and enzyme concentrations led to faster increase in release of peptide residues.

Zyactinase™ enzyme was also found to digest protein in myofibrillar and sarcoplasmic meat fractions at similar rates to that observed in whole meat. Large molecular weight proteins such as myosin and β and α-actinin were quickly digested while actin (42 kDa) was left seemingly untouched. During both myofilbrillar and sarcoplasmic hydrolysis low molecular weight proteins with molecular weight < 20kDa were produced. Connective tissue was hydrolysed by Zyactinase™ but to a much lesser extent than the myrofibrillar and sarcoplasmic fractions. The large molecular weight collagen protein moiety was found to decrease while smaller molecular weight proteins appeared. The collagen protein structure contributed to its resistance to hydrolysis. In SDS- PAGE of meat hydrolysates, some of larger molecular weight protein band still remain in shows that Zyactinase™ reacts moderately and can be consider as a specific enzyme.

Meat hydrolysis curve using Zyactinase™ enzyme had initially a rapid hydrolysis reaction rate followed by a slight reduction in reaction rate, followed by a levelling off of the reaction rate as time progressed. There was substrate inhibition occurring during the hydrolysis process and there was no enzyme inactivation occurring during the

hydrolysis process by Zyactinase™ enzyme. The additions of new substrate after the reaction reach plateau show the increase of production of NPN that may suggest the depletion of current substrate. This may further conclude that Zyactinase™ enzyme selectively digested the meat protein. Considering the fact that enzyme activation does not occur during the hydrolysis process the application of this enzyme on selectively meat hydrolysis industry may be economical and effective.

9.2 Recommendations for future work

In order to improve the current framework study and economic viability of the current process of hydrolysis using Zyactinase™ enzyme several studies are suggested:

To explore the effect of Zyactinase™ toward other protein materials to look for alternatives meat sources.
To identify the peptides in the meat hydrolysates and isolate or identify the bitter peptides to increase the acceptability of meat hydrolysate.
To determine the bioactive peptides that can be used for high-end application.
To study the effect of Zyactinase™ on functional properties of meat protein hydrolysate to be use as food ingredient/ application.
To study the acceptability of meat protein hydrolysate by Zyactinase™.
To scale up the current process as a production scale and study the stability of the meat hydrolysate produced by Zyactinase™

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Appendix A. 1.1

Table 1: Moisture content (g water per 100g sample) for hydrolysis at 50°C (Result are mean of 3 replicates, ± standard deviation)

Processing Time (Minute)	Enzymes concentration (% w)			
	Control 0%	1%	2%	4%
0	72.68 ± 1.04			
1	70.07 ± 0.41	71.69 ± 1.39	71.01 ±0.38	71.58 ± 1.30
30	70.60 ± 0.52	71.80 ± 0.57	70.32±1.24	70.49 ± 0.23
60	68.27 ± 2.63	71.08 ± 1.04	68.25±1.00	66.94 ± 0.26
90	66.85 ± 3.77	70.27 ± 0.42	67.10± 0.94	67.91 ± 0.35
120	65.44 ± 2.94	68.64 ± 1.35	66.23 ± 0.87	66.72± 1.35
180	65.91± 2.41	65.40± 0.92	65.30 ± 0.97	66.39± 1.05

Table 2: Moisture content (g water per 100g sample) for hydrolysis at 60°C (Result are mean of 3 replicates, ± standard deviation)

Processing Time (Minute)	Enzymes concentration (% w)			
	Control 0%	1%	2%	4%
0	72.68 ± 1.04			
1	72.91 ± 1.62	72.51± 0.20	72.26 ±0.91	72.91 ± 0.11
30	69.51 ± 0.50	71.31 ± 0.13	70.40±1.19	69.51 ± 0.10
60	66.94 ± 1.19	70.50 ± 0.20	69.21±0.54	66.94 ±0.38
90	64.75 ± 0.96	68.72± 0.14	67.97± 0.54	64.75 ± 0.21
120	65.83 ± 2.03	67.98 ± 0.58	66.72 ± 0.50	65.83 ± 0.17
180	65.33± 0.41	67.02 ± 0.33	65.92 ± 0.72	65.33 ± 0.30

Appendix A. 1.2

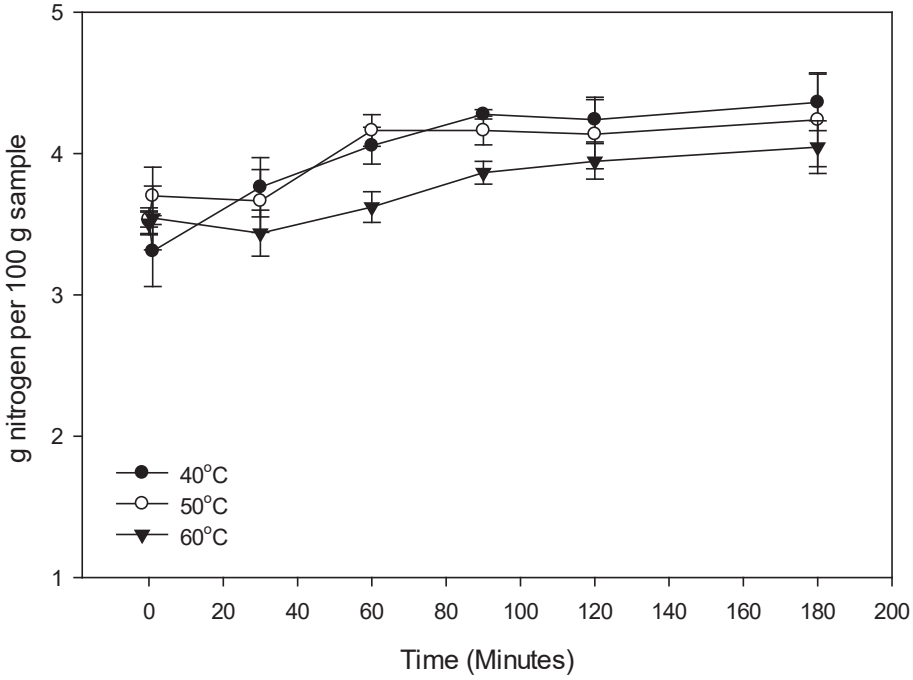

Figure 1: Total nitrogen content (Wet basis) in hydrolysate reactions at different temperature and no enzyme addition concentration

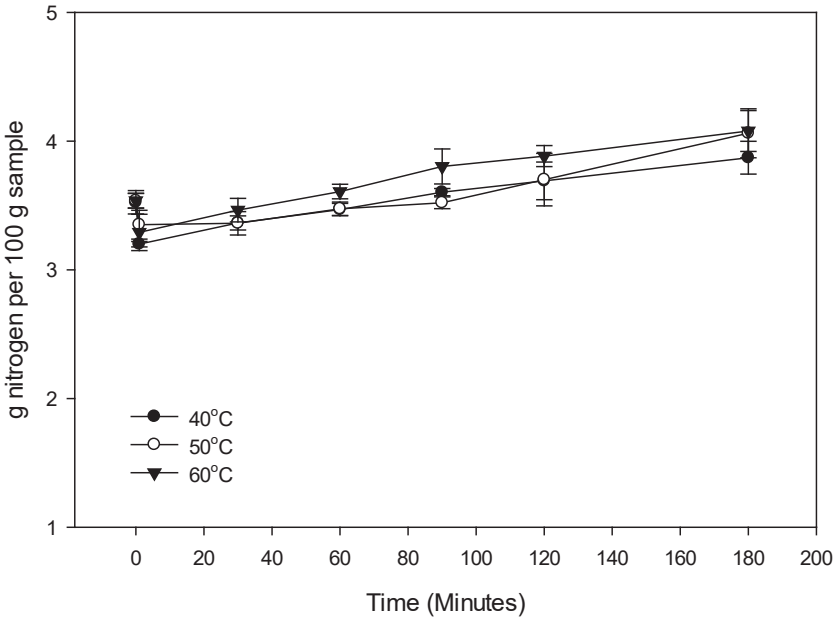

Figure 2: Total nitrogen content (Wet basis) in hydrolysate reactions at different temperature and 1% w/w concentration

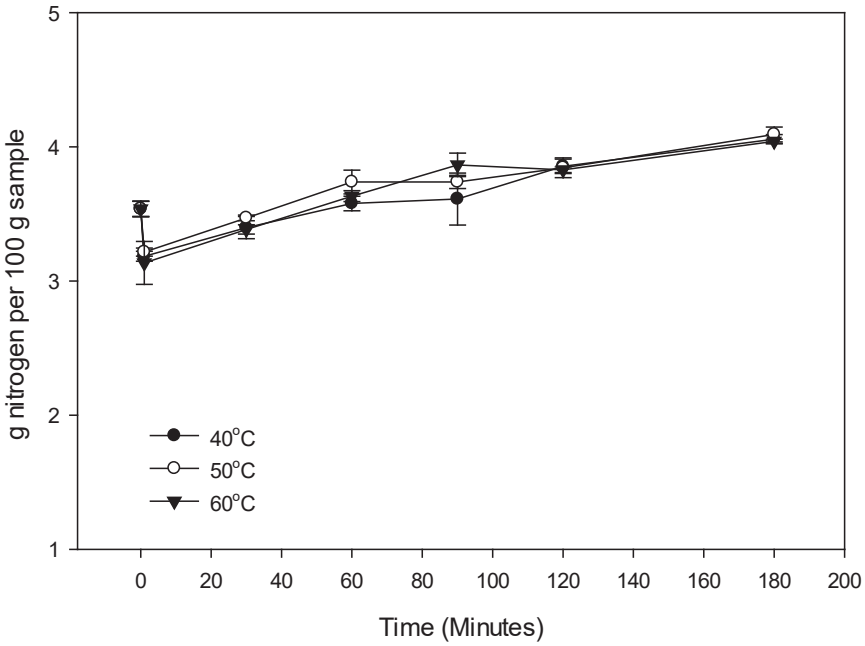

Figure 3 : Total nitrogen content (Wet basis) in hydrolysate reactions at different temperature and 2% w/w concentration

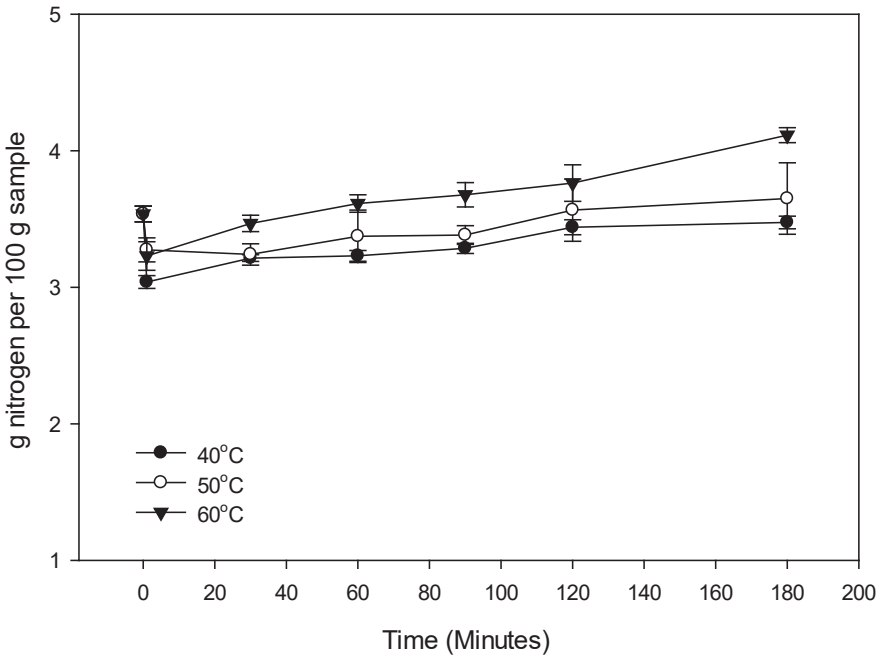

Figure 4 : Total nitrogen content (Wet basis) in hydrolysate reactions at different temperature and 4% w/w concentration

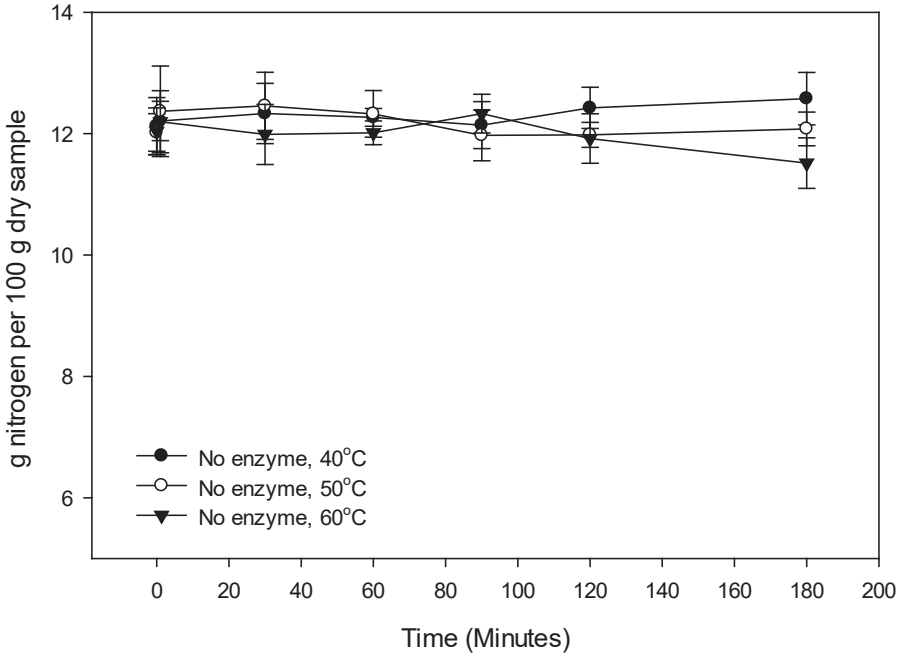

Figure 5 : Total nitrogen content (dry basis) in hydrolysate reactions at different temperature and no enzyme addition w/w concentration

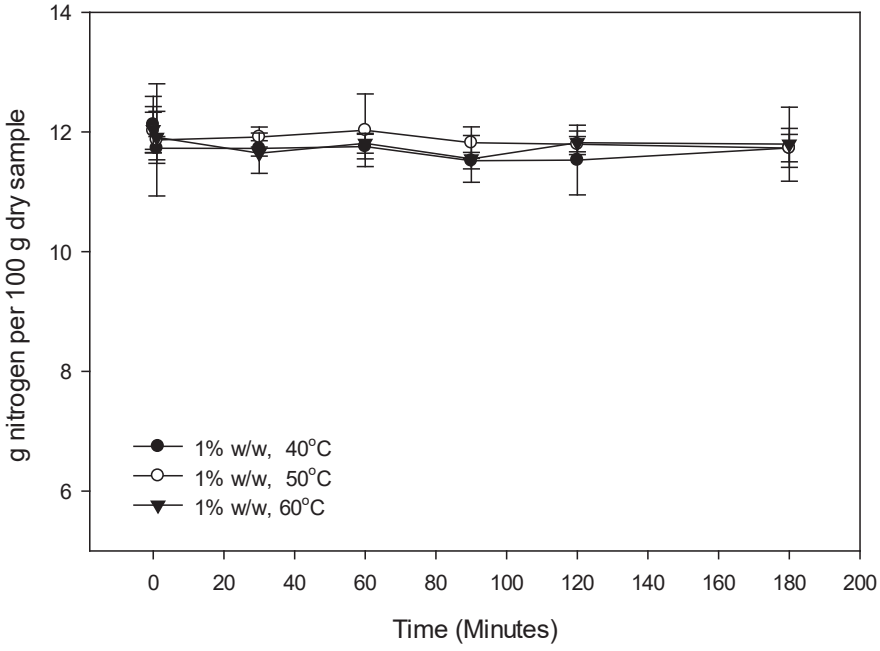

Figure 6 : Total nitrogen content (dry basis) in hydrolysate reactions at different temperature and 1% w/w concentration

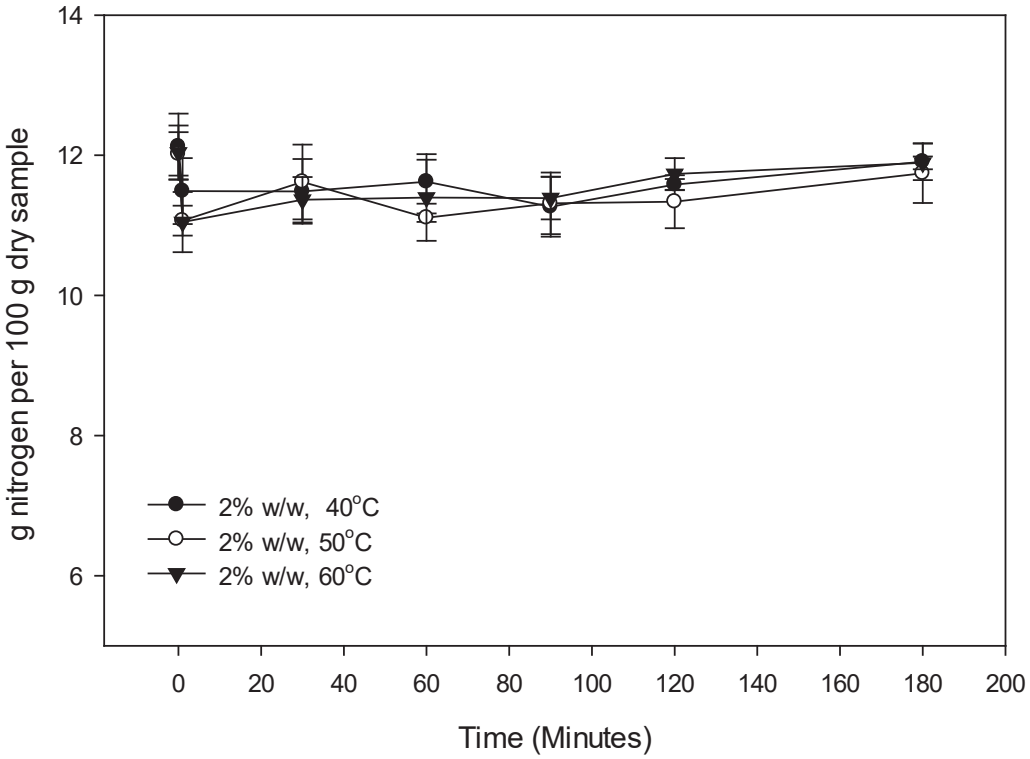

Figure 7 : Total nitrogen content (dry basis) in hydrolysate reactions at different temperature and 2% w/w concentration

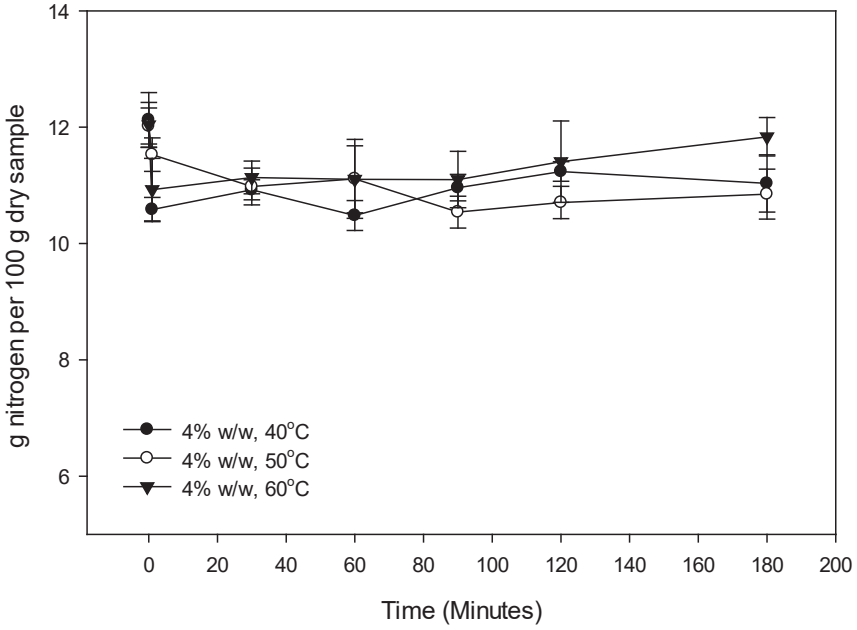

Figure 8 : Total nitrogen content (dry basis) in hydrolysate reactions at different temperature and 4% w/w concentration

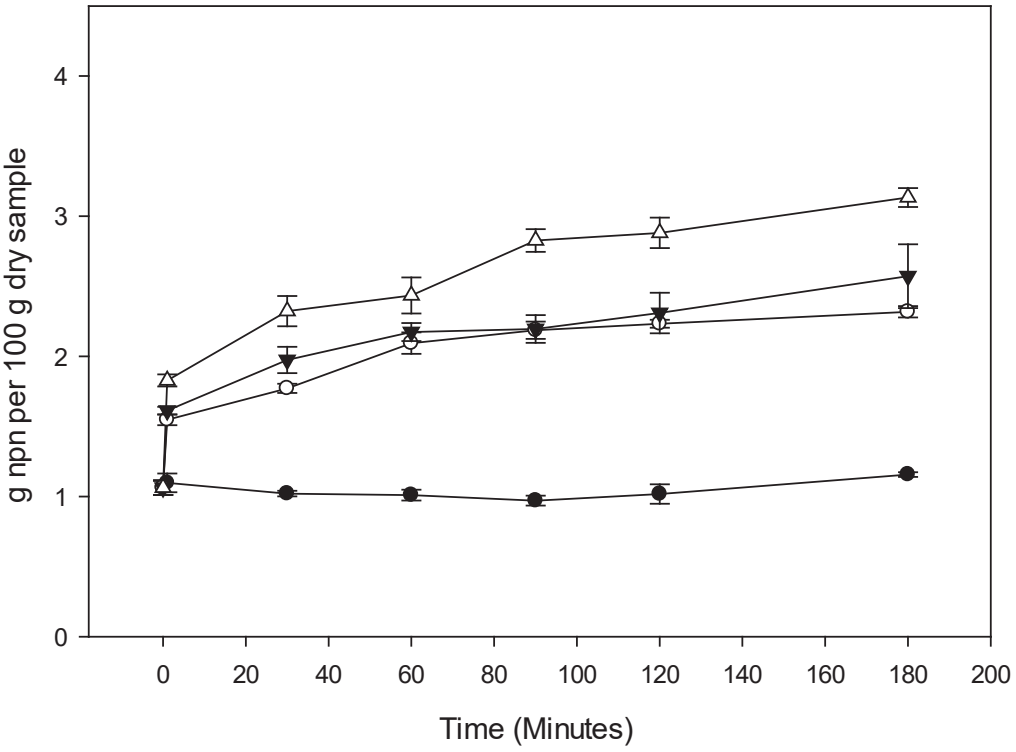

Figure 9: Total nitrogen content (dry basis) in hydrolysate reactions at different enzyme concentration and 40 C.

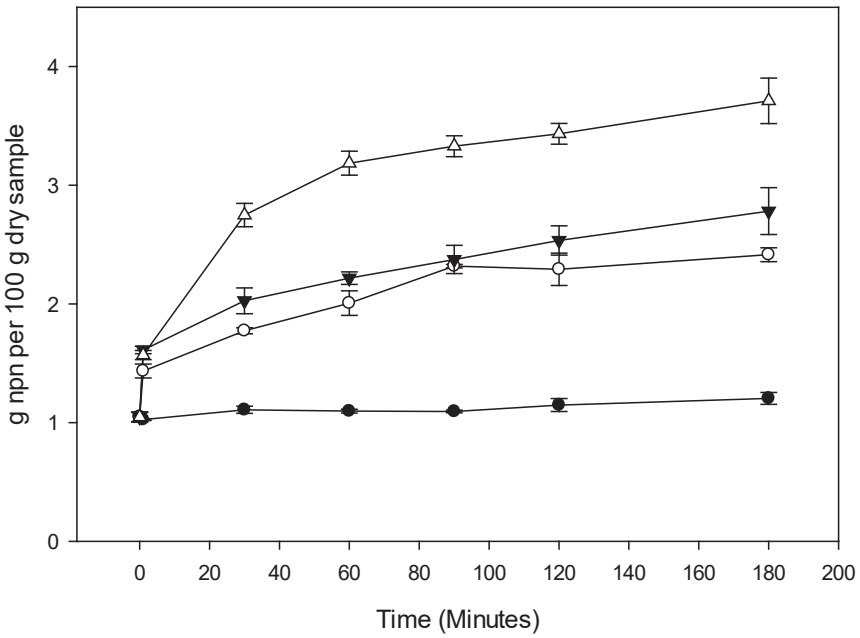

Figure 10: Total nitrogen content (dry basis) in hydrolysate reactions at different enzyme concentration and 50 C.

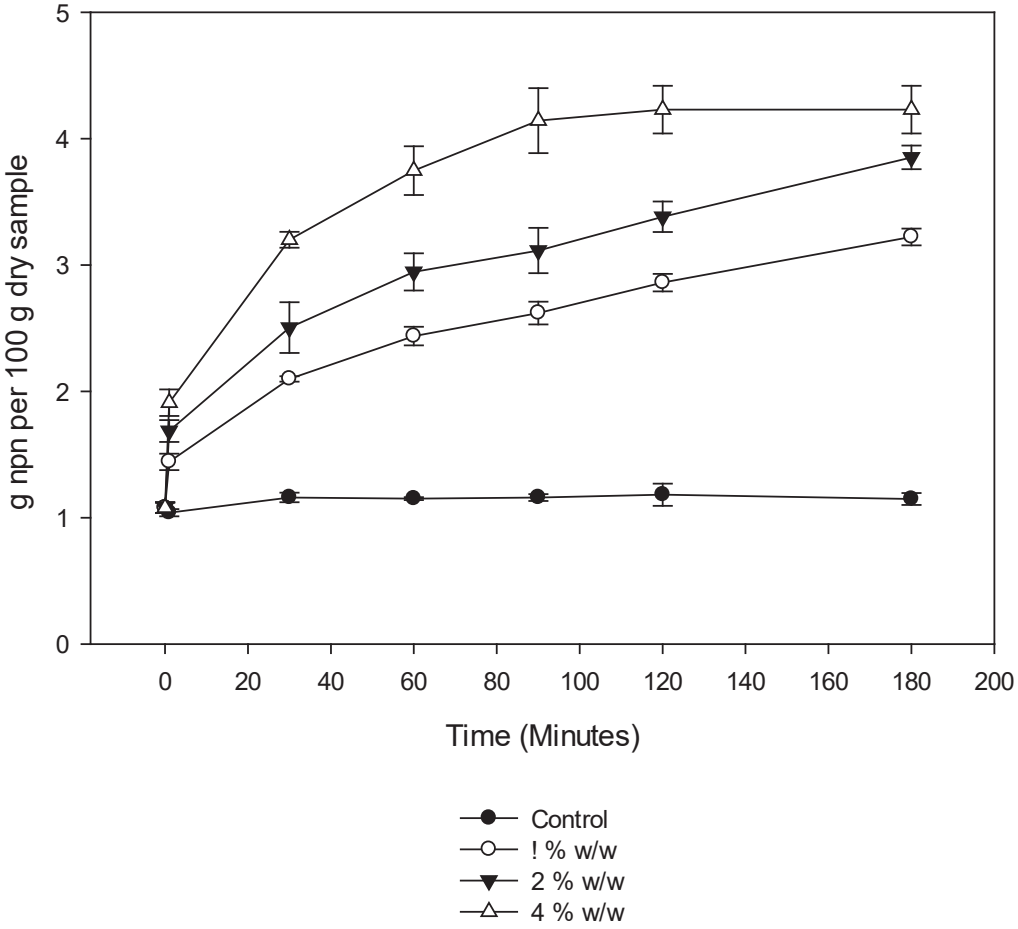

Figure 11: Total nitrogen content (dry basis) in hydrolysate reactions at different enzyme concentration and 60 C.

Table 4: Total nitrogen content (g/100g dry sample) of hydrolysate after 180 minutes hydrolysis at 40°C

Processing Time (Minute)	Enzymes concentration (% w)			
	Control 0%	1%	2%	4%
0	11.99 ± 0.28			
1	12.21 ± 0.32	11.73 ± 0.20	11.49 ± 0.47	10.69 ± 0.11
30	12.20 ± 0.32	11.73 ± 0.13	11.48 ± 0.46	10.96 ± 0.10
60	12.23 ± 0.16	11.76 ± 0.20	11.62 ± 0.31	10.80 ±0.38
90	12.54 ± 0.45	11.52± 0.14	11.27± 0.42	10.93 ± 0.21

120	11.95 ± 0.30	11.53 ± 0.58	11.58 ± 0.08	11.24 ± 0.17
180	12.31± 0.30	11.73 ± 0.33	11.68 ± 0.11	10.71 ± 0.30

Table 5: Total nitrogen content expressed as g /100 g (Dry basis) of hydrolysate at 50 °C

Processing Time (Minutes)	Enzymes concentration (% w)			
	Control 0	1%	2%	4%
0	12.44 ± 0.23			
1	12.37 ± 0.74	11.87 ± 0.94	11.06 ± 0.21	11.53 ± 0.29
30	12.45 ± 0.55	11.92 ± 0.17	11.62 ± 0.53	10.98 ± 0.32
60	12.33 ± 0.38	12.03 ± 0.61	11.11 ± 0.06	11.11 ± 0.68
90	11.97± 0.42	11.82 ± 0.26	11.32 ± 0.44	10.54 ± 0.27
120	11.98 ± 0.21	11.80 ± 0.13	11.34± 0.38	10.71 ± 0.28
180	12.01 ± 0.28	11.73 ± 0.23	11.54 ± 0.32	10.85 ± 0.43

Table 6: Total nitrogen content expressed as g /100 g (Dry basis) of hydrolysate at 60°C

Processing Time (Minutes)	Enzymes concentration (% w)			
	Control 0	1%	2%	4%
0	12.14 ± 0.44			
1	12.41 ± 1.42	11.91 ± 0.75	11.19 ± 1.01	10.86 ± 0.48
30	12.36 ± 0.85	11.73 ± 0.33	11.50 ± 0.43	11.21 ± 0.46
60	12.25 ± 0.47	12.40 ± 0.36	11.89 ± 0.84	11.29 ±0.72
90	11.91 ± 0.32	11.50 ± 0.51	11.18 ± 0.77	11.11 ± 0.53
120	11.34 ± 0.59	11.82 ± 0.32	11.69 ± 0.32	11.31 ± 0.93
180	11.82 ± 0.78	11.32 ± 0.57	11.32 ± 0.17	10.93 ± 1.07

Table 7: Non protein nitrogen (g/100g dry sample) content of hydrolysate at 40°C processing

Processing Time (Minutes)	Enzymes concentration (% w)			
	Control 0	1%	2%	4%
0	1.10 ± 0.04			
1	1.10 ± 0.07	1.69 ± 0.08	1.61 ± 0.03	1.83 ± 0.04
30	1.03 ± 0.02	2.07 ± 0.04	1.97± 0.09	2.32 ± 0.11
60	1.02 ± 0.03	2.12 ± 0.10	2.17 ± 0.06	2.43 ± 0.13
90	0.97 ± 0.03	2.19 ± 0.06	2.19 ± 0.09	2.83 ± 0.08
120	1.01 ± 0.07	2.23 ± 0.03	2.31 ± 0.14	2.88 ± 0.11
180	1.12 ± 0.03	2.26 ± 0.08	2.52 ± 0.17	3.13 ± 0.07

Table 8: Non protein nitrogen (g/100g dry sample) content of hydrolysate at 50 °C

Processing Time (Minutes)	Enzymes concentration (% w)			
	Control 0	1%	2%	4%
0	1.11 ± 0.05			
1	1.03 ± 0.008	1.44 ± 0.06	1.61 ± 0.03	1.57± 0.04
30	1.11 ± 0.03	1.73 ± 0.03	2.03± 0.10	2.75 ± 0.10
60	1.10 ± 0.02	1.95 ± 0.08	2.22 ± 0.05	3.19 ±0.10
90	1.09 ± 0.01	2.15 ± 0.14	2.37 ± 0.11	3.33 ± 0.09
120	1.15 ± 0.08	2.13 ± 0.09	2.53 ± 0.12	3.43 ± 0.09
180	1.20 ± 0.05	2.28 ± 0.02	2.77 ± 0.21	3.71 ± 0.19

Table 9: Non protein nitrogen (g/100g dry sample) content of hydrolysate at 60 °C

Processing Time (Minutes)	Enzymes concentration (% w)			
	Control 0	1%	2%	4%
0	1.10 ± 0.04			
1	1.07 ± 0.06	1.44 ± 0.07	1.69 ± 0.09	1.91 ± 0.10
30	1.19 ± 0.02	2.10 ± 0.02	2.50 ± 0.20	3.20 ± 0.06
60	1.17 ± 0.03	2.43± 0.07	2.94 ± 0.15	3.75 ± 0.19
90	1.12 ± 0.05	2.62 ± 0.09	3.11 ± 0.18	4.09 ± 0.20
120	1.17 ± 0.07	2.86± 0.07	3.37 ± 0.13	4.04 ± 0.06
180	1.20 ± 0.05	3.11 ± 0.07	3.79 ± 0.16	4.27 ± 0.23

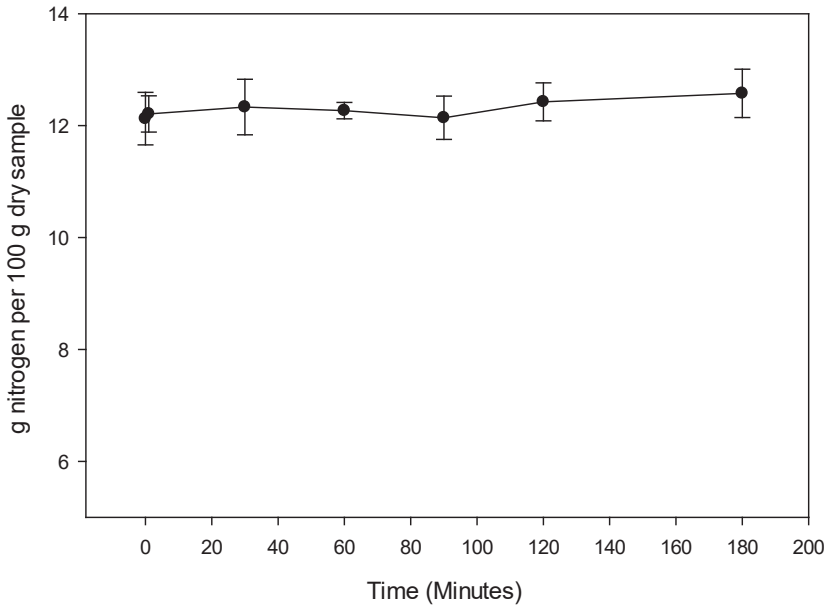

Figure 12: Total nitrogen content (dry basis) in hydrolysate reactions at 40°C without enzyme addition .

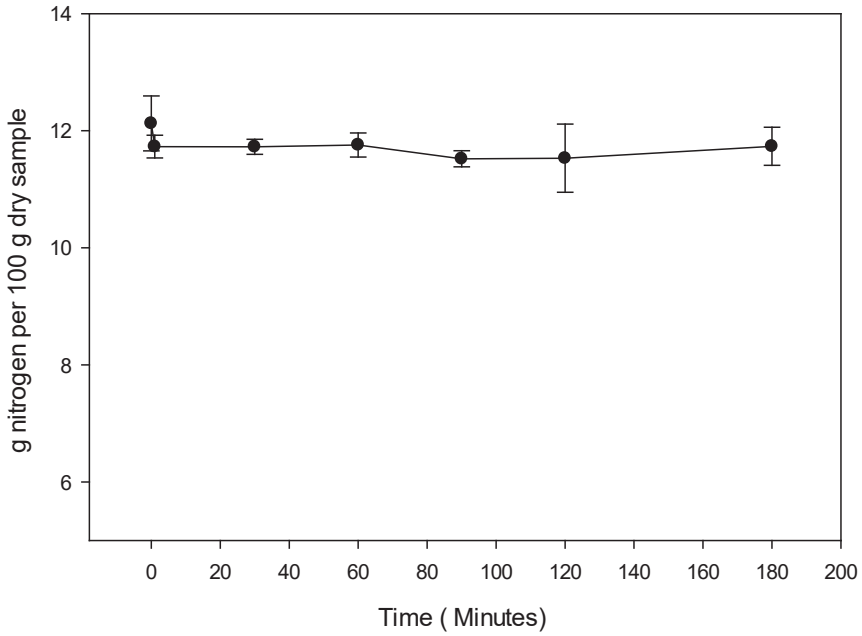

Figure 13: Total nitrogen content (dry basis) in hydrolysate reactions at 40°C with 1% enzyme concentration

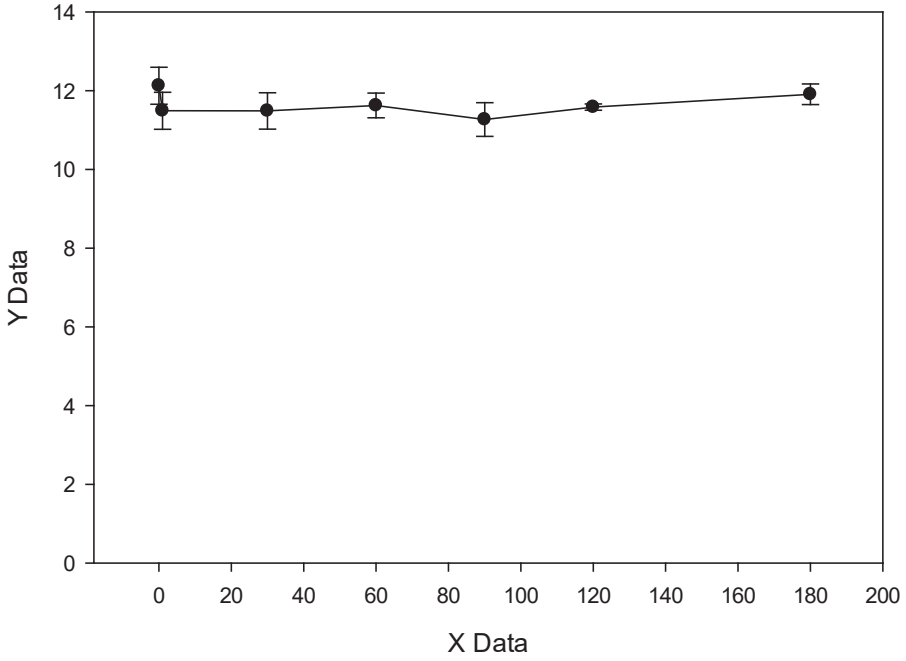

Figure 14: Total nitrogen content (dry basis) in hydrolysate reactions at 40°C with 2 % enzyme concentration

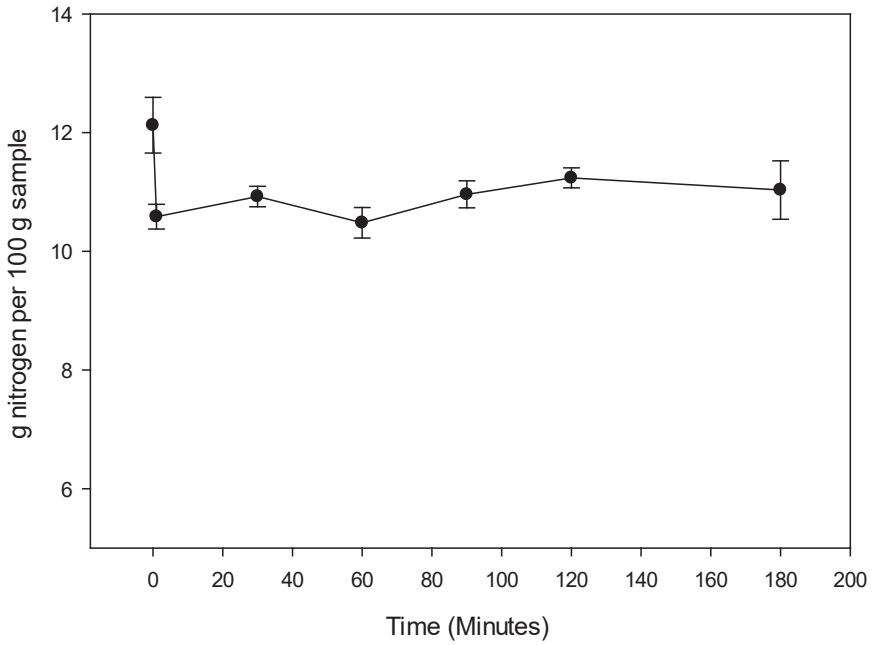

Figure 15: Total nitrogen content (dry basis) in hydrolysate reactions at 40°C with 4% enzyme concentration .

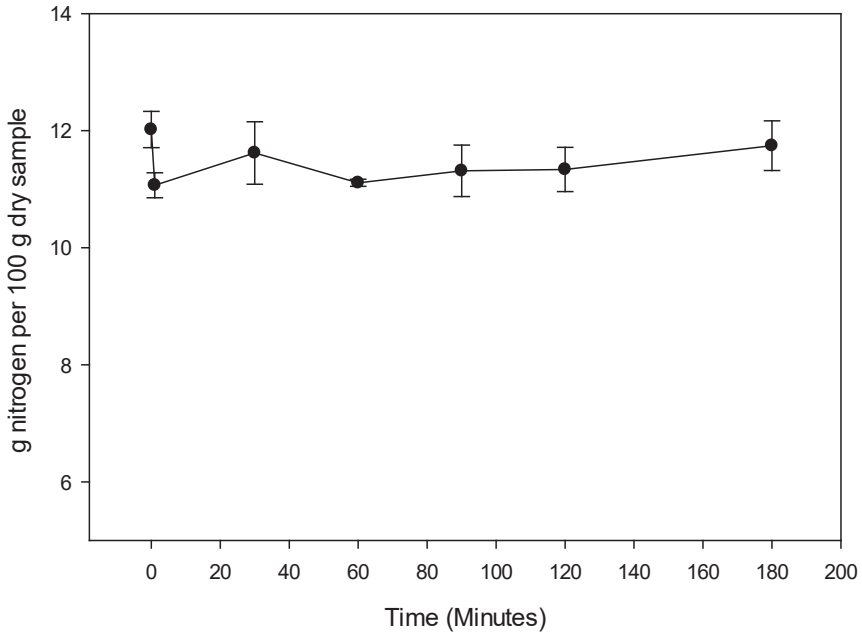

Figure 16: Total nitrogen content (dry basis) in hydrolysate reactions at 50°C with 2% enzyme concentration

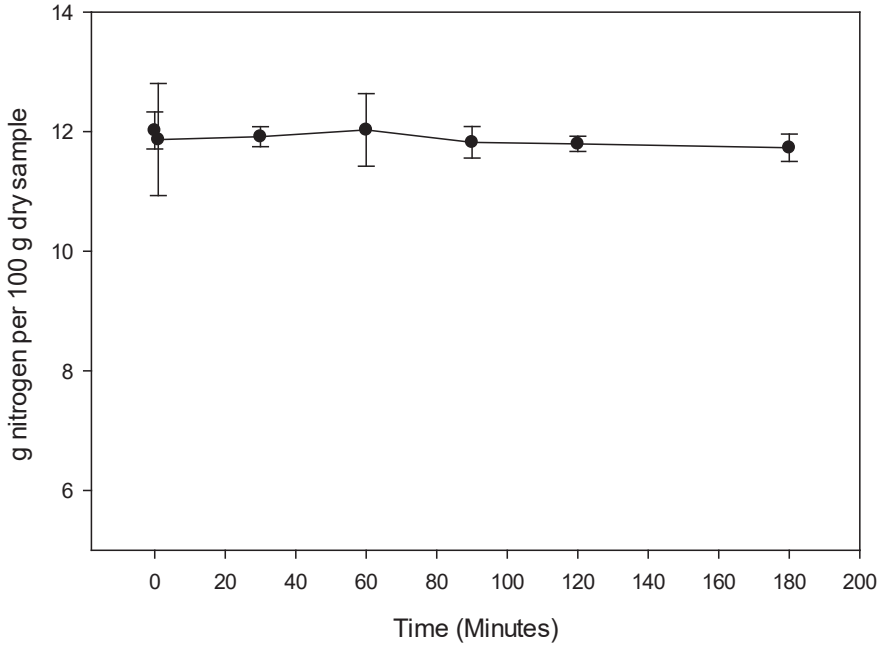

Figure 17: Total nitrogen content (dry basis) in hydrolysate reactions at 50°C with 1% enzyme concentration

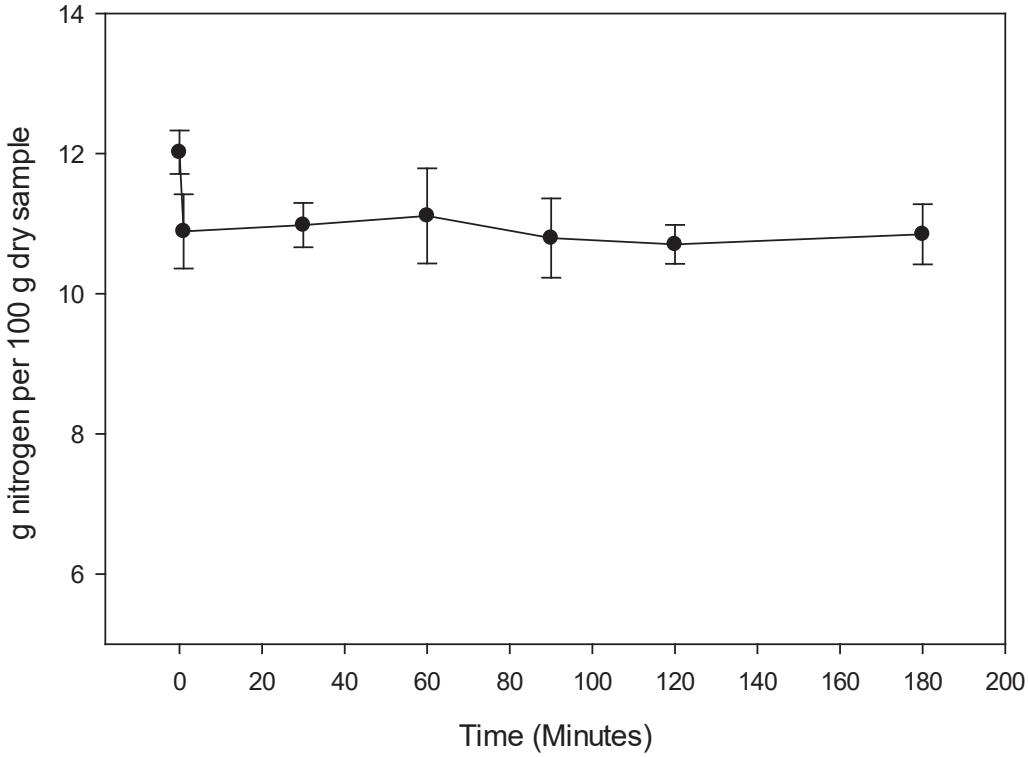

Figure 18: Total nitrogen content (dry basis) in hydrolysate reactions at 50°C with 4 % enzyme concentration

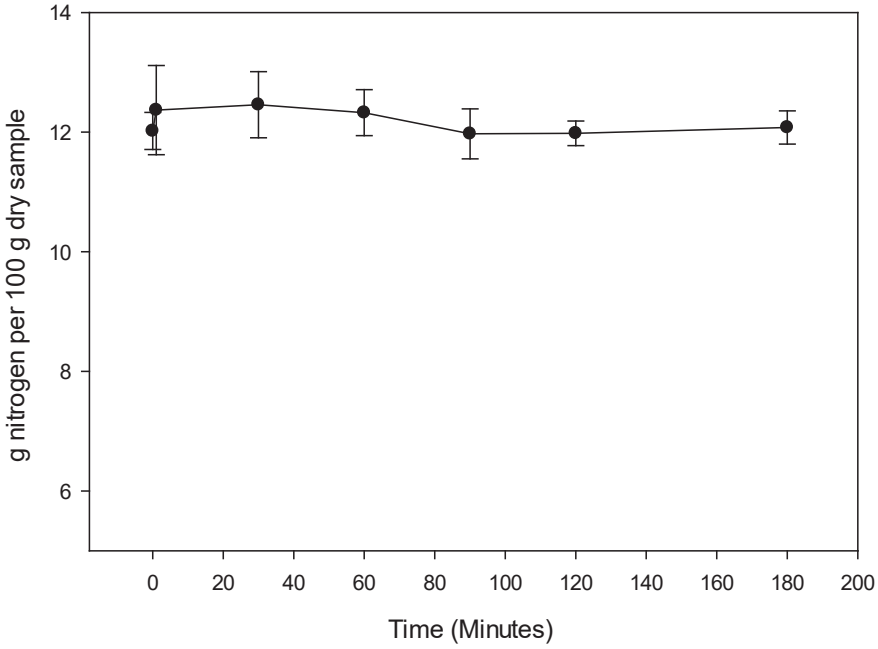

Figure 19: Total nitrogen content (dry basis) in hydrolysate reactions at 50°C with 0 % enzyme concentration

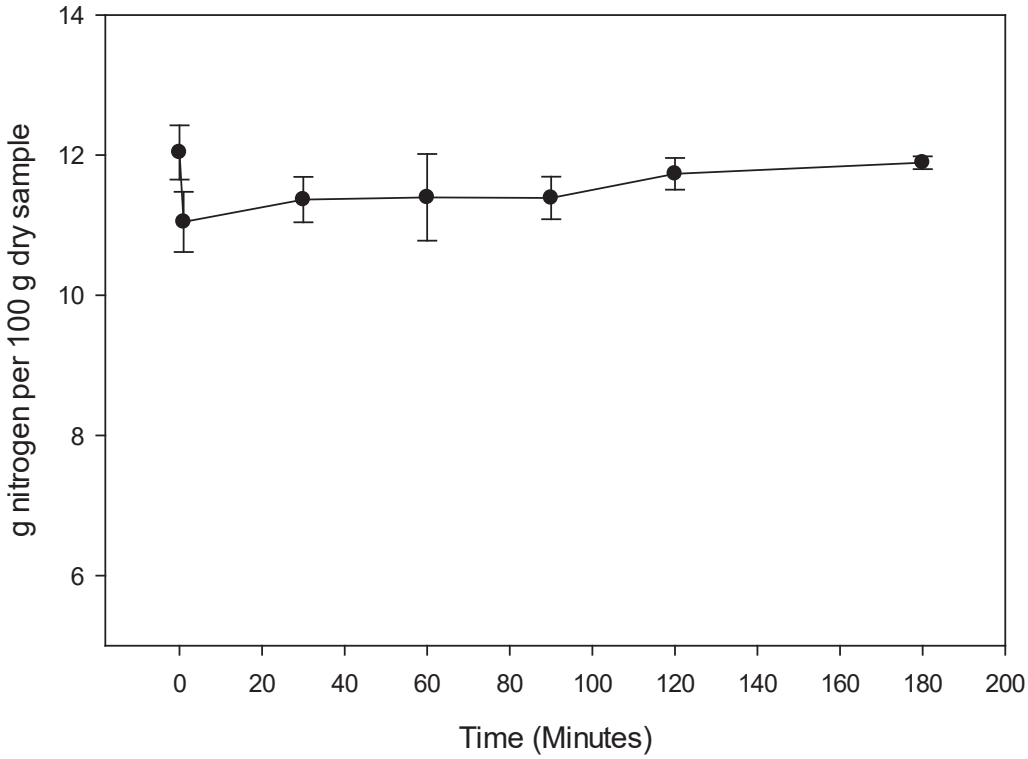

Figure 20: Total nitrogen content (dry basis) in hydrolysate reactions at 60°C with 2% enzyme concentration

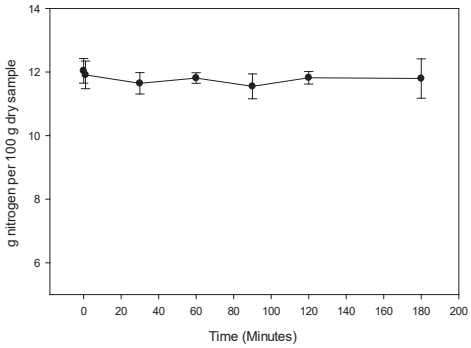

Figure 21: Total nitrogen content (dry basis) in hydrolysate reactions at 60°C with 1% enzyme concentration

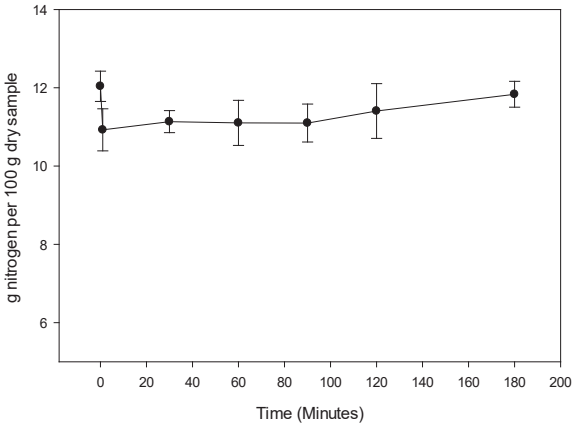

Figure 22: Total nitrogen content (dry basis) in hydrolysate reactions at 60°C with 4% enzyme concentration

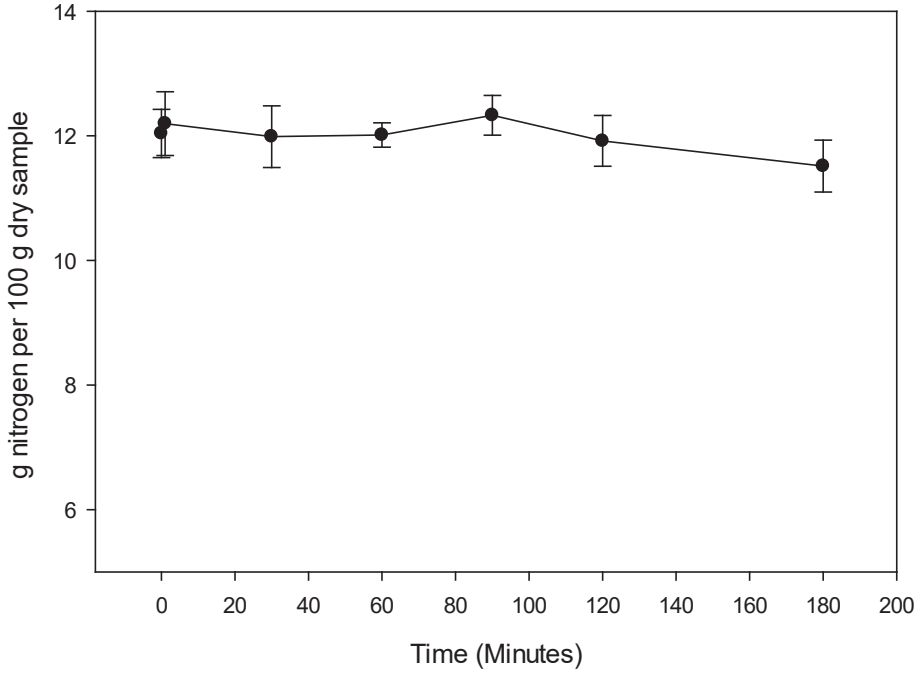

Figure 23: Total nitrogen content (dry basis) in hydrolysate reactions at 60°C with 4% enzyme concentration

Table 10: Non protein nitrogen (g/100g dry sample) protein content at different pH

Processing time (Minutes)	pH							
	2.0	**3.0**	**4.0**	**5.0**	**Control (5.6)**	**6.0**	**7.0**	**8.0**
1	0.91±0.06	1.51±0.06	2.32±0.19	2.36±0.15	2.35±0.11	2.41±0.05	2.42±0.19	**2.21±0.16**
30	0.98±0.08	1.90±0.08	4.68±0.12	4.64±0.24	4.46±0.17	4.09±0.07	3.37±0.12	**2.80±0.11**
60	0.99±0.05	1.92±0.06	5.50±0.15	5.43±0.15	5.24±0.17	4.95±0.07	3.81±0.10	**3.19±0.10**
90	0.96±0.09	1.99±0.14	5.86±0.12	5.99±0.11	5.58±0.27	5.44±0.07	4.14±0.13	**3.30±0.15**
120	**1.02±0.09**	**1.93±0.07**	**6.01±0.07**	**6.07±0.14**	**5.90±0.13**	**5.53±0.21**	**4.25±0.13**	**3.32±0.10**

Table 11: Non protein nitrogen (g/100g dry sample) content at different temperature

Processing time (Minutes)	Temperature (°C)					
	40	50	60	65	70	**75**
1	1.61 ± 0.03	1.61 ± 0.03	11.19 ± 1.01	1.61 ± 0.02	1.67±0.05	**1.61±0.05**
30	1.97± 0.09	2.03± 0.10	11.50 ± 0.43	2.62 ± 0.10	2.64 ±0.01	**2.39±0.03**
60	2.17 ± 0.06	2.22 ± 0.05	11.89 ± 0.84	3.07± 0.06	2.90±0.03	**2.34±0.01**
90	2.19 ± 0.09	2.37 ± 0.11	11.18 ± 0.77	3.17±0.04	3.02±0.01	**2.30±0.03**
120	2.31 ± 0.14	2.53 ± 0.12	11.69 ± 0.32	3.26±0.03	3.20 ±0.01	**2.29±0.01**
180	**2.52 ± 0.17**	**2.77 ± 0.21**	**11.32 ± 0.17**	**3.60 ±0.04**	**3.20±0.01**	**2.27±0.04**