Isolation, Partial Purification and Characterization of a Protease from Ginger *Zingiberaceae officinale* Roscoe

BY

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(M Sc./SCIE/55504/2005-06)

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ZARIA

July, 2011

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A project submitted to the Postgraduate School, Ahmadu Bello University in partial fulfillment for the award of M. Sc degree.

Department of Biochemistry
Ahmadu Bello University Zaria
Nigeria

July 2011

Declaration

I declare that the project entitled Isolation, partial Purification and Characterization of a protease from ginger *Zingiberaceae officinale* Roscoe (cv. Haliya indang) has been performed by me in the Department of Biochemistry under the supervision of Dr. E. Onyike. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project has been previously presented for another degree or diploma at any university.

______________________	__________________	__________
Name of student	Signature	Date

Certification

This project entitled **ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF A PROTEASE FROM GINGER** ***ZINGIBERACEAE OFFICINALE*** **ROSCOE (CV. HALIYA INDANG)** by Bassa Obed Yakubu meets the regulations governing the award of the degree of M. Sc. of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Name and Signature

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Chairman, Supervisory Committee

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Prof. Andrew J. Nok
Member, Supervisory Committee

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Head of Department

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Prof. A. A Joshua
Dean, Postgraduate School

Acknowledgment

This noble work could barely have seen the light of the day without the eminent contributions of many who directly or indirectly offered a helping hand.

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Lastly, I give God Almighty all the glory, honour and adoration for His mercies and grace upon me.

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Abstract

Protease from *Zingiberaceae officinale* Roscoe (cv. Haliya indang) was isolated, purified to homogeneity in a three step procedure involving ammonium sulphate fractionation, gel filtration, and ion-exchange chromatography. The partially purified enzyme was characterized. The enzyme was found to be made up of two sub-units designated 'ginger protease I and II (GPI & GPII); the subunits had specific activity of 173.88±5.6 and 131.54±3.7mg gelatin/min/mg protein respectively, with purification folds of 3.40 and 2.57 over crude extract. The molecular weights of 23.97 and 25.05 kDa were obtained by SDS-PAGE. The enzyme subunits had similar optimum temperature of 40°C, while retaining high activity at 46°C for GPI and 58°C for GPII suggesting GPI to be more heat labile. The subunits incubated at different temperatures showed highest stability at 40°C, with an optimum pH of 6.0 and 5.5, retaining significant activity within a pH range of 5.5-7.5 for GPI, and 5.0-7.5 for GPII. Mn^{2+}, Zn^{2+} inhibited the subunits mildly while Hg^{2+} had a severe inhibition on the subunits. The subunits were also severely inhibited by Iodoacetate (IA), a cysteine protease inhibitor. IA caused a mixed inhibition with K_I of 7.8mM and 18.8mM and K_i of 5.6mM and 13.5mM respectively. Time course of activity remained high for about 160 hours of storage at 4°C. The enzyme subunits demonstrated gelatin as its natural

substrate over other examined substrates with K_m of 0.8475 and 1.163mg/ml and V_{max} of 555.56 and 454.55mg gelatin/ml respectively. The properties of this enzyme meet the requirement needed for industrial application of the enzyme.

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CHAPTER ONE

1. 0 INTRODUCTION

Enzymes, especially proteases have become an important and indispensable part of the processes used in many applications by the modern food and feed industry to produce a large and diversified range of products for human and animal consumption. Enzyme technology has evolved to become an integral part of the food industry (Demir *et al,* 2007). Proteases have found applications in various industrial and medical uses. Several enzymes have been discovered, isolated and developed from various sources; plants, animals, bacteria and fungi. Presently, approximately 100 different biocatalytic processes are implemented in pharmaceutical, chemical, agricultural, and food industries and its applications continue to grow very rapidly (Bente, 2002). This technology has helped in processing food products. In spite of these successes, however, the vast potential of biocatalysis is yet to be fully realized. Bioactive agents from plants are one of the most intensive areas of natural product research today and this vast area is far from being exhausted. Biologically active plants with a broad range of therapeutic properties and rarely associated with adverse side effects, are exploited for economic benefits. The availability of plant material makes production of the enzyme feasible and allows extensive study of its various probable applications. Besides, interest in developing industrial raw materials (phytochemicals) from plant sources for

commercial purposes makes investigation into plants necessary and worthwhile. In the future the demand of amino acids in food, feed, pharmaceuticals, cosmetics, agriculture, will lead to further exploration of the potential of microorganisms, plants and enzymes to develop more efficient processes for amino acid production (Leuchtenberger *et al*., 2005).

Although proteases, used in industry have been obtained mostly from microorganisms, the unique substrate specificity of plant proteases is lacking in microorganisms or mammalian systems. Due to the nature of enzyme isolation and purification, many commercial enzyme preparations usually contain some unwanted side activities. However, plant-plant-based enzymes tend to be naturally free of unwanted side enzyme activities due to their unique specificity (Gary and Weiping, 2005). This factor makes the use of plants resources a valuable enzyme source serving increasingly significant roles in our processing systems. For instance, industrial utilization of cysteine protease family has received increasing interest due to a better understanding of their role in numerous important physiological and developmental processes. These natural processes are mimicked in-vitro and explored for industrial application.

1. 1 Statement of research problem

Zingiberaceae officinale has been the object of several chemical and pharmacological studies, it has long been employed for traditional

culinary/medicinal uses and its cholesterol-lowering effects has increased the populace that make use of it, and raised the rate of human consumption, previous reports have shown much literature about beneficial effects of ginger but, not much study on the protease has been reported (Adulyatham et al, 2000., Naveena et al, 2004). Unlike papain and bromelain which are used widely in industries, the enzyme in ginger has not been investigated widely enough to the extent of its meticulous application, usage and its prospective benefits have not been realized despite its large availability and usage.

1. 2 Aim

The aim of this work is to investigate the biochemical properties of the protease, zingibain, from one of the ginger cultivars 'Haliya Indang' within Northern Nigeria.

1. 3 Justification

The use of isolated proteases to modify the functional and nutritional properties of food proteins from underexploited sources could improve the quality and increase the quantity of proteins; thus, achieving the objectives of this project will reveal new values for this ancient herb and could have a positive effect on ginger and serve as a basis for effective and efficient

industrial application of the enzyme, increasing value to its benefit thus improving the food and feed industry.

1. 4 Specific objectives

1. Isolation and purification of protease
2. Characterization of the enzyme
    a. determination of optimum pH
    b. determination of optimum temperature
    c. stability characterization
    d. time course of inactivity
    e. inhibition studies
    f. determination of the effect of metal cations
    g. determination of molecular weight using SDS-PAGE

CHAPTER TWO

2.0 LITERATURE REVIEW

The use of higher plants and their extracts to treat infections is an age old practice in traditional medicine. This practice has been known for centuries in many parts of the world and varies from one region to another (Gong *et al*, 2004). Numerous plants and herbs have been exploited and their use is gaining more and more attention because quite a number of extracts from plants origin have shown valuable abilities and benefits (Badgujar and Mahajan, 2010).

2.1 Protease enzyme

Protease is the generic name given to a group of enzymes that catalyse the cleavage of peptide bonds of proteins. They form a large group of enzymes belonging to the class of hydrolases, they are ubiquitous in nature and perform a major role with respect to their applications in both physiological and commercial fields. Sixty (60) % of the enzymes used commercially are proteases (Badgujar and Mahajan, 2010). Proteases may be classified in a number of ways, though they are classified into two major categories: **exopeptidases**; that catalyse the cleavage of bonds at points removing an amino acid from either the C or N-terminus of the peptide, and **endopeptidases**; also synonymously referred to as **proteinases** they catalyse the cleavage within the protein producing two or more peptides. They could be

classified on the basis of the pH range over which they are active (acid, neutral, or alkaline), or their ability to hydrolyze specific proteins (keratinase, elastase, collagenase, etc) represent one of the three largest groups of industrial enzymes. Proteases can further be classified based on their similarity to well characterized proteinases such as pepsin, trypsin, or chemotrypin. Hartley in 1960 (Badgujar and Mahajan, 2010) described the most satisfactory system of classification presence of main catalytic amino acid residue in their active site, this classification recognized by IUBMB puts forward five groups of proteases namely: serine, aspartic, threonine, cysteine and metallo proteases. Proteases are widely found in plants, animals as well as in microorganisms intricately involved in many aspects of physiology and development (Kunert et al 2006).

2.2 Physiological functions of proteases

Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms (Sharmin and Rahman 2007). Proteases play a critical role in many physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis and development, inflammation, tumour growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins,

and transport of secretory proteins across membranes (Kunert et al 2006). Plant proteases have been identified to be involved in all aspects of the plant life cycle ranging from the mobilization of storage proteins during seed germination and protein remobilization upon the onset of leaf senescence, also responsible for the post-translational modification of proteins by limited proteolysis at highly specific sites, limited proteolysis resulting in the maturation of enzymes, controls the activity of enzymes, regulatory proteins and peptides, to the initiation of cell death and senescence programs (Schaller, 2004). In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell whereas intracellular proteases play a critical role in the regulation of metabolism. The study of proteases is valued because of their importance as reagents in laboratory, clinical, and industrial processes (Badgujar and Mahajan, 2010). In contrast to the multitude of the roles contemplated for proteases, our knowledge about the mechanisms by which they perform these functions is very limited.

2.3 Classes of proteases

There are five mechanistic classes of proteases recognized by the International Union of Biochemistry and Molecular Biology (IUBMB) 1984, classes of proteases in which serine, threonine, cysteine, aspartic or metallic groups play

a primary catalytic role. Serine, cysteine and threonine proteases are quite different from aspartic and metalloproteases (Schaller 2004). In the first three groups, the nucleophile in the catalytic centre is part of an amino acid residue, while in the second two groups the nucleophile is an activated water molecule. In cysteine proteases the nucleophile is a sulfhydryl group and the catalytic mechanism is similar to the serine proteases in which the proton donor is a histidine residue.

2.4 Applications of proteases

Besides, the endogenous roles played by proteases, commercial uses have also been identified. The role of endogenous proteases in their respective sources triggered the investigation of their application in various endeavours. For instance, research into the use of exogenous proteases investigated countless enzymes from plant, bacterial and fungal sources. Plant proteases have received special attention in the field of medicine and biotechnology due to their propertries of being active at very wide range of temperature and pH (Guiamet *et al*, 2007, Saroat *et al*, 2009). Paradigm now exists for production of industrial proteases from plant sources that allows cost effective use of these proteases in industrial processes. Processes demanding large amounts of protease are logical targets for plant production because increasing supply is fast and inexpensive (Elizabeth, 2002). However, only few exogenous plant

proteases have been classified as "Generally Recognised As Safe" (GRAS) by USDA's Food Safety Inspection Service (FSIS). Proteases from kiwi fruit (actinidin) and ginger are suggested to have shown potentials for future inclusion in meat systems (Haarasilta and Pullinen, 1992, Thompson et al, 1973). The performances of these enzymes depend on the plant source, the climate conditions for growth, and the methods used in its extraction and purification. Most of the enzymes that have been approved have a history in food and have not been shown to have negative side effects over long term use (Codex 1996). Homologous proteases are also found in other plant organs especially during senescence and massive protein degradation (Rowan and Buttle, 1994).

2.5 Cysteine proteases

In plants, proteases are involved in all aspects of the life cycle ranging from the mobilization of storage proteins during seed germination, the plant physiology and development as mentioned above, to the initiation of cell death and senescence programs (Fan and Wu, 2005), many of such proteases that play these roles are known to be cysteine proteases and occur in many plants and animals. They are found in fruits such as pineapple, and papaya, they account for some of the intense acidity in the juices of these fruits (Haarasilta and Pullinen, 1992). Analytical evidence have demonstrated that the proteases

conduct highly specific and selective modifications of proteins with their action being categorized into *limited proteolysis*; where a protease cleaves only one or a limited number of peptide bonds of a target protein leading to the activation or maturation of the formerly inactive protein referred to as zymogen e.g. conversion of prohormones to hormones, post-translational modification of proteins, maturation of enzymes, immune responses etc. and *unlimited proteolysis* where proteins are degraded into their amino acid constituents e.g. degradation of damaged, misfolded or potentially harmful proteins. They play roles such as intracellular protein turnover in lysosomes, in processing and activation of other proteins, in antigen processing and presentation and in bone remodelling. They share similar amino acid sequences and folds. Located predominantly in lysosomes, they exhibit/have the same active site geometry and catalyse processes via the same mechanism. They also exhibit optimal activity at weakly acidic pH, and predominantly show endopeptidases activity (Setyesh *et al*, 2003).

This cysteine protease family includes proteases such as papain from papaya, ficin from fig, bromelain from pineapple, several mammalian lysosomal cathepsins, the cytosolic calpains as well as several parasitic proteases and several others from plants and animals, papain being the best studied member of the family. Papain homologs are generally either lysosomal (vacuolar) or

secreted proteins (Tjendraputra et al, 2001). In plants, they are found in the vacuoles, but also in extracellular paces, as in the latex of papaya or fig. Plant cysteine proteases including papain from papaya (*Carica papaya*), bromelain from pineapple stem and actinidin from the Chinese gooseberry of kiwi fruit (*Actinidia chinensis*). Their activity is regulated, largely through their interaction with their endogenous inhibitors (cystatins and thyropsins) (Choi and Lausen, 2000). Cysteine proteases have attracted the interest of the pharmaceutical industries, since the discovery that they are promising drug targets for many diseases (Wiczk 2007). Cysteine cathepsins and other members of the papain family are now considered to be potential targets for the design and development of small molecule inhibitors as new therapeutics. Plant proteases such as papain and stem bromelain have been extensively used for medicine. The choice of enzymes for a given application is determined by the cost and availability as well as temperature and pH optima, substrate specificity, stability and sensitivity to inhibitors or activators (Simpson *et al,* 1984).

Papain was the first cysteine protease to be discovered in the latex and fruit of *Carica papaya* and served for a long time as a model for mechanistic and structural studies of cysteine protease (Drenth et al 1968). Upon review by Beers et al (2004), such enzymes were found to be involved in protein

degradation and N-mobilization during seed germination and leaf senescence, papain- like enzymes have been implicated in a number of different cell death events (Belenghi et al, 2003), they also contribute to plant resistance against pathogens and insects (Gary and Weiping, 2005). Much of the initial work on plant proteases focused on this class of enzymes, and they were believed to be the predominant class of proteases in plants (Schaller, 2004).

2.6 Ginger in Nigeria

The plant Ginger (*Zingiberaceae officinale*) Rosc. With voucher number 2261 of the Scitamineae order and Zingiberaceae family (Chevallier, 1996) is said to originate from India and also native to Africa and West India. It is an herbaceous perennial monocot herb cultivated in the rain forest (Cornell and Sutherland, 1969). Ginger is obtained from the rhizomes of Zingiber family and has no seeds; it is propagated vegetatively through budding from its knotty rhizome (Sutherland, 1981).

At harvest the rhizome is unearthed, washed, and sometimes boiled, then peeled. It has been used medicinally in the West for at least 2000 years (Cornell and Sutherland, 1969). It was traditionally used to warm the stomach and dispel chills (Murray, 1995). In the 18th century it was added to remedies to modify their action and to reduce their irritant effects upon the stomach and to reduce the toxicity of some herbs (Kathi *et al*, 1999).

Ginger has a long respected history as a spice. It is a tropical herb extensively grown for its pungently aromatic underground stem or rhizome (Bente, 2002). Nigeria is among the largest producers of ginger (the third largest exporter in the world after China and India). Ginger is produced in six states of the Federation namely, Kaduna, Nasarawa, Benue, Niger and Gombe with Kaduna as the major producer. Nigeria's production in 2005 was estimated at 110,000 metric tonnes (FAO) of which 10% is locally consumed as fresh ginger while 90% is dried primarily for the export markets.

Ginger possesses numerous medicinal actions and uses that it has been revered as a medical panacea for 5,000 years. It has a long history of both culinary and medicinal uses (Gong *et al*, 2004). In ancient China, ginger has commonly been used to cleanse and warm the body (Schon, 2006). Ginger is valued as a spice and medicinal plant from ancient times and is now used universally as a versatile spice and in traditional medicine as well as modern medicine (Haarasilta *et al*, 1992).

2.7 Chemical constituents of ginger

The pharmacologically active chemicals present in ginger include; the volatile oils having a yield of 2.4%, consist of 64.4% sesquiterpene, 6.6% carbonyl compounds, 5.6% alcohols, 2.4% monoterpene and 1.6% esters. The main sesquiterpene compounds are zingiberene (29.5%) and sesquiphellandrene (18.4%) (Oyenekwe, 1999). The aromatic compounds include; Toluene and ρ-Cymene, monoterpenes also comprise; α-Pinene, Camphene, β-Pinene,

Myrene, α-Phellandrene, δ-Terpinene, Terpinolene, Sabinene, Limonene, β-Phellandrene, while the sesquiterpenes consist of; bisabolene, α-Ylangene, Camphor, α-Gurjumene, Bergametene, δ-Elemene, α-Calacorene, γ-Calalorene, β- and γ-Elemene, Eremophyllene, β-Caryophyllene, (E)-farnesene, α-Humulene, Germacrene, Zingiberene, Farnesene, β-Sesquiphellandrene, α-Copaene, t-Muurolene, Vetivinene, Allaromadendrene, Perillene, Santalene, β-Farnesene, β-Selinene, Calamenene. A number of constituents not previously discovered in ginger oil were identified in Nigerian ginger, which include 2,6-dimethyl hepten-l-ol, α-gurjunene, linalool oxide, isovaler-alde-hyde, 2-pentanone, cadinol, α- and γ-calacorene, ere-mophyllene, t-muurolol, α-himachallene, α-cubebene acetic acid, pinanol, α-santalene, geranyl propionate, geranoic acid, (E,E)-farnesene, n-methyl pyrrole and geranic acid (Oyenekwe, 2000), proteolytic enzyme (zingibain), vitamin B_6, vitamin C, calcium, magnesium, phosphorus, potassium, linoleic acid, oleoresin consisting of essential oils and organically soluble resins is a viscous material that provides the flavour profile. (Yoshikawa, 1993, Schulick, 2001). The pungency of ginger is a characteristic attributable to the presence of gingerol compounds (6-, 8- and 10-gingerol), its decomposition product zingerone, and reduction product shagoal (Oyenekwe, 2000, Chane, 2003).

2.8 Zingibain

Ginger rhizome contains a digestive enzyme; **zingibain** (Adulyatham, 2001), whose effectiveness even exceeds that of papain (Thompson *et al*, 1973),

zingibain, a special kind of proteolytic enzyme, is known as a thiol proteinase (Naveena et al, 2004). Fresh ginger rhizome contains as much as two percent zingibain, which is believed to be as powerful an enzyme as the bromelain in pineapple or the papain in papaya (Schulick, 1996). Ginger protease from ginger rhizome, i.e. zingibain was first reported by Ichikawa et al in 1973 after they isolated it by chromatography on DEAE-cellulose column, they tentatively classified the enzyme as a cysteine protease based on their inhibition by thiol reagents, their relative molecular masses estimated by gel filtration chromatography were 29 and 31kDa. From their studies, on a comparative study of zingibain, with the popular proteolytic enzyme, papain Naveena et al, (2004) identified the tenderizing efficacy of zingibain to be superior and most preferred. Recent findings however have reported these proteases as having both proteinase and collagenase activities (Choi and Laursen 2000). The extracts have also been tested as a meat tenderizer (Micheal 2001, Antoine 2004). The ginger protease in an attempt to be characterized was separated by isoelectric focusing into three fractions, with p*I* values of 4.5, 4.6, and 4.8 respectively (Han-Tsung et al, 2009). All the fractions had a molecular weight of about 22.5kDa when sephadex G-100 gel chromatography column was used for the estimation. The ginger proteases were further separated into two fractions; P1 and P2 using an anion-exchange column, the ginger protease was significantly stabilized on addition of ascorbic acid and storage at $4^{o}C$, an inhibition test carried out indicated that P1 and P2 were cysteine proteases.

CHAPTER THREE

3. 0 MATERIALS AND METHODS

3. 1 Chemicals and Reagents

All the reagents used were of analytical grade obtained from Sigma or BDH (British Drug House) Poole, England unless specified otherwise. The reagents include; Sodium acetate, glacial acetic acid, disodium hydrogenphosphate dehydrate, sodium dihydrogenphosphate, trichloroacetic acid (TCA), di-ethylaminoethyl (DEAE) cellulose, sephadex G-75, Sodium potassium tartrate, copper sulphate, potassium iodide, sodium hydroxide, ethylenediaminetetra acetic acid (EDTA), sodium azide, Bovine saline albumin (BSA), gelatine, L-cysteine hydrochloride monohydrate, calcium chloride, sodium chloride, potassium chloride, polyacrylamide, Sodium deodocyl sulphate (SDS), Coomassie Brilliant Blue, ammonium persulphate, Tris, hydrochloric acid, acrylamide, bisacrylamide, absolute ethanol, formaldehyde, sodium carbonate, sodium ethylenediaminetetra acetic acid (Na_2EDTA), glutaraldehyde, sodium thiosulphate, n-butanol, glycine, silver nitrate, glycerol.

3. 2 Equipment and analytical instruments

UV/visible spectrophotometer (6405 Genway), quartz cuvette, Hamilton syringes, Electrophoretic equipment (Amersham pharmacia biotech EPS 601),

centrifuge (Labofuge 300), pH meter (Genway 3159), weighing balance (Metler balance), chromatographic column (30 x 1.6 cm), water bath (B & T A Searle Company), micro-pipettes, test tubes, pipettes, burette, volumetric flasks.

3. 3 Collection of samples

Fresh mature ginger rhizome was purchased from a locality designated within the geographical region by the herbarium department of Biological Sciences Ahmadu Bello University Zaria and verified by the department.

3. 4 Preparation of ginger powder

Fresh ginger rhizome were washed and allowed to drain. One hundred grams (100g) of the rhizome was weighed, sliced and dried in the oven at a temperature of 40°C (it was weighted periodically till constant weight was obtained and its moisture content was determined). The dried rhizome was then pulverized.

3. 5 Preparation of buffers

Buffers were prepared according to Gomori (1955) and the final pH was ascertained with a pH meter. Exactly 50 mM sodium acetate buffer, pH 4.5 containing 2 mM cysteine, 1 mM EDTA and 5 mM sodium tetrathionate

(Buffer 1) and 50 mM sodium phosphate buffer, pH 6.0 containing 2mM cysteine and 1mM EDTA 5 mM sodium tetrathionate (Buffer 2).

3. 6 Extraction and purification of protease enzyme

Unless otherwise stated all steps were performed with reagents and extract preserved and used at 4°C

Step 1: Preparation of crude extract. Pre-weighted portion was dried in an oven at 40°C and pulverized. Twenty grams (20g) of ginger powder was mixed with 30ml of buffer 1. The homogenate was centrifuged at 10,000g for 20 min at 4°C and the supernatant was preserved. The precipitate was re-extracted with 20ml of the same buffer and recentrifuged. The two supernatants were pooled and designated as crude extract.

Step 2: Ammonium sulphate salting out. Ammonium sulphate crystals was added to the pooled fractions progressively to saturation and the resulting suspension was collected after centrifugation and re-dissolved in 20ml sodium phosphate buffer with pH 7.2, while the supernatant was discarded if no activity is found in it.

Step 3: Chromatography on Sephadex G-10. The pooled fractions were applied to a *Sephadex G-10* column (30 x 1.6 cm) previously equilibrated with

phosphate buffer pH 7.2. The column was developed at a flow rate of 20ml/h and 5ml fractions were collected.

Step 4: Chromatography on DEAE-cellulose. The pooled active fraction was applied to a DEAE-cellulose column (30 x 1.6 cm) pre-equilibrated with phosphate buffer at pH 7.2. The adsorbed material was eluted with stepwise gradient concentrations of sodium chloride ranging from 0.0 to 0.4M prepared in the phosphate buffer. The column flow rate was set at 10ml/h and 5ml fractions were collected. The fractions eluted with high activity if consecutive were pooled.

Step 5: Chromatography on sephadex G-75. The pooled active fractions were individually applied to a *sephadex G-75* column (30 x 1.6 cm) previously equilibrated with phosphate buffer pH 7.2. The column was developed at a flow rate of 5ml/h and 3 ml fractions were collected. The protease was eluted with the same buffer.

3. 7 Determination of protein concentration

Protein concentration was measured by the Biuret method (Davies, 1988) using bovine serum albumin (BSA) as a standard. Protein concentration was determined by taking absorbance at A_{280}nm.

3. 8 Enzyme activity assays

Proteolytic activity was determined in triplicates according to Dominguez and Cejudo (1996) who modified the method of Holwerda and Rogers (1992) using gelatin as the substrate. Up to 50µl of crude extract was incubated with 500µl of 100mM sodium acetate buffer, pH 4.5, and 100µl of 3% (w/v) gelatin and adjusted to 1ml with distilled water. Assays were carried out at 37°C for 1 hour then stopped by the addition of 200µl of 20% (v/v) trichloroacetic acid (TCA). After the removal of precipitated protein by centrifugation (12,000g for 5 min at room temperature) the absorbance at A_{366}nm of the supernatant was determined using spectrophotometer. One unit of proteolytic activity is defined as mg gelatin hydrolyzed per hour under standard assay conditions i.e 1unit = 1milligram gelatin/hr.

3. 9 Sodium Deodocyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis under denaturing conditions was performed in 12.5% (w/v) acrylamide slab gel according to the method of Davis (1964) using a Tris-glycine buffer, pH 8.2 with protein bands visualized by silver staining of the gel. (John, 1996, Hames, 1998)

3. 10 Molecular weight determination

Molecular weight was estimated from SDS-PAGE; by interpolating from a linear logarithmic plot of relative molecular Mass versus the relative mobility (R_f) value with Progema molecular weight standards; Bovine Serum Albumin (67kDa), *T. aestivum* (60kDa), ovalbumin (43kDa), glyceraldehyde-3-phosphate (36kDa), Carbonic anhydrase (29kDa) and Soybean trypsin inhibitor (20.1kDa) used as marker proteins for the calibration curve (Laemmli 1970).

3. 11 Determination of temperature stability

Thermal stability was determined by pre-incubating 1.0mL of the crude enzyme extract at varying temperatures; 20°C, 37°C, 40°C, 50°C, 60°C, 65°C and 70°C in a water bath using a thermostat (sensitivity ± 0.01ºC). At 30 minutes interval, the preheated enzyme preparations was cooled to room temperature, added to the reaction mixture and incubated at 37°C for 30 minutes the enzyme activity determined. Enzyme kept at room temperature was taken as control. Corresponding blank at each temperature was run by adding TCA prior to addition of gelatin to the purified enzyme.

3. 12 Determination of optimum temperature

The protease activity of the purified enzyme was determined as a function of temperature. For this purpose 1.0 ml of the purified enzyme preparation was added to 0.3 ml of the substrate (1.0% gelatin) and incubated at varying temperatures; 20°, 37°, 40°, 50°, 60° and 65°C and after 60 minutes of incubation the reaction was arrested by addition of 0.6 ml of 0.1 M TCA solution and the residual activity determined. Corresponding blank at each temperature was carried out by adding TCA to the purified enzyme solution prior to mixing with substrate.

3. 13 Determination of optimum pH

The protease activity of the purified enzyme was determined as a function of pH. For this purpose 1.0 ml of the purified enzyme preparation was added to 0.3 ml of the substrate (1.0% gelatin) and incubated at varying pH; 4.5, 5.0, 5.5, 6, 6.5 and 7.0, 7.5, 8.0, and 8.5 (using 10mM acetate buffer, phosphate buffer, Tris-HCl buffer, glycine-NaOH buffer for preparing the pHs) and after 60 minutes of incubation the reaction was arrested by addition of 0.6 ml of 0.1 M TCA solution and the residual activity determined. Corresponding blank at each pH was carried out by adding TCA to the purified enzyme solution prior to mixing with substrate.

3. 14 Determination of storage Stability of Protease

Storage stability of protease was determined at 0°C, 4°C and at room temperature. The enzyme was preserved with sodium azide (NaN_3) and kept at room temperature. NaN_3 is a toxic chemical and was added to the enzyme solutions at a final concentration of 0.05%. The activity of the enzyme was checked after regular time intervals (5 days). Two other sets of enzyme preparations were kept at 0°C and 4°C and the activity of the enzyme was checked after five days intervals.

3. 15 Inhibition studies

The kinetics of inhibition of the most effective inhibitor was carried out using the method outlined by Singh *et al* (2003), the purified enzyme was pre-incubated with varying concentration (0.5-16mM) of inhibitor for 60 minutes at 37°C temperature and the residual enzyme activity was determined by the usual assay method. The tube without inhibitor was used as control. The necessary graphs were drawn to determine the pattern of inhibition and K_i.

3. 16 Effect of metal ions

The effect of different metal ions on the enzyme activities was determined by addition of the following salts; $CaCl_2$, $FeCl_3$, $MgCl_2$, $CoCl_2$, $CuSO_4$, $LiCl_2$, $ZnCl_2$, $MnSO_4$, to furnish their corresponding metal ions. (Patil and Shastri,

1985). **Note:** The relative activity of metal ions were determined taking the tubes without metals to have 100% activity (Fahmy and Mohammed, 2004) and t-test was used to determine the variation of the individual metal ions from the control.

3. 17 Effect of different compounds

To determine the class to which the proteases belong to, the following compounds; Iodoacetate (IA), Phenylmethylsulphonyl fluoride (PMSF), Soybean trypsin inhibitor (STI), Dithiothreitol (DTT), ethylenediaminetetra acitic acid (EDTA), and β-mercaptoethanol were incubated with the enzyme for 30 minutes and their residual activity measured. **Note:** The relative activity of compounds were determined taking the tubes without any of the compounds to have 100% activity (Fahmy and Mohammed, 2004) and t-test was used to determine the variation of the individual compounds from the control.

3. 18 Substrate specificity test

The substrate specificity was performed using the following proteins as substrates; Gelatin, Casein, Egg albumin, Collagen, and Hemoglobin.

CHAPTER FOUR

4.0 RESULTS

The results of the purification scheme of *Z. officinale* protease were summarized in Table 4.1 below. The crude extract contained 30.5±3.27mg of protein, with a total activity of 1559.25±132.5 enzyme units with a specific activity 51.12±2.3 unit/mg protein. Most of the enzymatic activity (82.91%) was recovered in the Ammonium sulphate salting out. From the elution profile of the chromatography on DEAE-cellulose column (Fig. 4.1), it can be seen that two peaks were eluted with the first eluted peak having a broad base, i.e. the protease was resolved into two active fractions far apart as confirmed on the SDS-PAGE profile (Fig. 4.17) which means that the protease is made up of two sub-units designated GPI and GPII. These fractions with relatively high proteolytic activity were separately pooled and dialysed. The final purified preparations were obtained by gel filtration on a Sephadex G-75 column (Fig. 4.2 and Fig. 4.3) had a 3.40 and 2.57 fold yield and specific activity of 173.88±5.6 and 131.54±3.7 unit/mg protein of GPI and GPII respectively.

From the SDS-PAGE, the enzyme migrated revealing two faintly distinct bands that were very close distant wise. The molecular weight of *Z. Officinale* protease subunits (GPI and GPII) estimated from Fig. 4.4 to be 23.97 and 25.05 kDa respectively.

A study of the pH effect on the activity profile of the enzyme shows that the enzyme subunits as shown (on Fig. 4.5) had optimal activity at weakly acidic pH of 6.0 and 5.5 respectively. The enzyme subunits retained about 60% of there activity within a the range 5.5-7.5 for GPI, and 5.0-7.5 for GPII.

The subunits had similar optimum temperature of 40^oC (Fig. 4.6) while retaining about 60% of activity at 46^oC for GPI and 58^oC for GPII suggesting GPI to be more heat labile. The enzyme pre-incubated at various temperatures while taking the residual activity after every 30 minutes revealed (Fig. 4.7a & b) that the enzyme units at various temperatures had a declining activity with time of incubation, but, at 40^oC, the rate of declination was least. The time course of activity (Fig. 4.8) showed that the subunits retained significant activity for about 7days (160hours), the activity declined drastically thereafter.

The effect of metal ions on the proteases is shown in Fig. 4.9. Cations Mn^{2+}, Zn^{2+}, had a mild inhibitory effect but Hg^{2+} strongly inhibited both subunits. The enzyme subunits were inhibited slightly by DDT and significantly by IA (Fig. 4.10).

The kinetic study of the enzyme subunits showed K_m of 0.8475 and 1.163 mg/ml and V_{max} of 555.56 and 454.55 mg/ml respectively (Fig.4.12 and Fig. 4.13). They both showed a mixed (competitive-non-competitive) pattern of inhibition with IA (Fig. 4.14a and Fig. 4.14b) with K_i of 6mM and 16mM respectively (Fig. 4.15a & b and Fig. 4.16a & b).

Table 4.1: Purification scheme for *Z. officinale* protease

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1} protein)	Fold purification	Recovery %
Crude extract	30.5±3.27	1559.25±132.5	51.12±2.3	1.00	100.00
Ammoniumsulphate salting out	15.4±0.9	1292.84±61.2	83.95±5.1	1.64	82.91
GPI on DEAE-cellulose	8.45±0.7	985.61±45.6	116.64±4.7	2.28	63.21
GPII on DEAE-cellulose	4.35±1.3	246.28±11.6	56.61±2.9	1.11	15.80
GPI on Sephadex G-75	3.98±0.5	692.03±28.7	173.88±5.6	3.40	44.98
GPII on Sephadex G-75	1.42±0.8	186.78±9.3	131.54±3.7	2.57	11.98

Note: Each value represents the average of three experiments ±SE. One unit of proteolytic activity is defined as mg α–amino acid liberated per hour under standard assay conditions.

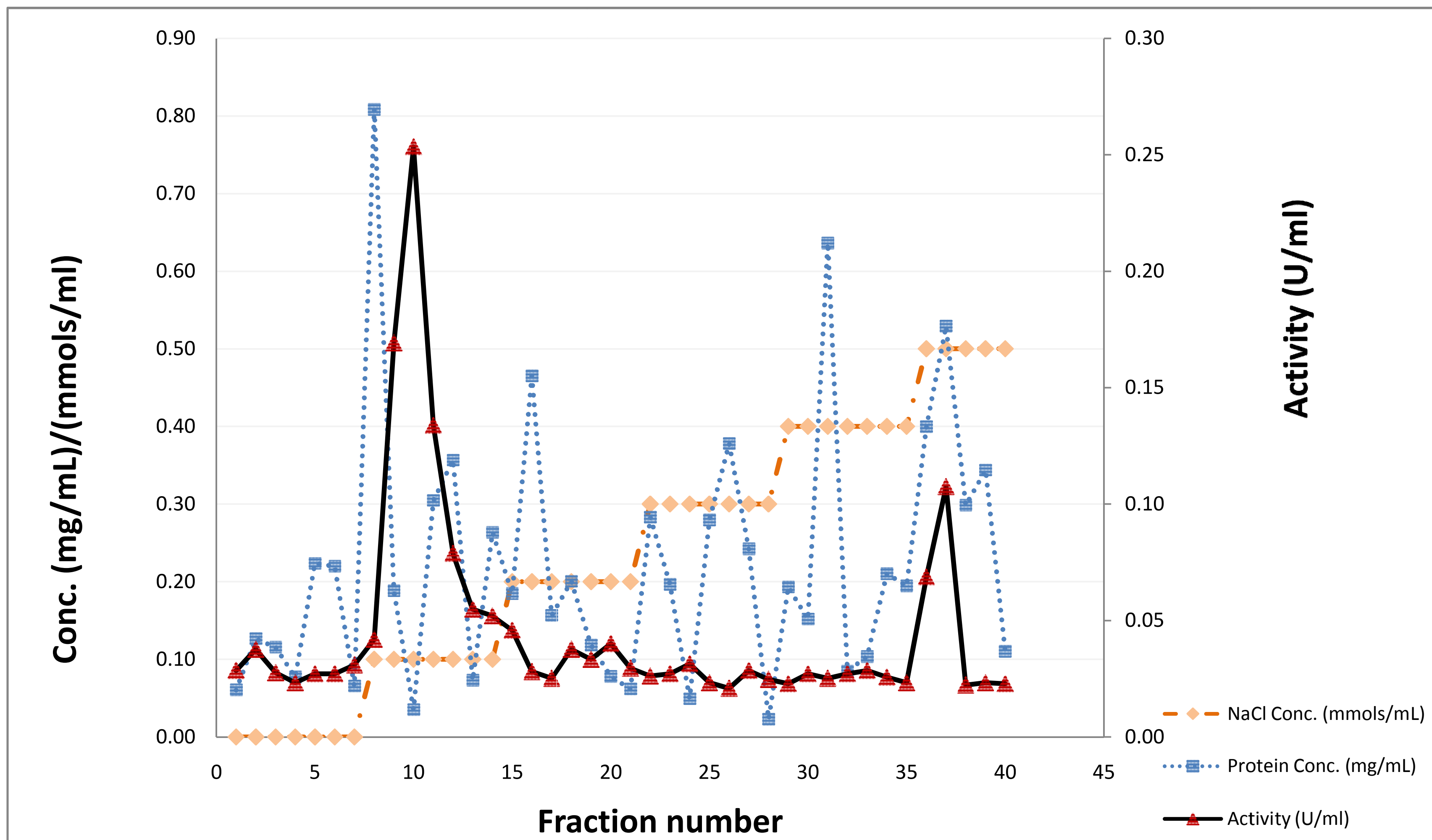

Figure 4.1: An elution profile for the chromatography of *Z. Officinale* protease on DEAE-cellulose
The column was equilibrated with 50mM sodium phosphate buffer, pH 7.2 containing 2mM cysteine, 1mM EDTA and5 mM sodium tetrathionate at a flow rate of 10 mlh^{-1} and 50 fractions were collected and tested for activity and protein concentration.

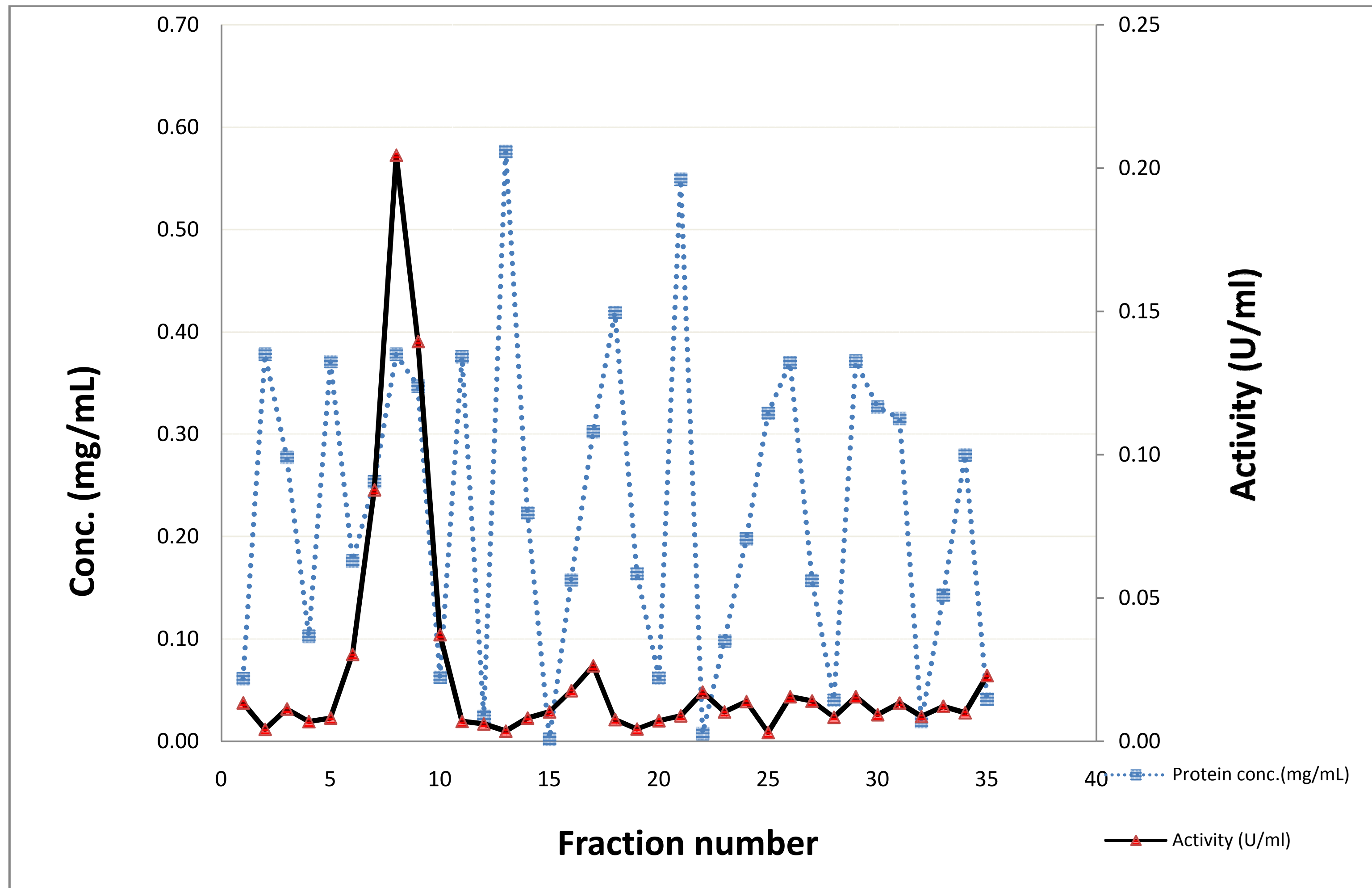

Figure 4.2: An elution profile for the gel filtration of the first pooled DEAE-cellulose active fractions of *Z. Officinale* protease (GPI) on Sephadex G-75 column (30 x 1.6 cm)

The column was equilibrated with 50mM sodium phosphate buffer, pH 7.2 containing 2mM cysteine 1mM EDTA and 5 mM sodium tetrathionate at a flow rate of 10 mlh^{-1} and 42 fractions were collected and tested for activity and protein concentration.

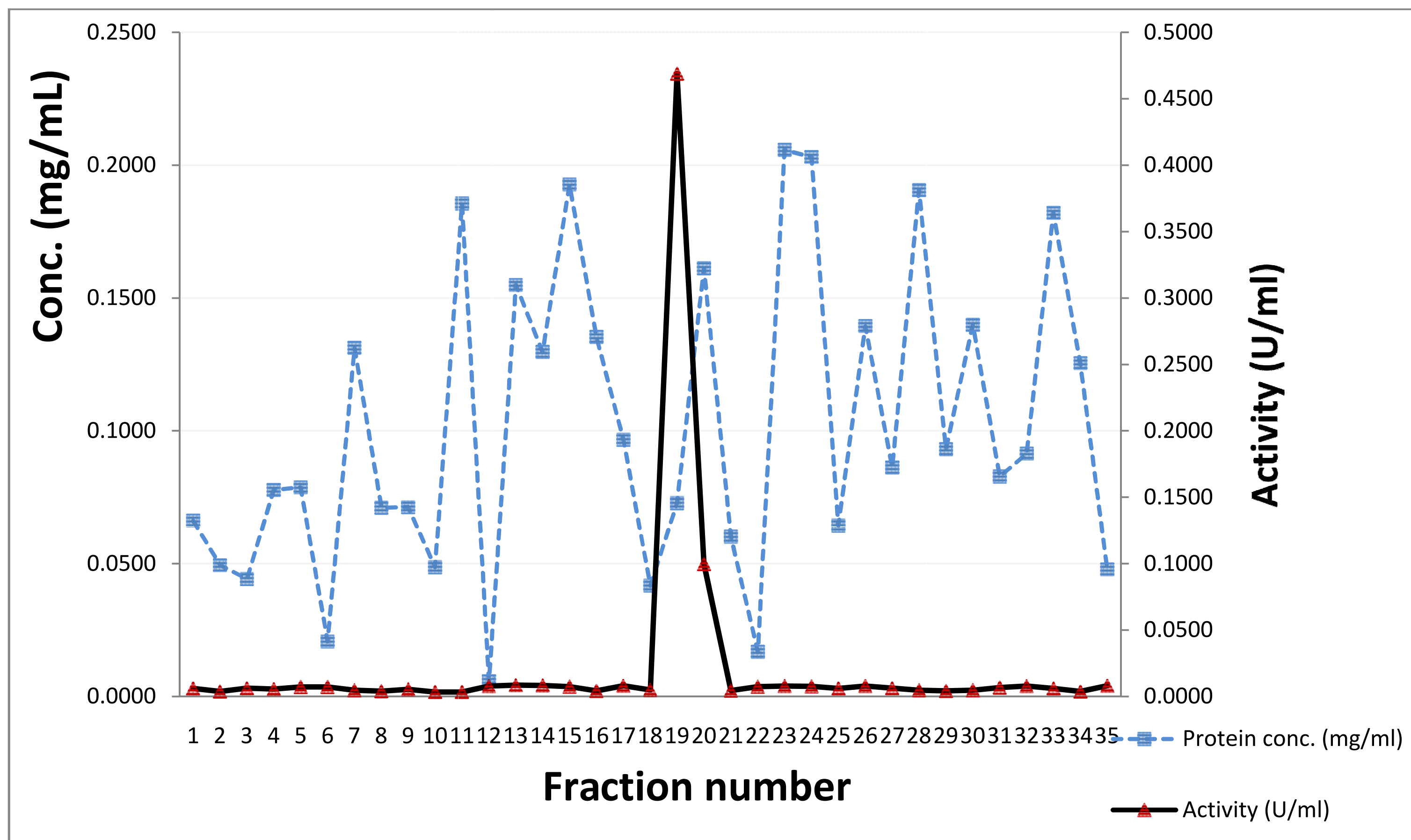

Figure 4.3: An elution profile for the gel filtration of the second pooled DEAE-cellulose active fractions of *Z. Officinale* protease (GPII) on Sephadex G-75 column (30 x 1.6 cm)

The column was equilibrated with 50mM sodium phosphate buffer, pH 7.2 containing 2mM cysteine 1mM EDTA and 5 mM sodium tetrathionate at a flow rate of 10 mlh^{-1} and 42 fractions were collected and tested for activity and protein concentration.

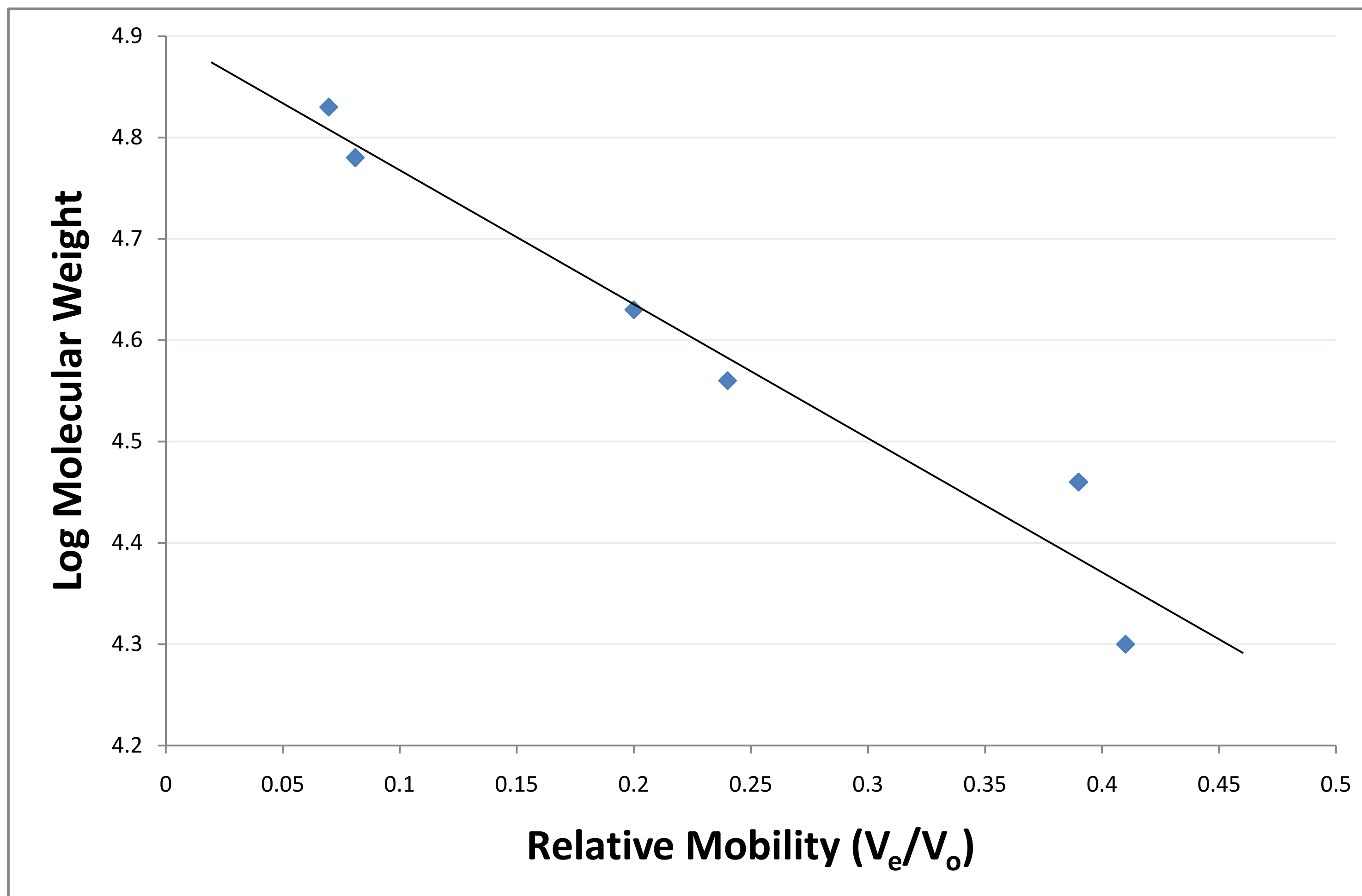

Figure 4.4: A Curve showing the log of Molecular Weight against the Relative mobility of the Standard Proteins used for the estimation of the Molecular Weight of *Z. Officinale* Protease

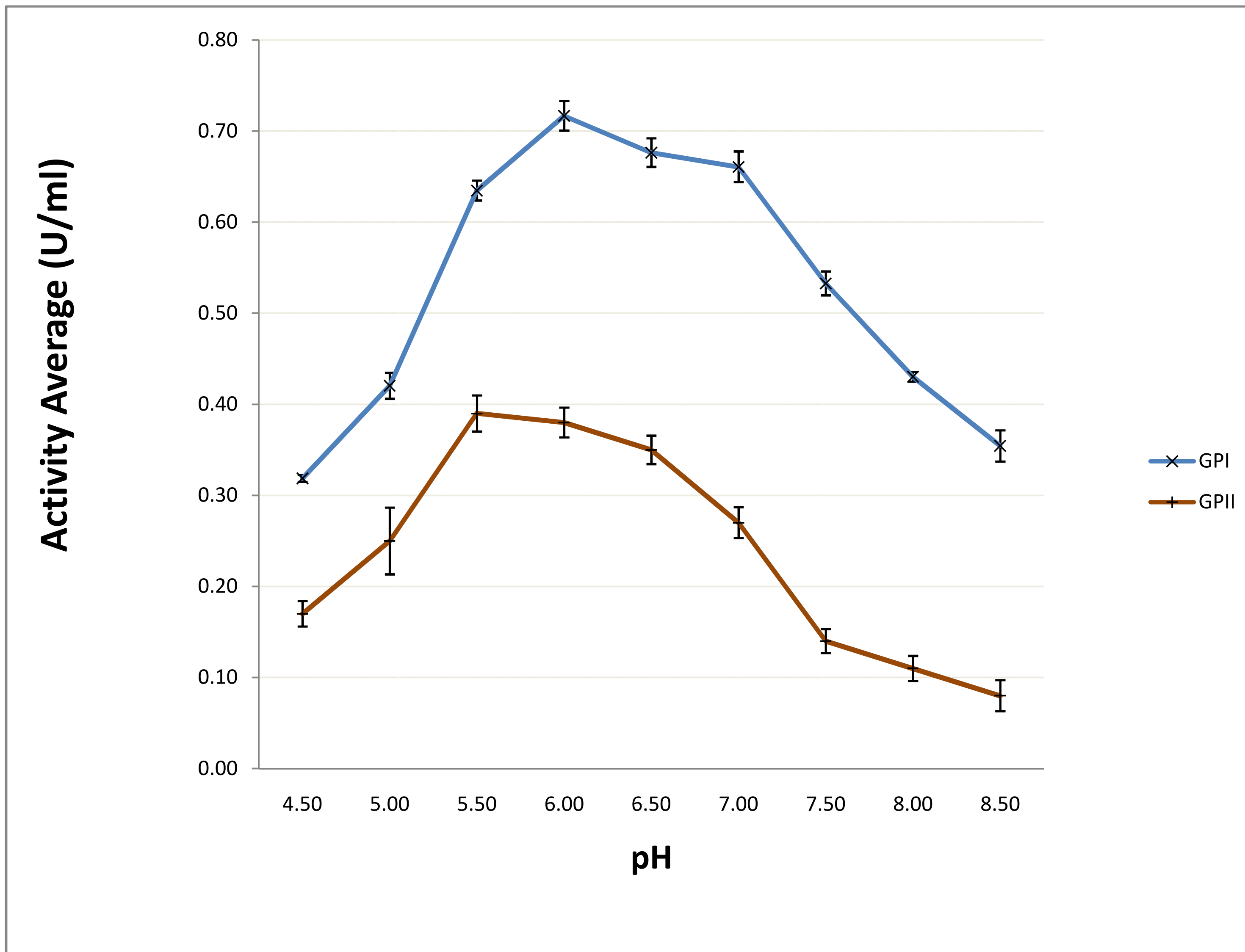

Figure 4.5: The effect of pH on *Z. Officinale* Proteases.

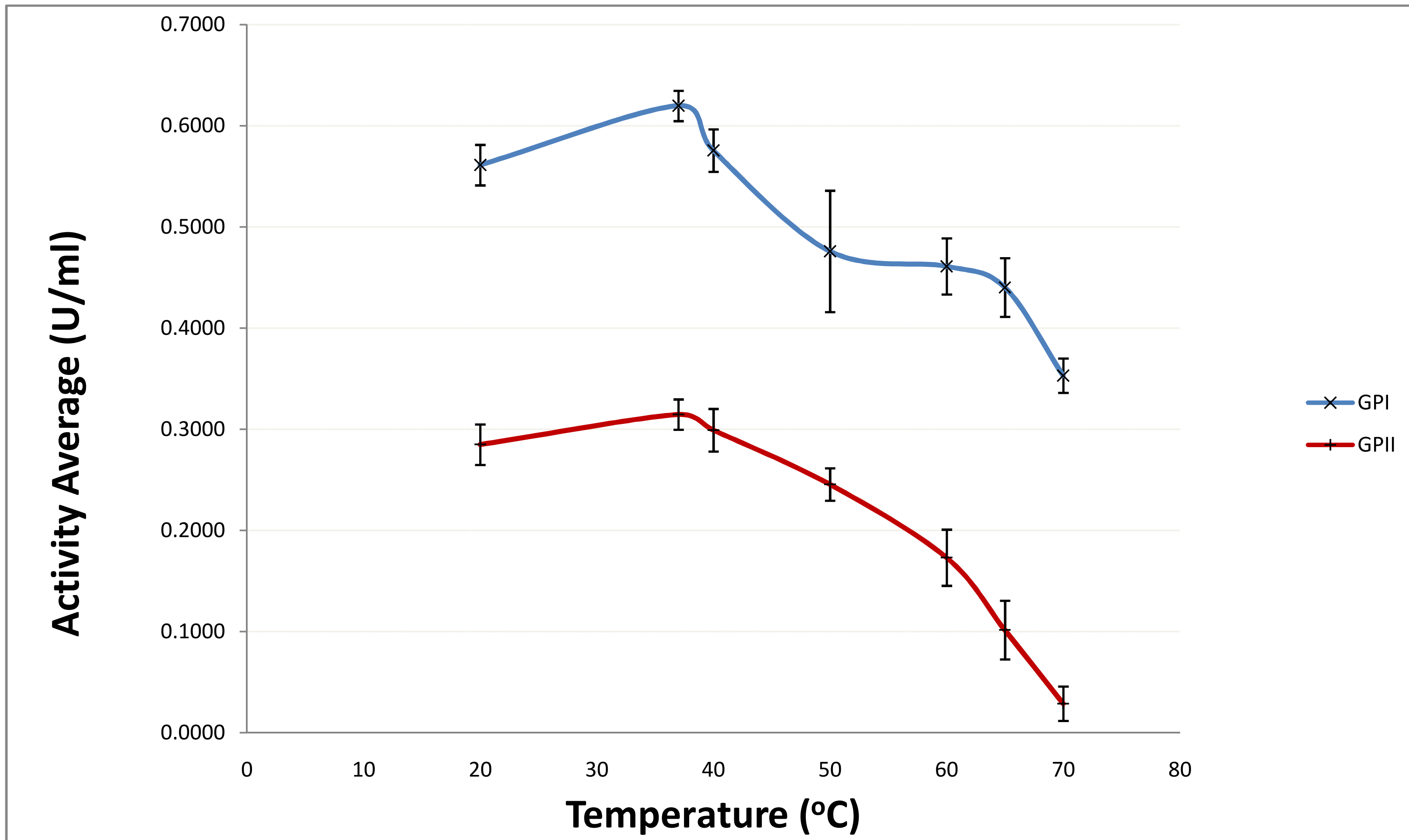

Figure 4.6: The effect of temperature on *Z. Officinale* Proteases.

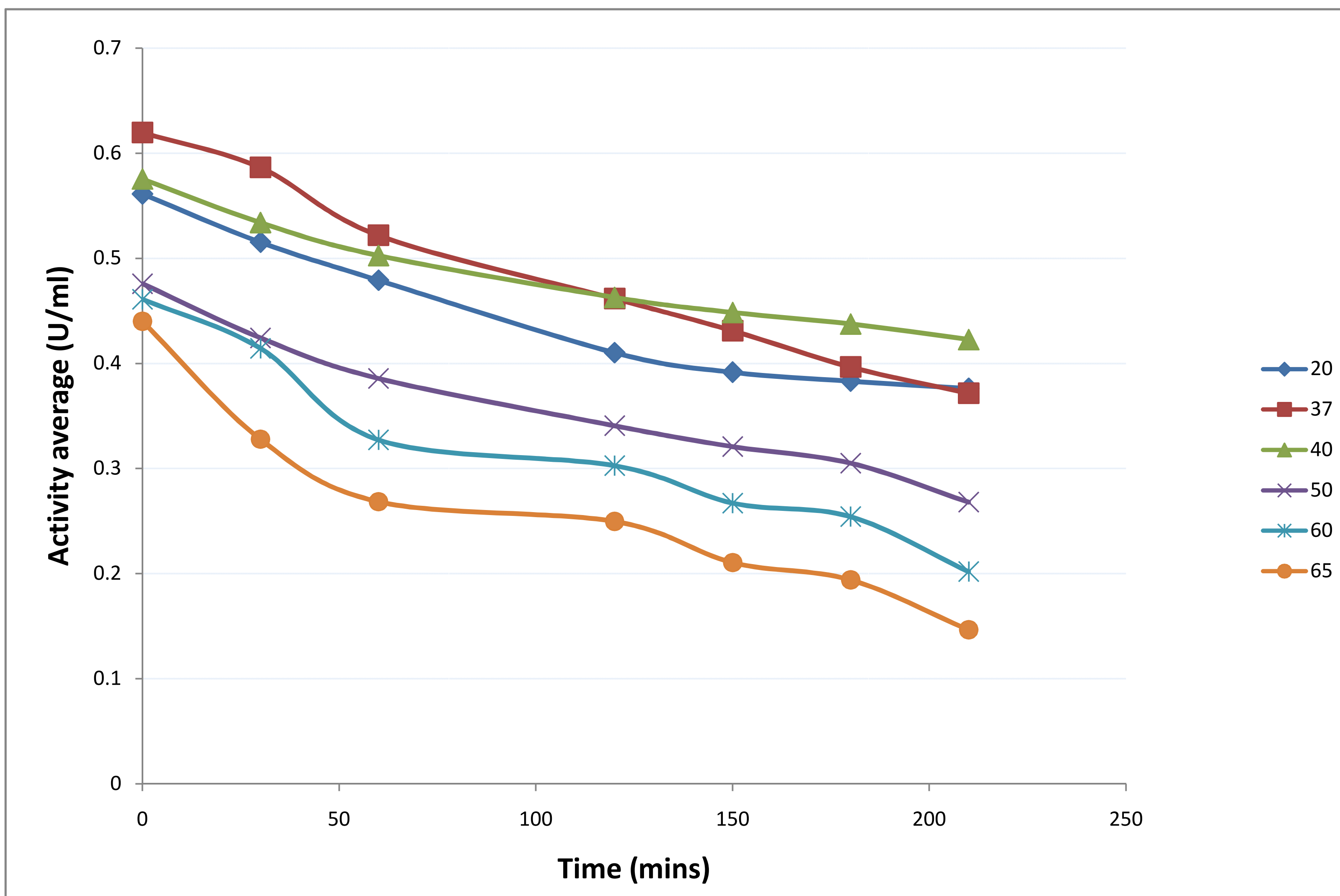

Figure 4.7a: The effect of temperature on the stability of *Z. Officinale* Protease (GPI).

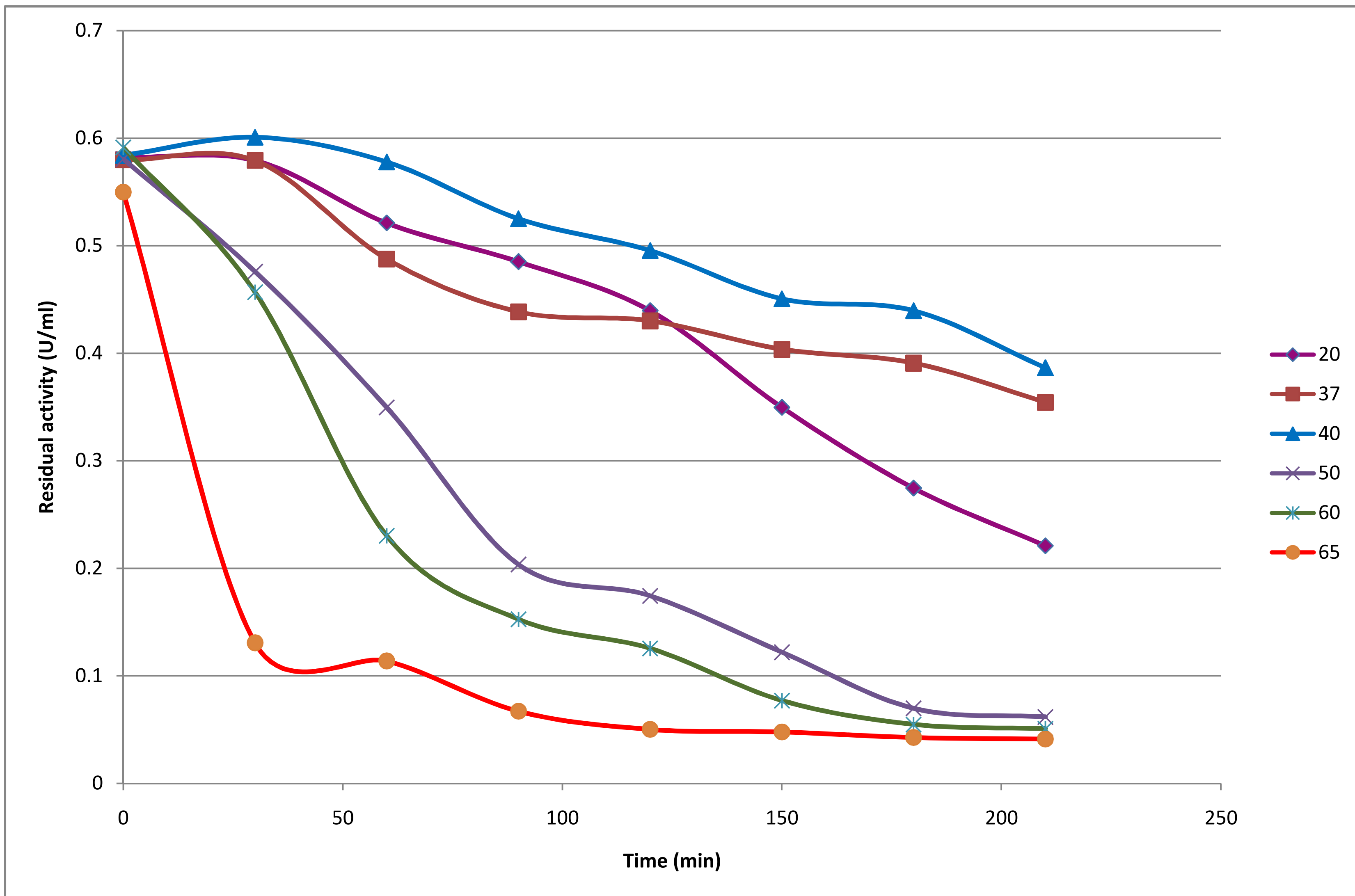

Figure 4.7b: Temperature stability of *Z. officinale* proteases (GPII)

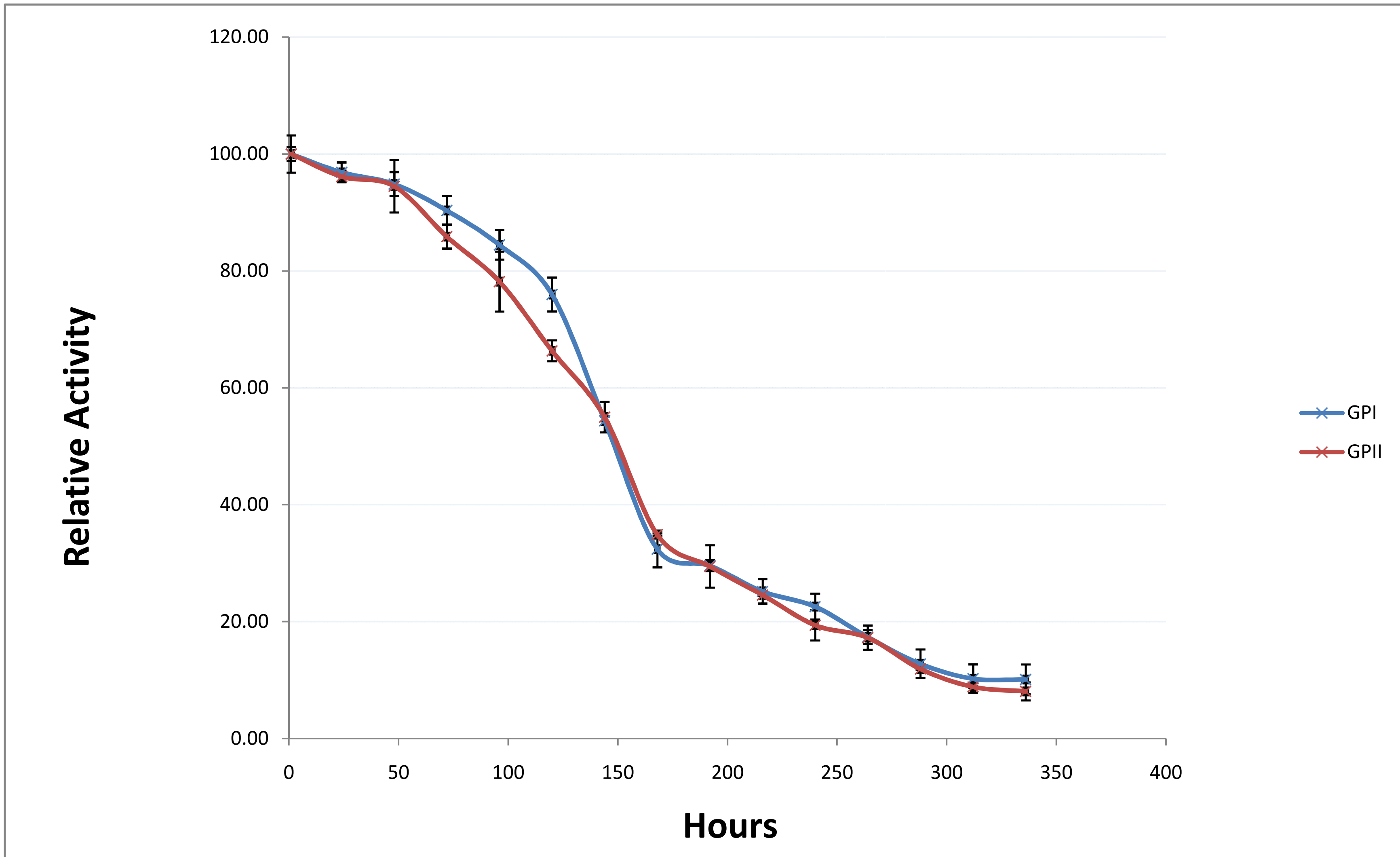

Figure 4.8: Time course of activity for Z. *Officinale* Proteases (GPI and GPII)

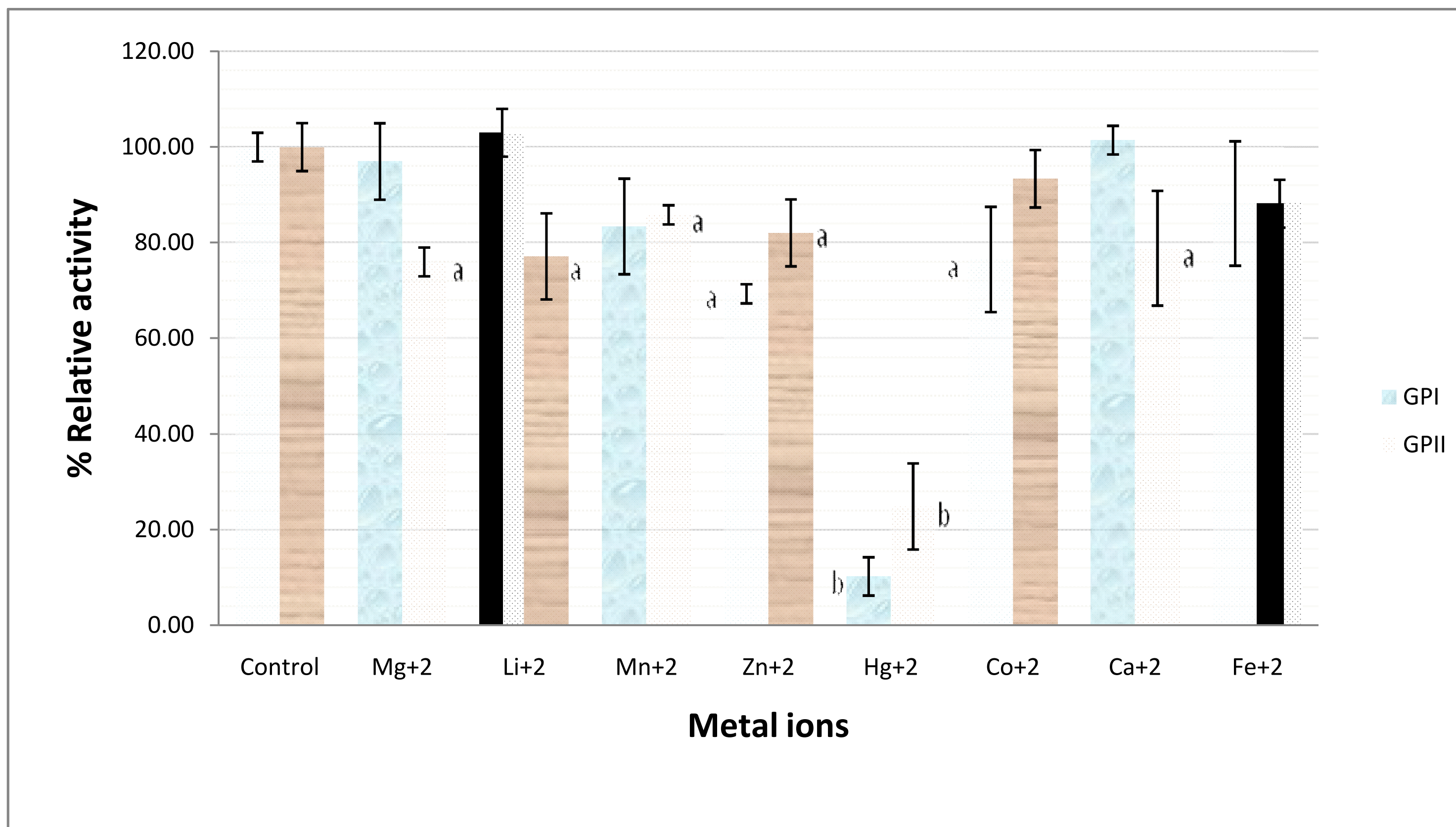

Figure 4.9: The effect of metal cat-ions on the proteases
Enzymes were pre-incubated for 15 min. at 37°C with 2mM of listed cations as final concentration prior to substrate addition. The activity of the control was without any metal ion added was taken as 100%. Each value represents the average of three experiments ±SE. a and b: differs significantly from the control at 95% and 66.7% level of confidence respectively

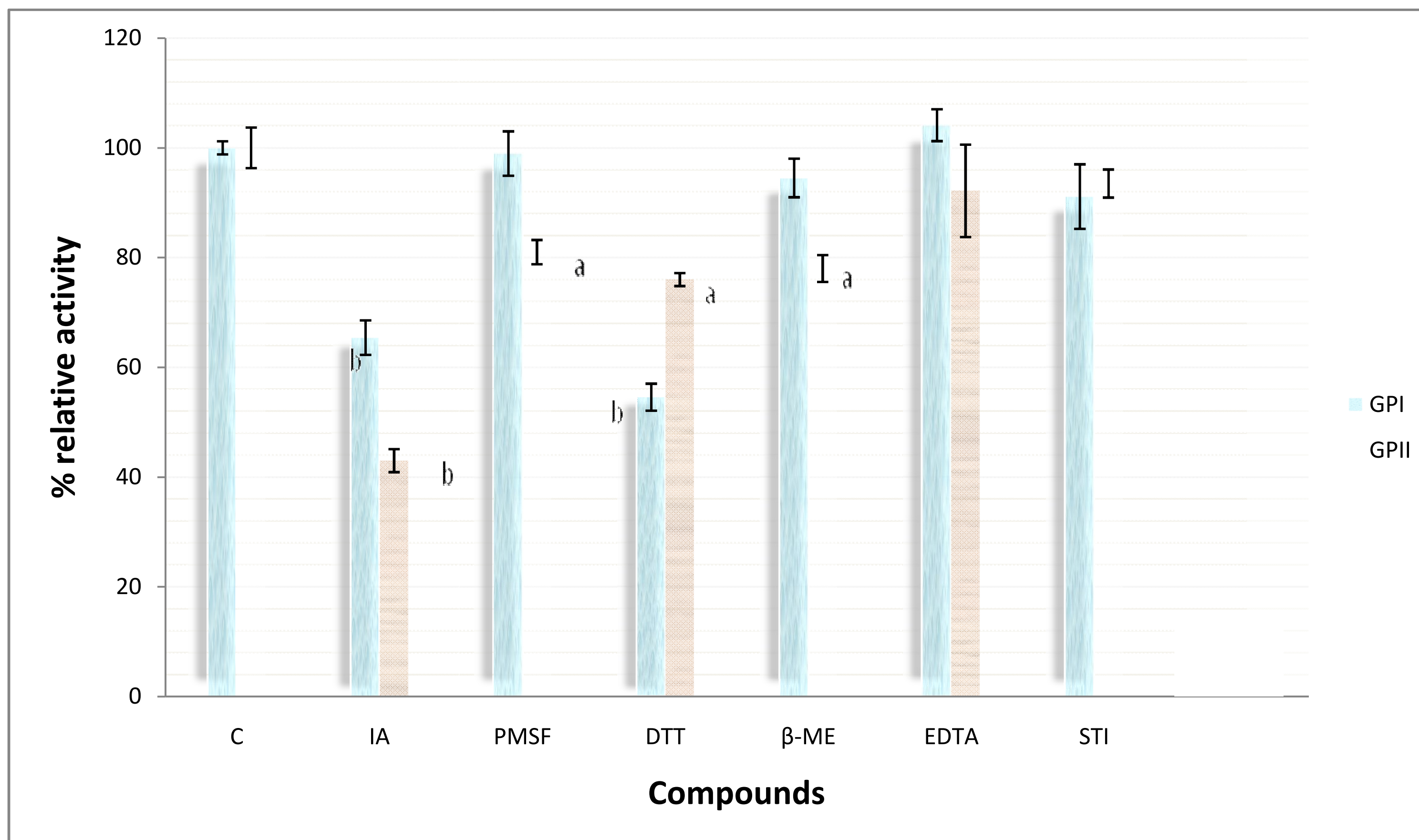

Figure 4.9: The relative activity of different compounds used on *Z. officinale* proteases (GPI and GPII) activity.
Note: Enzymes were pre-incubated for 15 min. at 37°C with 2mM of listed compounds as final concentration prior to substrate addition. The activity of the control (C) without any compound was taken as 100% activity. Each bar represents the average of three experiments ± SE; a and b differs significantly from its control at 95% and 66.7% level of confidence.

IA	= Iodoacetate	β-ME	= β-Mercaptoethanol
PMSF	= Phenylmethylsulphonylf	EDTA	= Ethylenediaminetetraacetic acid
DTT	= Dithiothreitol,	STI	= Soybean Trypsin Inhibitor

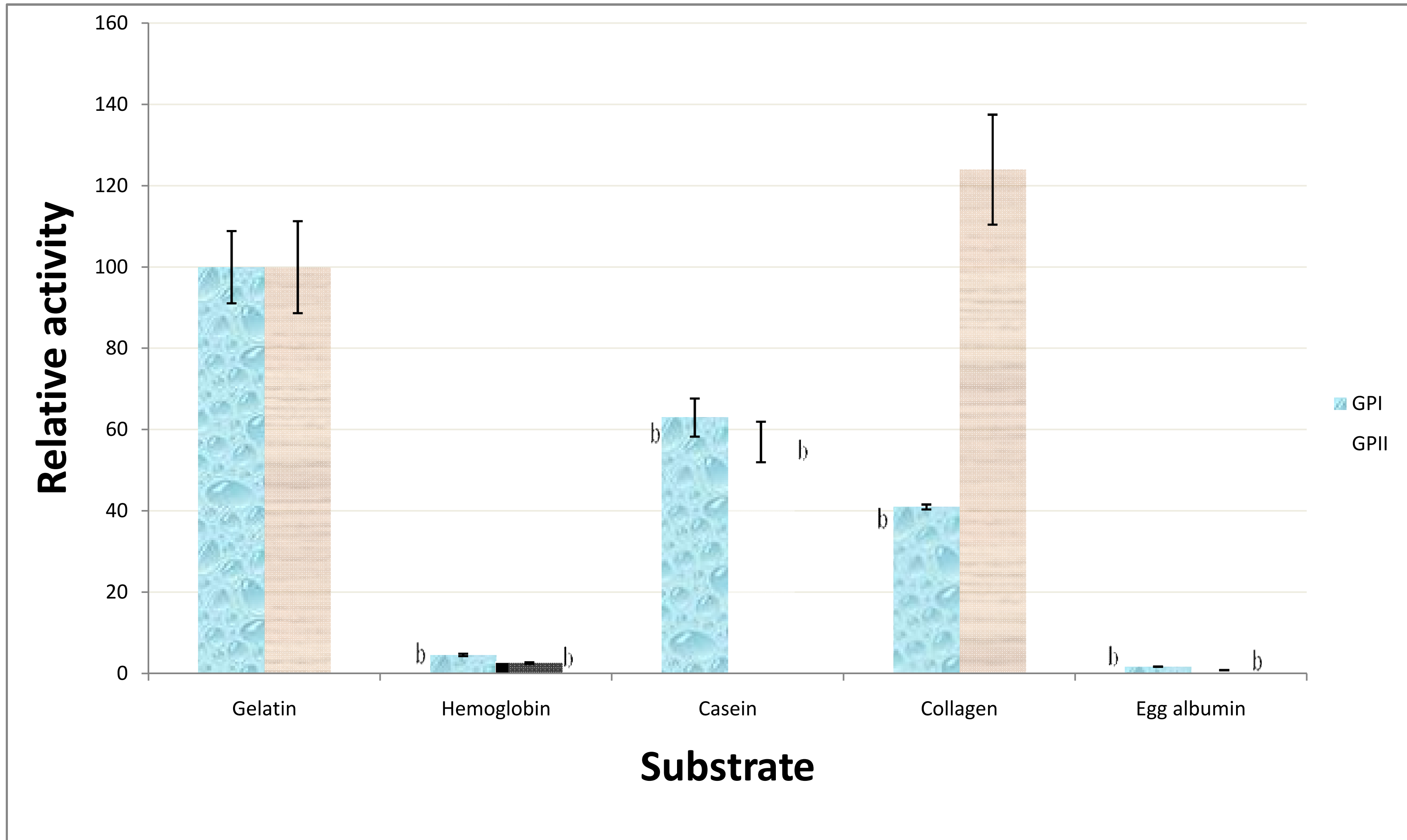

Figure 4.10: Relative activity of *Z. Officinale* Protease (GPII) toward different proteins substrates

Note: a and b: differs significantly from the control at 95% and 66.7% level of confidence respectively

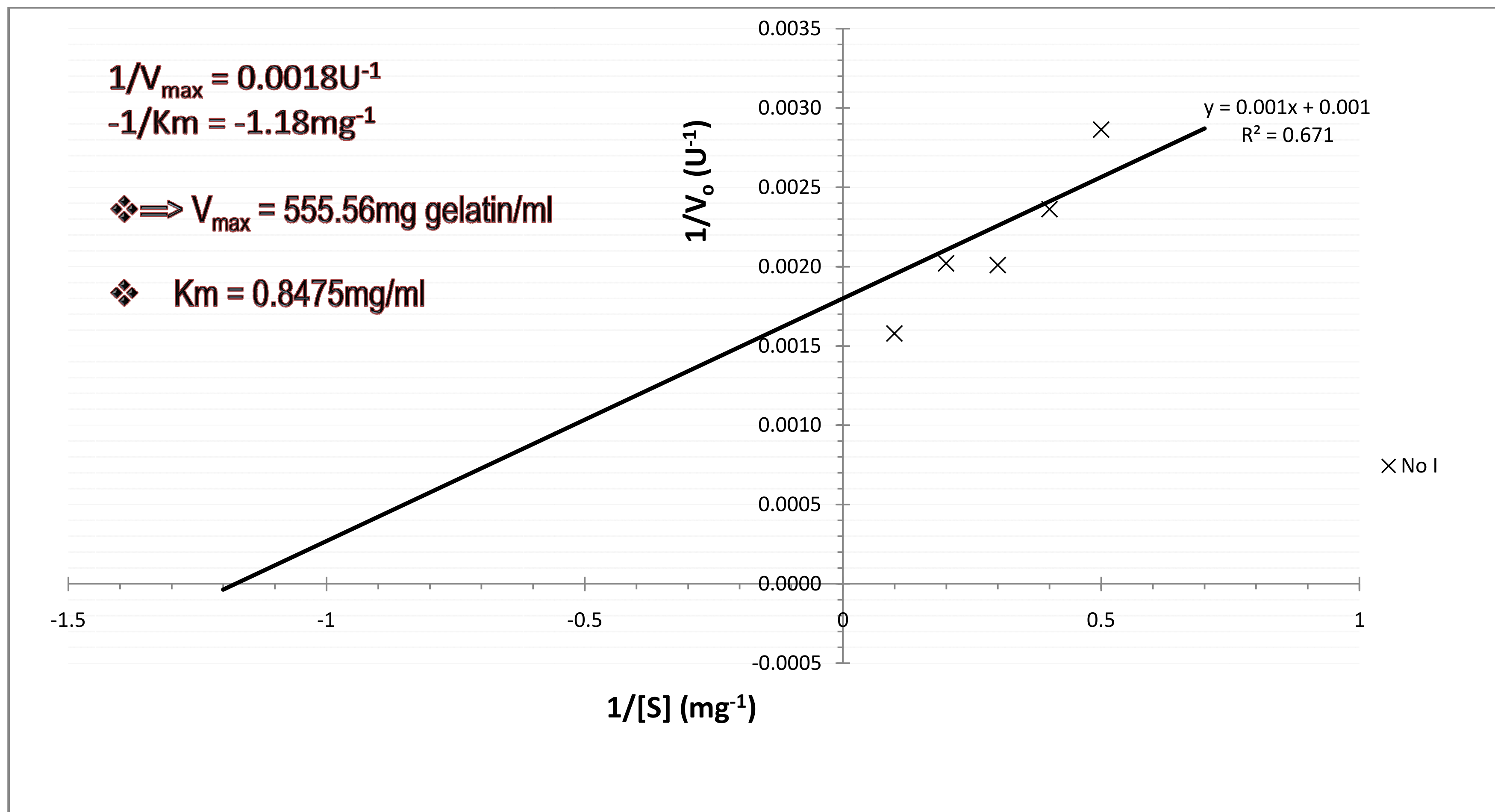

Figure 4.11: Double reciprocal plot (Lineweaver-burk plot) for determination of V_{max} and K_m using gelatin as substrate on *Z. Officinale* Protease (GPI)

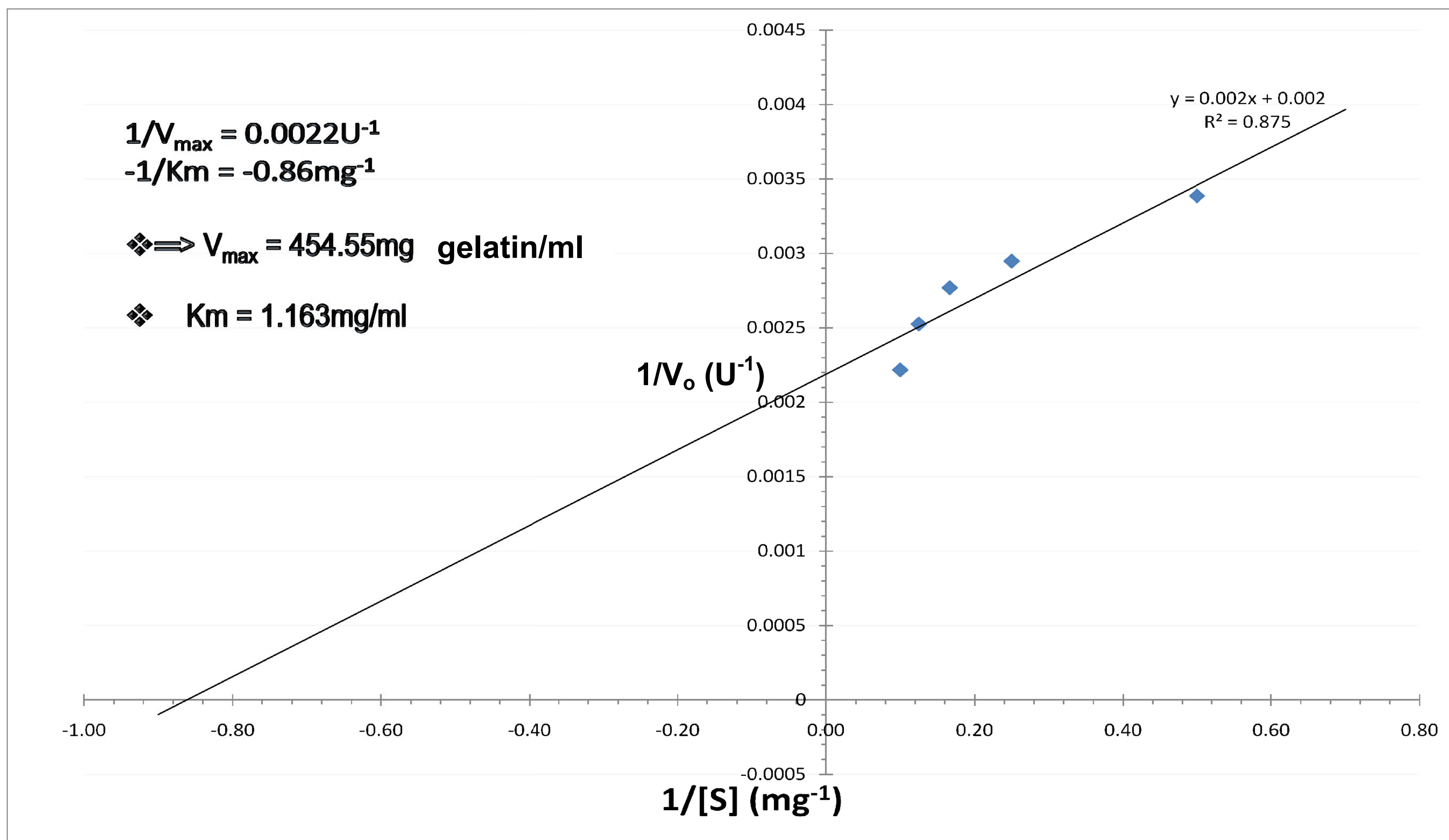

Figure 4.12: Double reciprocal plot (Lineweaver-burk plot) for determination of V_{max} and K_m using gelatin as substrate on *Z. Officinale* Protease (GPII)

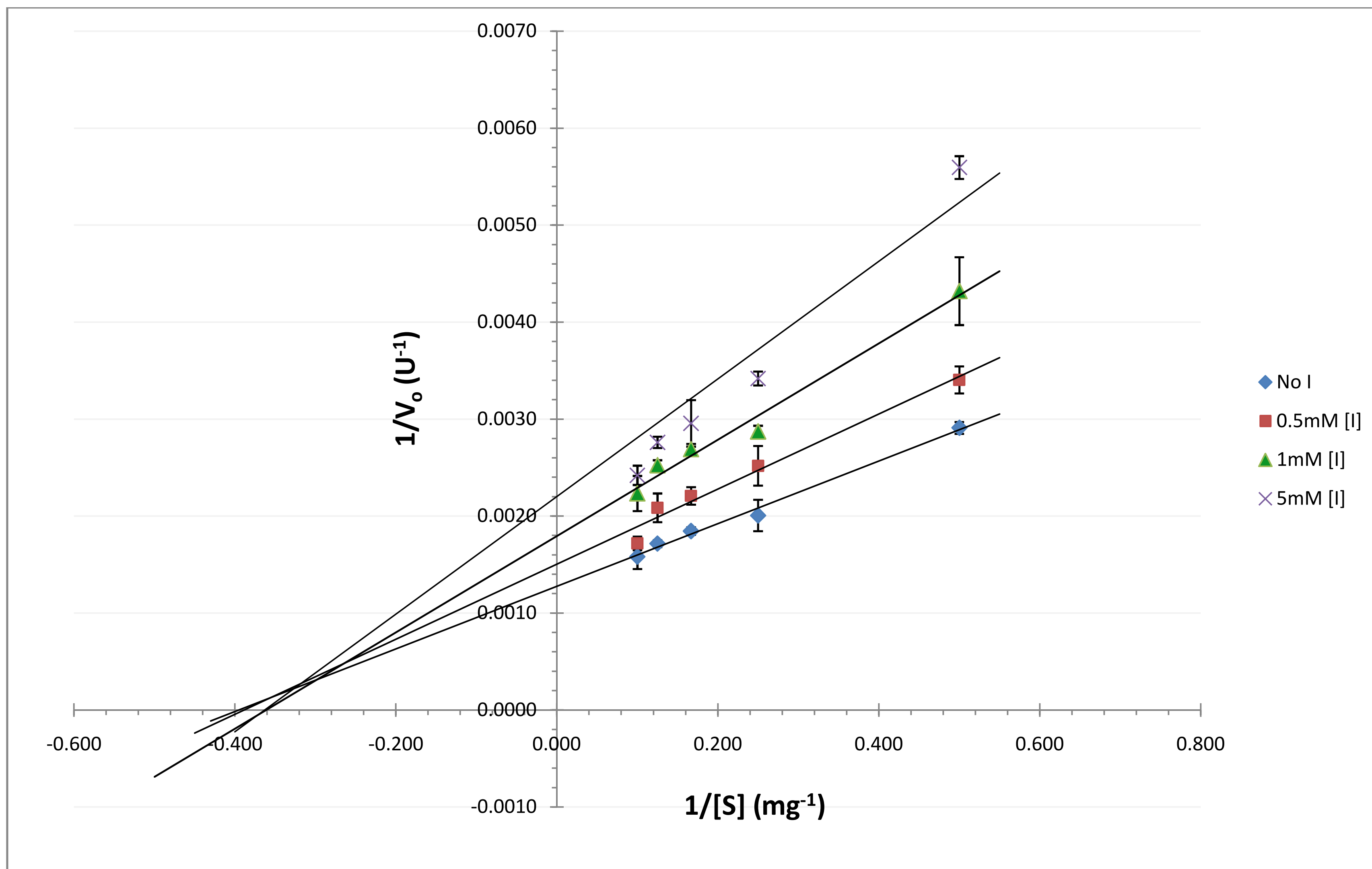

Figure 4.13a: Inhibition kinetics for *Z. Officinale* Protease (GPI) by Iodoacetate (IA), plot of reciprocal concentrations of casein (mg) and IA in concentrations indicated.

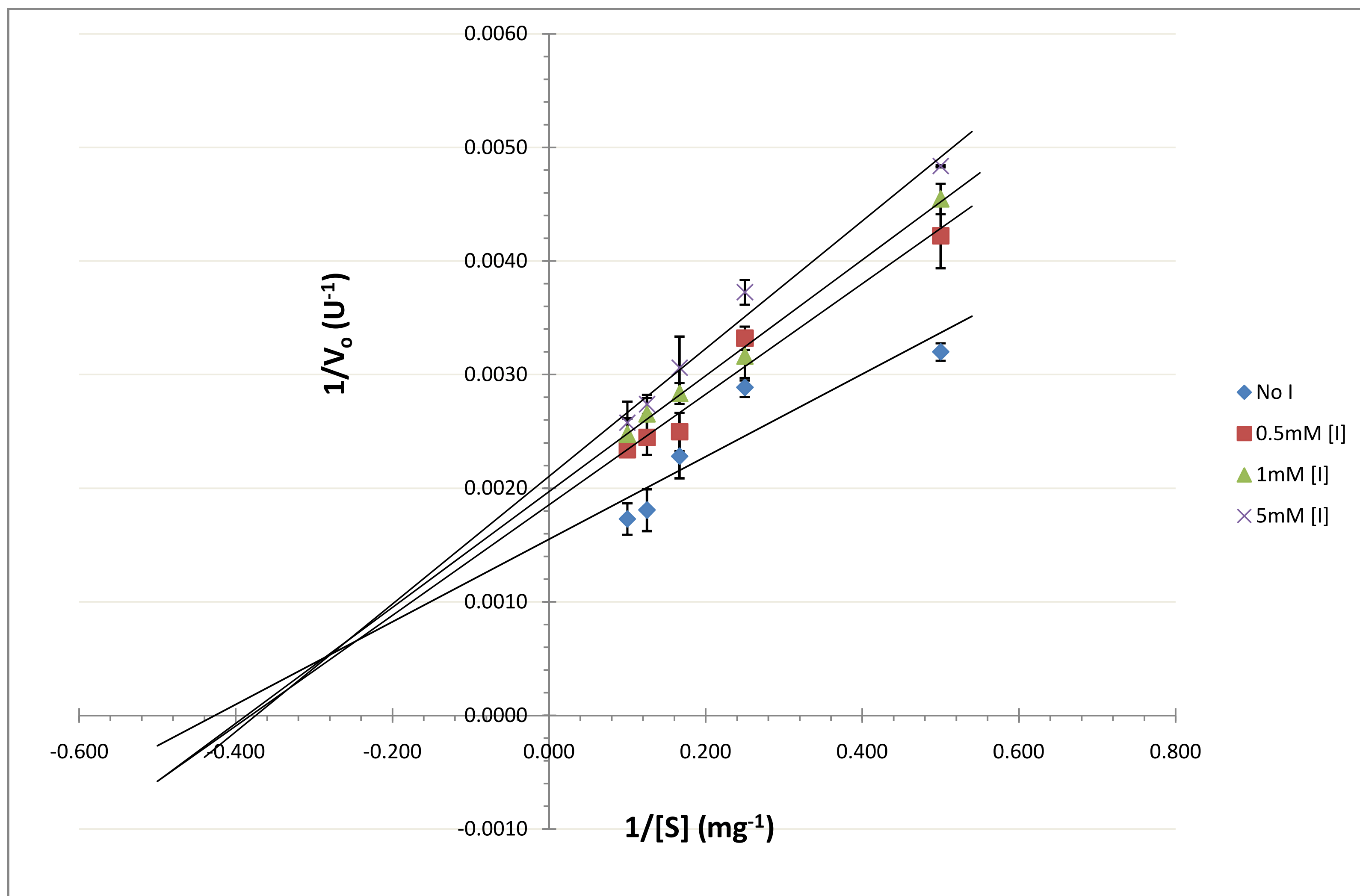

Figure 4.14b: Inhibition kinetics for *Z. Officinale* Protease (GPII) by Iodoacetate (IA), plot of reciprocal concentrations of casein (mg) and IA in concentrations indicated.

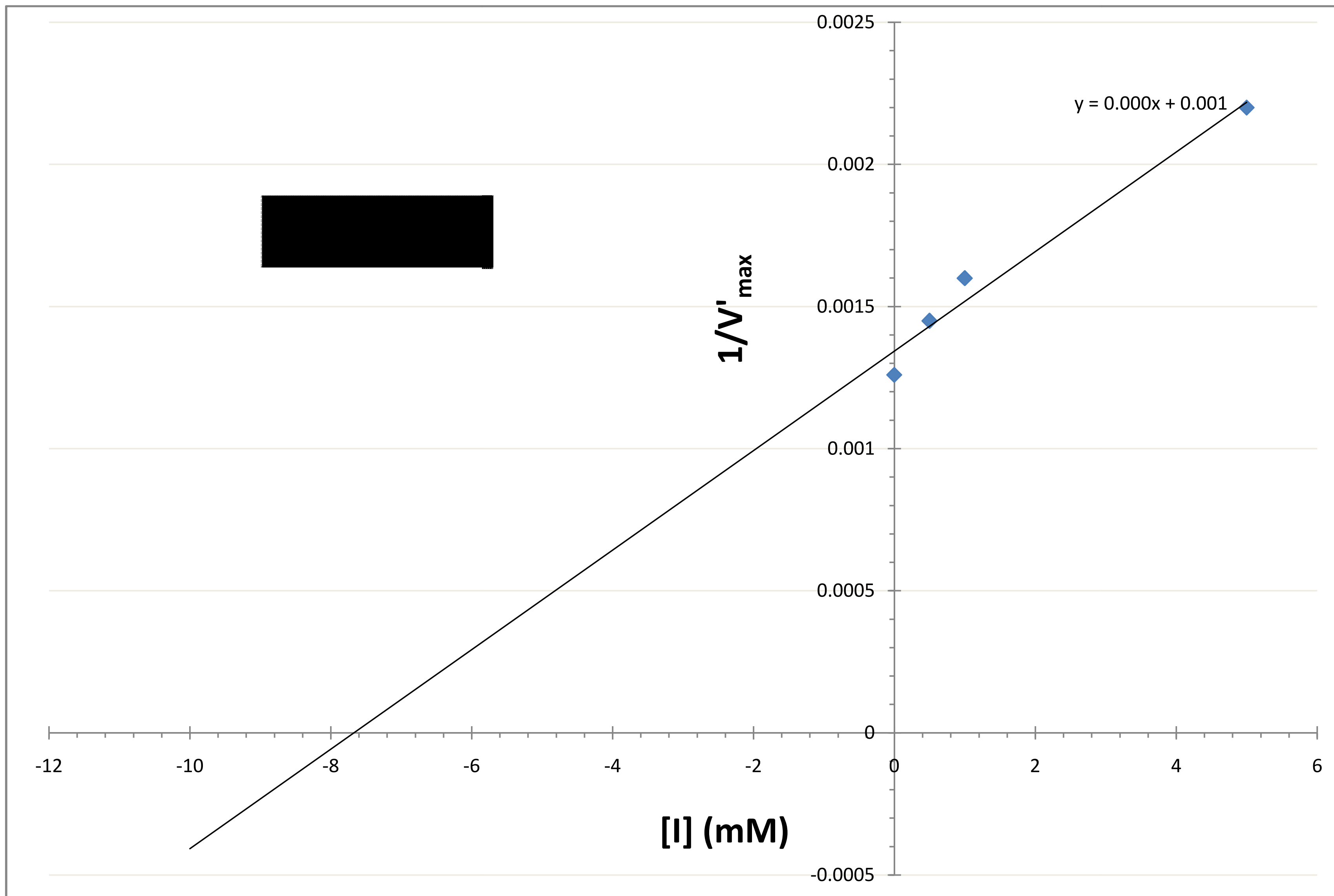

Figure 4.14a: Secondary plot to determine K_I for *Z. Officinale* Protease (GPI) by Iodoacetate

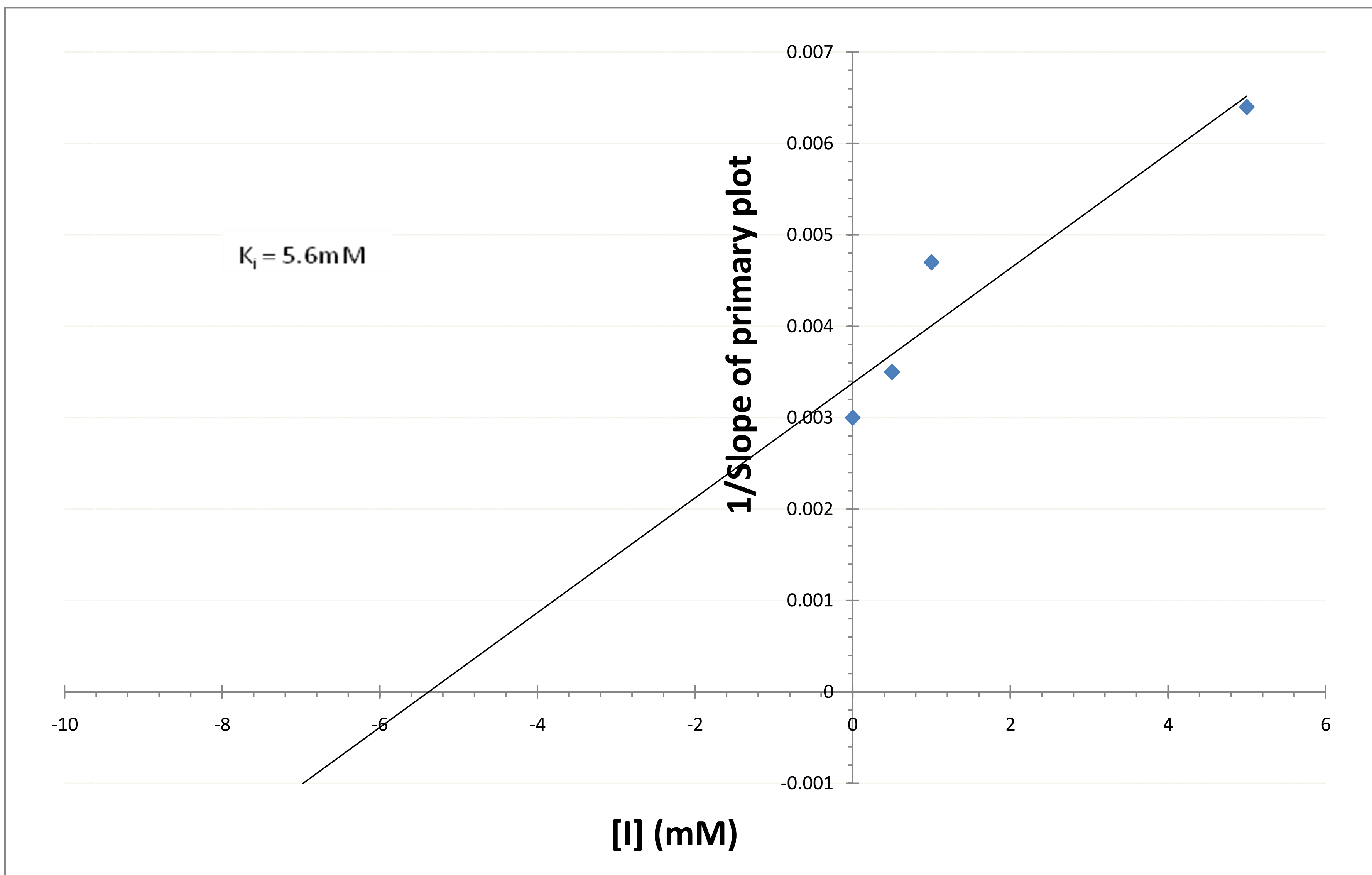

Figure 4.15b: Secondary plot to determine K_i for *Z. Officinale* Protease (GPI) by Iodoacetate

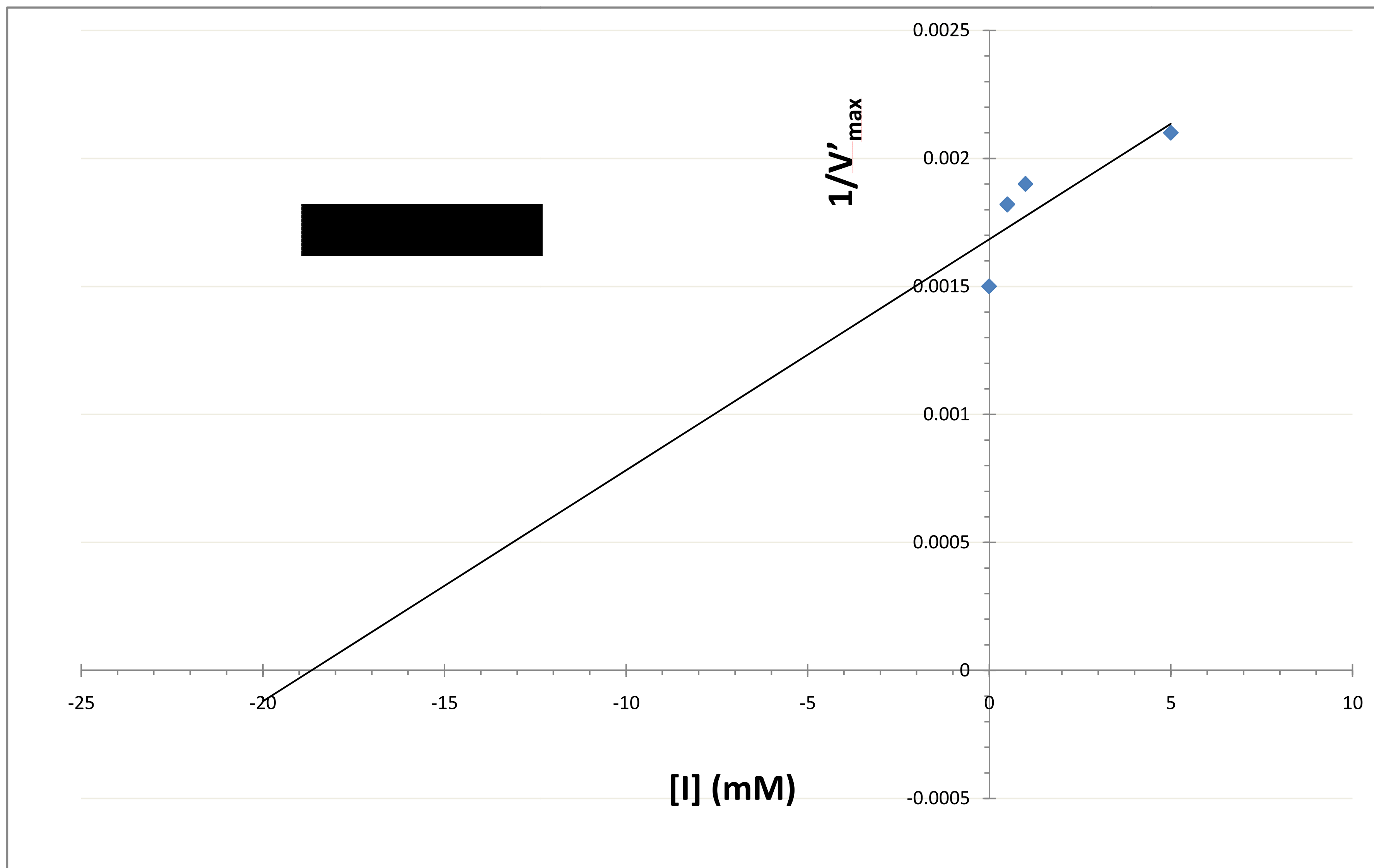

Figure 4.15a: Secondary plot to determine K_I for *Z. Officinale* Protease (GPII) by Iodoacetate

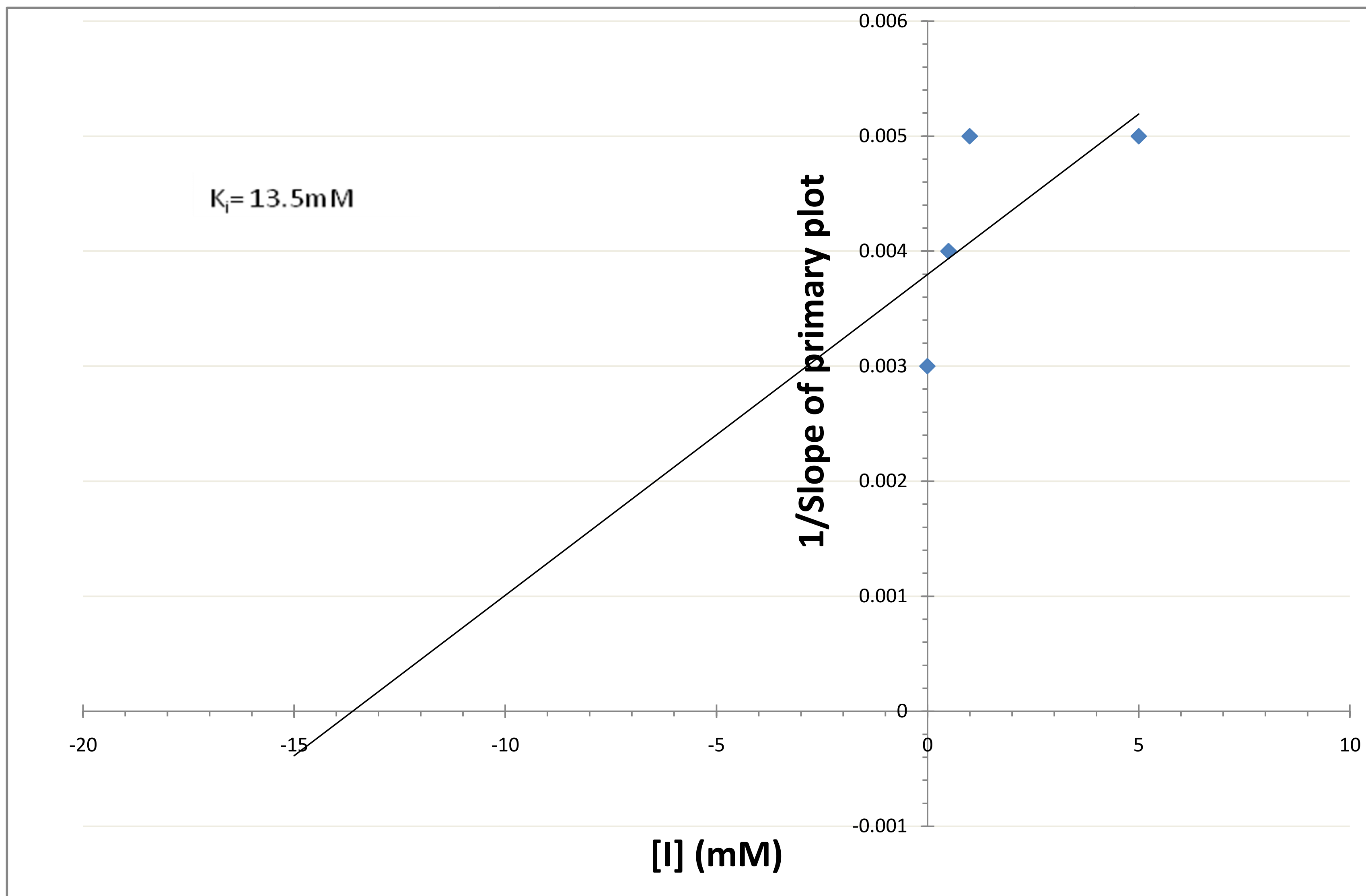

Figure 4.16b: Secondary plot to determine K_i for *Z. Officinale* Protease (GPII) by Iodoacetate

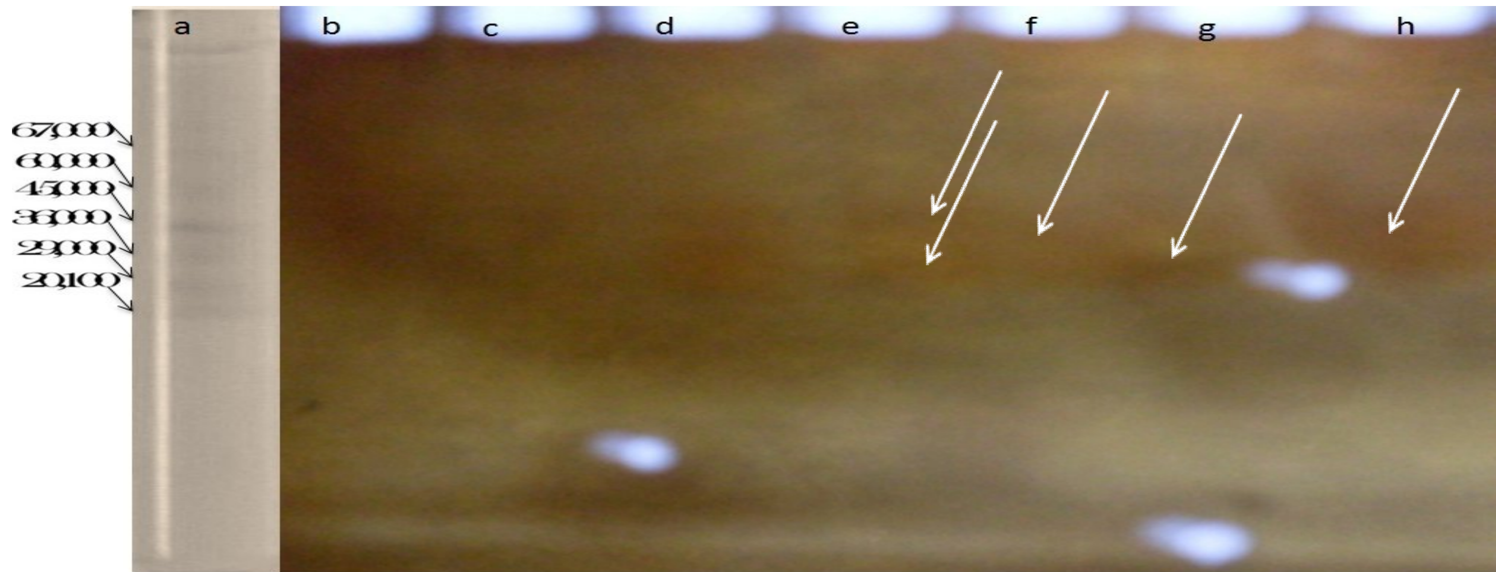

Figure 4.16: Electrophoretic patterns for Z. officinale proteases:

a- standard proteins,

b- crude extract,

c- dialysed fraction,

d- DEAE-Chromatography,

e- Sephadex G-10 of pooled active fractions ,

f- G-75 of GPI,

g- Sephadex G-75 of GPI,

h- Sephadex G-75 of GPII

CHAPTER FIVE

5. 0 DISCUSSION

The results obtained from the purification procedure indicated the effectiveness of the purification steps applied in the research. However, the yields of the enzyme after purification were found to be low. This might be due to the result of autolysis of the enzyme in the process of purification. In this study, we have demonstrated the feasibility of obtaining protease from ginger which can be explored. The purification procedure of *Z. Officinale* yielded an essentially homogeneous preparation with an overall recovery of 44.73% and 11.98% of GP-I and GP-II respectively. Under these experimental conditions an economic purification procedure combined with the ready availability of the plant material would make large-scale preparation of the enzyme feasible allowing an extensive study of its novel properties and hence probable applications. It was observed from SDS-PAGE that the purified proteases form *Z. Officinale* migrated as two distinct bands closely positioned, suggesting that it is probably made up of two subunits having very close molecular weight.

The estimated molecular masses of the subunits of *Z. Officinale* proteases (23.98 and 25.1 kDa) is fairly lower than that realized by Ichikawa 1973 (29

and 31kDa) but found to be in conformity with that of Han-Tsung et al, (2009) which was 22.5kDa, the little variation may have occurred due to the different approach in determining the molecular weight: the SDS-PAGE which was more precise gave a slight difference in the molecular weight of the sub-units. It was however largely lower than the molecular mass of *T. aestivum* cysteine protease (60kDa), and wheat grains (40-50kDa) but, was in the range of proteases classified to be cysteine proteases from; barley (29-37kDa), maize (12-36kDa), (Fahmy and Mohammed, 2004).

In the DEAE-cellulose column, GP-I being eluted first and GP-II later suggests that GPII is relatively the more acidic subunit. The acidic pH optimum of *Z. Officinale* protease indicates that it acts in an acidic cellular compartment such as the vacuole (Tjendraputra et al, 2001). Furthermore, enzyme activity within a pH range of 5.5-7.5 shows that the enzyme is more stable in an acidic medium and significantly decreases in the very alkaline pH range, possibly due to the denaturation of the enzyme, the range falls in a similar range of papain from *Carica papaya* with a pH range of 5.0-9.0 and stable up to 80°C (Fahmy and Mohammed, 2004, Mahajan and Badgujar 2010) and the protease from *Cucumis trigonus* (Roxb.) with a pH range of 5.2-7.6 and temperature range of 50-55°C employed in tenderizing buffalo meat (Naveena et al, 2004, Mahajan and Badgujar 2010).

In terms of storage stability, the activity of *Z. Officinale* protease remained significantly high for 7 days (160hours) of storage at 4^oC. This however can be improved as in the case of T. aestivum storage by lowering the temperature further; it retained almost constant activity for 150 days of storage at -20^oC when subjected to 15 freeze-thaw cycles (Fahmy and Mohammed, 2004).

The highly significant inhibition of *Z. Officinale* protease by iodoacetate (IA), a broad class inhibitor for protease subunits and the little or no inhibition exhibited by the others; phenylmethylsulphonyl fluoride (PMSF) (serine specific), ethylenediaminetetra acetic acid (EDTA) (metallo specific), and soybean trypsin inhibitor (STI) suggest that the purified enzyme from *Z. Officinale* protease likely belongs to a cysteine protease family.

Z. Officinale protease resists inhibition by proteinacious inhibitors such as STI inhibitor which is present in protein-rich foods such as Soybeans. Hence, it can be employed as a proteolytic enzyme in food industries especially in the meat and fish industries for tenderization to enhance quality of products (Gilbert and Raa, 1979, Shahidi and Kamil, 2001).

A number of natural proteins were tested as substrate for *Z. Officinale* protease, gelatin exhibited the highest activity, the activity decreased in the order; collagen, casein, hemoglobin and egg albumin had the least activity. *Z. Officinale* protease showed to have a narrow range of substrates. In the present study, the substrate

specificity indicated that it was active on gelatin and to a major extend on collagen. This suggests its prospective application in industries utilizing gelatin and collagen rich matter like as a meat tenderizer and meat related food industries and the hide and skin industries. These results are in agreement with those reported for plant proteases of the papain family having unique specificity of products, for instance papain is widely used in industries as a meat tenderizer and has also other application in the pharmaceutical, detergent, veterinary and food industry (Fahmy and Mohammed 2004, Mahajan and Badgujar 2010).

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY

The present results lead to several valuable conclusions about ginger proteases. First, a stable and homogenous protease can be prepared reproducibly from ginger extract. Secondly, the biochemical properties determined suggests its identity as a cysteine protease, thirdly, several inhibitors exhibited moderate or little inhibition and it showed selective substrate specificity. The purified enzyme was found to be a dimer with estimated molecular weight of 23.97 and 25.05 kDa. The enzyme subunits had similar optimum temperature of 40°C, with an optimum pH slightly acidic. The enzyme subunits showed high substrate specificity. They both exhibited a mixed (competitive-non-competitive) pattern of inhibition with IA. A comparison of the obtained data with previous studies suggests that ginger protease exhibits desirable properties suitable for industrial application, thus, offers potential for food industries.

6.2 CONCLUSIONS

The study showed a high similarity of properties with those of cysteine proteases thus, suggests that *Z. officinale* protease is likely of that family. The

enzyme subunits displayed quite similar biochemical characteristics and demonstrated gelatin as natural substrate, though, GPII exhibited high collagen activity. The properties of this enzyme reveal that it could be useful for industrial application.

6.3 RECOMMENDATIONS

The yield of the enzyme after purification were found to be low, to make this commercially viable, research is needed to improve the yield. This study has presented useful suggestions on the nature of *Z. Officinale* protease. However, for better understanding of their structure-function relationship further studies employing techniques like X-ray crystallography and nmr spectrometry need to be done to provide useful information and discover probable applications of ginger proteases (zingibain), consequently, realizing new potentials in biotechnological applications.

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APPENDIX

Preparation of Biuret reagent

2.25g of Sodium potassium tartrate, 0.75g of Copper sulfate .5H_2O and 1.25g Potassium iodide were dissolved in this order in 100ml of 0.2M NaOH. The volume was made up to 250ml with distilled water.

Preparation of solution of Bovine Serum Albumin (BSA)

10g of BSA was weighed and dissolved into 100ml of distilled water to make a standard solution of 10mg/ml

Buffer for extraction, dilution and assay:

Buffer: (Sodium phosphate, 100mM, pH 7), with cysteine (30mM) and EDTA (30mM). 8.9 g of di-sodium hydrogen phosphate dehydrate ($Na_2HPO_4 \cdot 2H_2O$) is dissolved in 450mL of distilled water and L-cysteine hydrochloride monohydrate (2.65g) and ethylenediaminetetra acetic acid (5.6g, EDTA) are added and dissolved. The pH is adjusted to 7.0 with 1M sodium hydroxide (40 g/L), and the volume is adjusted to 500mL. Sodium azide (0.2g) was added as a preservative. Store at 4°C.

Enzyme extraction and dilution:

Fresh ginger rhizome (2g) is homogenized in 30mL of Buffer and stirred for about 15 minutes at room temperature. The preparation is then strained and centrifuged (1,000*g*) for 15 minutes. The residue was re-suspended in 20mL of the Buffer and re extracted as explained above. The two extracts were pulled together to and designated the crude extract.

Table 1: Moisture content determination

Time (Hrs)	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Weight (g)	91.7	85.3	77.5	68.7	62.1	58.7	54.8	51.9	45.7	41.6	39.9	37.2	35.4	32.7	32.3	31.6	31.8

% moisture content = 100 – 31.8/100 = 68.2/100 = 0.682%

This original extract is then further diluted (1mL to 9mL of Buffer) until a concentration suitable for assay is obtained.

For the preparation of the standard curve, label three sets of test tubes with the numbers 1, 2, 3, 4, 5, and 6. Pipet distilled water, add the BSA stock, and then add the Biuret reagent as indicated in the chart below. Note that tube 1 serves as the reference tube.

Protein concentration determination

Specific volumes of the extract was pipetted as designated on the table below, and the volumes were topped to 1ml with distilled water.

2ml of biuret reagent was added to each tube and left to stand at 37°C for 15 minutes

Activity assay procedure

Pre-equilibrated enzyme solution (0.1ml of extract in 1ml of phosphate buffer pH 7.0) is added to pre-equilibrated substrate solution (1ml of 3% casein).

The solution is stirred and incubated at 37°C for 1hour.

The reaction is terminated by the addition of 0.5ml of 10% trichloroacetic acid (TCA) with vigorous stirring for 5 seconds.

The reaction tubes are allowed to equilibrate to room temperature for 5 minutes.

The absorbance read against the reaction blank at 366 nm.

Reaction blanks are prepared by adding the TCA to the substrate solution immediately before the enzyme preparation is added.

Note: One unit of proteolytic activity is defined as μg α–amino acid liberated per hour under standard assay conditions.

Determination of the Effect of pH on Protease Activity

Gelatine was dissolved in Tris-CL buffer solution and the enzyme assay was carried out within pH range (7.0 to 10.0) at 0.05M Concentration.

Determination of the Effect of Temperature on Protease Activity

For the determination of the effect of temperature, the reaction medium was incubated at varied temperatures and the protease activity was determined. The assay mixture, constituted by mixing 1.0mL of the pooled fractions with 0.2mL of 3% (w/v) gelatine, was incubated at different designated temperatures (incubated at 37°, 40°, 50°, 60°and 65°C temperatures) using a thermostat (sensitivity ± 0.01ºC). After 30 minutes incubation, the reaction was arrested by addition of 0.3mL of 5% (w/v) TCA. The protease activity was determined. Corresponding blank at each temperature was run by adding TCA to the pooled fractions prior to mixing with gelatine. Similar experiment was repeated with reaction mixture containing 9.8mM sodium carbonate.

Determination of Thermostability of Protease

The thermostability of the protease was determined in the range of 37° to 65°. For this purpose, the crude enzyme was incubated at 37°C, 40°C, 50°C, 60°C and 65°C for 30 minutes in a water bath. After cooling to room temperature

the preheated enzyme preparations were added to the reaction mixture and incubated at 37°C for 30 minutes to determine the enzyme activity. Enzyme kept at room temperature was taken as control.

Determination of Stability of Protease

Storage stability of protease was determined at 0°C, 4°C and at room temperature. The enzyme was preserved with sodium azide and kept at room temperature. NaN_3 is a toxic chemical and was added to the enzyme solutions at a final concentration of 0.05%. The activity of the enzyme was checked after regular time intervals (5 days). Two other sets of enzyme preparations were kept at 0°C and 4°C and the activity of the enzyme was checked after five days intervals.

Determination of the Effect of EDTA on Protease Activity

The protease after dialysis was preincubated with different concentration (0.5-16mM) of EDTA for 10 minutes of 37°C temperature and the residual enzyme activity was determined by the usual azocasein assay method. Control without EDTA was always used.

Determination of the Effect of metal cations on Protease Activity

The enzyme was preincubated for 15 minutes at 37°C with 2 mM of listed cations as final concentration prior to substrate addition. Activity of enzyme without added metal ions was taken as 100%.

Table 1: Protein concentration determination

Tube	H_2O (ml)	BSA (ml)	Biuret Reagent (ml)	ABS	ABS	ABS	ABS average (ml)	BSA Conc. (mg/ml)
1	1.0	0	2	0.00			0.00	0
2	0.9	0.1	2	0.0263	0.0259	0.0261	0.0261	1
3	0.8	0.2	2	0.0544	0.0538	0.0538	0.0540	2
4	0.7	0.3	2	0.086	0.0858	0.0682	0.0800	3
5	0.6	0.4	2	0.105	0.1045	0.1054	0.1049	4
6	0.5	0.5	2	0.1385	0.1382	0.1388	0.1385	5

Note: standard graph on the next page

Table 2: Unknown protein concentration calculations

Tube	Volume of unknown	ABS	ABS	ABS average	Amount of Protein (mg) [a]	Concentration (mg/ml)
a	0.01	0.017	0.015	0.016	0.556	55.6
b	0.02	0.033	0.031	0.032	1.185	59.25
c	0.1	0.124	0.184	0.154	6.815	68.15
d	0.2	0.278	0.293	0.285	Too high (off curve)	-- -- -- --

a Values obtained from the standard curve regression equation: Conc. = Abs./0.027

Average concentration of protein = $^{55.6 + 59.25 + 68.15}/_{3} = {}^{183}/_{3} = 61$ mg/ml

=› concentration of protein in the crude extract is 61±5.2mg/ml

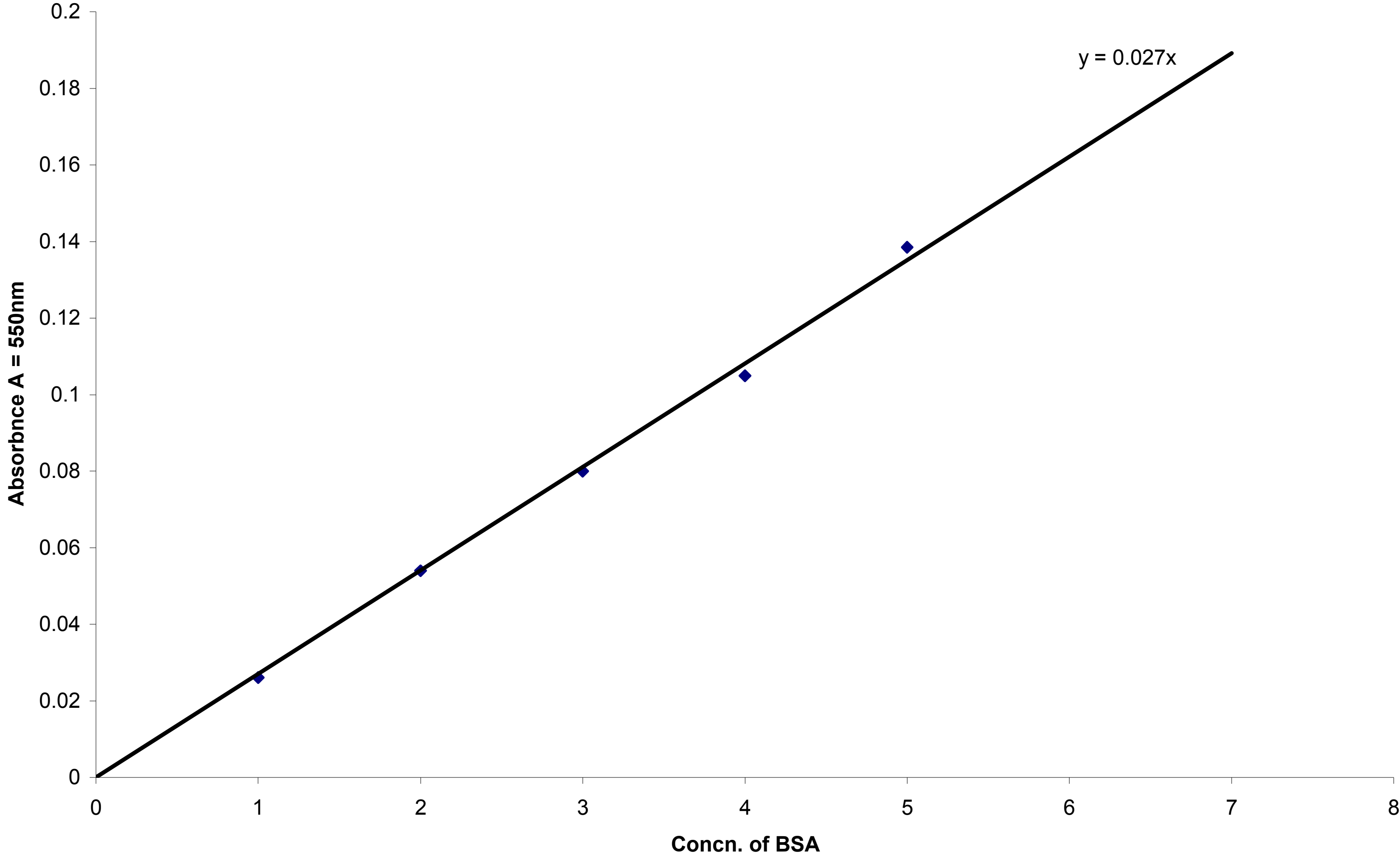
Table 3: Determination of amount of α–amino acid liberated per hour

Tube	Blank	1	2	3
Absorbance	0.00	0.144	0.093	0.137
Amount of α–amino acids liberated (mg)	-- --	0.0716	0.0472	0.0683

Note: Activity values were obtained from the standard curve regression equation: 478.38·Abs. + 2.73

The average amount of α–amino acid liberated per hour under standard assay conditions = $^{0.0716 + 0.0472 + 0.0683}/_{3} = 0.06237$ mg = 62.37μg

Standard curve



Preparation of stock solutions for Poly Acrylamide Gel Electrophoresis

Preparation of stock solutions for PAGE (as described by Laemmli) (John 1996)

Stock acrylamide Solution (30.8%T 2.7%C_{bis} solution)

60g of acrylamide and 1.6g of bisacrylamide was dissolved and made up to 200mL solution with distilled water.

Resolving gel buffer (1.5M Tris-Cl, pH 8.8)

36.3g of Tris was dissolved in 150mL distilled water, the pH adjusted to 8.8 with concentrated HCl and the solution was made up to 200mL with distilled water.

Stacking gel buffer (0.5M Tris-Cl, pH 6.8)

3g of Tris was dissolved in 40mL distilled water, the pH adjusted to 6.8 with concentrated HCl and the solution was made up to 50mL with distilled water.

10% Ammonium persulfate

0.1g of Ammonium persulfate was dissolved in 1mL distilled water.

10% SDS

5g of Tris was dissolved in 40mL distilled water, the pH adjusted to 6.8 with concentrated HCl and the solution was made up to 50mL with distilled water.

Electrophoresis buffer

6g of Tris, 28.8g of glycine and 1.0g of SDS were dissolved in distilled water and made up to 1L of solution.

Resolving gel overlay (0.375M Tris-Cl, 0.1% SDS, pH 8.8)

25mL of resolving gel is added to 1.0mL of 10% SDS and made u to 100ml with distilled water.

Treatment buffer (0.125M Tris-Cl, 4% SDS, 20%(v/v) glycerol, 0.2mL of DTT, 0.02% bromophenol blue, pH 6.8)

2.5mL of stacking gel buffer, 4mL of 10% SDS, 2.0mL of glycerol, 2.0mg of bromophenol blue and 0.31g of DTT were mixed with distilled water and made up to 10mL solution.

Tank buffer (0.025M of Tris, 0.192M glycine, 0.1% SDS, pH 8.3)

15.14g of Tris and 72.065g of glycine and 5g of SDS were all dissolved in distilled water and made up to 5L of solution.

Water-Saturted n-butanol

50mL of n-butanol was combined with 5mL of distilled water in a bottle and shaken. This mixture is allowed to settle (separate), while the upper phase is used to overlay gels.

Preparation of silver staining stock solutions

Silver stain fixing solution (40%(v/v) ethanol, 10% (v/v) acetic acid)

200mL of absolute ethanol, and 50mL of glacial acetic acid where mixed and diluted to 500mL by distilled water.

Sensitizing solution (30% (v/v) ethanol, 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulphate, 0.125% (v/v) glutaraldehyde)

150mL of absolute ethanol, 34g of sodium acetate and 1g of sodium petahydrate were dissolved in distilled water and made up to 500mL. At the point of use 0.5mL of 25% glutaraldehyde was added to 100mL of the above solution.

Silver solution (0.25% (w/v) silver nitrate, 0.015% (v/v) formaldehyde)

1g of silver nitrate was dissolved in distilled water and made up to 400mL. this was stored in a dark bottle at room temperature. 40µL of 37% formaldehyde was added to every 100mL of the silver nitrate solution at the point of use.

Developing solution (2.5% (w/v) sodium carbonate, 0.0074% (v/v) formaldehyde)

10g of sodium carbonate was dissolved distilled water, and the solution was made up to 400mL. 20µL of 37% formaldehyde was added to every 100mL of the silver nitrate solution at the point of use.

Stop solution (1.5% (w/v) Na_2EDTA)

7.5g of Na_2EDTA is dissolved in distilled water and made up to 500mL.

Preserving solution (30% (v/v) ethanol, 4% (v/v) glycerol)

150mL of absolute ethanol is added to distilled water, 20mL of glycerol was also added and the solution was made up to 500mL.

Silver staining protocol

The gel was stained by immersing the gel in ***silver staining fixing solution*** for one hour, then transferred to the ***sensitizing solution*** for about 30mins, then washed in distilled water for 5mins, after that, it was immersed in ***silver solution*** for about 20mins, then distilled water again for 2mins, followed by ***developing solution*** for 5mins afterward, into the ***stopping solution*** for 10mins, then to distilled water for 3mins and subsequently into the ***preserving solution*** where it is preserved.

ELUTION PROFILE OF DE 52 CHROMATOGRAPHY						
Fraction No.	**NaCl conc. mols/dm^3)**	**Absorbance at 366nm**	**Protein conc. Abs. at 545nm**	**Protein conc.**	**Activity (mU/ml) from std curve**	**Activity (U/fraction)**
1	0	0.071	0.612	11.33	0.0367	0.0183
2	0	0.094	0.628	11.63	0.0477	0.0238
3	0	0.1	0.762	14.11	0.0506	0.0253
4	0	0.089	0.658	12.19	0.0453	0.0227
5	0	0.097	0.754	13.96	0.0491	0.0246
6	0	0.068	0.544	10.07	0.0353	0.0176
7	0	0.158	0.973	18.02	0.0783	0.0392
8	0	0.209	1.375	25.46	0.1027	0.0514
9	0.1	0.241	1.77	32.78	0.1180	0.0590
10	0.1	0.164	0.638	11.81	0.0812	0.0406
11	0.1	0.144	1.37	25.37	0.0716	0.0358
12	0.1	0.023	1.83	33.89	0.0137	0.0069
13	0.1	0.018	0.79	14.63	0.0113	0.0057
14	0.1	0.163	3.69	68.33	0.0807	0.0404
15	0.1	0.287	0.579	10.72	0.1400	0.0700
16	0.1	0.295	0.6	11.11	0.1439	0.0719
17	0.2	0.377	0.838	15.52	0.1831	0.0915
18	0.2	0.288	0.54	10.00	0.1405	0.0703
19	0.2	0.072	1.04	19.26	0.0372	0.0186
20	0.2	0.003	1.15	21.30	0.0042	0.0021
21	0.2	0.032	1.33	24.63	0.0180	0.0090
22	0.2	0.036	1.11	20.56	0.0200	0.0100
23	0.2	0.026	1.86	34.44	0.0152	0.0076
24	0.2	0.036	0.86	15.93	0.0200	0.0100
25	0.3	0.047	0.82	15.19	0.0252	0.0126
26	0.3	0.082	2.5	46.30	0.0420	0.0210
27	0.3	0.096	0.93	17.22	0.0487	0.0243
28	0.3	0.086	1.71	31.67	0.0439	0.0219
29	0.3	0.098	0.82	15.19	0.0496	0.0248
30	0.3	0.101	0.61	11.30	0.0510	0.0255
31	0.3	0.107	0.79	14.63	0.0539	0.0270
32	0.3	0.11	0.79	14.63	0.0554	0.0277
33	0.4	0.098	1.11	20.56	0.0496	0.0248
34	0.4	0.102	2.47	45.74	0.0515	0.0258
35	0.4	0.101	0.39	7.22	0.0510	0.0255
36	0.4	0.092	1.11	20.56	0.0467	0.0234
37	0.4	0.119	1.22	22.59	0.0597	0.0298
38	0.4	0.117	2.08	38.52	0.0587	0.0294
39	0.4	0.126	2.43	45.00	0.0630	0.0315
40	0.4	0.127	1.33	24.63	0.0635	0.0317
41	0.5	0.129	0.54	10.00	0.0644	0.0322
42	0.5	0.156	0.47	8.70	0.0774	0.0387
43	0.5	0.131	0.61	11.30	0.0654	0.0327
44	0.5	0.12	0.58	10.74	0.0601	0.0301

45	0.5	0.113	0.75	13.89	0.0568	0.0284
46	0.5	0.067	0.48	8.89	0.0348	0.0174
47	0.5	0.122	0.77	14.26	0.0611	0.0305
48	0.5	0.114	0.85	15.74	0.0573	0.0286
49	0.5	0.114	0.36	6.67	0.0573	0.0286
50	0.5	0.117	0.52	9.63	0.0587	0.0294

Note: Activity = (Absorbance x 478.38 + 2.73)/100

GEL FILTRATION (G 100)					
Fraction No.	**Absorbance at 366nm**	**Protein conc. Abs. at 545nm**	**Protein conc.**	**Activity (U/ml) from std curve**	**Activity (U/fraction)**
1	0.039	0.025	0.46	0.0214	0.0107
2	0.029	0.001	0.02	0.0166	0.0083
3	0.084	0.01	0.19	0.0429	0.0215
4	0.019	0.009	0.17	0.0118	0.0059
5	0.348	0.013	0.24	0.1692	0.0846
6	0.964	0.065	1.20	0.4639	0.2319
7	0.035	0.008	0.15	0.0195	0.0097
8	0.133	0.016	0.30	0.0664	0.0332
9	0.029	0.015	0.28	0.0166	0.0083
10	0.046	0.023	0.43	0.0247	0.0124
11	0.268	0.031	0.57	0.1309	0.0655
12	0.293	0.031	0.57	0.1429	0.0714
13	0.046	0.001	0.02	0.0247	0.0124
14	0.008	0.073	1.35	0.0066	0.0033
15	0.044	0.023	0.43	0.0238	0.0119
16	0.037	0.001	0.02	0.0204	0.0102
17	0.02	0.006	0.11	0.0123	0.0061
18	0.026	0.094	1.74	0.0152	0.0076
19	0.026	0.011	0.20	0.0152	0.0076
20	0.018	0.015	0.28	0.0113	0.0057
21	0.012	0.003	0.06	0.0085	0.0042
22	0.014	0.044	0.81	0.0094	0.0047
23	0.045	0.017	0.31	0.0243	0.0121
24	0.026	0.011	0.20	0.0152	0.0076
25	0.053	0.001	0.02	0.0281	0.0140
26	0.021	0.001	0.02	0.0128	0.0064
27	0.069	0.045	0.83	0.0357	0.0179
28	0.054	0.034	0.63	0.0286	0.0143
29	0.059	0.045	0.83	0.0310	0.0155
30	0.106	0.012	0.22	0.0534	0.0267
31	0.055	0.02	0.37	0.0290	0.0145
32	0.089	0.001	0.02	0.0453	0.0227
33	0.081	0.021	0.39	0.0415	0.0207
34	0.026	0.008	0.15	0.0152	0.0076
35	0.035	0.009	0.17	0.0195	0.0097
36	0.034	0.012	0.22	0.0190	0.0095
37	0.012	0.009	0.17	0.0085	0.0042
38	0.033	0.018	0.33	0.0185	0.0093
39	0.049	0.017	0.31	0.0262	0.0131
40	0.047	0.015	0.28	0.0252	0.0126
41	0.035	0.001	0.02	0.0195	0.0097
42	0.018	0.003	0.06	0.0113	0.0057

Note: Activity = (Absorbance x 478.38 + 2.73)/100

Effect of metal cations

Cations	Absorbance			Activity (U/ml) from std curve			Activity Average	% Relative Activity	Std. Dev.	Std. Error
	Abs. 1	Abs. 2	Abs. 3	1	2	3				
Control	0.337	0.261	0.283	0.1639	0.1276	0.1381	0.1432	100.00	0.02	1.00
Mg^{+2}	0.093	0.086	0.081	0.0472	0.0439	0.0415	0.0442	30.86	0.00	0.01
Li^{+2}	0.382	0.309	0.297	0.1855	0.1505	0.1448	0.1603	111.91	0.02	0.37
Hg^{+2}	0.124	0.195	0.113	0.0620	0.0960	0.0568	0.0716	50.01	0.02	0.01
Zn^{+2}	0.274	0.122	0.166	0.1338	0.0611	0.0821	0.0923	64.48	0.04	0.13
Ca^{+2}	0.039	0.026	0.01	0.0214	0.0152	0.0075	0.0147	10.26	0.01	0.00
Co^{+2}	0.095	0.097	0.098	0.0482	0.0491	0.0496	0.0490	34.20	0.00	0.01
Mn^{+2}	0.324	0.273	0.297	0.1577	0.1333	0.1448	0.1453	101.45	0.01	0.88
Fe^{+2}	0.233	0.199	0.247	0.1142	0.0979	0.1209	0.1110	77.51	0.01	0.08

Effect of different compounds												
Inhibitor		**Absorbance**			**Activity (U/ml) from std curve**			**Activity Average**	**% Relative Activity**	**Std. Dev.**	**Std. Error**
		Abs. 1	Abs. 2	Abs. 3	1	2	3				
Control		1.054	1.029	1.049	0.5069	0.4950	0.5046	0.5022	100.00	0.01	1.00
Iodoacetate	(AI)	0.883	0.891	0.902	0.4251	0.4290	0.4342	0.4294	85.52	0.00	0.00
Phenylmethylsulphonyl fluoride	(PMSF)	0.912	0.956	0.933	0.4390	0.4601	0.4491	0.4494	89.49	0.01	0.00
Dithiothreitol	(DTT)	1.035	1.032	1.07	0.4979	0.4964	0.5146	0.5030	100.16	0.01	0.91
β-mercaptoethanol	(β-ME)	0.963	0.974	0.994	0.4634	0.4687	0.4782	0.4701	93.62	0.01	0.01
Ethylenediaminetetraacetic acid	(EDTA)	1.167	1.138	1.087	0.5610	0.5471	0.5227	0.5436	108.26	0.02	0.05
Soybean trypsin inhibitor	(STI)	0.919	0.946	0.956	0.4424	0.4553	0.4601	0.4526	90.12	0.01	0.00

Effect of Temperature								
Temp.	**Absorbance**			**Activity (U/ml) from std curve**			**Activity Average**	**Std. Dev.**
	Abs. 1	Abs. 2	Abs. 3	1	2	3		
Control	1.327	1.236	1.318	0.6375	0.5940	0.6332	0.6216	0.02
20°C	1.124	1.205	1.173	0.5404	0.5792	0.5639	0.5612	0.02
37°C	1.3	1.251	1.318	0.6246	0.6012	0.6332	0.6197	0.02
40°C	1.167	1.238	1.187	0.5610	0.5950	0.5706	0.5755	0.02
50°C	0.986	1.032	0.949	0.4744	0.4964	0.4567	0.4758	0.02
60°C	0.924	0.943	1.007	0.4448	0.4538	0.4845	0.4610	0.02
65°C	0.913	0.894	0.936	0.4395	0.4304	0.4505	0.4401	0.01

Temperature stability								
Tempt. (°C)	**Absorbance**			**Activity (U/ml) from std curve**			**Activity Average**	**Std. Dev.**
	Abs. 1	Abs. 2	Abs. 3	1	2	3		
Control	1.202	1.206	1.215	0.5777	0.5797	0.5840	0.5805	0.00
20	1.211	1.205	1.173	0.5820	0.5792	0.5639	0.5750	0.01
37	1.23	1.196	1.189	0.5911	0.5749	0.5715	0.5792	0.01
40	1.25	1.138	1.193	0.6007	0.5471	0.5734	0.5738	0.03
50	0.986	1.032	0.949	0.4744	0.4964	0.4567	0.4758	0.02
60	0.924	0.943	1.007	0.4448	0.4538	0.4845	0.4610	0.02
65	0.913	0.894	0.936	0.4395	0.4304	0.4505	0.4401	0.01
70	0.725	0.873	0.802	0.3496	0.4204	0.3864	0.3854	0.04

Effect of pH								
pH	**Absorbance**			**Activity (U/ml) from std curve**			**Activity Average**	**Std. Dev.**
	Abs. 1	Abs. 2	Abs. 3	1	2	3		
4.5	0.467	0.615	0.491	0.2261	0.2969	0.2376	0.2536	0.04
5.0	0.523	0.556	0.695	0.2529	0.2687	0.3352	0.2856	0.04
5.5	0.986	1.032	0.949	0.4744	0.4964	0.4567	0.4758	0.02
6.0	1.401	1.235	1.139	0.6729	0.5935	0.5476	0.6047	0.06
6.5	1.235	1.238	1.438	0.5935	0.5950	0.6906	0.6264	0.06
7.0	1.143	1.281	1.432	0.5495	0.6155	0.6878	0.6176	0.07
7.5	0.914	0.893	0.836	0.4400	0.4299	0.4027	0.4242	0.02
8.0	0.901	0.894	0.916	0.4338	0.4304	0.4409	0.4350	0.01
8.5	0.837	0.814	0.767	0.4031	0.3921	0.3696	0.3883	0.02