CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF STUDY

The *Carica papaya* plant belongs to the *Caricaceae* family, it is commonly called papaya. This plant is also known in different part if the world as papaw, paw-paw, kapaya, lapaya, tapaya, papayao, papaya, papaia, papita, lechosa, fruta bomba, mamon, mamona, mamao and tree melon. It is native to the Caribbean Coast of Central America (Milind and Gurditta, 2011). *Carica papaya* is cultivated in tropical and subtropical regions of the world (Lavanya *et al.,* 2018). Papaya is cultivated in the tropical and neo-tropical regions of the world between 32º North and South. Amongst the 31 species of the botanical family *Caricaceae* and the genera *Carica, papaya* specie is the most economically important and widely cultivated specie. *Carica papaya* is the most economically important and widely cultivated species amongst the 31 species of its botanical family (Nur, 2010).

During the early years of growth, papaya develops a single stem; with time in highly fertile soil, thereby promoting favourable growth condition, also heavy lateral branch develops. The leaves of a mature papaya plant are palmate with deep lobes, supported by smooth, hollow petioles (Nur, 2010). The stems, fruits and leaves contain copious amount of latex. It is crowded by terminal cluster of large and long stalked leaves, which rapidly grows and up to 20m of height (Banerjee, 1986).

Papaya species exist in three sex types: male, hermaphrodite and female. In commercial production, the hermaphrodite specie is most desired. A single gene having at least three alleles

controls the sex of the plant; a dominant allele for male plants, another dominant allele for hermaphrodite plants and a recessive allele for female plants.

Ripe papaya fruits have slight resemblance with melons; they are rich in retinol and ascorbic acid. They are rich sources of β-carotene, vitamin C, vitamin A and vitamin E, which are highly potent antioxidants; they possess minerals such as calcium, zinc, magnesium, manganese, potassium and iron; they contain also the phytochemicals and the B vitamins (Aravind *et al.,* 2013). Papaya is a good source of fiber. All the nutrients of papaya as a whole improve cardiovascular system, protect against heart diseases, heart attacks, strokes and prevent colon cancer and its enzymes are used in the treatment of arthritis (Milind and Gurditta, 2011). Papaya contains latex present in its unripe fruits as well the leaves and stems. *Carica papaya* contains biologically active compounds and enzymes (Tigist *et al.,* 2016).

Enzyme benefits the human health. Enzyme was first named in the late 19th century by Wurtz and Bouchut (1945) who purified partially the product from the sap of papaya; it was recognized as a constituent in the latex of tropical papaya fruit when named. From papaya latex, the endolytic plant cysteine protease enzyme – papain and chymopapain is isolated (Amri and Mamboya, 2012). Latex of the papaya contains a sulfhydryl protease –papain and chymopapain (Lavanya *et al.,* 2018). Papain (EC 3.4.22.2) and chymopapain are the most common important enzymes of papaya (Milind and Gurditta, 2011). Papain is of vital importance in biological processes in living organisms, it belongs to the papain superfamily, as it is has inherent proteolytic properties (Tsuge *et al.,* 1999). Papain basically is obtained by making incisions on the epicarp of unripe papaya, collecting and drying the latex which flows out. More active papain is gotten from a greener fruit (Amri and Mmaboya, 2012).

According to Menard *et al.,* (1990), papain preferentially cleaves peptide bonds involving basic amino acids, particularly arginine, lysine and residues following phenylalanine. In the 1990s were the precursors and inhibitors of papain studied (Vernet, 1991). The functionality of papain is due to its unique structure which helps in the comprehension of the mechanism of the proteolytic enzyme and its usefulness for a variety of purposes (Amri and Mmaboya, 2012). Papain and chymopapain is soluble in water, their solution has good temperature stability; the solution stability has high dependence on the pH. Papain solutions are unstable under acidic condition such as pH 2.8 and will result in significant loss of activity; they are however stable at a between a pH of 5.5 - 5.9 (Nur, 2010).

Papain is utilized in food processing especially in the tenderization of meat. The protease has the ability to dissolve dead tissue without damaging any living cell; it is used as commercial meat tenderizers. Papaya fruit and papain are used in wound care. Papain is a digestive enzyme of papaya that effectively treats the causes of trauma, allergies and sports injuries (Tigist*et al.,* 2016). Papain has high proteolytic activity and it is applied extensively in the fields of food and medicine. According to Chaudhari (1996), some studies indicate the enzyme helps in the prevention of diabetic-dependent-heart-disease. Papaya leaves traditionally are wrapped around meat and act a tenderizer. Due to the efficiency and less destructive nature of the proteolytic enzymes – papain and chymopapain, when compared to other proteases is the commonly used enzyme in cell isolation procedures on certain tissues (Nur, 2010). Papain is a constituent of contact lens solution. Papain is used in clarifying beer, synthesis of chewing gum and in pharmaceutical industry as digestive medicine. In a study by Huet *et al.,* (2006), traditional medicine through the administration of papain and chymopapain extract from papaya leaves is shown to be effective against the nematodes as papain causes damage to the cuticles of the

nematodes. The combination of papain with other proteolytic enzyme is useful in enzyme-therapy for cancer treatment.

1.2 STATEMENT OF RESEARCH PROBLEM

Papain enzyme generally is a sulfylhudryl protease obtained from *Carica papaya*. Several research carried out on the enzymes of the papaya plant focus on its extraction and application. The enzyme functions in the tenderization of flesh and also enhances the healing of wound. For those who experience poor wound healing will not have to depend on other materials that are expensive, sophisticated which might not be readily available. There are a number of papaya trees on the campus of Mountain Top University (MTU). The present study focuses on the enzymes in the leaf of the tree on the campus.

1.3 AIM AND OBJECTIVES OF STUDY

The aim of this study is to extract, purify and characterize papain enzyme from the leaves of *Carica papaya*.

The specific objectives of this study are:

a) To extract papain from Papaya leaves in MTU.
b) To purify the extracted papain.
c) To determine the papain concentration at each phase of purification.
d) To determine the enzyme activity at each purification phase.
e) To characterize the purified papain.

1.4 SCOPE OF STUDY

In order to achieve the objectives, the following procedure were taken:

a) Grinding the leaves in a mortar with pestle followed by homogenization in a warring blender.
b) Precipitation of the protein with 70% ammonium sulphate solution.
c) Dialysis of the homogenate against distilled water.
d) Passing the dialysate through DEAE-Cellulose column chromatography.
e) Gel filtration of the fractions from DEAE-Cellulose column.
f) Determination of the molecular weight, the effect of pH, temperature, substrate concentration and Mg^{2+} on the enzyme.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1.0 INTRODUCTION

Papaya grows in a wide range of climate; papaya production for extraction of papain, and as well chymopapain as served an income source to the industry and as well farmers. Papain enzyme (EC 3.4.22.2) is revealed to be enzymatic proteins of significant biological and economic importance. It is through their unique structure they are functional; they help to explain the mechanism of its proteolysis which makes it valuable for a variety of purposes. Further researches on papain enzyme in understanding the specificity, structural effect that brings about various thermodynamic pathways is of critical importance. Papain is found naturally in papaya which is a versatile plant having number of uses and enzymatic properties.

2.1.1 TAXONOMY OF *Carica papaya*

As stated by Kaliyaperumal *et al.,* (2014), the taxonomy of *Carica papaya* is as shown on the table below;

Table 2.1: Taxonomy of *Carica papaya* plant.

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Brassicales
Family	Caricaeae
Genus	*Carica*
Species	*Papaya L.*

2.1.2 ORIGIN OF *Carica papaya*

The *Carica papaya* plant belongs to the *Caricaceae* family, commonly known as papaya (Chaudhari, 1996). This plant is also known in different part if the world as papaw, paw-paw, kapaya, lapaya, tapaya, papayao, papaya, papaia, papita, lechosa, fruta bomba, mamon, mamona, mamao and tree melon. It is native to the Caribbean Coast of Central America (Milind and Gurditta, 2011). *Carica papaya* is cultivated in tropical and subtropical regions of the world (Lavanya *et al.*, 2018). Papaya is cultivated in the tropical and neo-tropical regions of the world between 32º North and South. Amongst the 31 species of the botanical family *Caricaceae* and the genera – *Carica*, the *papaya* specie is the most economically important and widely cultivated specie.

2.1.3 ECOLOGY OF *Carica papaya*

Carica papaya is reported to tolerate annual precipitation of 6.4 dm to 42.9 dm (mean of 42 cases = 19.2), annual temperature of 16.2°C - 26.6°C (mean of 42 cases = 24.5), and pH of 4.3 to 8.0 (mean of 33 cases = 6.1) (Kaliyaperumal *et al.*, 2014). Papaya is a tropical plant, killed by frost; it does not tolerate shade, water logging, or strong winds, and may require irrigation in dry regions. It recuperates very slowly from serious root or leaf injury. It can be grown well below 1,500 m in well-drained, rich soil of pH 6 - 6.5 (Duke, 1983).

2.1.4 MORPHOLOGY OF *Carica papaya*

Papaya is a small, frost-tender, succulent, broadleaved evergreen tree that bears papaya fruits throughout the year. Each tree typically has a single, unbranched, non-woody trunk bearing the scars of old leaf bases. The trunk is topped by an umbrella-like canopy of palmately lobed leaves. Large, fleshy, melon-like fruits (papayas) hang in clusters attached to the trunk top just

under the leaf canopy. Papaya typically grows to 6 m – 20 m tall (container plants to 10m tall) and is most noted for its edible melon-like fruit. It is crowded by terminal cluster of large and long stalked leaves, which rapidly grows and up to 20m of height (Banerjee, 1986). Papaya tree sometimes branches due to injury and it contains white latex in all parts. The stem is cylindrical, 10 cm - 30 cm in diameter, hollow with prominent leaf scars and spongy-fibrous tissue. It has an extensive rooting system (Orwa *et al.,* 2009). The leaves are spirally arranged, clustered near apex of the trunk; petiole is up to 1 m long, hollow, greenish or purplish-green; lamina orbicular, 25 cm - 75 cm in diameter, palmate, deeply lobed, broadly toothed, glabrous and prominently veined (Orwa *et al.,* 2009). However, several distinct varieties of papaya have been mentioned (Richharia, 1957), which vary in shape and size of fruits, height of plants, etc.**.** The shape of papaya plant is as that palm leaves with large monoaxial palms. Ripe papaya fruit resemble melons and they are rich in vitamin A and C. During the early years of growth, papaya develops a single stem; with time in highly fertile soil and favourable growth condition, heavy lateral branch develops. The leaves of a mature papaya plant are palmate with deep lobes, supported by smooth, hollow petioles. The stems, fruits and leaves contain copious amount of latex and the plant undergoes photosynthesis.

Fig 2.1: Morphology of *Carica papaya.* Papaya leaves arrangement like an umbrella-like canopy and melon-like unripe fruits hang in clusters attached to the trunk top just under the leaf canopy.

2.1.5 SEX TYPES OF PAPAYA PLANT

Papaya species exist in three sex types: male, hermaphrodite and female. In commercial production, the hermaphrodite specie is most desired. In papaya, a single gene having at least three alleles controls the plant sex; a dominant allele for male plants, another dominant allele for hermaphrodite plants and a recessive allele for female plants. The species plants are typically dioecious (hermaphroditic), and maple trees are uncommon. Hermaphrodite trees (flowers with male and female parts) are the commercial standard, producing a pear shaped fruit. These plants are self-pollinated. Flowers tiny, yellow, funnel-shaped, solitary or clustered in the leaf axils, of 3 types; female flowers 3 - 5 cm long, large functional pistil, no stamens, ovoid-shaped ovary; male flowers on long hanging panicles, with 10 stamens in 2 rows, gynoecium absent except for a pistillode; hermaphrodite flowers larger than males, 5-carpellate ovary; occurrence depends on

the season or age of the tree (Orwa *et al.*, 2009). The female flowers give way to smooth-skinned green fruits that ripen to yellow-orange with a yellow to pinkish-orange flesh and central cavity of pea-sized black seeds.

2.2 CONSTITUENTS OF PAPAYA

Protein and alkaloids are present in appreciable amount in Papaya leaves; amino acids in moderate quantity; carbohydrates steroids and glycosides as a detectable label. Alkaloids, tannins, phenolics and flavonoids are present in papaya leaf in appreciable quantity; carbohydrates, glycosides, saponins, proteins, amino acids, steroids and terpenoids in detectable amount in the papaya leaf (Kaliyaperumal *et al.*, 2014). Kaliyaperumal *et al.*, 2014 stated constituents of different parts of the papaya plant in his study, it is as shown below;

Table 2.2: List of constituents present in various parts of papaya (Kaliyaperumal *et al.*, 2014)

NO.	Categories	Phytoconstituents	Plant Part(s)
1	Enzymes	Papain, chymopapain A and B, endopeptidase papain III and IV glutamine cyclotransferase, peptidase A and B and lysozymes.	Unripe fruit (Latex)
2	Carotenoids	β carotene, crytoxanthin, violaxanthin, zeaxanthin.	Fruits
3	Alkaloid & Enzyme	Carposide, and an enzyme myrosin.	Roots
4	Glucosinolates	Benzyl isothiocynate, benzylthiourea, β-sitosterol, papaya oil, caricin and an enzyme myrosin.	Seeds
5	Minerals	Calcium, potassium, magnesium, iron, copper, zinc.	Shoots and Leaves
6	Monoterpenoids	4-terpineol, linalool, linalool oxide.	Fruits

7	Flavoniods	Quercetin, myricetin, kaempferol.	Shoots
8	Alkaloids	Carpinine, carpaine, pseudocarpine, vitamin C and E, choline, carposide.	Leaves and Heartwood
9	Vitamins	Thiamine, riboflavin, niacin, ascorbic acid, α-tocopherol.	Shoots and Leaves
10	Carbohydrates	Glucose, sucrose, and fructose.	Fruits

2.3 PAPAIN PRECURSOR

From the cells of insect infected with recombinant baculovirus, it has been expressed that the precursor of the cysteine protease papain is propapain (EC 3.4.22.2). Propapain is a zymogen with molecular weight of 39 kDa, a glycosylated whivh is processed in-vitro to active enzyme, papain of molecular weight 24.5 kDa (Vernet *et al.,* 1990). Hg^{2+} has the ability to stabilize propapain. Enzymatically inactive N-glycosylated papain precursor is secreted by the host *Spodoptera frugiperda* cells when infected with the recombinant Baculovirus. Activation of the propapain could be achieved in-vitro to yield per liter of the culture about 400 nmol of active papain. The recombinant active mature papain is found to be enzymatically indistinguishable from natural papain amino acid residue. According to *Vernet et al.,* (2000), under the control of the α-factor promoter in the yeast *Saccharomyces cerevisiae*, a synthetic propapain gene is expressed. Absolute processing of the intracellular zymogen– propapain into active mature papain could be achieved in vitro. Mutagenesis and the efficient screening of papain mutants are advantages of the yeast expression system over the baculovirus/insect cell system.

Propapain is purified through affinity, size-exclusion and ion-exchange chromatography procedures. The processing of propapain is inhibited by cysteine protease inhibitors. The papain precursor has the ability to undergo autoproteolytic cleavage– this statement is based on

biochemical and kinetic analysis of the activation and processing of propapain that has been studied.

2.4 PAPAIN ENZYME

Enzymes are macromolecular-biological organic catalyst that increases the rate of biochemical reactions by decreasing the activation energy, without it being used up or changed at the end of the reaction (Trivedi *et al.,* 2013). They are mostly protein in nature; some RNAs are enzymes as well. Reactions occur at much higher rate when enzymes are present. Enzyme benefits the human health.

Enzyme was first named by Wurtz and Bouchut (1940). The term papain was first introduced by Wurtz and Brochut (19440) in describing the proteolytic principle in papaya latex. Papain was first extracted from the dried latex of Carica papaya. Papain may be obtained from bacteria, plant, vertebrates and invertebrates. Wurtz and Bouchut (1940) partially purified the product from the sap of papaya; it was recognized as a constituent in the latex of tropical papaya fruit when named. In the group of cysteine protease, papain family accounts the largest of it all, they are proteolytic enzymes.

Proteolytic enzymes are groups of enzyme that catalyzes the hydrolysis of peptide bond in a protein molecule. Papain has high proteolytic activity and it is applied extensively in the fields of food and medicine. The active site geometry was evaluated and the three-dimensional structure was determined to a 1.65Å resolution in the 1980s. According to Menard *et al.,* (1990), papain preferentially cleaves peptide bonds involving basic amino acids, particularly arginine, lysine and residues following phenylalanine. In the 1990s the precursors and inhibitors of papain were studied (Vernet, 1991). The functionality of papain is due to its unique structure which

helps in the comprehension of the mechanism of the proteolytic enzyme and its usefulness for a variety of purposes (Amri and Mmaboya, 2012).

2.4.1 PROPERTIES, STRUCTURE AND FEATURES OF PAPAIN

Papain enzyme are globular protein consisting of a single polypeptide chain with three disulfide bridges and sulfuhydyl group necessary for activity of the enzyme, and an accession number 1CVZ with molecular weight of 23,406 Da (Amri and Mamboya, 2012). Pure chymopapain (EC 3.4.22.6) enzyme has a molecular mass of 24,700Da. Papain consists of 212 amino acid with four disulfide bridges and it contains amino acid residues that enhances catalysis in the following positions Gln19, Cys25, His158 and His159 (Mitchel *et al.*, 1970; Robert *et al.*, 1974; Tsuge *et al.*, 1999). Papain emzyme consists of two well-defined domains which provide for studies, an outstanding system in understanding the folding-unfolding behavior of proteins (Edwin *et al.*, 2002).

Papain is stable and active under a wide range of conditions. Cohen *et al.*, (1986) reported that at elevated temperatures papain remains stable. According to Nur (2010), the optimum pH for maximum activity of papain enzyme is around 6.0 to 7.0. The optimum pH for the activity of papain ranges from 3.0-9.0 as this varies with different substrate (Edwin and Jagannadham, 2000; Ghosh, 2005). To high concentrations of denaturing agents, such as 8M urea or organic solvent like 70% EtOH, papain unlike most enzyme is defiant.

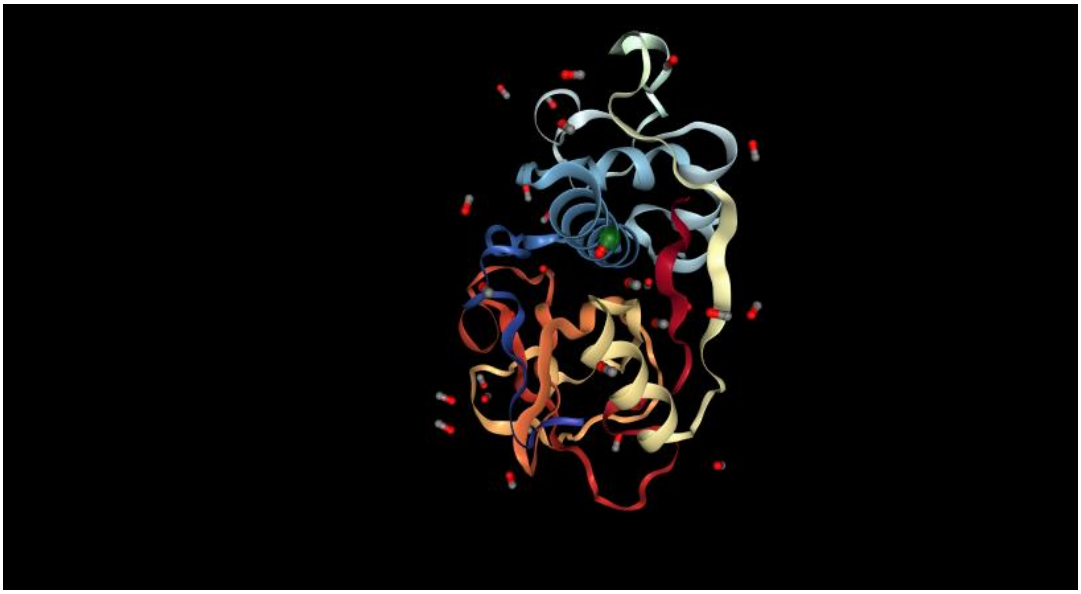

Fig. 2.1: Quaternary protein structure of the papain enzyme. A display of the papain subunits and ligands (represented in ball and stick model). Source: rcsb.org/3-dview/9papa *(RCSB Protein Data Bank).*

The three disulfide bridges in which the papain enzyme is folded along creates a strong interaction amongst the side chains as this also contributes to the stability of the enzyme (Edwin and Jagannadham, 2000; Tsuge *et al.,* 1999). A cleft between the two distinct structural domain in the three-dimensional structure contains the active site of the enzyme. Papain molecule has an all α domain and an anti-parallel β-sheet domain (Kamphuis *et al.,* 1984; Madej *et al.,* 2012). In the presence of SDS, the conformational behavior of papain in aqueous solution was studied and was reported to show high α-helical content and unfolded structure of papain due to strong electrostatic repulsion (Huet *et al.,* 2006). Further research in understanding the specificity, the structure, the effect on addition of inhibitors, low pH, metal ions and fluorinated alcohols is of critical importance (Huet *et al.,* 2006; Naeem *et al.,* 2006).

2.4.2 HYDROPHOBICITY OF PAPAIN

The hydrophobic interactions have an influence on the conformation of protein, hence its functionality. The hydrophobic amino acids are alanine, leucine, methionine, valine and Isoleucine is responsible for the hydrophobicity of papain enzyme. The overall folding pattern of a protein could be indicated by the relative hydrophobicity or hydrophilicity values of the amino acids in its protein sequence (Amri and Mamboya, 2012). The major thermodynamic force which drives protein folding is the interaction between the hydrophobic and hydrophilic amino acids of the papain molecule. As reported by Stevenson and Storer (1991), papain and chymopapain is more stable in hydrophobic solvents; it catalyzes reactions under a variety of conditions in organic solvents with its substrate. Native proteins have a hydrophobic core and a polar group on the exterior aqueous medium. The hydrophobic core enhances the stability of the tertiary structure of the enzyme by hydrophobic interaction while the outer polar surfaces interact with the exterior aqueous medium (Wang *et al.,* 2006).

2.4.3 MECHANISM OF PAPAIN FUNCTIONS

The mechanism of function of papain is made possible through the Cys-25 portion of the active site which attacks the carbonyl carbon in the backbone of the peptide chain to release the amino terminal portion, making the protein break apart. Asparagine-175 helps to re-orient the imidazole ring of His-159 to allow the deprotonation of Cys-25 by His-159 for the peptide bonds to be broken (Amri and Mambayo, 2012). Although far apart within the chain, these three amino acids are in close proximity due to the folding structure. Nucleophilic attack by Cys-25 on the carbonyl carbon of a peptide backbone takes place (Menard *et al.,* 1990; Tsuge *et al.,* 1999). Peptidyl and non-peptidyl N-nitrosoanilines could act as an effective inhibitor to the activity of papain (Guo *et*

al., 1996; 1998). The formation of a stable S-NO (Snitroso-Cys25) bond in the active site leads to the inactivation of papain (Xian *et al.,* 2000).

2.4.4 STABILITY OF PAPAIN

Papain is soluble in water. Prior to its use, it is diluted in buffer containing 5mM cysteine. Papain is stabilized by the following agents; EDTA, cysteine and dimercaptopropanol. The solution of papain is stable to several denaturing agents and its full activity can be maintained after re-crystallization in 70% methanol and in 8M urea solution. Papain solution has good temperature stability; the solution stability has high dependence on the pH. Papain solutions are unstable under acidic condition such as pH 2.8 and will result in significant loss of activity; they are however stable at a between a pH of 5.5 - 5.9.

2.5 METHODS OF EXTRACTION OF PAPAIN

2.5.1 PAPAIN FROM PAPAYA LATEX

Prior to this time, papain commercial products accessible to the market are extracted from the latex of unripe papaya fruit. The extracted papain further undergo purification by salt precipitation and drying. Developments in the purification of papain without the use of high salt concentrations or thiol containing substances is stated by the use natural inhibitors present in latex resulting in crystallized papain (Monti *et al.,* 2000). Higher purity of papain is attained in shorter time compared to salt precipitation; this purification of papain from fresh latex involves an aqueous two-phase extraction.

Latex of the green papaya contains higher papain concentration and enzymatic activity. The typical technique for papain extraction from papaya involves making incisions on the unripe fruit

for the collection of latex; further processing through drying results in papain powder. Conversely, the effect of making incisions on the unripe green fruit would result in lower market value.

2.5.2 PAPAIN FROM PAPAYA LEAF

Different studies show that the leaves of papaya contain papain. Several studies carried out on papain require the leave from the papaya plant to dry for about 5-7days. The utilization of the leaves and other parts of papaya tree has maximized the benefits and usefulness from the plant. Methods of obtaining papain from papaya leaf are chemically possible. Proteolytic assay of papain is made by using casein as substrate (Tigis *et al.*, 2016). The ultarsonication, enzyme assisted and the blending method are some of the methods utilized in the extraction of papain (Nur, 2010) from the leaves of papaya. According to Tigis *et al.*, (2016), at 60^{o}C for 60mins, sonicated papain extract shows maximum absorbance compared to the ground papain sample.

Purification of papain extracted from fresh papaya leaf could be by two-step salt precipitation (Tigis *et al.*, 2016). However, on the two-step salt precipitaton high purity of papain cannot be gotten.

2.6 METHODS OF EXTRACTION

2.6.1 EXTRACTION OF RAW MATERIAL

In developing the method for the extraction of papain enzyme from leaves and the preparation of other biologically active compound, the first step to take is to blend or crush the leaves through the aid of a blender. By blending, cell disruption will take place hence releasing the intracellular product. Cell wall disruption, which accelerates mass transfer and extraction kinetics, can be

obtained by mechanical or enzymatic treatment, or a combination of both. Some of other used method of extracting plant material is hot water extraction, subcritical water extraction, supercritical fluid extraction and solvent extraction. In order to minimize the use of chemical compound in the extraction process, blending using distilled water, ultrasonication and enzyme assisted extraction is used. In addition, extraction of enzyme protein requires a more delicate or softer technique than mentioned previously. Enzyme is prone to denaturation, a high temperature operating condition or excessive use of organic solvent would affect the efficiency of the extraction process. The amount of papain enzyme isolated from grinded leaves of papaya is higher than that of sonicated papaya. Papain enzyme is found to be more efficient in sonicated leaf sample indicating relative purity of the enzyme isolated from sonicated samples to that isolated from the grinded sample (Nur, 2010). The activity of papain is affected by conditions such as temperature, pH, substrate concentration, metal ions and cofactors. Enzymes activity is affected by temperature. The optimum temperature for the isolated papain enzyme is about 60°C. The enzyme concentration of papaya latex is higher than the leaf (Sarote *et al.*, 2006). Papain extraction from papaya leaf is simpler than extraction from the latex part; the latex extract contains less contaminant (Sarote *et al.*, 2006).

2.6.1.1 ULTRASONICATION EXTRACTION

Ultrasound technology is used in many fields; food processing, process improvement and biological homogenization. This method is used to disrupt prokaryotic and eukaryotic cells. The vibrations created cause mechanical shearing of the cell wall.

A study was once carried out and it was reported that cell damage is evaluated using transmission electron microscopy as it was evident in the plasmolysis that occured in the cells of

the leaves after ultrasonic-assisted extraction of Epimedium C from Epimedium fresh leaves. It could thereby be deduced that ultrasound has destructive effects on the structures of leaf tissue, the cell walls and its biomolecules.

2.6.1.2 ENZYME ASSISTED EXTRACTION

Enzyme catalyzes a wide range of biochemical reaction. The application of enzyme is not restricted to catalysis alone, but it is also utilized in the extraction of plant cells intracellular material. Compared to other methods of extraction which require mechanical action - blending, milling and ultrasonication, the enzyme extraction is more environmental friendly and economical. In the industry, the enzyme-assisted extraction is been applied in the oil extraction from seeds and also fruits.

In cosmetic and pharmaceutical industry in which the use of enzyme is more promising, the cost associated with enzyme treatment can be neglected and can be used to extract more valuable oils. Although enzyme extraction often is treated as extra process or as a pre-treatment of raw material, the incorporation of enzyme treatment can provide a valuable method to reduce the use of conventional methods present in some industrial disadvantages such as plant security problems, emissions of volatile organic compounds up to the atmosphere, high-operation costs and poor quality products caused by high processing temperatures (Nur, 2010). Hence, enzyme extraction is perceived a tool for extraction.

2.6.1.2.1 MECHANISM OF ENZYME ACTION

Papain enzyme are located in the intracellular compartment of the plant cell. It is therefore desired to ensure papain is released to the solution for better extraction and higher yield obtained. The use of cell wall degrading enzyme is suggested to degrade the cell wall of plant tissue

containing cellulose, hemi-cellulose and pectin (Nur, 2010). Enzymes such as glucanase and pectinase weaken and break down the cell wall rendering the intracellular materials more accessible for extraction. The enzyme will treat the cell wall as the substrate and digest the material comprising the cell wall.

Although the field of interest of enzyme assisted extraction is in the degradation of plant cell wall, the use of enzyme is not limited to extraction of plant material process only. In the analysis of catechin a type of flavonoids in the milk-tea beverages, the use of enzyme hydrolase is capable to release the catechins from their conjugated forms. In another invention to improve a process for obtaining instant tea, the treatment of the tea leaves with the tannase and cell wall digesting enzyme leads to a soluble tea with a unique biopolymer profile. It was once reported that the use of enzymes release and modify plant cell wall biopolymers thus creates a product which contribute to flavor and acid stability of the instant tea. Moreover, in the extraction of oil it is well established that oil bodies in plant are trapped in the meshwork of proteins and cellulose/hemicelluloses structures, thus the treatment of enzyme will eventually assist in disbanding the structures.

2.6.1.2.2 ENZYMES USED IN THE EXTRACTION

Different desired material may affect the type of enzyme to be use for pretreatment. When it comes to extracting oil from oil seed or kernels, the enzymes used are usually enzyme cocktail or a mixture of enzyme. The most common ingredient of enzyme cocktail includes cellulase, protease and pectinase. Scientists discovered that in the enhancement of grape seed oil extraction, four types of enzyme are used namely the cellulase, protease, xylanase and pectinase, it has also been reported that other works relating to the use of plant leaves applied the use of

cellulase, beta-glycosidase and pectinase. However in the work of tea protein extraction, the use of the enzymes cocktail is quite different, incorporating the use of alcalase and protamex (Shen *et al.,* 2008).

The type of enzyme used would depend greatly on the working material. If the desired material contains is inside a rigid cellulose cell wall, the perfect matching enzyme will be the combination cellulase and pectinase. Cellulase will randomly splits cellulose chains into glucose while pectinase will breakdown or transform the pectin that stabilizes the cell wall (Cinar, 2005).

2.7 CHARACTERIZATION OF PAPAIN

2.7.1 EFFECT OF PRESSURE ON PAPAIN

A study by Gomez *et al.*, (1999) on the effects of high pressure on papain activity and structure showed that papain progressively is inactivated by increasing pressures in both phosphate (pH 5·0 and 6·8) and Tris (pH 6·8) buffer. In all system, the effect of pressures up to 600 MPa is minimal and temperature independent. At 800 MPa, signficant losses are found. These losses at 800 MPa were more marked when pressure treatment is at 60°C compared to when at 20°C.

2.7.2 ACTIVATION OF PAPAIN

In a study by Werner (1945), the thiosulfate activation of milk clotting action of 6 ferments of the papain group was checked. The results are as presented on the table below. Cysteine, KCN, and Na_2S are activators used in the milk clotting action of papain. Cysteine activates the stability and activity of immobilized papain (Homaei, 2010). In this study, sodium thiosulfate activated all six studied papainase preparations in their gelatin splitting, peptone splitting, and milk clotting activity.

Table 2.3: Activation of milk clotting activity of different ferments of the papain groups (Werner ,1945).

Ferment	Activator	Clotting Time (minutes)
Papain		1.25
Papain	Thiosulfate	0.57
Chympopapain		12.00
Chymopapain	Thiosulfate	0.83
Ficin		2.75
Ficin	Thiosulfate	0.42
Ficin	Cysteine	0.46
Bromelin		15.00
Bromelin	Thiosulfate	6.30
Tabernamontanain		2.33
Tabernamontanain	Thiosulfate	0.42
Calatropain		1.75
Calatropain	Cysteine	1.68

The table below shows a list of activators of papain and their percentage activity. According to Sanner and Pihl (1963), below are list of activators of papain and the enzyme activity percentage. The activity of papain was measured at pH 6.56 in the presence of increasing concentrations of the activators. The data in the table are the maximal values obtained. The maximal activity found with cysteine was set equal to 100%.

Table 2.4: Activators of papain and their enzyme activity (Sanner and Pihl, 1963).

Activator	Enzyme activity
Cysteine	100
Cysteamine	103

Dimethylcysteamine	103
2,3-Dimercaptopropanol	88
Penicillamine	72
Potassium cyanide	85

2.7.3 INACTIVATION/INHIBITION OF PAPAIN

Urea and guanidine hydrochloride are inactivators of papain. This inactivation is not accompanied by change in the secondary structure as judged by optical rotation experiments; this does not define inactivation. Though minute change is found in the specific rotation of papain in 6 M urea, it is almost completely inactivated; however it is reversibly inactivated at this concentration. As judged by optical rotation measurements, guanidine hydrochloride, 5 M, irreversibly inactivates papain, produces a maximally unfolded molecule (Hill *et al.*, 1959).

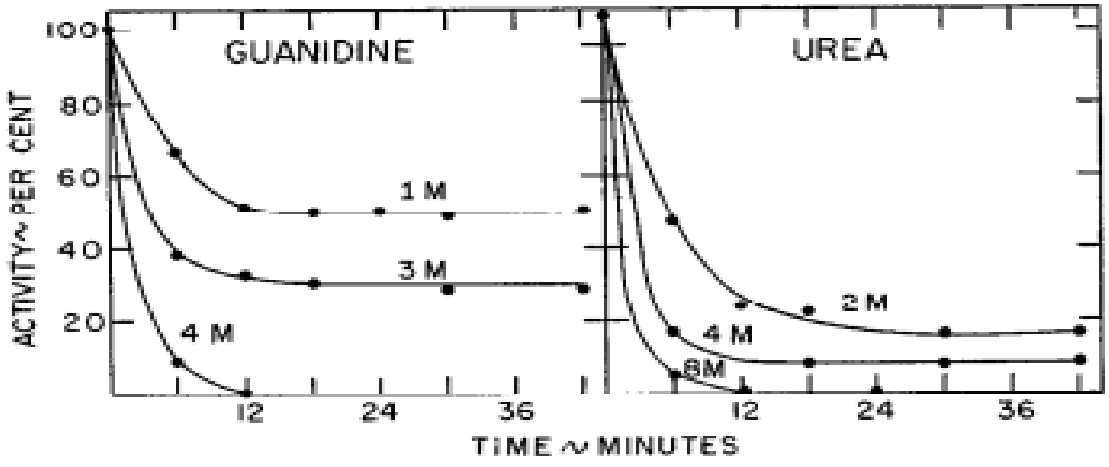

Figure 2.3: Time dependence of guanidine hydrochloride and urea inactivation. The proteolytic coefficient is calculated as a function of time at the concentration of denaturing agent shown. The results of this study were expressed as per cent of the activity of the papain preparation in water **(**Hill *et al.*, 1959**).**

2.7.3.1 KETONE AND ALDEHYDES INHIBITS PAPAIN ACTIVITY

Extremely potent competitive inhibitors of papain are aldehydes, structurally related to the acyl portion of substrates. Approximately 1 mole of acetyl-L-phenylalanylaminoacetaldehyde at pH 5.5 and 25°C, is bound per mole of papain, with K_i value of 4.6 X 10^{-8} M. Against inactivation by N-ethylmaleimide, aldehydes protect the enzyme.

The diazomethyl ketone of z-Phe and z-Phe-Phe inactivate papain through a mechanism different from chloromethyl ketone by a stoichiometric reaction at the active centre thiol of papain; the pH dependence of their inactivation of papain is different, the rate increases with decreasing pH (Leary *et al*., 1997).

2.7.3.2 FICIN AND PAPAIN INHIBITOR FROM CHICKEN EGG WHITE

Fossum and Whitaker (1968) studiedteh action of protein inhibitor purified from chicken egg white on papain and ficin. The findings of their study are as follows; the inhibitor slightly inhibited bromelain, but it did not inhibit proteolytic enzymes from a number of bacteria, trypsin and chymotrypsin; a mole of the protein inhibitor reacts with a mole of either ficin or papain; irrespective of the substrate used– casein or *p*-nitrophenyl benzyloxycarbonyl-glycinate, the purified inhibitor is equally effective. Also, they observed that on combination of the inhibitor with ficin, the effect of pH, between pH 4 and 9 was very little. Approximately ~1×10^{-9}M was the dissociation constant of the ficin-inhibitor complex and after 30 minutes of boiling at a pH of 4 and 9 respectively, 10% and 60% of the inhibitory activity was lost. By gel filtration, the molecular weight of the inhibitor was determined to be 12.7kDa.

Carino and Whitaker (1973) studied some properties of the ficin-papain inhibitor from avion egg white. Through polyacrylamide disc gel electrophoresis, the inhibitor from chicken egg white was purified. The dissociation constant of the ficin-inhibitor complex gotten was $1.47 \pm 0.68 \times 10^{-8}$M. Changes in pH, ionic strength, and temperature did not affect the extent of complex function; this infers that the forces involved in the complex formation appear to mainly be hydrophobic.

Also, Bolter (1993) researched on the induction of papain inhibitors in leaves of *Lycopersicon esculentum* by methyl jasmonate. He stated that high level of soluble protein that inhibited papain was found in the leaves of *Lycopersicon esculentum* exposed to gaseous methyl jasmonate for 24 hours at 30°C under the condition of constant lighting. The molecular mass of the protein was determined through chromatographic analysis; it was determined to be between 80,000D to 90,000D. The inhibitor activity in the plant treated for less than 18 hours increased with time, the levels remained constant for up to 4 days, after which time activity decreased. The study also identified that at a faster rate, the youngest leaf lost its activity compared to the older. Alao, treatment of the palnt with Methl Jamonate induced inhibitor activityat a significantly lower rate in the dark. A cross reaction between the polyclonal antibodies raised to purified potato tuber skin cysteine proteinase inhibitors (CPI) and the tomato inhibitor, suggested that the tomato papain inhibitor and the potato CPI are closely related. In this study, it was observed that there was no papain inhibitor activity in extracts from wounded tomato leaves, nor was there any immunological reaction with antibodies raised to potato tuber skin CPI.

Rhodes and Hoff (1984) isolated protein crystals from potato tubers which were inhibitors of cysteine proteinases such as papain, chymopapain, and ficin. The molecular weight of the inhibitor as determined by gel filtration at pH 4.3 or SDS-PAGE was 80,000Da. The inhibitor

was evaluated at pH 8.4 in a linear concentration under non-denaturing conditions; two bands of approximately 320,000Da to 350,000Da were formed. Gel filtration in the presence of varying amounts of papain suggested that the monomer combines with four papain molecules. The inhibitor contained no cysteine.

According to Schechter and Berger (1968), one of the subsites of the papain enzyme active site - namely "S_2" interacts specifically with phenylalanine residues. The evidence to this is that: (a) The peptide's susceptiblity to hydrolysis is enhanced due to the presence of Phenylalanine in positions three or higher from the C-terminal of the substrate; (b) The enzymic attack is directed by Phenylalanine to the bond next-but-one to it towards the C-terminal; (c) The presence of Phenylalanine as the second residue from the C-terminal in peptides are strong competitive inhibitors of papain. Schechter and Berger (1968) assumed that part of the active sites (S_1, S_2, etc.) of papain is being occupied by these inhibitors in the same manner as substrates. These findings could be useful, by means of X-ray analysis of papain-inhibitor complex, to depict the orientation of peptide chains bound to the active site of the enzyme.

2.8 USEFULNESS OF PAPAYA AND PAPAIN ENZYME

2.8.1 PAPAYA - A NUTRACEUTICAL FOR HEALTH BENEFITS

All parts of the papaya plant including the fruit which is usually ingested possess medicinal properties. Nutraceuticals enhance the immune system and ensures the well-being of man. Papaya has so many benefits due to its high content of Vitamins, proteolytic enzymes like papain and chymopapain which possess anti-oxidant properties. The methanolic extract of the papaya seeds and 2, 3, 4- trihydroxytoluene displayed significant antifungal activity against *Aspergillus flavus, Candida albicans* and *Penicillium citrinium* (Singh and Ali, 2011). *Carica papaya* is used

in treatment of diseases like amenorrhoea, eczema, cutaneous tubercles, glandular tumors, blood pressure, dyspepsia, constipation, expel worms and stimulate reproductive organs (Aravind et al., 2013). Chymopapain, a product of papaya, is commercially produced as a drug for sciatic pain. Papaya latex is used as a vermifuge having bacteriostatic effects on a number of infectious organisms (Karunamoorthi *et al.,* 2014).

2.8.2 PAPAYA – A NUTRACEUTICAL TO PREVENT ILLNESSES

Papaya can be considered a medicinal food. Papaya as a nutraceutical plant, helps in the control of diseases, particularly neurodegenerative diseases and helps in the management of AIDS (Bonuccelli, 2012). Papaya ingestion could bring about a boost in the immune system and could thereby reduce viral load of HIV patients; it is a potential source for the cure of AIDS virus (Karunamoorthi *et al.,* 2014). Reactive oxygen species causes oxidative stress which could induce cell damage, cause diseases such as Alzhemier and cancer and also promotes aging process. Femented papaya preparation could serve as a potential benefit as a nutraceutical and food supplements displaying antioxidant, anti-inflammatory, immunostimulatory activities (Aruoma et al., 2010).

2.8.3 BIOLOGICAL ACTIVITIES OF PAPAYA AND PAPAIN ENZYME

2.8.3.1 Treatment of Digestive System Disorders

As a traditional remedy for GIT disorders, papaya fruit is used in the treatment of the disorder (Karunamoorthi *et al.,* 2014). In the treatment for certain intestinal and digestive disorders, papain is being used. Muss *et al.,* (2013) reported that a papaya extract called Caricol® used in a double blind placebo controlled study design helps in the maintenance of an healthy digestive tract. Also Mantok (2005) reported that tea prepared with the green papaya leaf, promotes

digestion and aids treatment of disorders such as indigestion, arteriosclerosis, high blood pressure, obesity and overweight and weakening of the heart.

2.8.3.2 Anxiolytic and antioxidant activity

Papaya when ingested cause a relieve from stress and other disease conditions occur. Kebebew and Shibeshi (2013) showed in their study that papaya has anxiolytic and sedative effect. Similarly, a study by the same scientists in the year 2013 showed that papaya had antioxidant activity. The study conducted by da Silva *et al.* (2010) corroborates the notion that papain, the enzyme purified from the latex of unripe papaya is a promising source of potential antioxidant.

2.8.3.3 Anticancer property

Papaya contains natural compounds with both anti-tumor and pesticidal properties (Karunamoorthi *et al.,* 2014). The papaya leaf tea or extract is used in tumor destruction (Last, 2008). Tumor cell growth is inhibited through the stimulation of anti-tumor effects by identified components of papaya extract (Otsuki *et al.*, 2010). Such components include tocopherol, lycopene and flavonoid. Papaya has a direct antitumor effect on cancer cells, and its thereby useful in cancer therapy (Karunamoorthi *et al.,* 2014).

2.8.3.4 Treatment of Dengue Fever

Dengue fever is also called the breakbone fever caused by a virus (Flaviviridae). It is transmitted through the infective bite of the *Aedes aegypti* and *Aedes albopictus*. Dengue fever is controlled by the use of drugs, by personal protection and also through vector control. Dengue, a mosquito-borne infection has become a major international public health challenge. Dengue is predominant in urban and semi-urban areas of tropical and sub-tropical regions. *Carica papaya* leaf extract

has haemostatic and other medicinal properties that could be utilized in the treatment of dengue (Lavanya *et al.,* 2018).

Reports have claimed that there is a possible treatment of dengue virus by the use papaya leaf juice (Karunamoorthi *et al.,* 2014). Karunamoorthi *et al.* (2014) reported a study conducted by Ahmad *et al.*, (2011) to investigate the efficacy of papaya leaves extracts against Dengue Fever Virus (DFV) in a 45 year old patient. A 25 ml aliquot of the aqueous extract was administered daily, twice for five consecutive days. Significant increase in the Platelets count (PLT), White blood cells (WBC) and Neutrophils (NEUT) was observed in the study. The blood sample analysis demonstrates strong potential activity against DFV by aqueous extract of papaya leaves.

2.8.3.5 Anti-malaria activity

Contrary to the statement by Karunamoorthi *et al.* (2014) that malaria is often referred to as a disease of poverty and a cause of poverty, malaria is a disease caused by the female *Anopheles* mosquito. Malaria is treated by pharmaceutical combinations such as Arthemeter and Lumenfanthrine. Mature leaves and fruits of papaya are widely used to treat malaria (Adjanohoun, 1996). The seed of the papaya plant also shows antimalaria activities (Bhat and Surolia, 2001). Ngemenya *et al.*, (2004) recorded the antiplasmodial activity of the leaves and seeds of *Carica papaya*. All these are vivid indications of the antimalaria property of the papaya plant.

2.8.3.6 Enhances Wound Healing

Wound healing deals with the re-generation of the dermis, and it is aided by blood clotting factors promoted by Vitamin K. Papaya epicarp extracts have shown to be beneficial for wound treatment. Recent study conducted by Anuar *et al.*, (2008) confirms that ripe fruits promote the

healing of wound. Topical treatment of pediatric infected burns by the application of papain and chymopapain is effective for prevention of infection and roviding a granulating wound (Starley, 1999).

2.8.3.7 Meat Tenderizer

Rural villages in Nigeria make use of the Papaya leaves to tenderize meat. It was once assumed that papain is the enzyme used to catalyze the tenderization of meat, and the Iron present in the wire guaze on which the meat is being smoked serves as a co-factor activator of the enzyme. However, Sluyterman (1967) reported that active papain inactivation is enhanced by Fe^{2+} and Cu^{2-} and is retarded by EDTA in the absence of cysteine.

Purified papaya enzymes−papain, chymopapain and papaya peptidase A are meat tenderizers; Chymopapain is the primary contributor for tenderization because it constitutes the major protease in the mixture and it has higher thermostability and more favorable action at the meat's natural pH than papain or papaya peptidase (Kang and Warner, 1974).

2.8.3.8 Treatment of Thrombocytopenia

A characteristic feature of dengue fever is thrombocytopenia. A study for the evaluation of platelet augmentation activity of *Carica papaya* leaf aqueous extract in mice with cyclophosphamide induced thrombocytopenia and showed significant platelet count increase and decrease in clotting time (Lavanya *et al.*, 2018). Papaya extract active components such as papain, tocopherol, ascorbic acid, chymopapain, cystatin, flavonoids, cyanogenic-glucosides and glucosinolates inhibits the immune mediated platelet destruction, as well as suppresses the bone marrow caused by virus and stabilizes the membrane of infected cells, hence it hastens recovery

through natural processes, increasing the platelet count and preventing the complication of thrombocytopenia.

According to Lavanya *et al.,* (2018), papain enzyme posess some of the stated pharmacological functions−Antimicrobial, anti-amoebic, anthelminitic, antimalarial, antifungal, hepatoprotective, diuretic, immunomodulatory and female antifertility

2.9 APPLICATION OF PAPAIN

Papaya leaves traditionally are wrapped around meat and act a tenderizer. Due to papains efficiency and less destructive nature, when compared to other proteases is the commonly used enzyme in cell isolation procedures on certain tissues (Nur, 2010). Papain is a constituent of contact lens solution. Papain is used in clarifying beer, synthesis of chewing gum and in pharmaceutical industry as digestive medicine. In a study by Huet *et al.,* (2006), traditional method is shown to be effective against the nematodes as papain causes damage to the cuticles of the nematodes. The combination of papain with other proteolytic enzyme is useful in enzyme-therapy for cancer treatment.

2.9.1 Papain in Medical Uses

Papain in tissue that lack α-1-antitripsine plasmatic antiprotease which inhibits proteolysis acts as a debris-removing agent (Flindt, 1979). This occurs through the cleavage of polypeptide chains or hydrolysis of collagen cross-linkages. These cross-linkages ensure stability to the collagen fibrils, which become weaker when exposed to the papain gel (Beeley *et al.,* 2000). Papain enzyme has a long history of being used to treat sports injuries, other causes of trauma and allergies (Dietrich, 1965). Papain protease heals faster minor injuries than with placebos. Also, athletes that make use of the enzyme supplements are able recover faster from cuts

(Trickett, 1964; Dietrich, 1965). Papain has been reported to be of potential usefulness in biochemical excavation procedures for dentin (Piva *et al.,* 2008). Mansfield *et al.,* (1985) reported papain to have significant analgesic and anti-inflammatory activity against symptoms of acute allergic sinusitis like headache and toothache pain without any adverse-side effects.

2.9.2 Papain Uses in Drug Design

Some characteristics are shared between papain and the physiologically important mammalian cysteine protease; the nearly identical folding pattern around the active site has been useful for drug design (Meara and Rich, 1996). Thus, papain is reported to be useful as an experimental model structure to understand the inhibition mechanism of newly developed specific inhibitors of cathepsin L; thus, papain superfamily has antioxidant properties useful in preventing certain types of illnesses (Tsuge *et al.,* 1999; Gayosso-Garcia *et al.,* 2010). Since most of the amino acid residues that are involved in the binding to papain are conserved in cathepsin L, this publicly available high resolution structure has provided an excellent model for the successful design of highly active and specific cathepsin L inhibitors (Katunuma *et al.,* 1999). Papain is also reported to be used as a surrogate enzyme in a drug design effort to obtain potent and selective inhibitors of cathepsin K, a new member of the papain superfamily of cysteine proteases that is selected and highly expressed in osteoclasts (LaLonde *et al.,* 1998). Papain is also reported to be useful as catalyzed (co) oligomerization of α-amino acids (Schwab *et al.,* 2012).

2.9.3 Industrial Uses and Pharmaceutical Application

As stated previously, papain is used as a tenderizing agent. Protease enzymes are used to modify the proteins in charge of tenderness (myofibrillar proteins and the connective tissue proteins); the enzyme has extensively been used as an ingredient in the brewery product and meat processing

industry (Khanna and Panda, 2007). As a proteolytic enzyme, papain is used in combating dyspepsia and similar digestive disorders of the gastrointestinal tract (Huet *et al.,* 2006). Pharmaceutical industries produce in gel-dosage form, papain as it possesses antifungal, antibacterial and anti-inflammatory properties (Chukwuemeka and Anthoni, 2010).

2.10 CONSUMPTION OF PAPAIN DURING PREGNANCY

During pregnancy a number of factors could cause abortion and as well miscarriages. Ingestion of ripe papaya fruit has no effect on pregnancy; however an ingestion of unripe papaya fruit could be regarded as unsafe during pregnancy (Adebiyi *et al.*, 2002). It induces miscarriage in susceptible pregnant woman. The unripe fruit contains much more latex compared to the ripe papaya. The implication of this is that high intake of papain also could induce miscarriage in susceptible pregnant women. Adaikan and Adebiyi (2004) found in their study that crude papaya latex contains papain and chymopapain, which causes uncontrolled uterine contractions which leads to abortion (Cherian, 2000).

2.11 MARKET OPPORTUNITY AND COMMERCIALIZATION OF PAPAIN

Papain is beneficial to the industries. Papain is extracted from papaya which is a tropical and herbaceous succulent plant which possesses self supporting stems that grows in all tropical countries and many sub-tropical regions of the world (Jaime *et al.*, 2007). Papain is available round the year; therefore there seem no limitation in its production (Amri and Mamboya, 2012). However, in some countries such as Malaysia, papain cannot be manufactured upon the suitable climate as papaya cannot be grown (Nur, 2010). In the world today, papain is manufactured, distributed and sold as food ingredient. Papain yield of 8.17 g per fruit and highest papain of 686.29 g per plant in a period of 6 months has been recorded by a well managed papaya

production (Kamalkumar *et al.*, 2007; Reddy *et al.*, 2012). Papain is used in industries for diverse processes due to its high export demand. Latex is the major raw material of papain. The leaves of papaya are of significance as it is used in the treatment of asthma, hypertension and malaria. The good prospect for papain in the market has brought about an income source to farmers, manufacturers and this also increases in the economy of a country.

CHAPTER THREE

3.0 MATERIALS AND METHODOLOGY

3.1 MATERIALS

The equipments, instruments and glassware used in this study are as follows;

Analytical weighing balance; Warring blender; Cold-refrigerator centrifuge; Refrigerator; UV-Visible Spectrophotometer; Water bath; Dialysis bag; Pipette filler; Stop watch; Filter paper; Funnel; Spatula; Test tube racks; Measuring cylinder, Beakers; Stirring rod; Column; Test tubes and Pipette.

The following chemicals of analytical grade were used;

Sodium Hydroxide (NaOH); Magnesium Chloride ($MgCl_2$); Sodium Chloride (NaCl); Hydrochloric acid (HCl); Disodium Hydrogen phosphate (Na_2HPO_4); Sodium dihydrogen phosphate (NaH_2PO_4); ammonium per sulphate ($NH_4S_2O_8$); Ammonium sulphate ($NH_4)_2SO4$); Tyrosine and Copper Sulphate ($CuSO_4.5H_2O$); all from British Drug Houses (BDH), while Casein; DEAE cellulose; Sephadex G-25; Perchloric acid; Folin C reagent were from Sigma. Egg albumin was obtained from the Mountain Top University cafeteria.

3.2 EXPERIMENTAL PLANT

Two *Carica papaya* leaves weighing 173.08g and 162.72g each were used in this study. The leaves were obtained from the College of Basic and Applied Sciences, Mountain Top University, Ogun State premises. Immediately after obtaining these leaves from its tree, they were cut and washed using distilled water. The leaves were wiped dry.

3.3 METHODS

3.3.1 EXTRACTION OF CRUDE ENZYME

PRINCIPLE

Protein analysis begins with isolation and purification. However, extraction of a protein requires the disruption of the cell and as well homogenization. Thos process is often followed by differential centrifugation and, if the protein is a component of an organelle, by density gradient centrifugation. Before extraction occurs, the selection of the source of which the protein is isolated from is required. The source of isolation used in this study was the leaves of *Carica papaya.*

PROCEDURE

Fifty gram of the papaya leaves were cut into pieces and transferred into 100ml of 0.1N ice-cold phosphate buffer pH 5.5 in five places. They were homogenized with the aid of a warring blender; the homogenate was pooled and filtered, the residue weighing 105.80g was discarded. The 500ml filtrate obtained was centrifuged at 3000rpm for 10 minutes at 4°C using the cold-refrigerated centrifuge. The supernatant was discarded and the sediment obtained was rinsed with 1ml of cold 0.1M phosphate buffer pH 5.5; this extract served as the crude enzyme source. The extract was kept at 4°C for 10 minutes. The enzyme activity was determined as reported by Akinwande and Kusimo (1996) with slight modification and the protein determination was as reported as well using the method by *Lowry et al.,* (1951).

3.3.2 PURIFICATION THROUGH AMMONIUM SULPHATE PRECIPITATION

PRINCIPLE

Protein molecules contain hydrophilic and hydrophobic amino acids. In aqueous medium, hydrophobic amino acids form protected areas while hydrophilic amino acids form hydrogen bonds with surrounding water molecules (salvation layer).

When proteins are present in the salt solution – Ammonium sulfate, some of the water molecules in the salvation layer are attracted by salting. When the salt concentration gradually increases, the number of H_2O molecules in the salvation layer gradually decreases until protein molecules coagulate forming a precipitate; this is known as “salting out”.

As different proteins have different compositions of amino acids, different protein precipitate at different concentrations of the salt solutions.

The purification procedure through this method is based on the principle in which proteins are precipitated out of solution on the addition of saturated ammonium sulphate solution.

PROCEDURE

Ammonium sulphate (70% saturation) was prepared and added to the crude extract. The mixture was left overnight in the refrigerator at 4°C after which the supernatant was gently separated from the precipitate by centrifuging at 3000 rpm for 5 minutes at 4°C.

3.3.3 DIALYSIS

PRINCIPLE

Proteins are routinely separated from low molecular-weight impurities by dialysis. When a dialysis bag (an artificial semi-permeable membrane) containing a cell extract is suspended in water or a buffered solution, small molecules pass out through the membrane pores.

PROCEDURE

The precipitate containing the protein was dissolved in cold 0.1M phosphate buffer pH 5.5 and was dialyzed against distilled water overnight to remove the salt. Total protein content and enzyme activity were determined on the dialysate.

3.3.4 PURIFICATION THROUGH A DEAE - CELLULOSE COLUMN

PRINCIPLE

This is a form of ion - exchange chromatography. It separates proteins on the basis of their charge. It is an anionic-exchange resin, consisting of positively charged materials binding reversibly with a protein's negatively charged group. It is used to separate large molecules such as proteins which cannot penetrate the closely linked structure of the resin. DEAE (Diethylaminoethyl) is a weak anion exchanger with a positive charge on it.

$$-CH_2CH_2^-(NH)^+(C_2H_5)_2$$

Diethylaminoethyl (DEAE)

After proteins which do not bind to the resin have been removed, the protein of interest is recovered by washing the column with either a strong salt solution or changing the pH of the wash buffer.

PROCEDURE

To 1 litre of 0.1N HCl, 50g of DEAE-Cellulose was added and stirred thoroughly, left in the acid for an hour after which the acid was replaced with 1 litre of 0.1N NaOH. The mixture was stirred thoroughly and left also for an hour.

The alkaline solution was discarded and the resin was washed continuously with distilled water until neutral to litmus. The resin was packed into a column (0.85 X 31cm) with the eluting buffer - 0.1M Phosphate buffer pH 5.5.

A 3ml aliqout of the dialysate was applied on the packed column. Thirty milliliters of the discontinuous gradient of 0.0M, 0.1M, 0.2M, 0.4M and 2M NaCl in 0.1M phosphate buffer pH 5.5 was carried out and 3ml fractions of each gradient solution were collected into ten test tubes.

The absorbance of each fraction at 280nm was taken using Jenway 7205 spectrophotometer; the enzyme activity was determined using the method reported by Akinwande and Kusimo (1996) with slight modification.

The graph of fraction number against absorbance was plotted using excel statistical package and three peaks were obtained. The fractions in the peak were plotted. The three protein peaks were labeled A, B and C.

3.3.5 PURIFICATION THOROUGH A SEPHADEX G-25 COLUMN

PRINCIPLE

This is a size-exclusion chromatography or the gel-filtration chromatography. It separates proteins on basis of their molecular weight. The column is packed with the porous resin (gel) through which particles in an aqueous solution flow and is separated according to size.

Molecules larger than the gel pores are excluded and therefore move through the column quickly. Molecules that are smaller than the gel pores diffuse in and out the ores, so their movement through the column is retarded. The elusion time determines the protein size.

PROCEDURE

Weight of 4.5g Sephadex G-25 was dissolved in 100ml of distilled water and placed over the boiling water bath for five hours for the beads to swell. The gel was packed into a column (0.85 X 31cm) with the eluting phosphate buffer fractions were collected.

A 2ml aliquot of protein fractions A, B and C each obtained from DEAE-Cellulose column chromatography were pooled and applied to the packed column and was run through with the mobile phase – 0.1M phosphate buffer pH 5.5, and 3ml fractions of the sample were collected into twenty test tubes.

The absorbance of each fraction at 280nm was taken using Jenway 7205 spectrophotometer; the enzyme activity was determined using the method previously reported Akinwande and Kusimo (1996) with slight modification.

The graph of fraction number against absorbance was plotted using Microsoft excel statistical package. Two peaks were obtained; the fractions in each peak were pooled and labeled D and E.

3.3.6 ASSAY PROCEDURE FOR ENZYME ACTIVITY

To 4ml of substrate (1% casein), 1ml of 0.1M phosphate buffer pH 5.5 and 1ml of the extracted papain were added and incubated at 37°C water bath for 15 minutes. The reaction was stopped by adding 1.0ml of 4.2% perchloric acid. 1ml of copper reagent was then added and placed in the water bath for exactly 20 minutes. The mixture was cooled and 1ml of Folin-C reagent was added; followed by 7ml of distilled water. The mixture was properly mixed and the absorbance at 280nm was read against blank.

Using the natural substrate method, 1.0 unit of enzyme is equivalent to 1.0µmol of casein hydrolysed per minute at 37°C. The Concentration of tyrosine liberated was determined by the derived equation below:

CALCULATION

Enzyme activity was calculated using the following equations:

Papain $activity$ (µmol/min/mL) = $\frac{Abs/min \times V}{1000 \times v \times l \times \varepsilon}$

Where $Abs.\min^{-1}$ = $\frac{Absorbance\ value\ at\ 280nm}{Total\ reaction\ time}$

1000 = $factor\ introduced\ for\ the\ expression\ of\ papain\ activity\ in\ \mu mol\ /min/\ mL$

$V = total\ reaction\ volume$

ε= $molar\ extinction\ coefficient\ of$ tyrosine

v = $volume\ of\ enzyme$ used

l = $light\ path\ lenght$ ($1cm$)

Specific activity for papain was calculated using the expression:

Papain specific $activity$ (µmol /min/mL) = $\frac{Papain\ activity\ (\mu mol/min/mL)}{Protein\ concentration(mg/mL)}$

3.3.7 DETERMINATION OF PROTEIN CONCENTRATION

Protein Concentration in the crude and purified enzyme preparation was determined as previously reported by *Lowry et al.*, (1951).

PROCEDURE

To 1ml of test sample, 4ml of the alkaline solution and 1ml of copper solution were added. The mixture was allowed to stand at room temperature for 10 mins.

A volume of 0.5ml diluted Folin-C reagent was added rapidly and mixed. The mixture was left to stand for 30 minutes and the absorbance at 280nm was read against blank.

The concentration of the protein was read off the calibration curve of standard egg albumin (0.2mg/ml).

3.3.8 CHARACTERIZATION OF PAPAIN ENZYME

In the characterization of the enzymes from *Carica papaya*, the effect of substrate concentration, pH, temperature and Magnesium ion concentrations were determined.

3.3.8.1 EFFECT OF TEMPERATURE

Optimum temperature of the enzymes were determined by carrying out the enzyme assay procedure at 20°C, 30°C, 40°C, 50°C, 60°C and 70°C.

A 1ml aliquot of the DEAE-Cellulose and Sephadex G-25 purified enzyme solution were used.

3.3.8.2 OPTIMUM pH DETERMINATION

Optimum pH for the enzyme was determined by carrying out the assay procedure with buffers of pH 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5.

A 1ml aliquot of the DEAE-Cellulose and Sephadex G-25 purified enzyme solution were used.

3.3.8.3 EFFECT OF SUBSTRATE CONCENTRATION

The effect of Substrate concentration on the enzyme activity was determined. 2mg/ml, 4mg/ml, 6mg/ml, 8mg/ml and 10mg/ml casein were used as substrate for the enzyme assay. 1ml of the DEAE- Cellulose and Sephadex G-25 purifed enzyme solutions were used.

Michaelis constant (K_m) and the V_{max} for the enzymes were determined from the Lineweaver-Burk plot of the data obtained from the enzyme assay.

3.3.8.4 EFFECT OF VARIOUS CONCENTRATION OF Mg^{2+}

The effect of Mg^{2+} from $MgCl_{2(aq)}$ on the substrate concentration was determined. The assay procedure was carried out with 10mmol, 20mmol, 40mmol and 80mmol of Mg^{2+} against varying substrate (casein) concentrations of 2mg/ml, 4mg/ml and 6mg/ml. A 1ml aliquot of the Mg^{2+} solution was added to the Sephadex G-25 purified enzyme alone.

CHAPTER FOUR

4.0 RESULTS

4.1 SUMMARY OF PURIFICATION OF ENZYME

Table 4.1 below shows the summary of the purification of the papain enzyme. There is a decrease in value of the volume, activity, protein activity and yield of the enzymes through the purification phases and an increase in values of the specific activity and purification fold. It shows that by a 3- fold purification procedure, the yields and specific activities of the enzyme from the papaya leaf in different pools varies. Specific activity with the percentage yield of enzyme in pool A, B and C obtained from DEAE-Cellulose and enzyme in D and E from Sephadex G-25 column are as represented below. The highest specific activity with purification fold of the enzymes of the papaya plant is attributed to the enzyme in Pool E.

Table 4.1: Summary of purification of papain enzyme

	Volume (ml)	**Activity (µmol/min/ml)**	**Protein Concentration (mg/ml)**	**Specific Activity (µmol/min/mg)**	**Purification Fold**	**Yield (%)**
Crude extract	100	0.7778	1.9899	0.3909	———	100
Dialysate	90	0.7072	1.4494	0.4879	1.2481	72.84
DEAE-Cellulose						
A	30	0.3375	0.6276	0.5378	1.3757	31.54
B	30	0.3906	0.7939	0.4920	1.2586	39.90
C	30	0.2281	0.3877	0.5883	1.5050	19.48
Sephadex G-25						
D	9	0.3750	0.7265	0.5162	1.3205	36.51
E	12	0.1250	0.1947	0.6420	1.6424	9.78

4.2 PURIFICATION THROUGH DEAE-CELLULOSE COLUMN CHROMATOGRAPH

Elution pattern of the enzyme from *Carica papaya* by gradient mobile phase solution through the DEAE-Cellulose gave three peaks at fractions (1-10), (11-16) and (22-32) (Figure 4.1). The three peaks suggests that the papaya leaves contain three enzymes.

Figure 4.1a shows the plot of the optical density against tube number of the dialysate through DEAE-Cellulose column chromatography. This shows also that there are three enzymes (protein) present in the leaf of papaya. The figure 4.1b also is a plot of the optical density against tube number of the dialysate through DEAE-Cellulose column chromatography eluted by a gradient solution as mobile phase.

4.3 PURIFICATION THROUGH SEPHADEX G-25 COLUMN CHROMATOGRAPHY

The enzyme in pool A, B and C were passed through Sephadex G-25 column.

Figure 4.2 is a plot of the optical density against tube number of the dialysate through Sephadex G-25 Column chromatography. The result shows the elution pattern of the enzymes by an isocratic mobile phase solution which gives two peaks labeled D from fractions (1-4) and E from fractions (8-12) (Figure 4.3). This suggests that the papaya leaves contain two enzymes of high molecular weight.

4.4 CHARACTERIZATION OF EXTRACTED PAPAIN ENZYME

4.4.1 EFFECT OF TEMPERATURE

4.4.1.1 Effect of Temperature on the Enzyme Activity in Pool A

Figure 4.3 shows the effect of temperature on the activity of the enzymein pool A. The result shows the optimum temperature for the activity of the enzyme in pool A is 59°C.

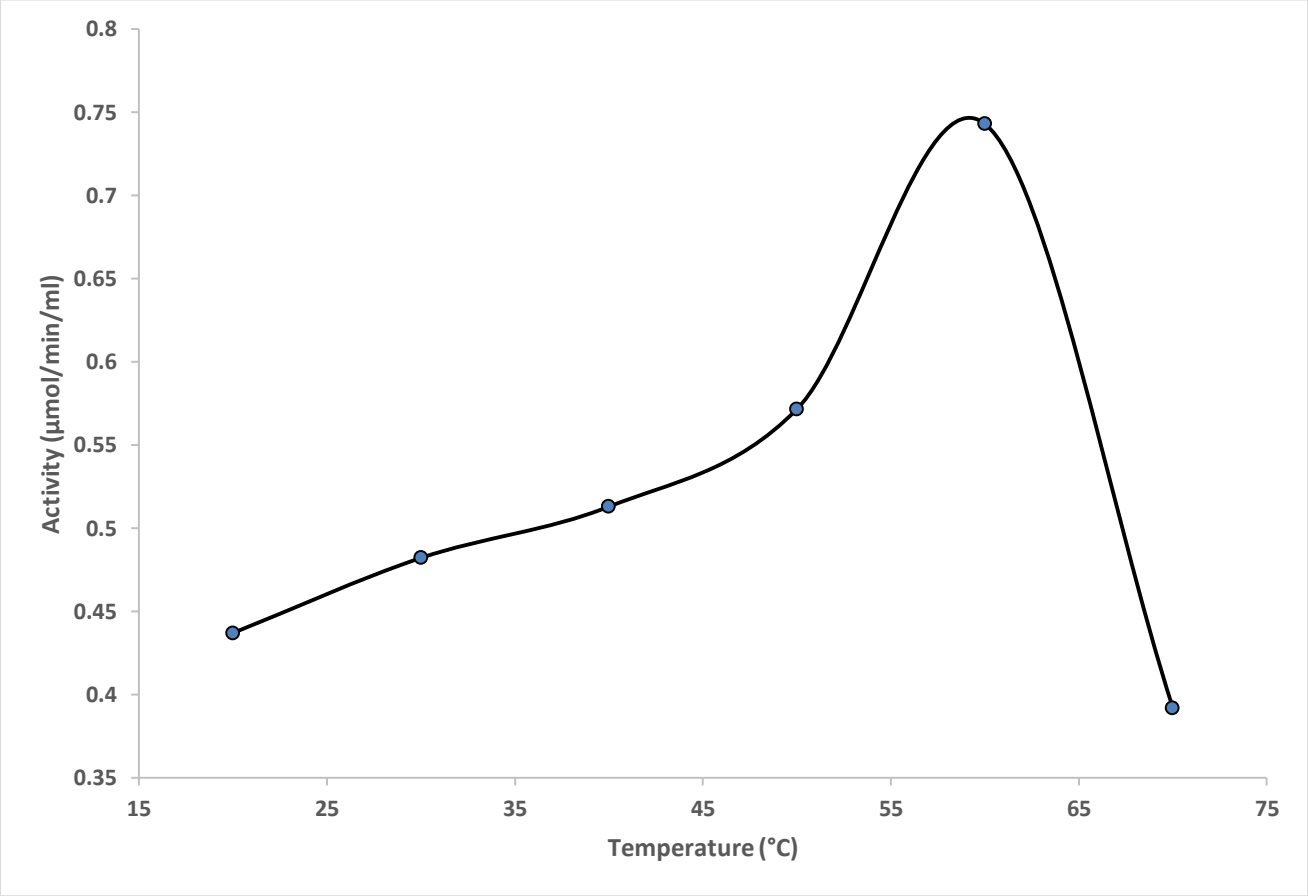

Figure 4.3: Effect of temperature on the enzyme activity in pool A.

4.4.1.2 Effect of Temperature on the Enzyme Activity in Pool B

Figure 4.4 shows the effect of temperature of the enzyme activity in pool B. The result shows that the optimum temperature for the activity of the enzyme in pool A is 58℃.

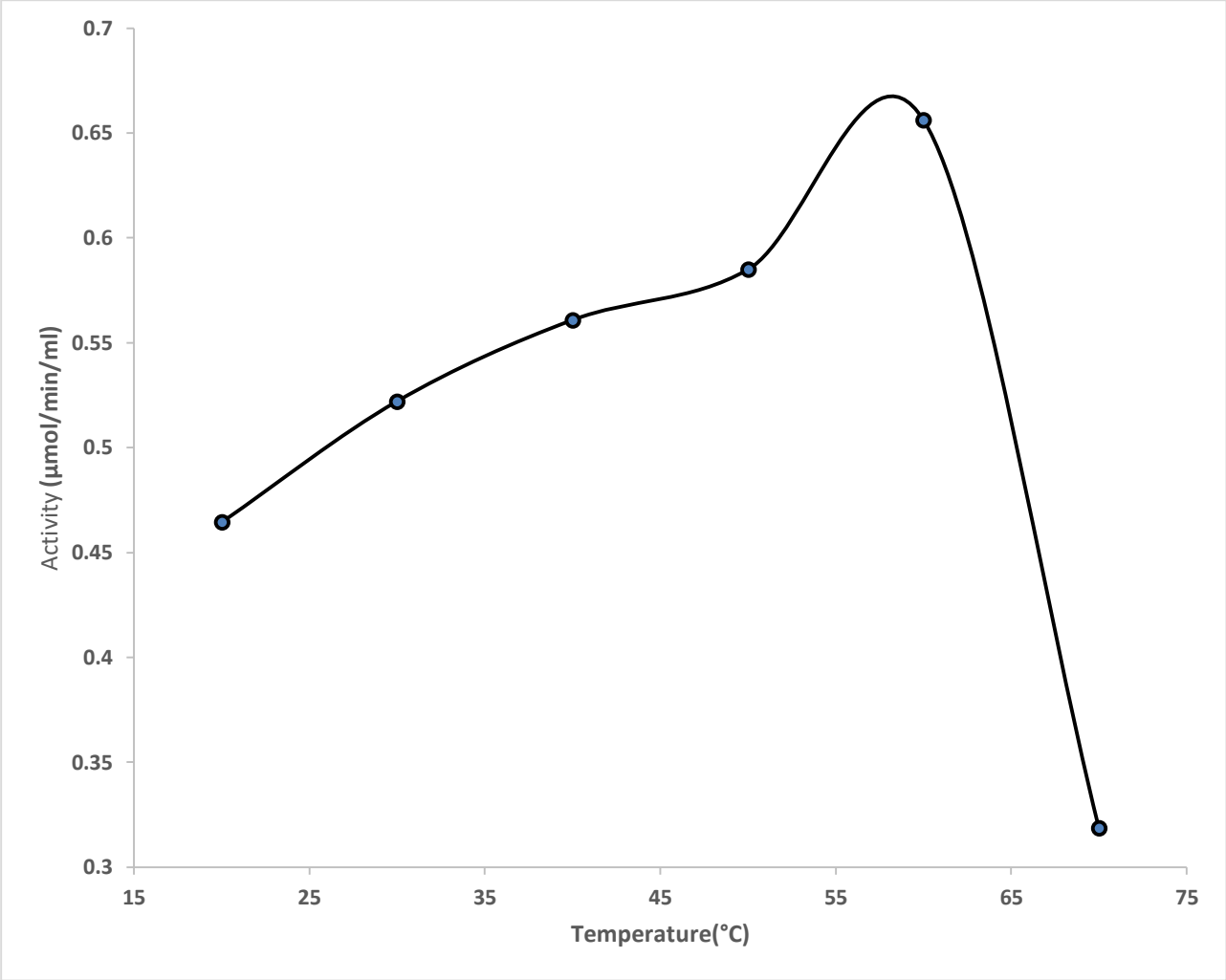

Figure 4.4: Effect of temperature on the enzyme activity in pool B.

4.4.1.3 Effect of Temperature on the Enzyme Activity in Pool C

Figure 4.5 shows the effect of temperature of the enzyme activity in pool C. The result shows that the optimum temperature for the activity of the enzyme in pool C is 59°C.

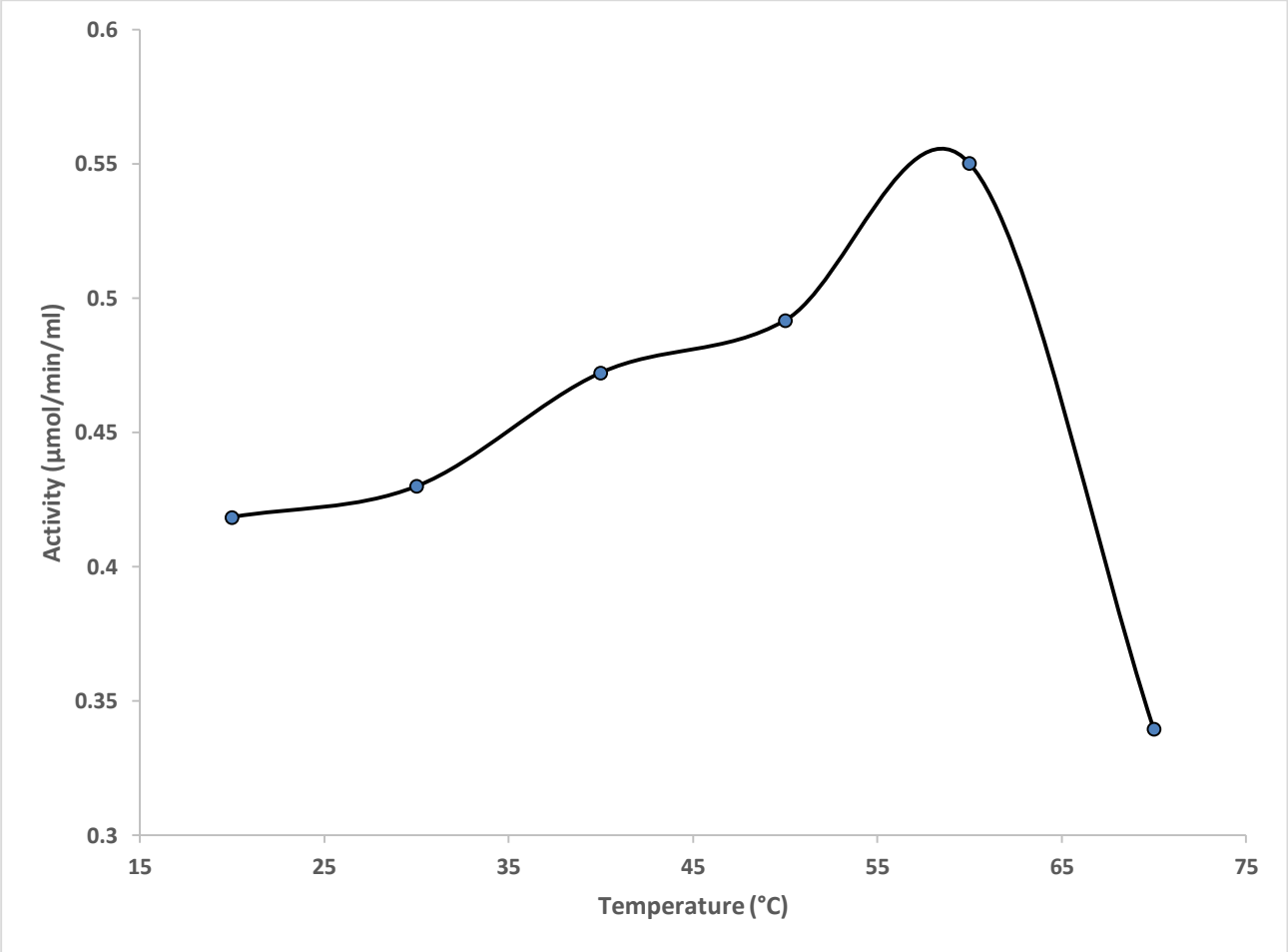

Figure 4.5: Effect of temperature on the enzyme activity in pool C

4.4.1.4 Effect of Temperature on the Enzyme Activity in Pool D

Figure 4.6 shows the effect of temperature of the enzyme activity in pool D. The result shows that the optimum temperature for the activity of the enzyme in pool D is 59°C.

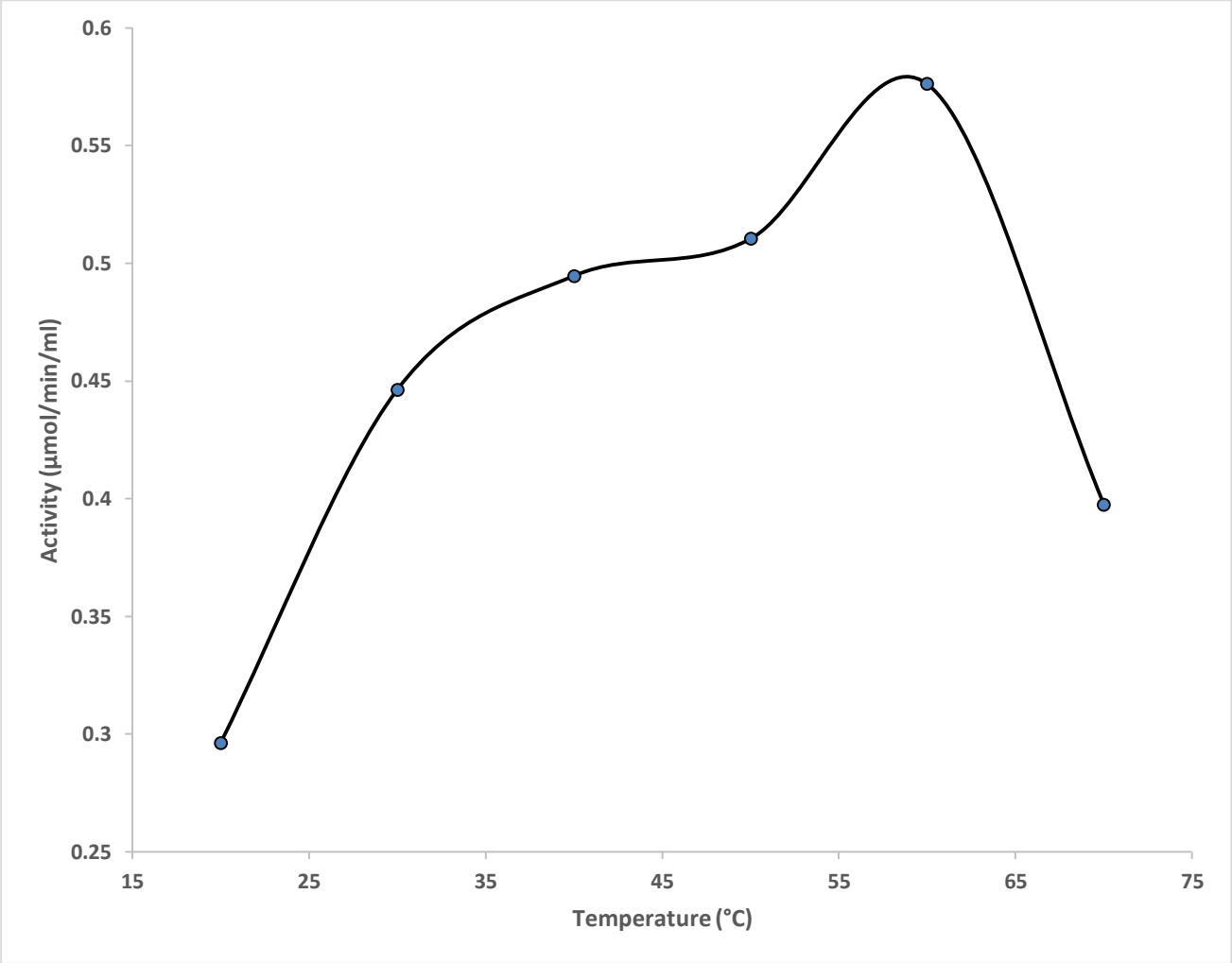

Figure 4.6: Effect of temperature on the enzyme activity in pool D.

4.4.1.5 Effect of Temperature on the Enzyme Activity in Pool E

Figure 4.7 shows the effect of temperature of the enzyme activity in pool E. The result shows that the optimum temperature for the activity of the enzyme in pool A is 50°C.

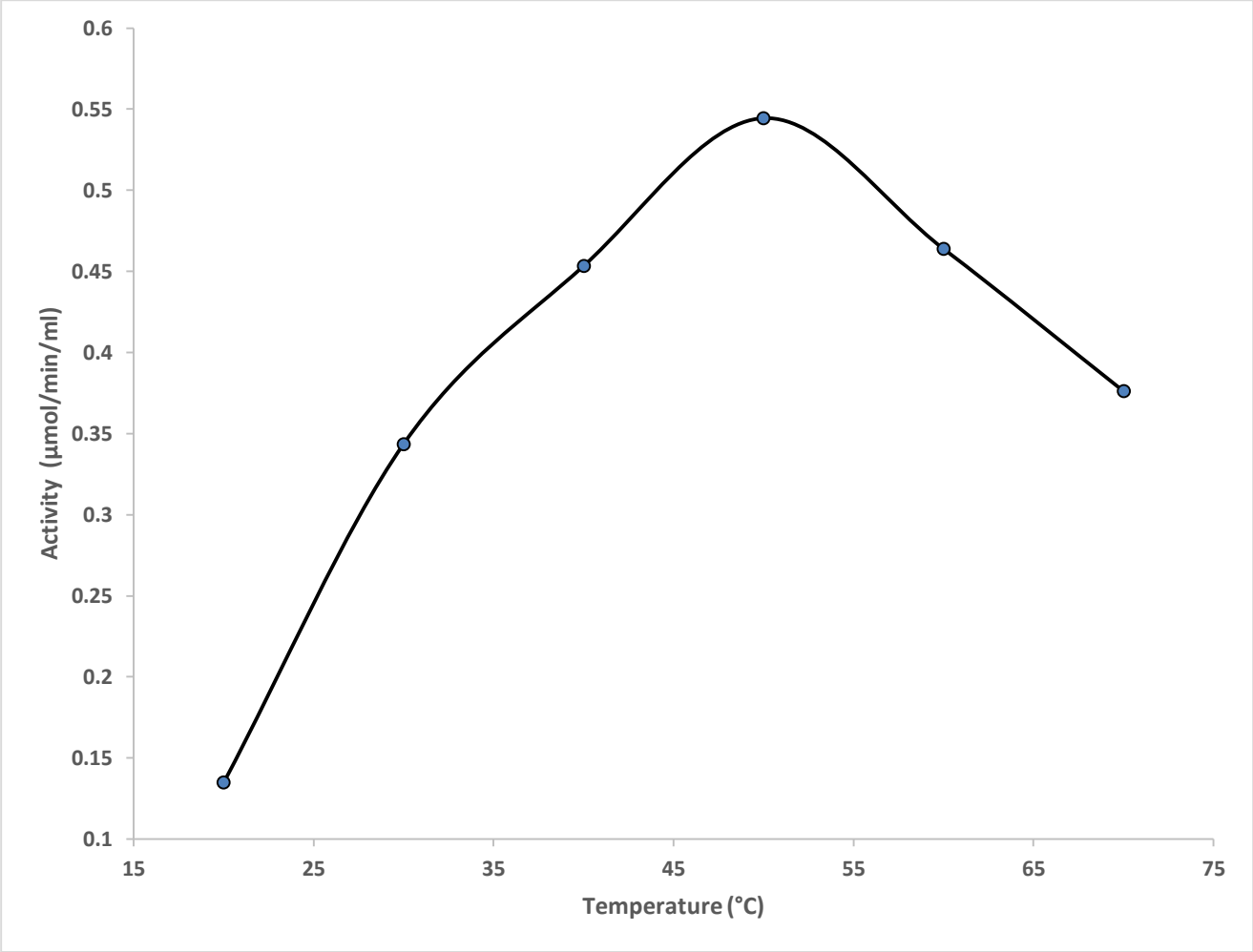

Figure 4.7: Effect of temperature on the enzyme activity in pool E.

4.4.2 EFFECT OF pH

4.4.2.1 Effect of pH on the Enzyme Activity in Pool A

Figure 4.8 shows the effect of pH of the enzyme activity in pool A. The result shows that the optimum pH for the activity of the enzyme in pool A is 5.5.

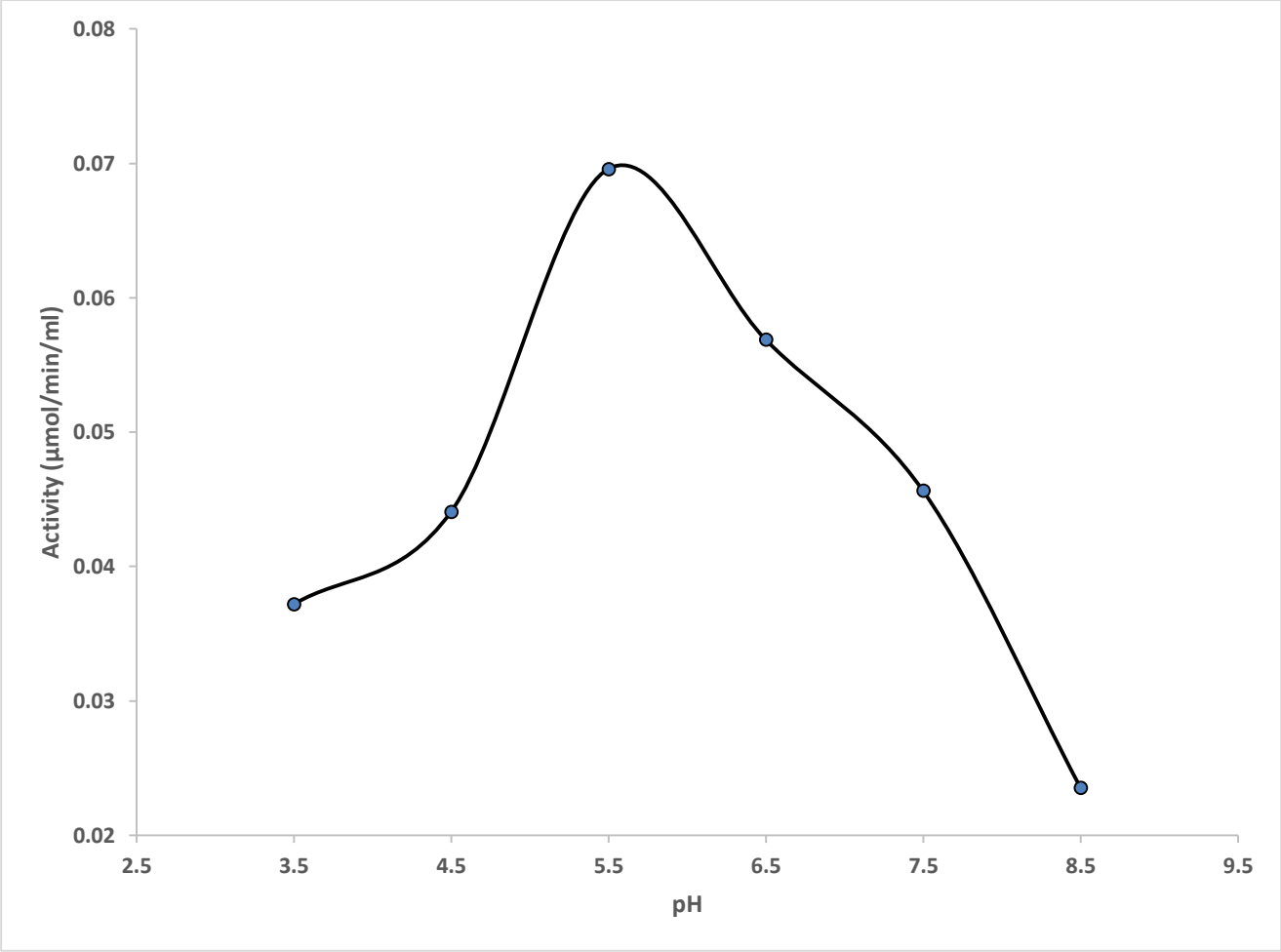

Figure 4.8: Effect of pH on the enzyme activity in pool A.

4.4.2.2 Effect of pH on the Enzyme Activity in Pool B

Figure 4.9 shows the effect of pH of the enzyme activity in pool B. The result shows that the optimum pH for the activity of the enzyme in pool B is 6.7.

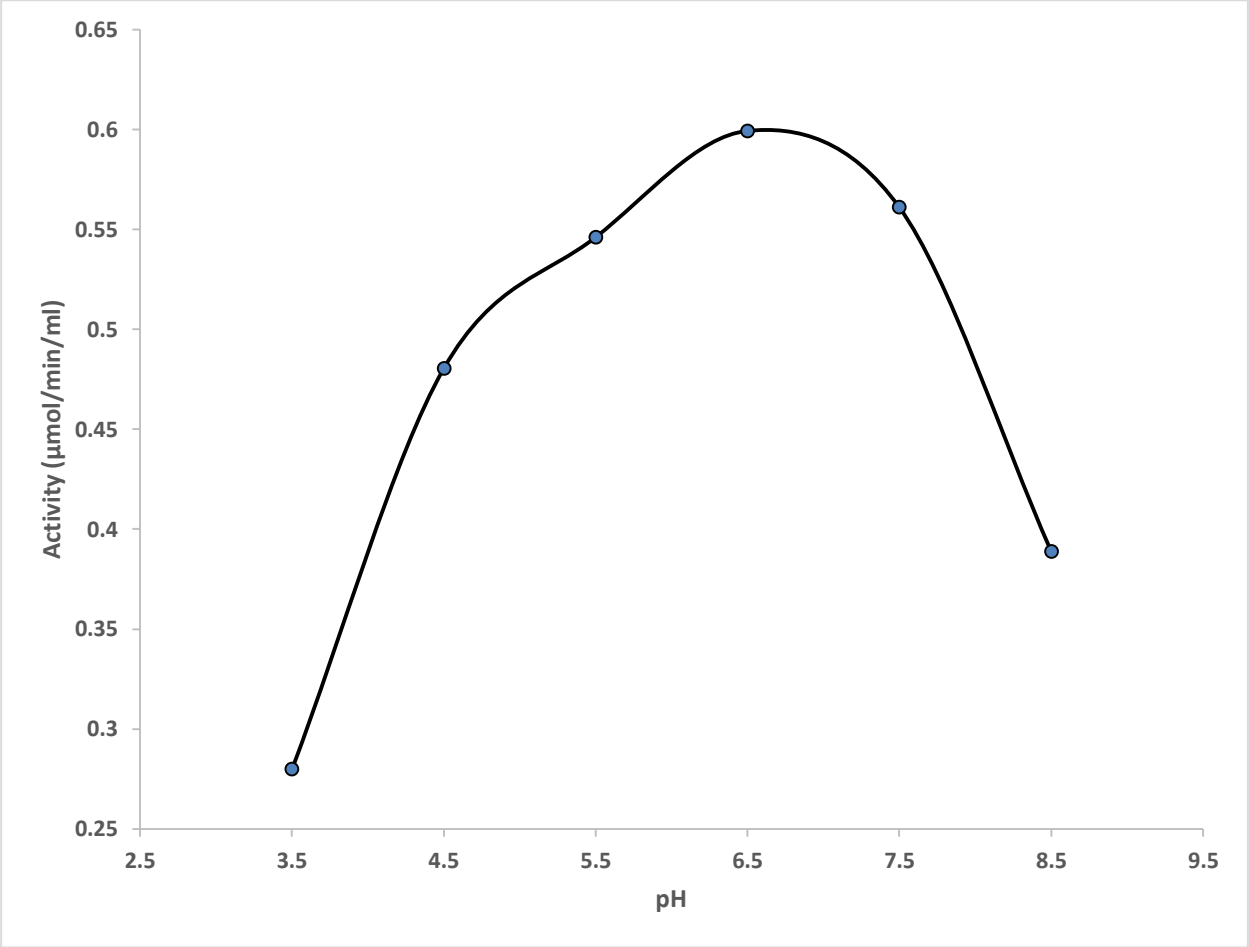

Figure 4.9: Effect of pH on the enzyme activity in pool B.

4.4.2.3 Effect of pH on the Enzyme Activity in Pool C

Figure 4.10 shows the effect of pH of the enzyme activity in pool C. The result shows that the optimum pH for the activity of the enzyme in pool B is 4.5.

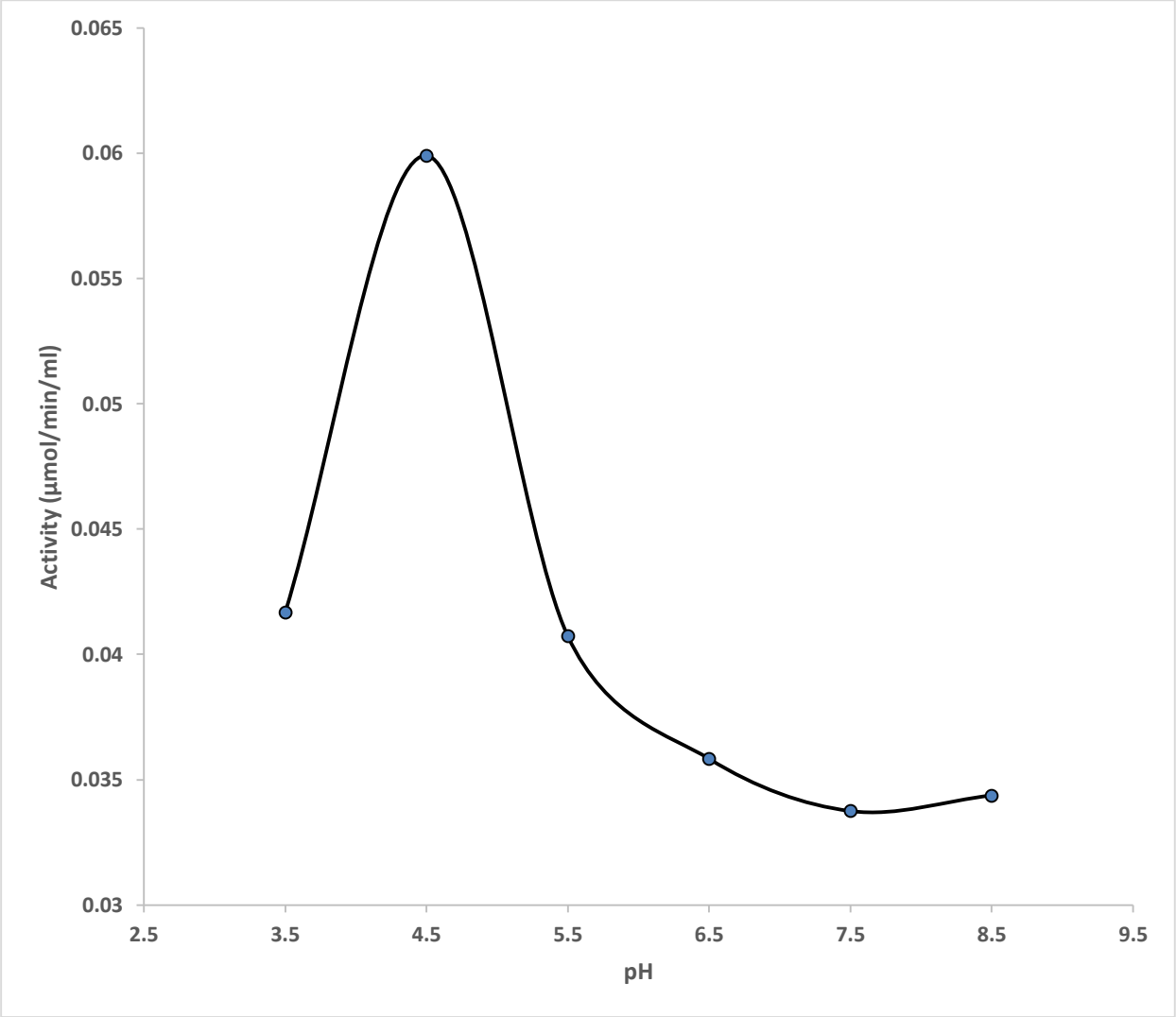

Figure 4.10: Effect of pH on the enzyme activity in pool C.

4.4.2.4 Effect of pH on the Enzyme Activity in Pool D

Figure 4.11 shows the effect of pH of the enzyme activity in pool D. The result shows that the optimum pH for the activity of the enzyme in pool B is 4.5.

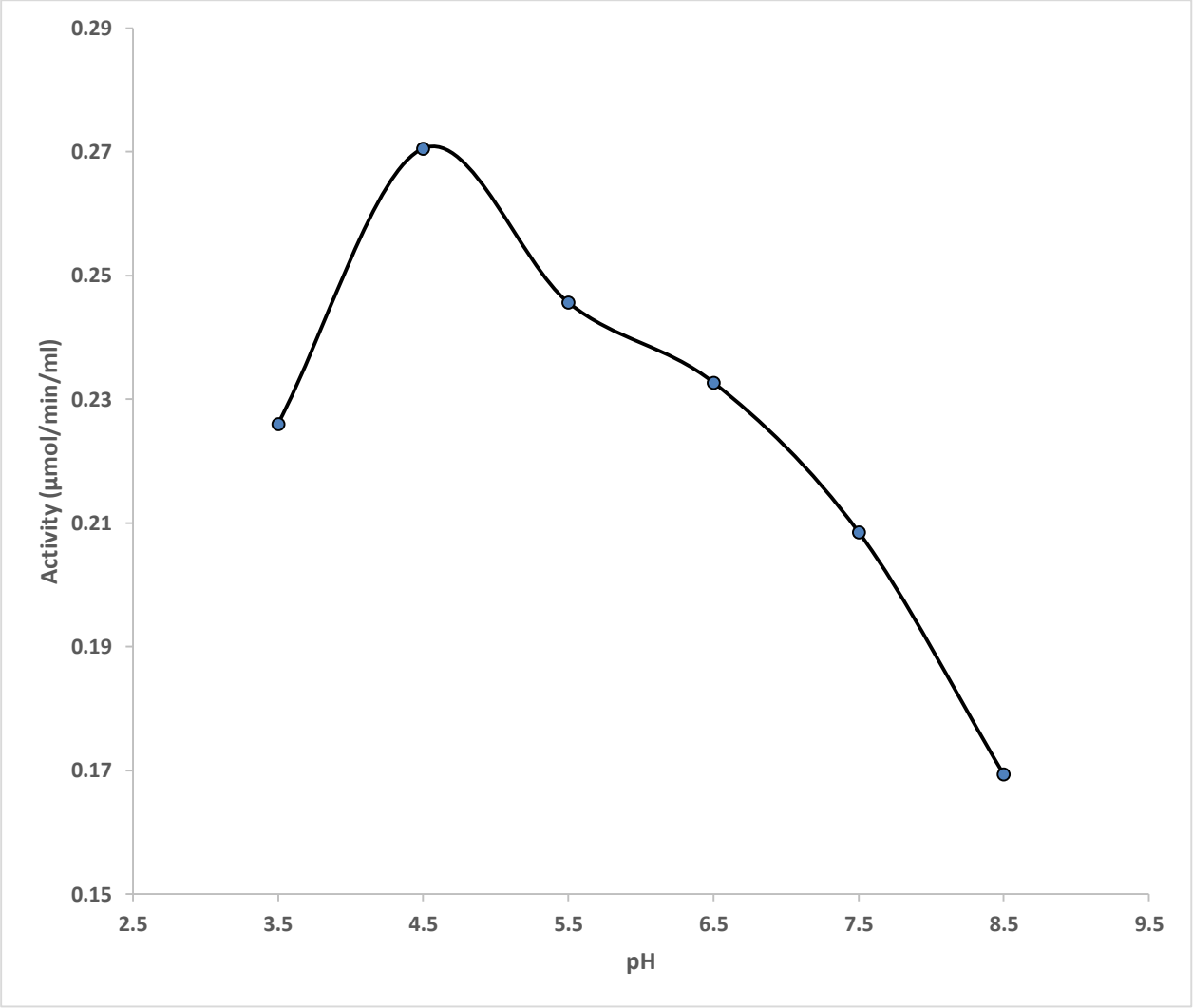

Figure 4.11: Effect of pH on the enzyme activity in pool D.

4.4.2.5 Effect of pH on the Enzyme Activity in Pool E

Figure 4.9 shows the effect of pH of the enzyme activity in pool E. The result shows that the optimum pH for the activity of the enzyme in pool B is 5.5.

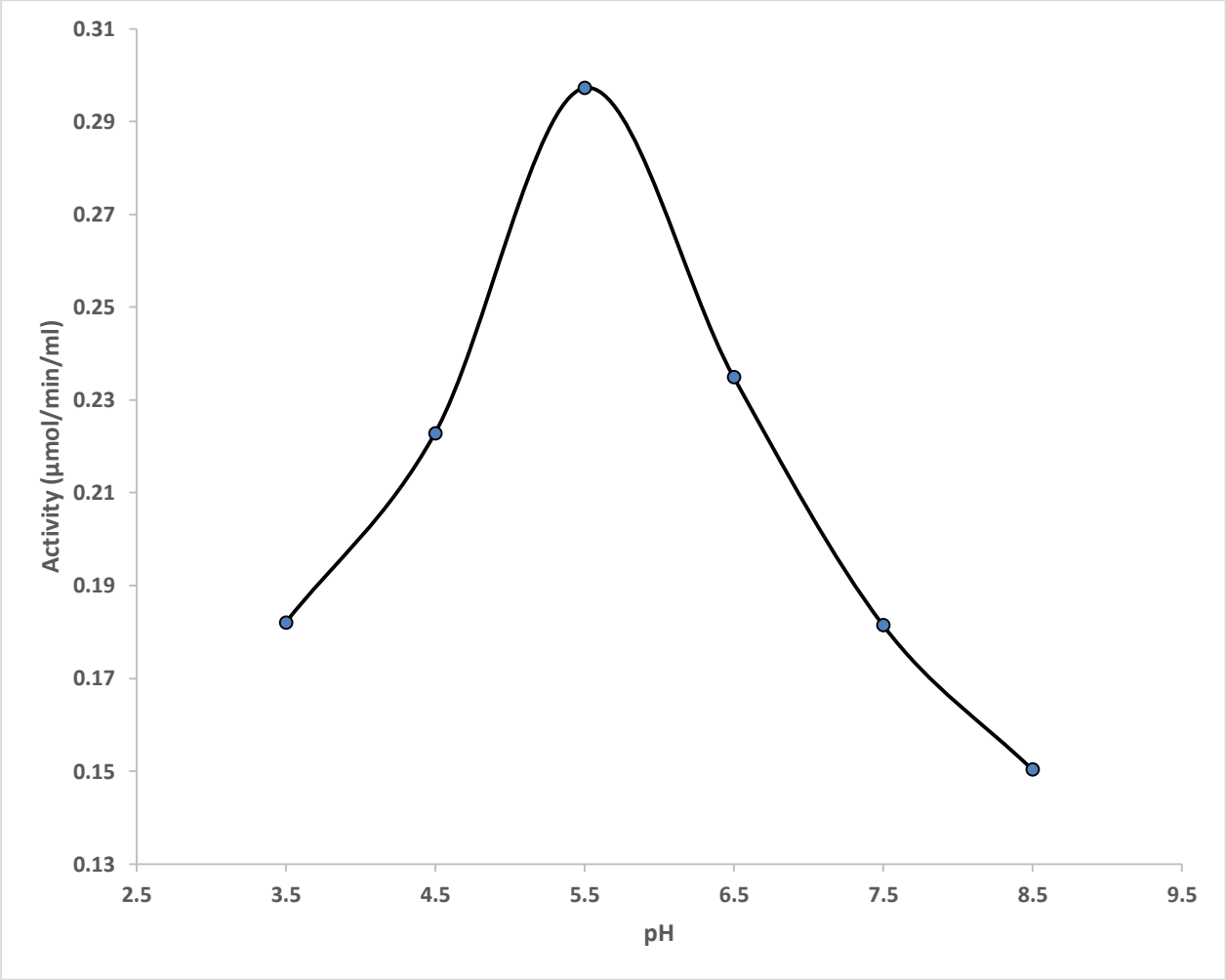

Figure 4.12: Effect of pH on the enzyme activity in pool E.

4.4.3 EFFECT OF SUBSTRATE CONCENTRATION ON ENZYME ACTIVITY

The graphs were plotted using the Lineweaver - Burk plot. Below are the results of the effect of various substrate (casein) concentration on the various pooled fractions gotten from the *Carica papaya* leaves;

4.4.3.1 Effect of Substrate Concentration on the Activity of the Enzyme in Pool A

Figure 4.13 shows the line-weaver Burk plot of reciprocal of velocity of the enzyme in pool A against reciprocal of casein concentration. The result shows the K_m value is 8.70mg/ml and the V_{max} is 0.20.

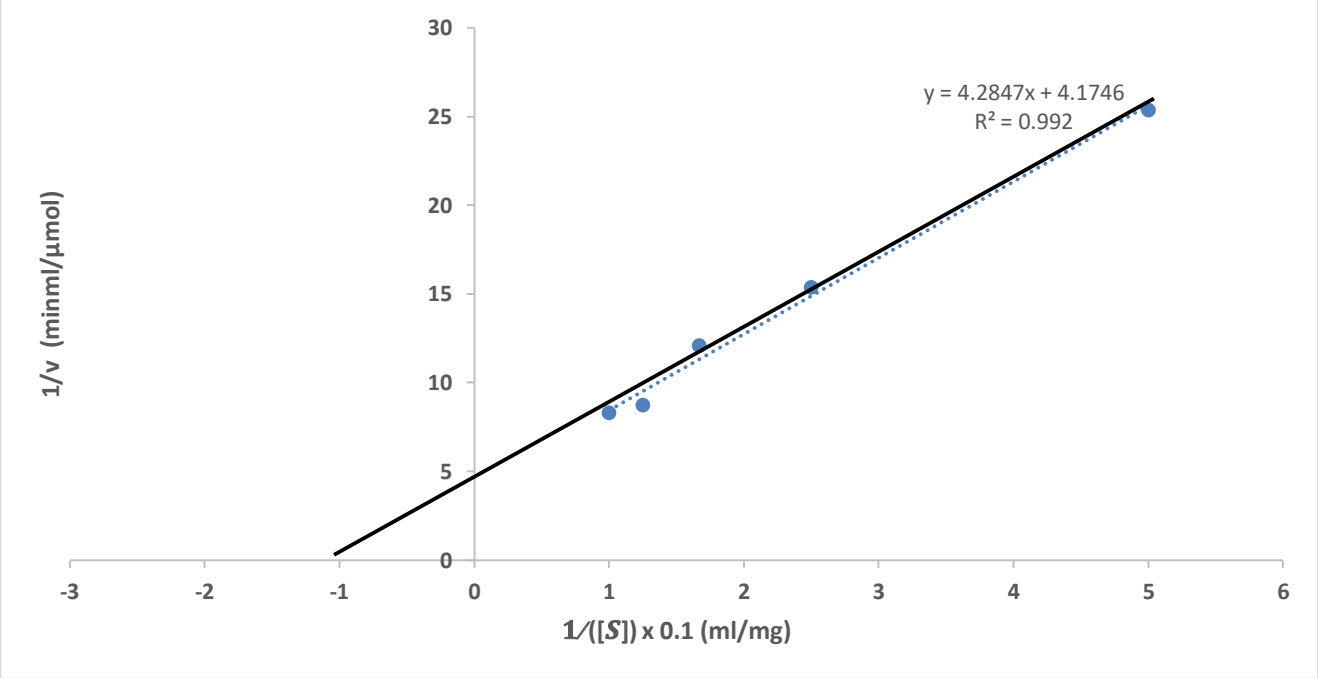

Figure 4.13: Line-weaver Burk plot of reciprocal of velocity of enzyme in pool A against reciprocal of casein concentration.

4.4.3.2 Effect of Substrate Concentration on the Activity of the Enzyme in Pool B

Figure 4.14 shows the line-weaver Burk plot of reciprocal of velocity of the enzyme in pool B against reciprocal of casein concentration. The result shows the K_m value is 1.47mg/ml and the V_{max} is 0.059.

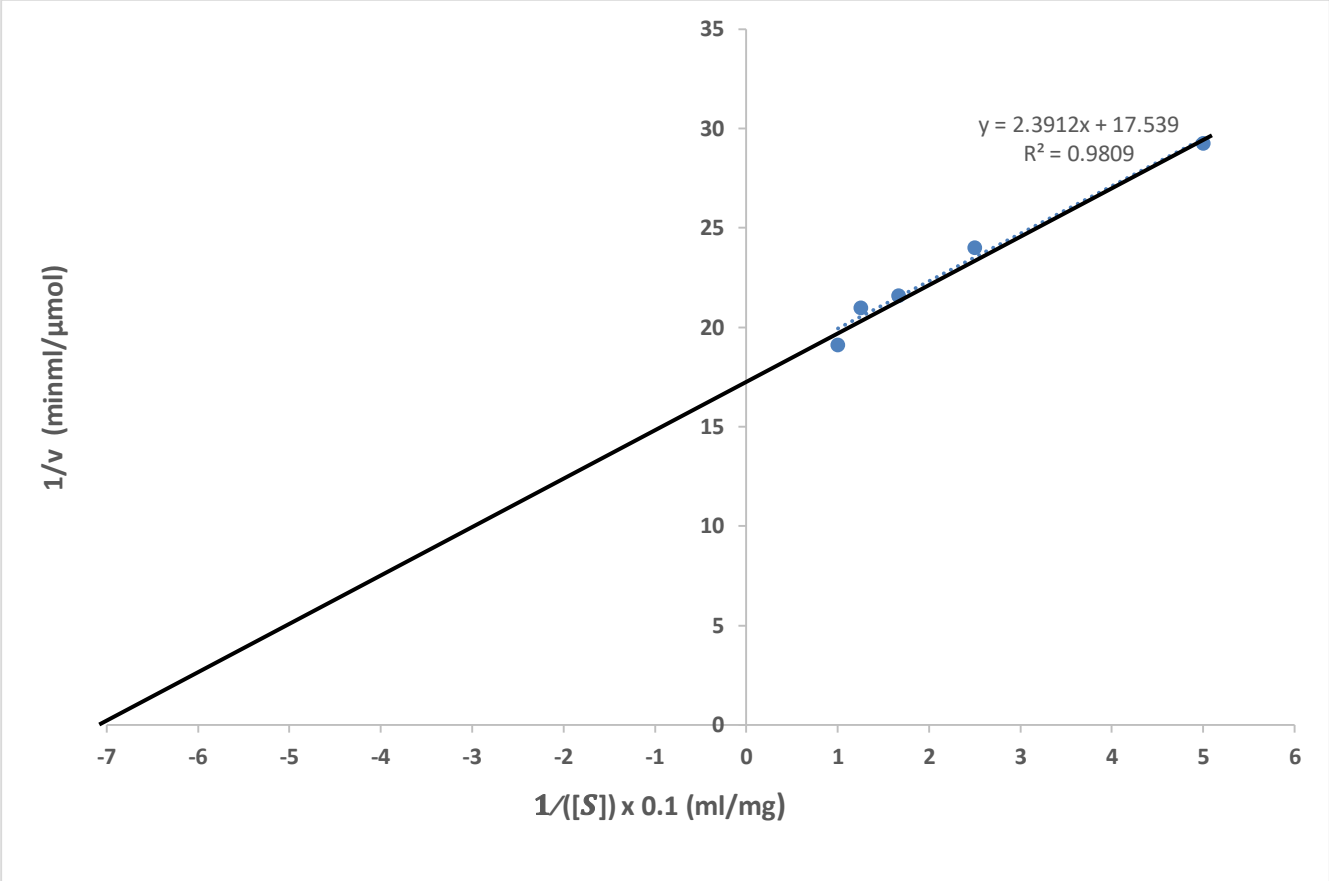

Figure 4.14: Line-weaver Burk plot of reciprocal of velocity of enzyme in pool B against reciprocal of casein concentration.

4.4.3.3 Effect of Substrate Concentration on the Activity of the Enzyme in Pool C

Figure 4.15 shows the line-weaver Burk plot of reciprocal of velocity of the enzyme in pool C against reciprocal of casein concentration. The result shows the K_m value is 1.52mg/ml and the V_{max} is 0.042.

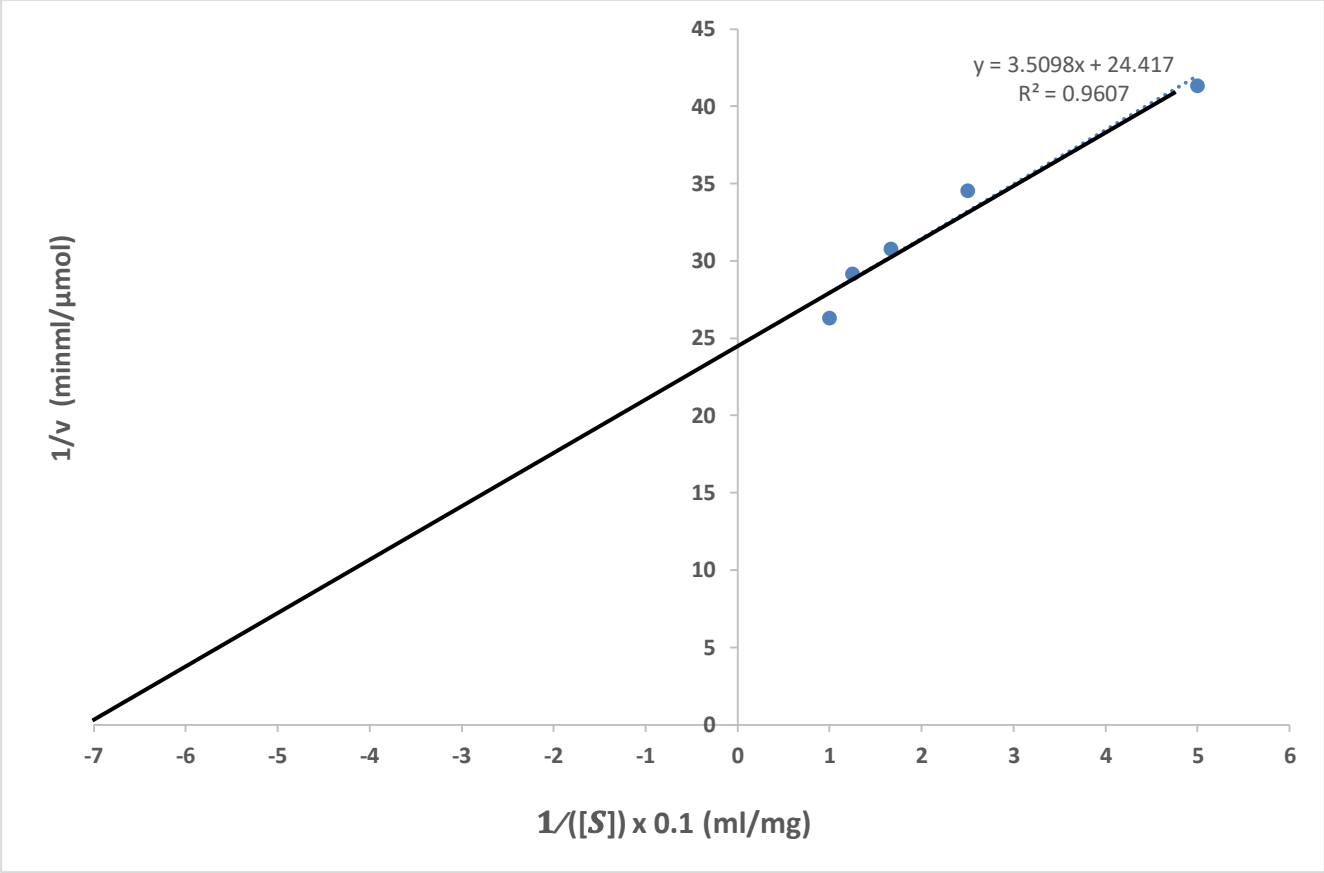

Figure 4.15: Line-weaver Burk plot of reciprocal of velocity of enzyme in pool C against reciprocal of casein concentration.

4.4.3.4 Effect of Substrate Concentration on the Activity of the Enzyme in Pool D

Figure 4.16 shows the line-weaver Burk plot of reciprocal of velocity of the enzyme in pool D against reciprocal of casein concentration. The result shows the K_m value is 5.00mg/ml and the V_{max} is 0.33.

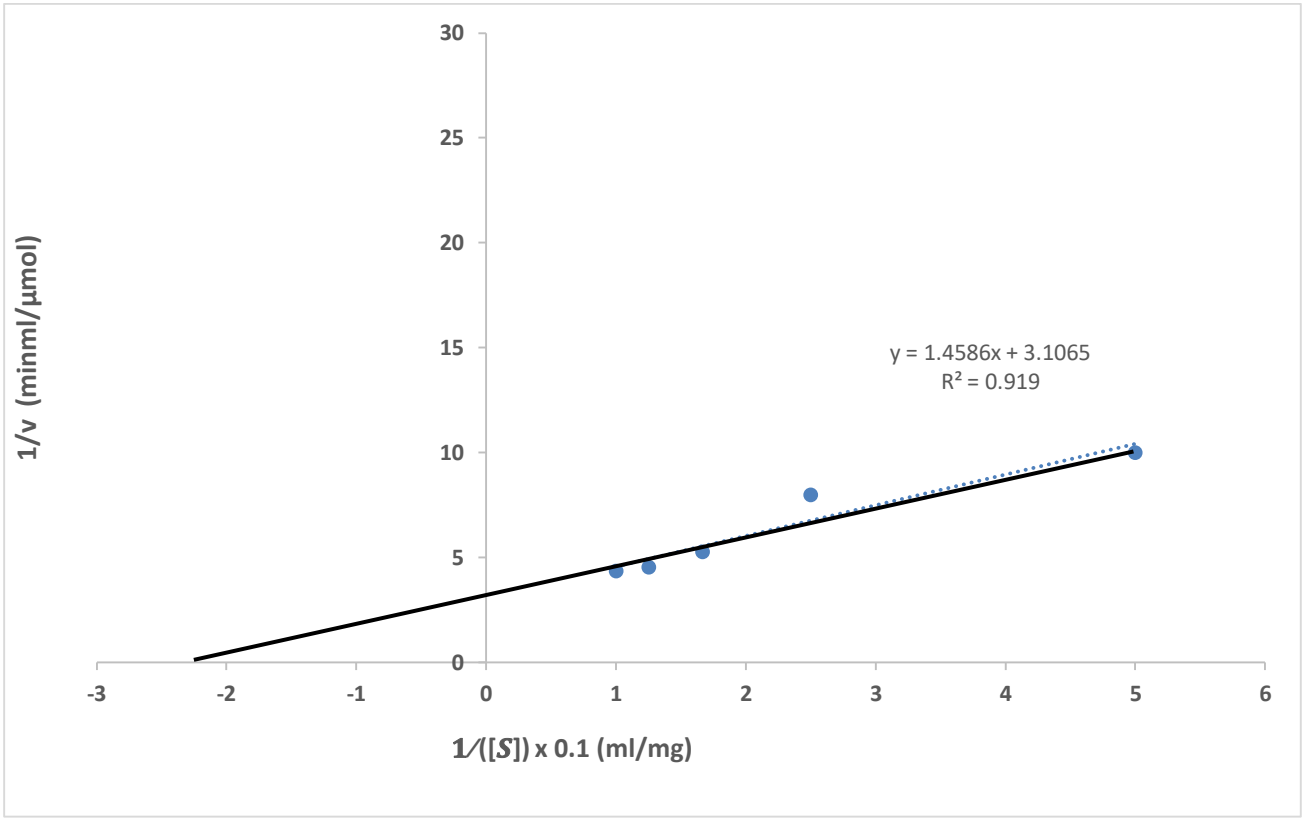

Figure 4.16: Line-weaver Burk plot of reciprocal of velocity of enzyme in pool D against reciprocal of casein concentration.

4.4.3.5 Effect of Substrate Concentration on the Activity of the Enzyme in Pool E

Figure 4.17 shows the line-weaver Burk plot of reciprocal of velocity of the enzyme in pool E against reciprocal of casein concentration. The result shows the K_m value is 1.25mg/ml and the V_{max} is 0.42.

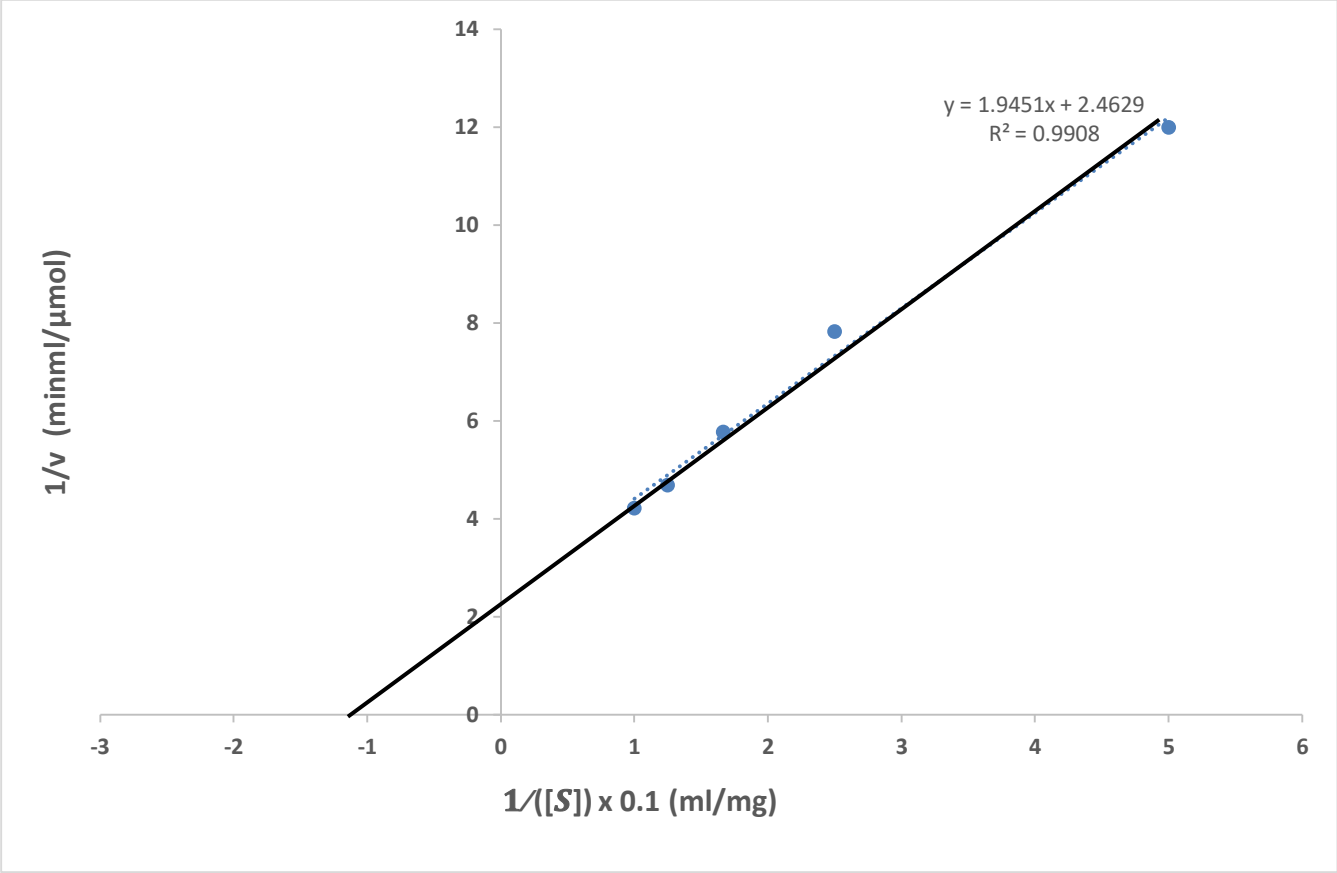

Figure 4.17: Line-weaver Burk plot of reciprocal of velocity of enzyme in pool E against reciprocal of casein concentration.

4.4.4 EFFECT OF VARIOUS CONCENTRATIONS OF Mg^{2+} ON ENZYME ACTIVITY

The results of the effect of various Mg^{2+} concentrations on enzymes gotten from the *Carica papaya* leaves are as represented as follows;

4.4.4.1 Effect of Mg^{2+} on the Activity of the Enzyme in Pool D at 2mg/ml Substrate (Casein) Concentration

Figure 4.18 shows the effect of Mg^{2+} on activity of the enzyme in pool D at 2mg/ml casein concentration. At 10mM concentration of the Mg^{2+}, the enzyme in pool D is significantly activated. The metal ion at different concentration activates the enzyme.

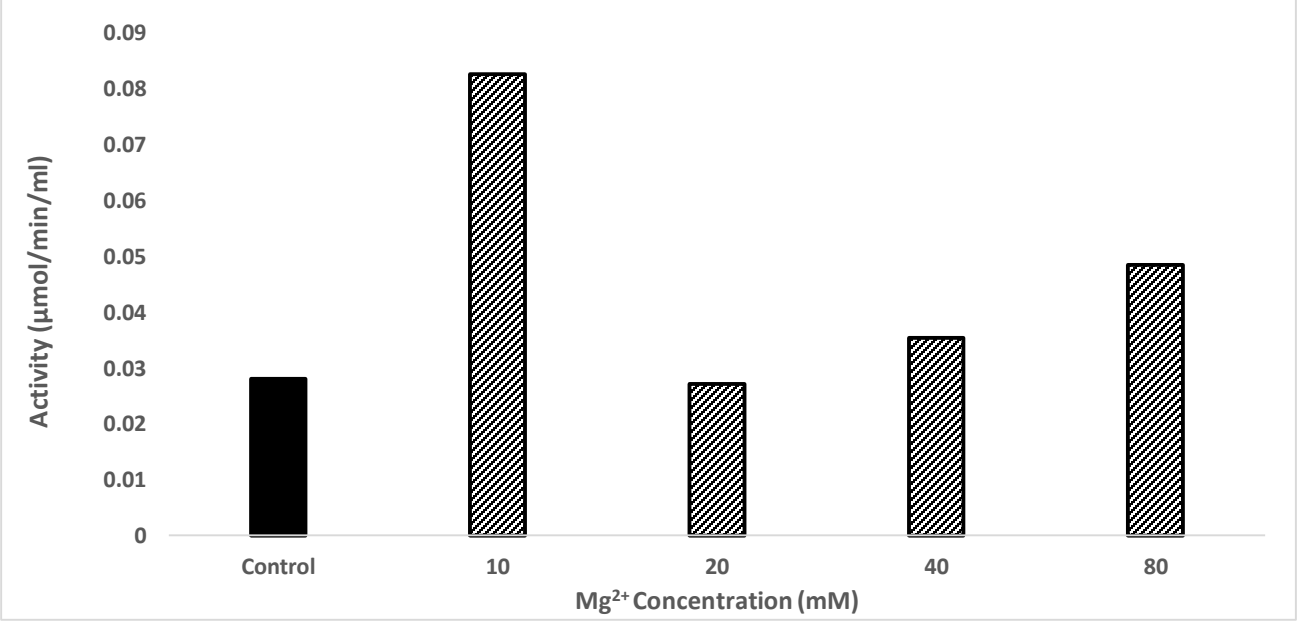

Figure 4.18: Effect of Mg^{2+} on activity of the enzyme in pool D at 2mg/ml casein concentration. I (Control): Papain activity in presence of casein only. II (10mM): Papain activity in presence of casein and 10mM [Mg^{2+}]. III (20mM): Papain activity in presence of casein and 20mM [Mg^{2+}]. IV (40mM): Papain activity in presence of casein and 40mM [Mg^{2+}]. V (80mM): Papain activity in presence of casein and 80mM [Mg^{2+}].

4.4.4.2 Effect of Mg^{2+} on the Activity of the Enzyme in Pool D at 4mg/ml Substrate (Casein) Concentration

Figure 4.19 shows the effect of Mg^{2+} on activity of the enzyme in pool D at 4mg/ml casein concentration. At 10mM concentration of Mg^{2+}, the enzyme in pool D is significantly activated. The metal ion at different concentration activates the enzyme.

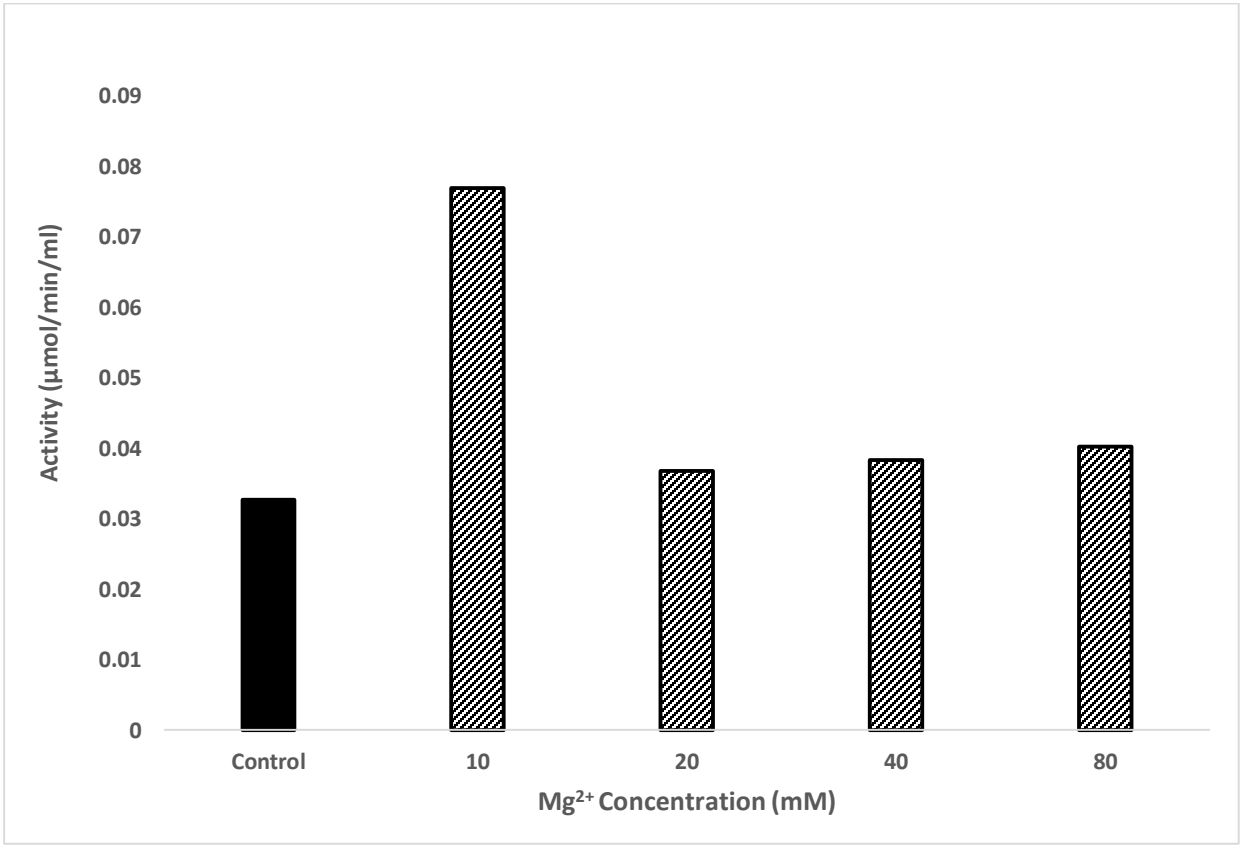

Figure 4.19: Effect of Mg^{2+} on activity of the enzyme in pool D at 4mg/ml casein concentration. I (Control): Papain activity in presence of casein only. II (10mM): Papain activity in presence of casein and 10mM [Mg^{2+}]. III (20mM): Papain activity in presence of casein and 20mM [Mg^{2+}]. IV (40mM): Papain activity in presence of casein and 40mM [Mg^{2+}]. V (80mM): Papain activity in presence of casein and 80mM [Mg^{2+}].

4.4.3.3 Effect of Mg^{2+} on the Activity of the Enzyme in Pool D at 6mg/ml Substrate (Casein) Concentration

Figure 4.20 shows the effect of Mg^{2+} on activity of the enzyme in pool D at 6mg/ml casein concentration. At 10mM and 80mM concentrations of Mg^{2+}, the enzyme in pool D is significantly activated. The metal ion activates the enzyme in pool D.

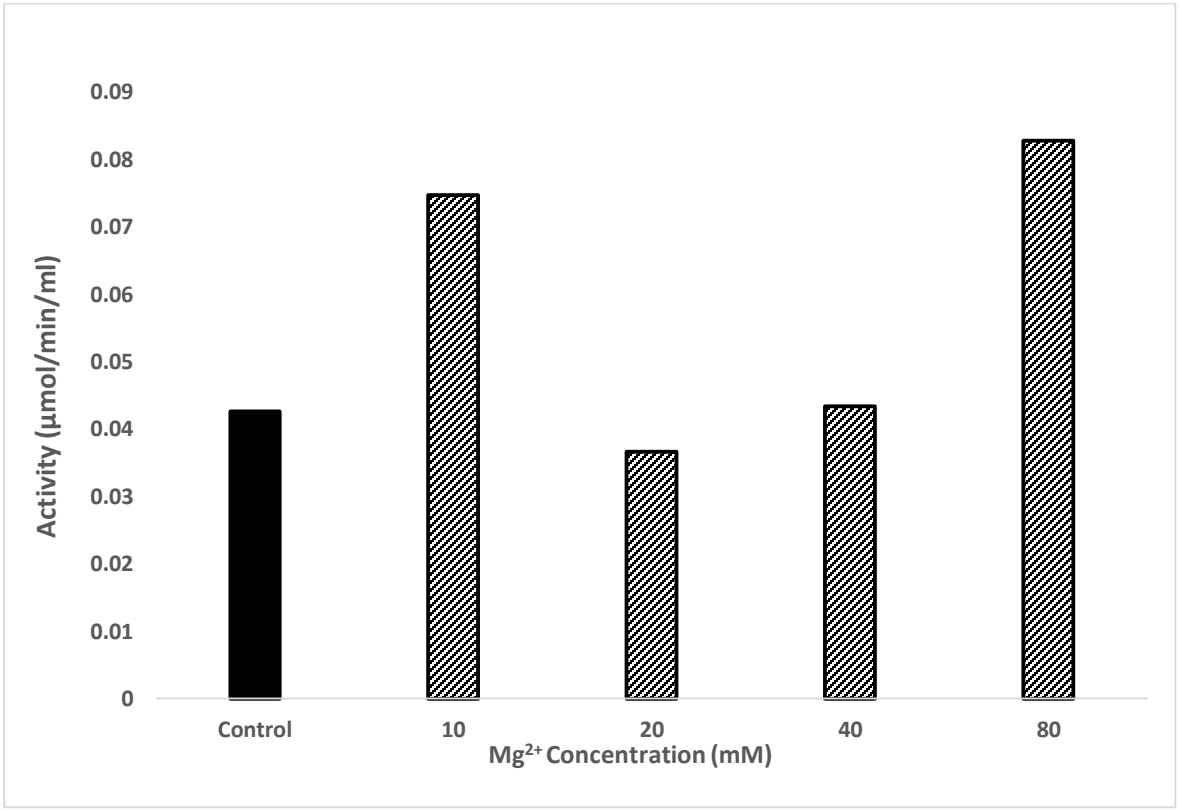

Figure 4.20: Effect of Mg^{2+} on activity of the enzyme in pool D at 6mg/ml casein concentration. I (Control): Papain activity in presence of casein only. II (10mM): Papain activity in presence of casein and 10mM [Mg^{2+}]. III (20mM): Papain activity in presence of casein and 20mM [Mg^{2+}]. IV (40mM): Papain activity in presence of casein and 40mM [Mg^{2+}]. V (80mM): Papain activity in presence of casein and 80mM [Mg^{2+}].

4.4.4.4 Effect of Mg^{2+} on the Activity of the Enzyme in Fraction E at 2mg/ml Substrate (Casein) Concentration

Figure 4.21 shows the effect of Mg^{2+} on activity of the enzyme in pool E at 2mg/ml casein concentration. All concentrations Mg^{2+} significantly increase the activity of the papain enzyme. The metal ion activates the papain pool E enzyme.

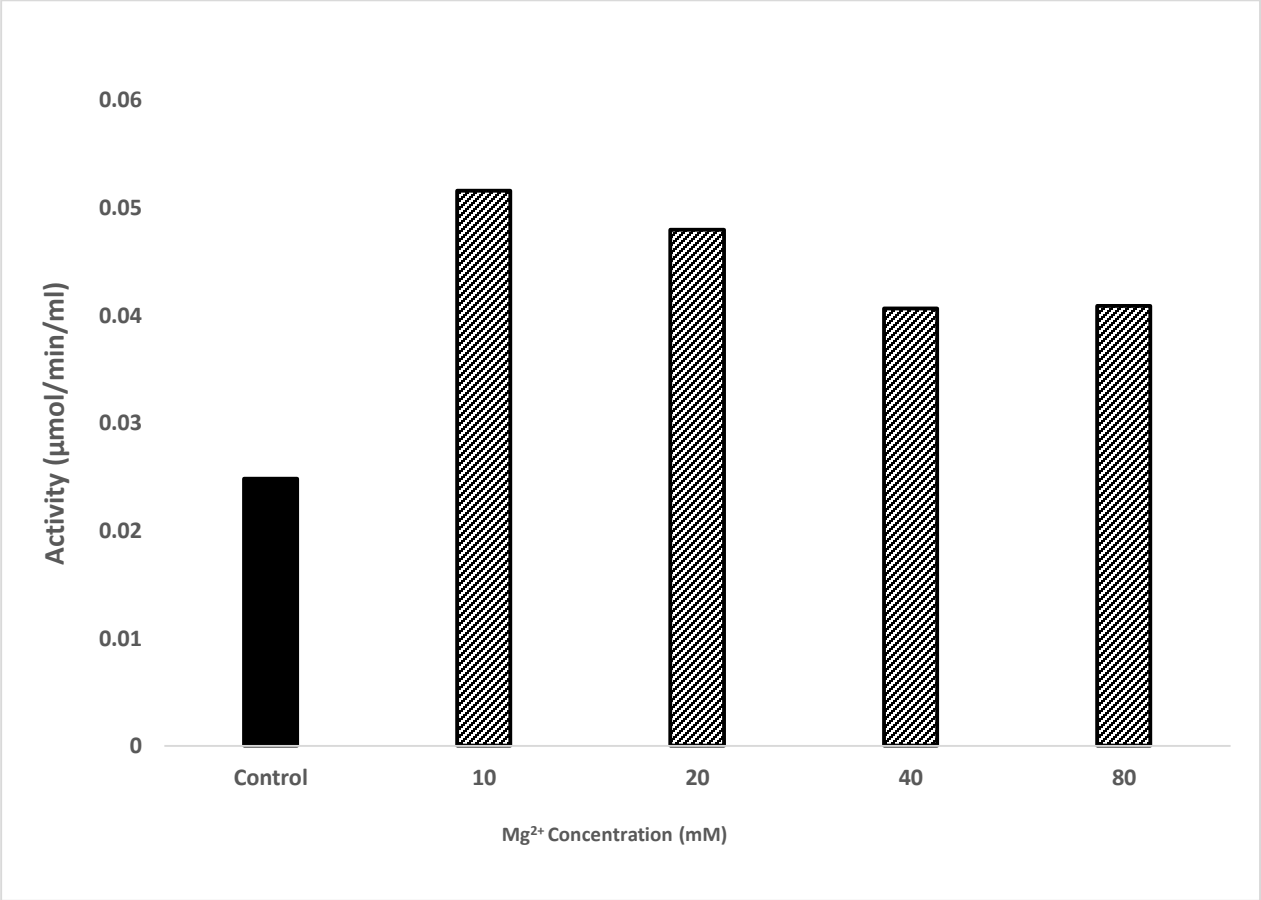

Figure 4.21: Effect of Mg^{2+} on activity of the enzyme in pool E at 2mg/ml casein concentration. I (Control): Papain activity in presence of casein only. II (10mM): Papain activity in presence of casein and 10mM [Mg^{2+}]. III (20mM): Papain activity in presence of casein and 20mM [Mg^{2+}]. IV (40mM): Papain activity in presence of casein and 40mM [Mg^{2+}]. V (80mM): Papain activity in presence of casein and 80mM [Mg^{2+}].

4.4.4.5 Effect of Mg^{2+} on the Activity of the Enzyme in Pool E at 4mg/ml Substrate (Casein) Concentration

Figure 4.22 shows the effect of Mg^{2+} on activity of the enzyme in pool E at 4mg/ml casein concentration. All concentrations of Mg^{2+} significantly increase the activity of the papain enzyme.

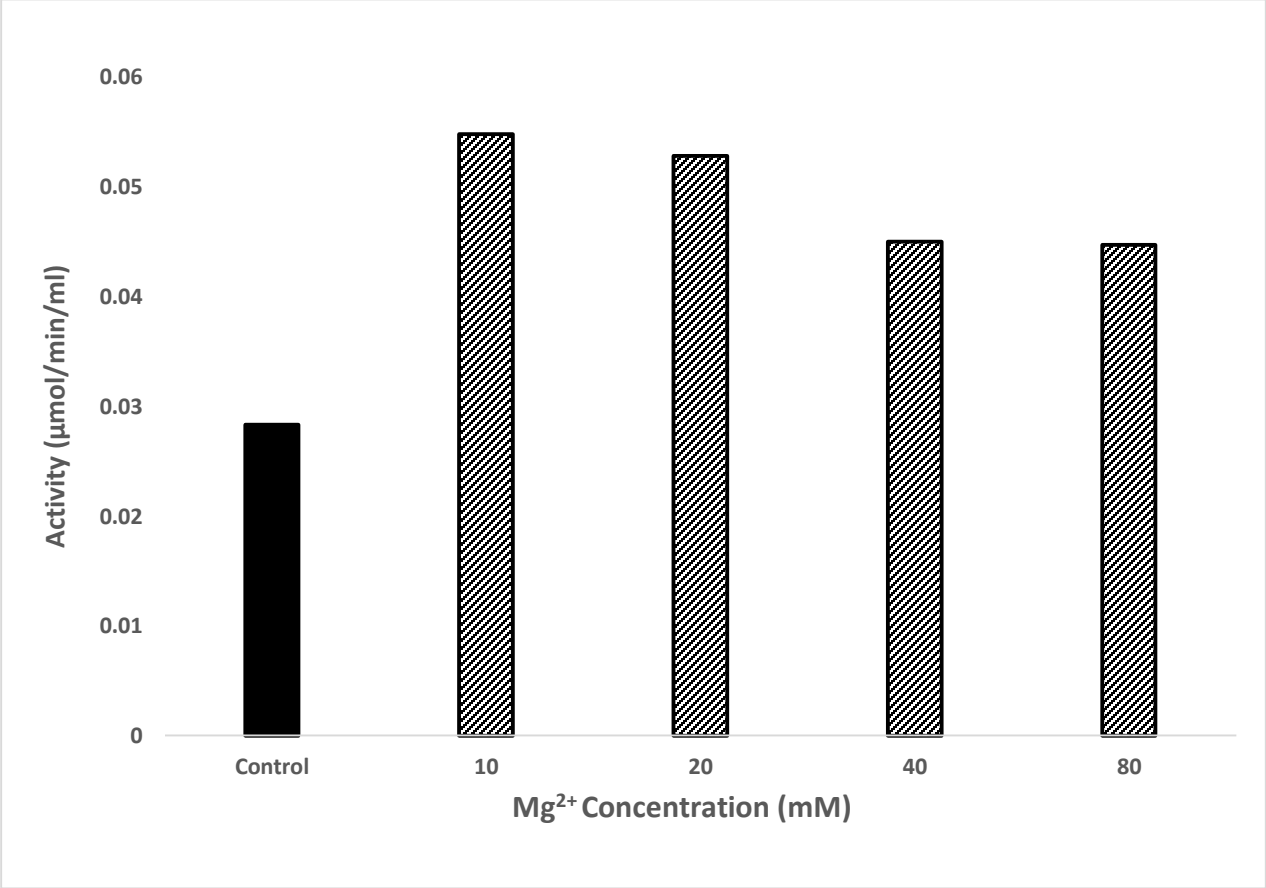

Figure 4.22: Effect of Mg^{2+} on activity of the enzyme in pool E at 4mg/ml casein concentration. I (Control): Papain activity in presence of casein only. II (10mM): Papain activity in presence of casein and 10mM [Mg^{2+}]. III (20mM): Papain activity in presence of casein and 20mM [Mg^{2+}]. IV (40mM): Papain activity in presence of casein and 40mM [Mg^{2+}]. V (80mM): Papain activity in presence of casein and 80mM [Mg^{2+}].

4.4.4.6 Effect of Mg^{2+} on the Activity of the Enzyme in Pool E at 6mg/ml Substrate (Casein) Concentration

Figure 4.23 shows the effect of Mg^{2+} on activity of the enzyme in pool E at 6mg/ml casein concentration. All concentrations of Mg^{2+} significantly increase the activity of the papain enzyme.

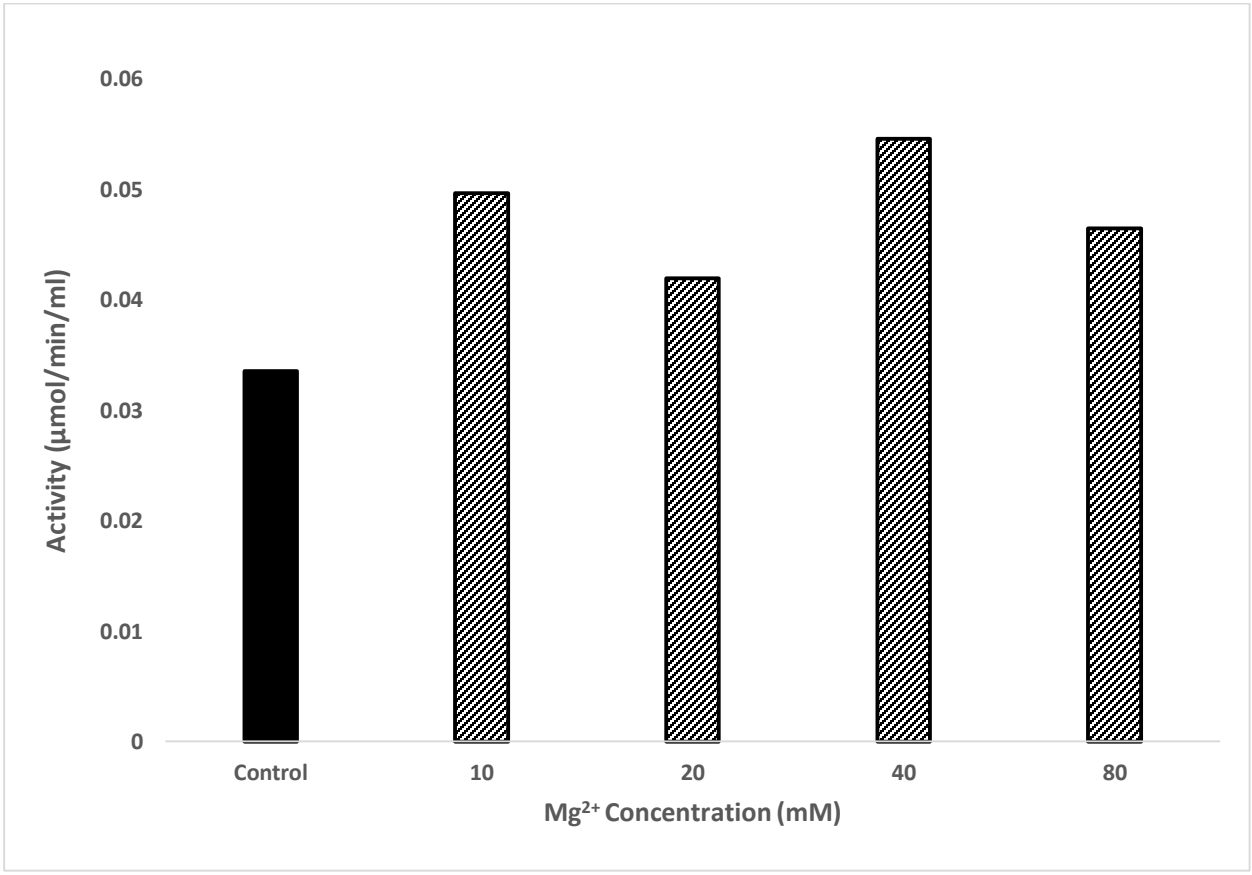

Figure 4.23: Effect of Mg^{2+} on activity of the enzyme in pool E at 6mg/ml casein concentration. I (Control): Papain activity in presence of casein only. II (10mM): Papain activity in presence of casein and 10mM [Mg^{2+}]. III (20mM): Papain activity in presence of casein and 20mM [Mg^{2+}]. IV (40mM): Papain activity in presence of casein and 40mM [Mg^{2+}]. V (80mM): Papain activity in presence of casein and 80mM [Mg^{2+}].

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Papain (EC 3.4.22.2) and Chymopapain (EC 3.4.22.6) are enzymes of the papaya plant. Papain is a globular protein family consisting of a single polypeptide chain with three disulfide bridges and sulfuhydryl group necessary for activity of the enzyme, and an accession number 1CVZ with molecular weight of 23,406 Da (Amri and Mamboya, 2012), while pure chymopapain enzyme has a molecular mass of 24,700Da as estimated by SDS-PAGE (Khan and Polgar, 1983). Further research in understanding the specificity, structure, effect of addition of inhibitors, low pH, metal ions and fluorinated alcohols is of critical importance (Huet *et al.,* 2006; Naeem *et al.,* 2006). In this study, the enzymes from *Carica papaya* were characterized.

This study shows that by a 3- fold purification procedure, yields and specific activities of the enzymes from the papaya leaf in different pools varies (Table 4.1). Specific activity with the percentage yield of enzyme in pool A, B and C obtained from DEAE-Cellulose are 0.5378 with a 72.84% yield, 0.4920 with a 31.54% yield, and 0.5883 with a 39.90% yield respectively. The specific activities with percentage yield of the enzyme in pool D and E from Sephadex G-25 column are 0.5162 with a 36.51% yield and 0.6420 with a 9.78 % yield respectively. The highest specific activity with purification fold of the enzymes of the papaya plant is attributed to the enzyme in Pool E.

Elution pattern of the enzymes from *Carica papaya* by gradient mobile phase solution through the DEAE-Cellulose gave three peaks at fractions (1-10), (11-16) and (22-32) (Figure 4.1a and 4.1b). The three peaks suggests that the papaya leaves contain three enzymes. The enzyme in

pools A, B and C were passed through Sephadex G-25 column and the elution pattern of the enzymes by an isocratic mobile phase solution gave two peaks labeled D from fractions (1-4) and E from fractions (8-12) (Figure 4.2). This suggests that the papaya leaves contain two enzymes of high molecular weight. Chymopapain (EC 3.4.22.6) and papain (EC 3.4.22.2) are enzymes of the papaya plant with a molecular weight of 24,700Da (Amri and Mamboya, 2012) and 23,406 Da (Khan and Polgar, 1983) respectively.

According to *Tigis et al.,* (2016), at 60°C, sonicated papain extract shows maximum absorbance compared to the grinded papain sample. Sarote *et al.,* (2006) affirmed that the optimum temperature for isolated papain enzyme is about 60°C. From this study, the optimum temperature of the purified papain enzymes ranges from 50°C – 59°C. The result presented in Figures 4.3 and 4.5 indicates that the enzyme in pools A and D are active over a broad temperature values with the same optimum temperature at 59°C. The precision of this report agrees with that gotten from previous studies. Also, the temperature-dependence profile of the enzyme in pool B (Figure 4.4) shows its optimum activity is at 58°C similar in value to the enzyme in pool C (Figure 4.5). The optimum temperature of the enzyme in pool E is 50°C (Figure 4.6).

The optimum pH for the activity of papain ranges from 3.0 – 9.0 depending on the substrate used (Edwin and Jagannadham, 2000; Ghosh, 2005). Papain solution is unstable under acidic conditions (pH 2.8) and will result in significant loss of activity; the enzyme is however stable from the range of pH 5.5 – 5.9 (Nur, 2010). From this study, the optimum pH of the papain enzymes is within the range 4.5 – 6.6. The result presented in Figures 4.8 and 4.12 indicates that the enzyme in Pool A and E are active at optimum pH 5.5. Also, the pH-dependence profile of the enzyme in Pool C (Figure 4.10) shows that its optimum activity is at 4.5 similar to the optimum pH of the enzyme in Pool D (Figure 4.11). The optimum pH of the enzyme in pool B is

6.6 (Figure 4.9). The result of this study falls between the range of the optimum pH as reported in previous studies. Within the pH 5.0 – 7.5 in the presence of cysteine containing substrates, the, specific activity of the papain is at maximum (Kimmel and Smith, 1954).

The kinetics of the enzymes purified from papaya were determined in this study. Lineweaver-burk plot of the reciprocal of velocity against the reciprocal of substrate concentration was used in determining the K_m (Michealis-Menten constant) and V_{max} (Maximum velocity) values of the purified enzymes. In this study, the K_m values and the V_{max} of the enzymes in Pools A, B, C, D and E are 8.70mg/ml and 0.20 (Figure 4.13), 1.47mg/ml and 0.059 (Figure 4.14), 1.52mg/ml and 0.042 (Figure 4.15), 5.00mg/ml and 0.33 (Figure 4.16), and 1.25mg/ml and 0.42 (Figure 4.17) respectively. Mole and Horton (1973) reported in their study that the Michealis constant of papain using the substrate α-N-benzoyl-L-arginine-p-nitroanilide is 3mM. The Michealis-Menten constant of papain increase with the organic solvent concentration compared to results from water (Fernandez *et al.*, 1991).

Thiosulfonate activates enzymes of the papain family (Werner, 1945). $Mg2^+$ activates rhodonese enzyme from the gut segment of *Hyperiodrilus adricanus* at different substrate concentrations (Adeyanju *et al.,* 2014). Mg^{2+} in various metabolic pathways has a wide range of activity in enzymes, serving as a co-factor. The effect of the metal ion on the enzyme activity in Pool D was studied at different concentrations of various casein concentrations. At a concentration of 10mM of Mg^{2+}, the activity of the enzyme in pool D at substrate concentrations 2mg/ml (Figure 4.18), 0.4mg/ml (Figure 4.19) and 6mg/ml (Figure 4.20) is significantly increased; also at a concentration of 80mM of Mg^{2+}, the activity of the enzyme in pool D at 6mg/ml substrate concentration (Figure 4.20) significantly increases. Generally, the different [Mg^{2+}] increases the activity of the enzyme in pool D. Also, the effect of the metal ion on the enzyme activity in Pool

E was studied at different concentrations of various casein concentrations. All concentrations of Mg^{2+} significantly increase the activity of the enzyme in pool E at substrate concentrations 2mg/ml (Figure 4.21), 4mg/ml (Figure 4.22) and 6mg/ml (Figure 4.23). The conclusion from this result is consistent with the study by Kaul *et al.*, (2002), in their study Ca^{2+} and Mg^{2+} increases the activity of the papain enzyme at substrate concentration 1×10^{-3}M.

5.2 CONCLUSION

The findings from this study showed that purified papain enzyme from *Carica papaya* has optimum active at 50°C - 59°C and pH 4.5 – 6.6; its activity is further enhanced by addition of Mg^{2+}. Within the range 1.47mg/ml – 8.70mg/ml of casein, the enzyme-catalyzed reaction is half its maximum velocity and the maximum velocity of the enzymes ranges from 0.042µmol/ml/min – 0.4167µmol/ml/min. It is hoped that the values of the parameters obtained from this study could be put to use by the industry in the production of papain and also for household and medical use in storing the product.

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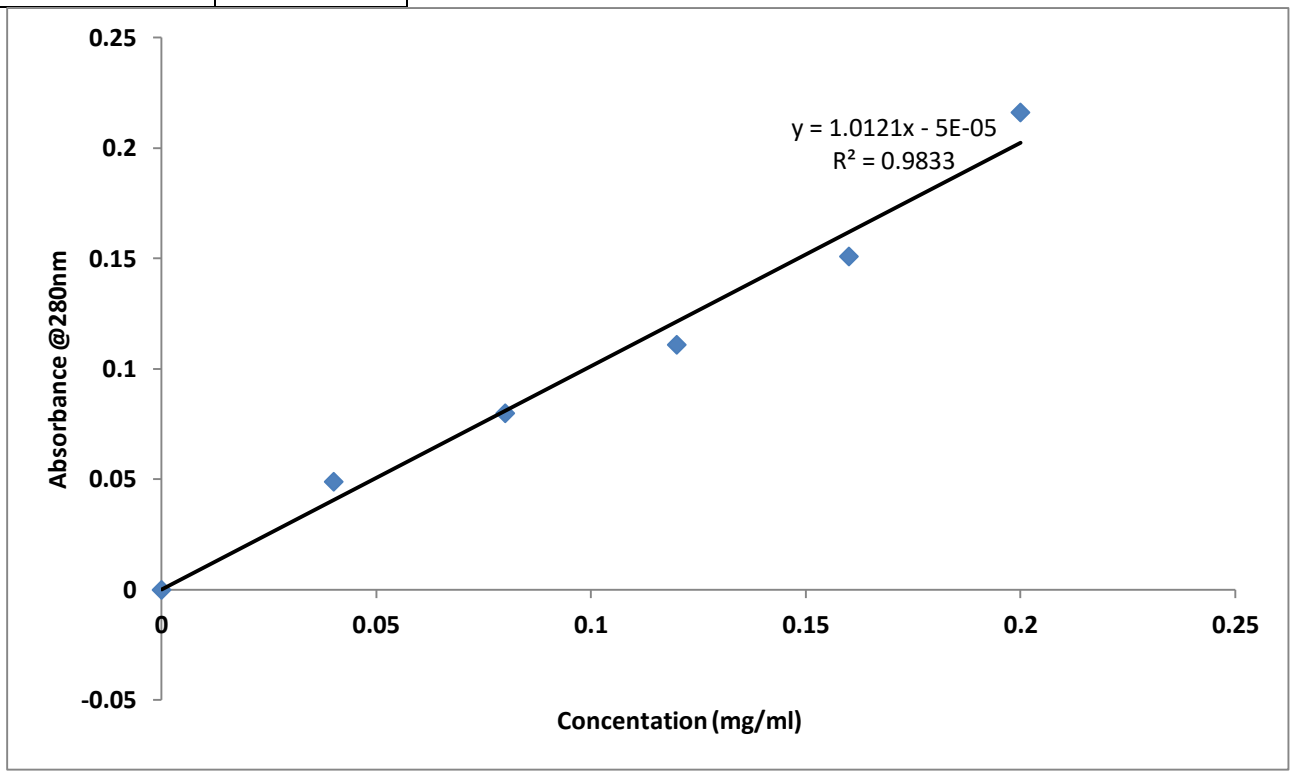
APPENDIX I

STANDARD CURVE

1. Standard Curve of Egg-Albumin

Concentration (mg/ml)	Absorbance @280nm
0	0
0.04	0.049
0.08	0.08
0.12	0.111
0.16	0.151
0.2	0.216



APPENDIX II

TABLES OF THE PURIFICATION RESULTS

1. DEAE-Cellulose Purified Enzyme With the Use of Gradient Solution

MOBILE PHASE	TUBE NO	Abs_1	Abs_2	Abs_3	Absorbance
0.0M (Phosphate buffer)	1	0.208	0.232	0.228	0.222667
	2	0.711	0.71	0.71	0.710333
	3	1.086	0.986	0.995	1.022333
	4	1.032	1.146	1.059	1.079
	5	0.687	0.703	0.702	0.697333
	6	0.751	0.762	0.769	0.760667
	7	0.645	0.647	0.649	0.647
	8	0.488	0.488	0.487	0.487667
	9	0.376	0.386	0.38	0.380667
	10	0.282	0.282	0.28	0.281333
0.1M NaCl	11	0.373	0.38	0.38	0.377667
	12	1.249	1.257	1.253	1.253
	13	1.108	1.112	1.113	1.111
	14	0.699	0.686	0.685	0.69
	15	0.461	0.469	0.469	0.466333
	16	0.327	0.335	0.334	0.332

	17	0.326	0.334	0.333	0.331
	18	0.29	0.296	0.296	0.294
	19	0.305	0.311	0.311	0.309
	20	0.32	0.326	0.323	0.323
0.2M NaCl	21	0.111	0.109	0.099	0.106333
	22	0.077	0.068	0.067	0.070667
	23	0.474	0.484	0.481	0.479667
	24	0.465	0.47	0.478	0.471
	25	0.717	0.716	0.714	0.715667
	26	0.729	0.735	0.735	0.733
	27	0.538	0.524	0.502	0.521333
	28	0.399	0.406	0.404	0.403
	29	0.37	0.403	0.402	0.391667
	30	0.321	0.328	0.335	0.328
0.4M NaCl	31	0.34	0.341	0.34	0.340333
	32	0.198	0.206	0.209	0.204333
	33	0.27	0.238	0.24	0.249333
	34	0.19	0.197	0.2	0.195667
	35	0.204	0.208	0.205	0.205667
	36	0.183	0.174	0.183	0.18
	37	0.141	0.146	0.147	0.144667
	38	0.113	0.121	0.113	0.115667
	39	0.088	0.08	0.083	0.083667

	40	0.052	0.082	0.078	0.070667
2M NaCl	41	0.048	0.061	0.06	0.056333
	42	0.115	0.12	0.122	0.119
	43	0.115	0.124	0.122	0.120333
	44	0.019	0.022	0.023	0.021333
	45	0.01	0.01	0.01	0.01
	46	0.076	0.044	0.034	0.051333
	47	0.008	0.015	0.015	0.012667
	48	0.074	0.07	0.07	0.071333
	49	0.067	0.067	0.069	0.067667
	50	0.142	0.156	0.164	0.154

2. Sephadex G-25 Purified Enzyme with Isocratic Solution

TUBE NO	**Abs_1**	**Abs_2**	**Abs_3**	**Absorbance**
1	0.647	0.716	0.89	0.751
2	0.988	1.275	1.34	1.201
3	0.374	0.386	0.366	0.375333
4	0.256	0.249	0.245	0.25
5	0.232	0.243	0.232	0.235667
6	0.283	0.236	0.221	0.246667

7	0.14	0.163	0.16	0.154333
8	0.131	0.133	0.132	0.132
9	0.379	0.377	0.368	0.374667
10	0.368	0.348	0.35	0.355333
11	0.156	0.157	0.158	0.157
12	0.061	0.064	0.046	0.057
13	0.154	0.166	0.163	0.161
14	0.168	0.167	0.164	0.166333
15	0.154	0.158	0.157	0.156333
16	0.153	0.158	0.153	0.154667
17	0.128	0.134	0.129	0.130333
18	0.128	0.133	0.132	0.131
19	0.121	0.128	0.126	0.125
20	0.134	0.136	0.133	0.134333

APPENDIX III

TABLES OF THE CHARACTERIZATION RESULTS

1. Effect of Temperature on the Enzyme Activity in Pool A

Temp(°C)	Abs_1	Abs_2	Abs_3	Absorbance	Activity (µmol/min/ml)
20	1.343	1.478	1.375	1.398667	0.437083
30	1.717	1.582	1.33	1.543	0.482188
40	1.599	1.611	1.715	1.641667	0.513021
50	1.797	1.829	1.862	1.829333	0.571667
60	2.357	2.41	2.367	2.378	0.743125
70	1.203	1.277	1.284	1.254667	0.392083

2. Effect of Temperature on the Enzyme Activity in Pool B

Temp(°C)	Abs_1	Abs_2	Abs_3	Absorbance	Activity (µmol/min/ml)
20	1.531	1.46	1.468	1.486333	0.464479
30	1.672	1.626	1.715	1.671	0.522188
40	1.724	1.794	1.867	1.795	0.560938
50	1.81	1.851	1.955	1.872	0.585
60	2.097	2.107	2.097	2.100333	0.656354
70	1.012	1.012	1.036	1.02	0.31875

3. Effect of Temperature on the Enzyme Activity in Pool C

Temp(°C)	Abs_1	Abs_2	Abs_3	Absorbance	Activity (µmol/min/ml)
20	1.221	1.385	1.411	1.339	0.418438
30	1.377	1.371	1	1.375667	0.429896
40	1.498	1.512	1.523	1.511	0.472188
50	1.569	1.564	1.587	1.573333	0.491667
60	1.755	1.762	1.765	1.760667	0.550208
70	1.101	1.017	1.142	1.086667	0.339583

4. Effect of Temperature on the Enzyme Activity in Pool D

Temp(°C)	Abs_1	Abs_2	Abs_3	Absorbance	Activity (µmol/min/ml)
20	0.983	0.96	0.9	0.947667	0.296146
30	1.423	1.451	1.41	1.428	0.44625
40	1.56	1.631	1.558	1.583	0.494688
50	1.626	1.633	1.641	1.633333	0.510417
60	1.842	1.819	1.87	1.843667	0.576146
70	1.379	1.216	1.222	1.272333	0.397604

5. Effect of Temperature on the Enzyme Activity in Pool E

Temp(°C)	Abs_1	Abs_2	Abs_3	Absorbance	Activity (µmol/min/ml)
20	0.44	0.431	0.425	0.432	0.135
30	1.201	1.373	1.197	1.1	0.34375
40	1.46	1.465	1.428	1.451	0.453438
50	1.827	1.639	1.762	1.742667	0.544583
60	1.475	1.491	1.489	1.485	0.464063
70	1.298	1.35	0.965	1.204333	0.376354

6. Effect of pH on the Enzyme Activity in Pool A

pH	Abs_1	Abs_2	Abs_3	Absorbance	Activity (µmol/min/ml)
3.5	0.116	0.123	0.118	0.119	0.037188
4.5	0.139	0.141	0.143	0.141	0.044063
5.5	0.232	0.221	0.215	0.222667	0.069583
6.5	0.181	0.184	0.181	0.182	0.056875
7.5	0.147	0.147	0.144	0.146	0.045625
8.5	0.08	0.073	0.073	0.075333	0.023542

7. Effect of Temperature on the Enzyme Activity in Pool B

pH	Abs_1	Abs_2	Abs_3	Absorbance	Activity

					(µmol/min/ml)
3.5	0.904	0.896	0.887	0.895667	0.279896
4.5	1.546	1.555	1.512	1.537667	0.480521
5.5	1.506	1.816	1.921	1.747667	0.546146
6.5	1.907	1.94	1.907	1.918	0.599375
7.5	1.753	1.807	1.827	1.795667	0.561146
8.5	1.236	1.288	1.211	1.245	0.389063

8. Effect of Temperature on the Enzyme Activity in Pool C

pH	**Abs_1**	**Abs_2**	**Abs_3**	**Absorbance**	**Activity (µmol/min/ml)**
3.5	0.128	0.137	0.135	0.133333	0.041667
4.5	0.191	0.194	0.19	0.191667	0.059896
5.5	0.132	0.131	0.128	0.130333	0.040729
6.5	0.112	0.117	0.115	0.114667	0.035833
7.5	0.105	0.11	0.109	0.108	0.03375
8.5	0.107	0.111	0.112	0.11	0.034375

9. Effect of Temperature on the Enzyme Activity in Pool D

pH	Abs_1	Abs_2	Abs_3	Absorbance	Activity (µmol/min/ml)
3.5	0.712	0.729	0.729	0.723333	0.226042
4.5	0.862	0.868	0.867	0.865667	0.270521
5.5	0.784	0.799	0.775	0.786	0.245625
6.5	0.745	0.742	0.747	0.744667	0.232708
7.5	0.674	0.686	0.642	0.667333	0.208542
8.5	0.554	0.54	0.532	0.542	0.169375

10. Effect of Temperature on the Enzyme Activity in Pool E

pH	Abs1	Abs2	Abs3	Absorbance	Activity (µmol/min/ml)
3.5	0.572	0.586	0.59	0.582667	0.182083
4.5	0.714	0.716	0.71	0.713333	0.222917
5.5	0.918	0.972	0.964	0.951333	0.297292
6.5	0.755	0.759	0.742	0.752	0.235
7.5	0.574	0.586	0.583	0.581	0.181563
8.5	0.485	0.476	0.483	0.481333	0.150417

11. Effect of Substrate Concentration on Enzyme Activity in Pool A

[S] (mg/ml)	**Activity (µmol/min/ml)**	$1/_{[S]}$	$1/_{v}$
10	0.120521	0.1	8.297321
8	0.11476	0.125	8.713838
6	0.082813	0.1666667	12.07547
4	0.065114	0.25	15.3576
2	0.039454	0.5	25.3459

12. Effect of Substrate Concentration on Enzyme Activity in Pool B

[S] (mg/ml)	**Activity (µmol/min/ml)**	$1/_{[S]}$	$1/_{v}$
10	0.052292	0.1	19.12351
8	0.047632	0.125	20.9941
6	0.046303	0.1666667	21.5971
4	0.041645	0.25	24.0123
2	0.034167	0.5	29.26829

13. Effect of Substrate Concentration on Enzyme Activity in Pool C

[S] (mg/ml)	**Activity (µmol/min/ml)**	$1/_{[S]}$	$1/_{v}$
10	0.038021	0.1	26.30137
8	0.034271	0.125	29.17933
6	0.0325	0.1666667	30.76923
4	0.028934	0.25	34.5612
2	0.024186	0.5	41.3456

14. Effect of Substrate Concentration on Enzyme Activity in Pool D

[S] (mg/ml)	**Activity (µmol/min/ml)**	$1/_{[S]}$	$1/_{v}$
10	0.228958	0.1	4.367607
8	0.22001	0.125	4.545248
6	0.1897	0.1666667	5.271481
4	0.125	0.25	8
2	0.1	0.5	10

15. Effect of Substrate Concentration on Enzyme Activity in Pool E

[S] (mg/ml)	**Activity (µmol/min/ml)**	$^{1}/_{[S]}$	$^{1}/_{v}$
1	0.236771	0.1	4.223493
8	0.21309	0.125	4.692853
6	0.1732	0.1666667	5.773672
4	0.12769	0.25	7.831467
2	0.024792	0.5	12

16. Effect of Mg^{2+} on the Activity of the Enzyme in Pool D at 0.2% [Casein]

[S] (mg/ml)	**[Mg2+] (mM)**	**Activity (µmol/min/ml)**
2	Control	0.028021
	10	0.082604
	20	0.027188
	40	0.035416
	80	0.048438

17. Effect of Mg^{2+} on the Activity of the Enzyme in Pool D at 0.4% [Casein]

[S] (mg/ml)	[Mg2+] (mM)	Activity (µmol/min/ml)
4	Control	0.032708
	10	0.076979
	20	0.036771
	40	0.038333
	80	0.040208

18. Effect of Mg^{2+} on the Activity of the Enzyme in Pool D at 0.6% [Casein]

[S] (mg/ml)	[Mg2+] (mM)	Activity (µmol/min/ml)
6	Control	0.042604
	10	0.074688
	20	0.036667
	40	0.043333
	80	0.082813

19. Effect of Mg^{2+} on the Activity of the Enzyme in Pool E at 0.2% [Casein]

[S] (mg/ml)	[Mg2+] (mM)	Activity (µmol/min/ml)
2	Control	0.024792
	10	0.051563
	20	0.047916
	40	0.040625
	80	0.040833

20. Effect of Mg^{2+} on the Activity of the Enzyme in Pool E at 0.4% [Casein]

[S] (mg/ml)	[Mg2+] (mM)	Activity (µmol/min/ml)
4	Control	0.028333
	10	0.054792
	20	0.052813
	40	0.045
	80	0.044688

21. Effect of Mg^{2+} on the Activity of the Enzyme in Pool E at 0.6% [Casein]

[S] (mg/ml)	**[Mg2+] (mM)**	**Activity (µmol/min/ml)**
6	Control	0.033542
	10	0.049688
	20	0.041979
	40	0.054583
	80	0.046458