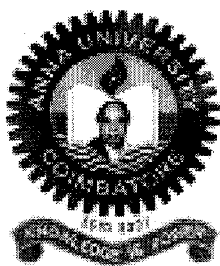

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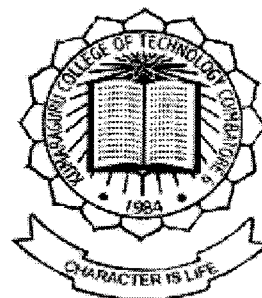

SCREENING AND CHARACTERIZATION OF PROTEOLYTIC ENZYME IN BANANA PEELS

A PROJECT REPORT

Submitted by

PAVITHRA.D (0710204030)

in partial fulfillment for the award of the degree

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BONAFIDE CERTIFICATE

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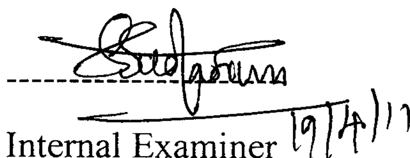

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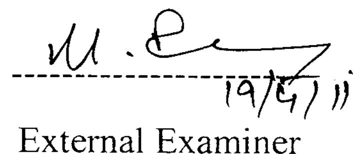

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Abstract

ABSTRACT

The present study was carried out with the objective to screen different varieties of the banana peel for the protease activity, characterize the enzyme and partially purify the protease enzyme. Protease activity was assayed by spectrophotometric method using tyrosine as the standard. High protease activity was found in the variety Sevvazhi (*Musa spp* – Red Banana-AAA) with specific activity ranging from 14-16 U/mg. The protease enzyme was found to be most active at pH 7.0 and the optimum temperature for its activity was observed at 30°C. The K_m and V_{max} value were found to be 43 μM and 10.59 U/mg. The protease activity was found to increase with the increase in the enzyme concentration. The enzyme activity gradually increased and attained saturation level with the increase in substrate concentration. Protease was inhibited by the by 1,10-Phenanthroline and EDTA. Metal ions also affected the protease activity to a greater extent. Out of the metal ions used Fe^{2+} and Hg^{2+} lowered the enzyme activity with increase in metal ion concentration. Cu^{2+}, Ca^{2+} and Na^{+} ions were found to enhance the protease activity with increase in the concentration of the ion. The enzyme was partially purified by ammonium sulphate fractionation and dialysis and the purification fold was found to be 1.5 and 1.606 respectively. The native PAGE zymogram was performed for the crude extracts and dialysed sample by using gelatin as a substrate in the resoliving gel. Clear bands were observed in the gel which proves that the extract contains proteolytic enzyme. SDS PAGE was performed for the peel extract and the molecular weight was found to be 32 kDa. Collagenase assay was performed for the enzyme extract obtained from the banana peel and the specific activity was found to be 17.17±0.26 U/mg.

Contents

Table of contents

CHAPTER	TITLE	PAGE NO
1	**Introduction**	1
2	**Objective**	5
3	**Literature review**	6
	3.1Human papilloma virus	6
	3.2 Genital warts	7
	3.3 Signs and Symptoms	7
	3.4 Treatment of warts	7
	3.4.1 Anti-proliferative agents	7
	3.4.1.1 Podophyllin or podophyllotoxin	7
	3.4.1.2 5-Flurouracil	8
	3.4.1.3 Cidofovir	8
	3.4.2 Cryotherapy	8
	3.4.3 Destructive or excision therapy	9
	3.4.3.1 Tricholoro acetic acid	9
	3.4.3.2 Scalpel, curette or scissor excision	9
	3.4.3.3 Laser therapy	9
	3.4.3.4 X-ray therapy	10

	3.4.3.5 Photodynamic therapy	10
	3.4.4 Immunomodulators or vaccines	10
	3.4.4.1 Interferons	10
	3.4.4.2 Imiquimod	10
	3.4.4.3 Dinitrochlorobenzene	11
	3.4.4.4 Therapeutic vaccines	11
	3.5 Disadvantages of physical and chemical treatment of genital warts	11
	3.6 Proteases	12
	3.7 Mechanism of action of protease	14
	3.8 Characterization of protease	17
	3.9 Purification of protease	17
	3.10 Applications of protease	18
	3.10.1 Food industry	18
	3.10.2 Detergent industry	19
	3.10.3 Leather industry	19
	3.10.4 Cosmetics	19
	3.11 Use of enzymes in treating skin diseases	20
	3.12 Advantages of enzyme treatment	20
	3.13 Banana	21
	3.13.1 Banana peel	22
	3.13.2 Uses of banana peels	25

4	**Materials and Methods**	26
	4.1 Banana varieties	26
	4.2 Enzyme extraction	26
	4.3 Enzyme assay	26
	4.4 Protein estimation	29
	4.5 Characterizaion of protease	30
	4.5.1 Effect of temperature on protease activity	30
	4.5.2 Effect of pH on protease activity	30
	4.5.3 Effect of substrate concentration on protease activity	30
	4.5.4 Effect of enzyme concentration on protease activity	31
	4.5.5 Effect of inhibitors on protease activity	31
	4.5.6 Effect of metal ions on protease activity	31
	4.6 Partial purification of protease	31
	4.6.1 Ammonium sulphate fractionation	31
	4.6.2 Dialysis	32
	4.7 Native PAGE zymography	32
	4.8 SDS PAGE	32
	4.9 Assay for collagenase	33
5	**Results and discussion**	34
	5.1 Enzyme source	34
	5.2 Enzyme assay	34
	5.3 Screening for high protease activity in different	34

	varieties of banana peel	
	5.4 Characterization of protease	35
	5.4.1 Effect of temperature on protease activity	35
	5.4.2 Effect of pH on protease activity	36
	5.4.3 Effect of substrate concentration on protease activity	36
	5.4.4 Effect of enzyme concentration on protease activity	37
	5.4.5 Effect of inhibitors on protease activity	38
	5.4.6 Effect of metal ions on protease activity	39
	5.4.7 Effect of SDS on protease activity	43
	5.5 Partial purification of protease	43
	5.5.1 Crude extract	44
	5.5.2 Ammonium sulphate fractionation	44
	5.5.3 Dialysis	44
	5.6 Native PAGE zymography	45
	5.7 SDS PAGE	46
	5.8 Collagenase assay	47
6	**Summary and Conclusion**	49
	Appendices	50
	References	53

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
3.1	Nutrient composition of banana peels	23
3.2	Amino acid composition of banana peels	23
3.3	Minerals composition of *Musa sapientum* peel	24
3.4	Proximate composition and anti-nutritional content of *Musa sapientum* peel	25
4.1	Assay for protease	27
4.2	Color development for protease assay	28
4.3	Assay for L-tyrosine standard	29
5.1	Protease activity and recoveries in the stages of purification	45

LIST OF FIGURES

FIG. NO	TITLE	PAGE NO.
3.1	Crystal structure of the cysteine peptidase papain in complex with its covalent inhibtor E-64	13
3.2	Crystal structure of bovine chymotrypsin.	13
3.3	Crystal structure of Trypsin, a typical serine protease.	14
3.4	Mechanism of action of serine protease	15
3.5	Mechanism of action of cysteine proteases	16
5.1	Comparison of Protease activity in different varieties of banana peel	34
5.2	Effect of temperature on protease activity	35
5.3	Effect of pH on protease activity	36
5.4	Effect of Substrate Concentration on protease activity	37
5.5	Effect of Enzyme concentration on protease activity	38
5.6	Effect of EDTA on protease activity	39
5.7	Effect of 1,10-Phenanthroline on protease activity	39
5.8	Effect of Fe^{3+} on protease activity	40
5.9	Effect of Hg^{2+} ions on protease activity	41
5.10	Effect of Cu^{2+} ions on protease activity	41
5.11	Effect of Ca^{2+} ions on protease activity	42
5.12	Effect of Na^{+} ions on protease activity	42
5.13	Effect of SDS on protease activity	43
5.14	Native PAGE zymogram	46
5.15	SDS PAGE	47

Chapter 2
Objectives

2. OBJECTIVES

- To screen the different varieties of banana peel for protease activity.
- To characterize the protease enzyme obtained from a variety of banana peel having high protease activity.
- To partially purify the protease enzyme and study its applications

Chapter 3
Literature Review

3. LITERATURE REVIEW

3.1 Human Papilloma Virus (HPV)

Human Papillomavirus (HPV) is an epitheliotropic, double stranded, circular DNA virus from Papovavirus family, which is found to infect cells in the basal layer of squamous epithelium. Thus, infection caused by HPV is found in various body sites, such as anogenital tract, skin, conjunctiva, larynx, tracheobronchial mucosa, esophagus and oral cavity (Saini *et al.*, 2010). On the basis of their association with disease types, papillomaviruses are classified into high-risk (HR) and low-risk (LR) types. HR-HPV types (HPV 16, 18, 31, 35, 39, 45, 51, 52, 56, 59, 66, 68, 69 and 73) are often associated with high grade lesions and invasive cancer, whereas the LR-HPV types (HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108) are mainly found in low grade lesions, genital or skin warts and condyloma accuminata. HPV 16 and HPV 18 are the most prevalent oncogenic genotypes as two together are responsible for more than 80 per cent of total HPVassociated cancerous lesions (Bharti *et al.,* 2009). It is generally accepted that HPV E6 and E7 function as the dominant oncoproteins of 'high-risk' HPVs by altering the function of critical cellular proteins (Fehrmann and Laimins, 2003). Expression of the E6 and E7 proteins, as a consequence of viral integration is paramount to the establishment and maintenance of the tumorigenic state. HPV E6 has been shown to interact with and enhance the degradation of p53 by the ubiquitin pathway, which plays an important role in cell cycle control and apoptosis in response to DNA damage, while HPV E7 disables the function of the retinoblastoma tumour suppressor protein Rb. It has been shown that both HPV E6 and E7 interact with the host cell targeting a plethora of key host cellular proteins that are involved in apoptosis and malignant cellular transformation (Mammas *et al.,* 2008). Infection with Human Papilloma Virus may be latent or sub-clinical and may also be the contributory factor for cervical and genital cancers (Steinberg *et al.,* 1983).

3.2 Genital warts

Warts are benign skin papillomas caused by an epidermotropic DNA virus (human papilloma, HPV). They are very common and can affect many different sites including the face, hands, feet and genitalia (Fabbrocini *et al.,* 2001). Genital warts also known as Condylomata Acuminata or Venereal warts, is one of the most common types of sexually transmitted diseases. Genital warts are soft, wart-like growths on the skin and mucus membranes of the genitals in men and women (Storck, 2010). They look like small, flesh colored bumps or have a cauliflower-like appearance. Genital warts may be as small as 1 to 2 mm in diameter, smaller than the width of a ballpoint pen refill or may multiply into large clusters (Sabni, 2005). Genital warts are generally described by 1 of the following 4 phenotypic categories: classic condyloma acuminata, which are pointed or cauliform; keratotic warts with a thick, horny surface resembling common skin warts; papular smooth warts; and flat warts, frequently observed on the cervix (Gunter, 2003).

3.3 Signs and symptoms

The signs and symptoms of genital warts include:

- Tiny, gray, pink or red swellings in the genital area that grow quickly.
- Several warts cluster together that take a cauliflower like appearance.
- Itching or burning in the genital area.

34 Treatment of warts

3.4.1 Anti-proliferative agents

3.4.1.1 Podophyllin or Podophyllotoxin

Podophyllum is a resin obtained from *Podophyllum emodi* or *Podophyllum peltatum,* containes many biologically active lignin compounds including Podophyllotoxin (podofilox), α-peltatin, and 4-demethyl-podophyllotoxin (Beutner *et al.,* 1998). Moist areas such as on the

glans of the uncircumcised penis, the vagina and vulva, the perineal area, and groin, are best treated with 25% resin of podophyllum (Rees, 1985). Podofilox is thought to be most active against genital warts (Walter, 1974). Severe systemic toxicity may arise after topical use of podophyllin, most commonly when it is applied in large volumes, and death, intrauterine death, teratogenicity and a variety of neurological and other complications have all been described (Fraser *et al.*, 1993).

3.4.1.2 5-Fluorouracil

5-Fluorouracil (5-FU) is a cytotoxic agent. It is a pyrimidine antagonist that acts via inhibition of nucleic acid synthesis. 5-FU in the form of a 5%cream has been available for a number of years (Mansell *et al.*, 1975). Topical 5-fluorouracil may be successful at times for urethral meatal warts, but if used on the face or somewhere else, it may cause post inflammatory hyperpigmentation and the cure for planar or flat warts is not particularly high (Rees, 1985).

3.4.1.3 Cidofovir

Cidofovir is an acyclic nucleoside phosphonate with a broad spectrum of activity against DNA viruses. Cidofovir is only licensed as an intravenous agent for the treatment of cytomegalovirus retinitis. Cidofovir's anti-HPV activity was first described in a report of three AIDS cases with severe, relapsing anogenital lesions where complete clearance with topical 1% Cidofovir was reported. Two problems substantially limit any attempts to use Cidofovir for use in individual patients: (1) the need for expert pharmacy input and dedicated facilities in making up topical preparations as the agent is toxic, and (2) the cost of a single intravenous unit dose (in the UK, 375 mg in 5 ml, £655) (Snoeck *et al.*, 2001).

3.4.2 Cryotherapy

Cryotherapy involves light freezing the warts at intervals of two weeks for one to six treatments. This therapy appears to be more effective for multiple warts (Rees, 1985). Various methods of freezing can be employed. Most commonly in the anogenital region, spray guns are used to apply liquid nitrogen. Another system used is the cryoprobe which is a closed system using either gaseous nitrous oxide or liquid nitrogen. Freezing is applied to each wart usually for

10–20 seconds so that the wart and a small margin of surrounding normal skin are frozen. Discomfort is mild to moderate so that anaesthesia is rarely required. Freezing should lead to erythema, some lesional micro-oedema and necrosis. Typically, the freeze time required to obtain a good cure rate does not lead to scarring (Godley *et al.*, 1987; Stone *et al.*, 1990; Abdullah *et al.*, 1993).

3.4.3 Destructive or excision therapy

3.4.3.1 Trichloroacetic aicd (TCA)

Various acids may be used for warts, but there is a great danger of scarring. sulphuric, nitric, diacetic, and trichloroacetic acids have all been used, but the safest is trichloroacetic acid. Even this may leave post inflammatory hyperpigmentation on the extremities. TCA has no systemic effects and is safe in pregnancy. Studies using TCA have reported clearance rates of 70–81% after six applications and recurrence rates of 36% (Godley et al., 1987; Abdullah et al., 1993).

3.4.3.2 Scalpel, curette or scissor excision

Electrosurgery is a very popular method of wart removal. Scarring is likely possible unless the operator is highly skilled. Dermal curette is mostly used to remove exophytic warts (Rees, 1985). Surgical excisions, especially wedge excisions are not appropriate for warts on pressure-point or weight-bearing surfaces. The patient has a double problem if warts recur in the scar (Jablonska, 1984).

3.4.3.3 Laser therapy

Vulvar and penile condylomata and intraepidermal neoplasia (bowenoid lesions) are best treated, when the laser is used, with the carbon dioxide laser in the vaporizing mode (Ferenesey, 1984). However, recurrence rates of genital warts after laser or other surgical therapy are similar to those with other treatment modalities (Beutner and Ferenczy, 1997).

3.4.3.4 X-ray therapy

X-rays are also most effectively used in treating planar warts by single exposure at 60 KV. For warts 12 mm in diameter 1800 rads are given, 2000 rads for 8-10 mm, 2200 rads for 6-8 mm. It is important to pare out the dead wart 3 weeks later (Rees, 1985).

3.4.3.5 Photodynamic therapy

Photodynamic therapy (PDT) combines the administration of a photosensitiser and the subsequent irradiation of wavelengths that, after being absorbed by the chromophore, lead to the destruction of the target tissue (Fabbrocini, 2001). Topical d-aminolaevulinic acid (ALA) is applied to the wart, since it is capable of penetrating the disrupted epidermis (Svanberg, 1994). PDT is found to be more effective in removing the warts without causing any pain to the patient.

3.4.4 Immunomodulators or Vaccines

3.4.4.1 Interferons

Interferons (IFN) are cytokines with anti-viral, immunoregulatory and anti-proliferative properties. They have been used successfully in other viral infections and have been evaluated in the context of HPV-related disease. Interferons have been administered topically, intralesionally, subcutaneously and intramuscularly (Beutner and Ferenczy, 1997). Interferon therapy has never really gained a therapeutic niche. It is expensive with a potential for adverse effects and no real advantage in terms of efficacy. It is, therefore, not recommended for routine clinical practice (Bonnez *et al.*, 1995).

3.4.4.2 Imiquimod

Imiquimod is a member of a new class of imidazoquinolines and has shown potent immunomodulating, anti-viral and anti-tumour activities in animal models. Its mechanism of action in clearance of genital warts involves tissue production of IFN-α, -β, -γ and tumour necrosis factor-α (Tyring *et al.*, 1998). Imiquimod is a more expensive treatment option costing £110 for 8 weeks treatment.

3.4.4.3 Dinitrochlorobenzene (DNCB)

DNCB is described to be most effective in curing genital warts (Georgala *et al.,* 1989). However, the recognition of mutagenicity induced by DNCB precludes its further use (Hengge and Ruzicka, 2004).

3.4.4.4 Therapeutic vaccines

Autogenous vaccines have been used for decades, both by dermatologists and gynecologists. Warts from the patient are ground up, suspended in saline, passed through a porcelain filter, and inactivated with 0.4% formalin. On theoretical grounds, this is objectionable because such vaccines contain DNA, and might alter host DNA to render it oncogenic (Lacey *et al.*, 1999).

3.5 Disadvantages of physical and chemical treatment of Genital warts

Though many treatment methods are available for treating genital warts, these methods are found to be disadvantageous in many aspects.

- Electrosurgery and other excision methods are more painful treatment techniques and it has to be performed after administering local anesthesia to the patients.
- Laser therapy and X-ray therapy is also not performed since radiations can cause oncogenic effects.
- Immunomodulators treatment is expensive and efficacy is also not satisfactory when compared to chemical methods.
- Treatment with anti-proliferative agents is found to be less effective since those agents can cause inflammations in the skin surrounding the warts, hyper-pigmentation of the skin and can cause some neurological disorders.

P 3400

3.6 Proteases

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation (Gupta *et al.*, 2002). Proteases are the single class of enzymes, which occupy a pivotal position with respect to their application in both physiological and commercial fields. They catalyze the cleavage of peptide bonds in other proteins (Mala *et al.,* 1998). Protease is an enzyme that conducts proteolysis, that begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein.

Classification of proteases

Proteases are currently[update] classified into six broad groups:

- Serine proteases (EC 3.4.21)
- Threonine proteases (EC 3.4.25)
- Cysteine proteases (EC 3.4.22)
- Aspartate proteases (EC 3.4.23)
- Metalloproteases (EC 3.4.24)
- Glutamic acid proteases

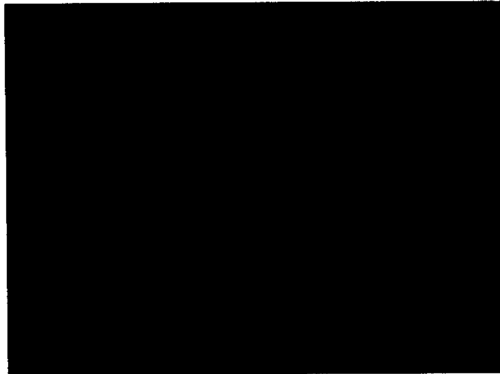
Five catalytic types of proteases can now be recognized, in which serine, threonine, cysteine, aspartic, or metallo groups play primary roles in enzyme catalysis. The serine, threonine, and cysteine proteases are catalytically very different from the aspartic and metalloproteases in that the nucleophile of the catalytic site is part of an amino acid, whereas it is an activated water molecule in the other two groups. In cysteine proteases the nucleophile is the sulfhydril group of a Cys residue and the catalytic mechanism is similar to that of serine type proteases in that the proton donor is a His residue (Priolo *et al.,* 2000).

ucture of protease

Fig 3.1: Crystal structure of the cysteine peptidase papain in complex with its covalent inhibtor E-64

Fig 3.2: Crystal structure of bovine chymotrypsin. The catalytic residues are shown as yellow sticks

Fig 3.3: Crystal structure of Trypsin, a typical serine protease.



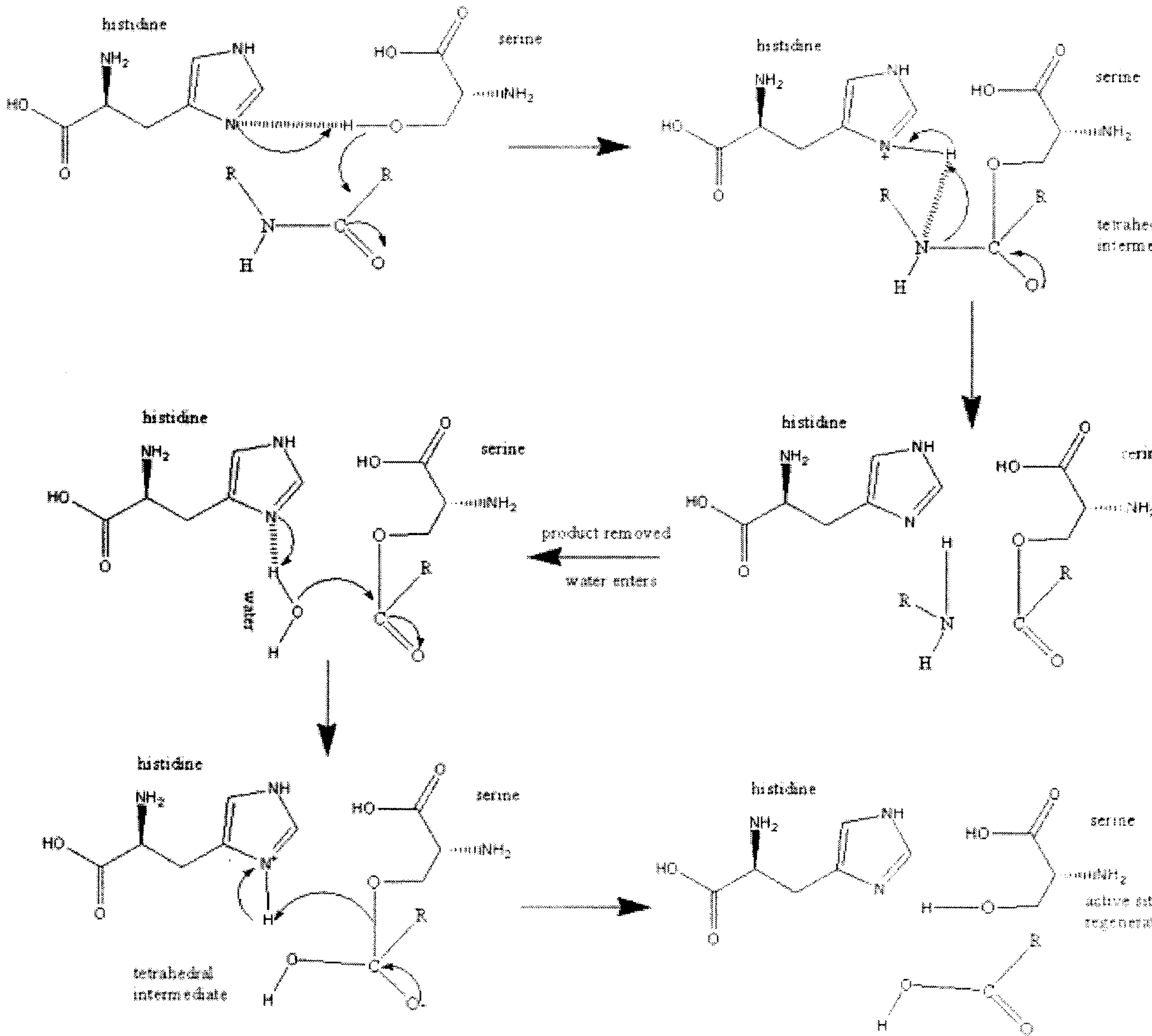
7 Mechanism of action of protease

Mechanism of action of serine proteases

Basically, the carbon of the scissile bond is first bonded to the O of the -OH group of rine 195 and the other end gets the H from that group and is released. A water molecule then mes along and gives its H to the remaining O of the serine 195's OH group, taking the carbon hich was attached there in return.

The purpose of the Aspartate 102 and Histidine 57 groups (the so-called oxyanion hole) to stabilise these transfers, creating a charge-relay system. In the middle of each of these two eps a tetrahedral intermediate is formed, which is just a half-way house between the start and d points of the steps. The tetrahedral intermediate is the target for inhibitors, as it is the ansition state always more tightly bound than the substrates or products.

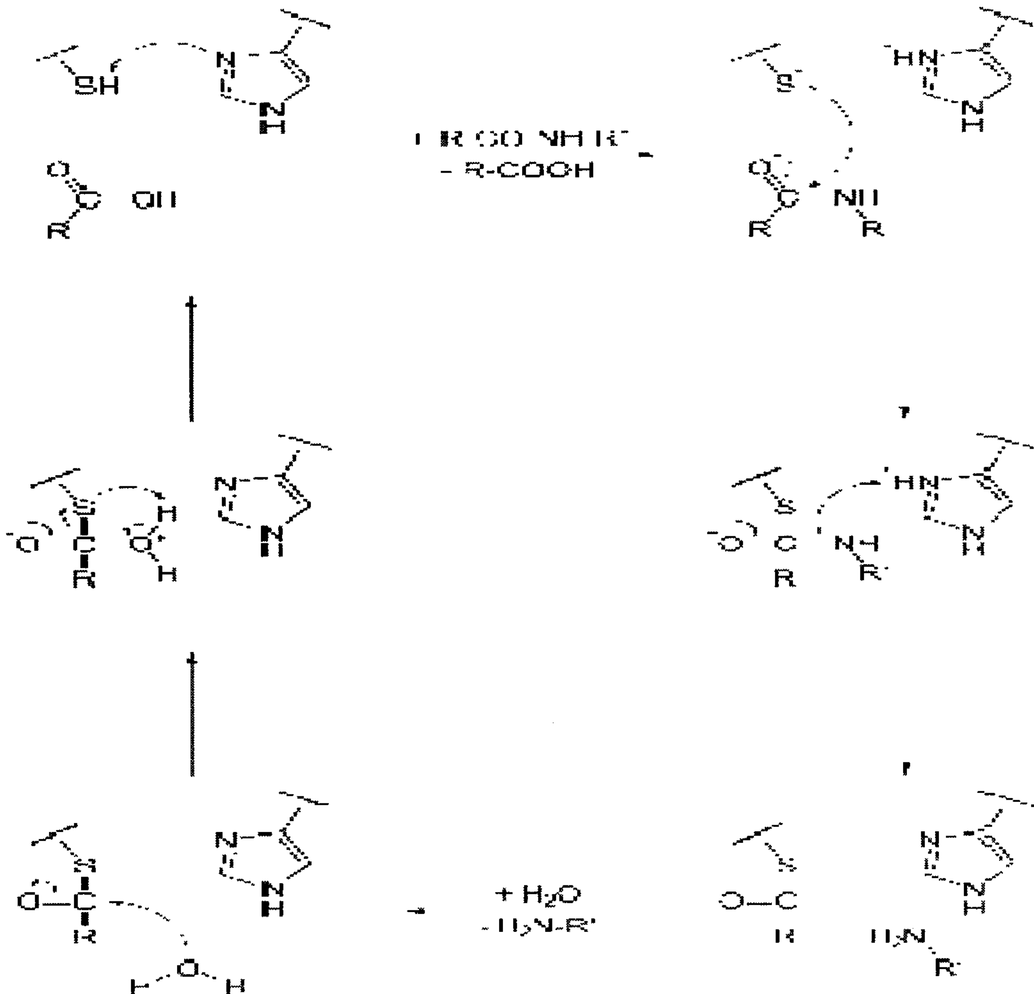
Fig 3.4: Mechanism of action of serine protease



Mechanism of action of cysteine proteases

Cysteine proteases act very much like serine proteases with the -SH group of the cysteine acting like the OH group of the serine.

Fig 3.5: Mechanism of action of cysteine protease



3.8 Characterization of protease

Proteases, which firmly maintain first place in the world market of enzymes, play an important role in biotechnology, given that proteolysis changes the chemical, physical, biological, and immunological properties of proteins. Proteolytic enzymes of plant origin have received special attention in the field of medicine and industry due to their property of being active over a very wide range of temperature and pH (Lahl and Brown, 1994). The optimum pH range for the cysteine protease isolated from the Araujia hortorum fruits was found to be 8.0-9.5 (Priolo *et al.*, 2000). Protease obtained from the fermented melon seeds had an optimum pH of 6.6-7.6 (Evans *et al.*, 2009). Protease obtained from the Lapsi fruits had a maximum activity at a temperature of 30°C (Prajapati *et al.*, 2009). Similarly, protease obtained from the Araujia horotorum fruits had an optimum temperature at 40°C (Evans *et al.*, 2009). Most of the proteases that are used in the field of medicine have an optimum pH ranging from 6.0-8.0 and optimum temperature at 30-40°C.

The effect of several potential inhibitors has been studied for many proteases. 1,10-Phenanthroline was found to be the potent inhibitor for protease isolated from dry seeds of grass pea (Ramakrishna *et al.*, 2010). Slight inhibition was observed with EDTA for the type of collagenase protease isolated from file fish (Kim *et al.*, 2002), whereas no inhibition with EDTA was observed for metalloprotease isolated from dry seeds of grass peas (Ramakrishna *et al.*, 2010). The effect of metal ions has been studied for many proteases, most effective metal ions that affect the enzyme activity are Mercuric chloride, Ferric chloride (Kim *et al*, 2002 and Ramakrishna *et al.*, 2010). Ca^{2+} ions are found to be potent enhancers of proteolytic activity (Kim *et al.*, 2002).

3.9 Purification of protease

Crude enzyme extract is usually purified by Ammonium sulphate or Acetone precipitation followed by dialysis to remove the metal ions from the enzymes, since metal ions are known to inhibit the enzyme activity. High yield of enzyme is obtained only if the enzyme is purified by following column chromatography technique. Protease obtained from germinating horse gram seeds were initially purified by ammonium sulphate precipitation and the purification fold was found to be 2.44. Followed by ammonium sulphate precipitation and dialysis the

samples were purified by running through DEAE-Cellulose column and CM-Sephacel and the purification fold was found to be 28.27, 118.36 respectively (Jinka *et al.,* 2009). Similarly, collagenase protease obtained from the Mackeral fish were purified by Acetone precipitation, DEAE Sephadex A-50, Sephadex G-100, DEAE Sephacel, Sephadex G-75 and the purification fold was found to be 2.6, 11.5, 23.3, 26.4 and 39.5 respectively (Park *et al.,* 2002).

3.10 Applications of protease

Proteases are physiologically necessary for living organisms as such they are ubiquitous being found in a wide diversity of sources such as plants, animals and micro-organisms (Barrett, 1994). Proteases play a prominent role in plant physiology, being the catalysts of important processes like hydrolysis of storage proteins during seed germination, activation of proenzymes, degradation of defective proteins, etc. (Rudenskaya *et al.,* 1998). Proteases are involved in a multitude of physiological reactions from simple digestion of food proteins to highly-regulated cascades (e.g., the blood-clotting cascade, the complement system, apoptosis pathways, and the invertebrate prophenoloxidase-activating cascade). Bacteria also secrete proteases to hydrolyse (digest) the peptide bonds in proteins and therefore break the proteins down into their constituent monomers. Bacterial and fungal proteases are particularly important to the global carbon and nitrogen cycles in the recycling of proteins, and such activity tends to be regulated in by nutritional signals in these organisms (Sims, 2006).

3.10.1 Food industry

Rennet (mainly chymosin), obtained from the fourth stomach (abomasum) of unweaned calves has been used traditionally in the production of cheese. papain from the leaves and unripe fruit of the pawpaw (*Carica papaya*) has been used to tenderise meats (Jaivoot *et al.*, 2002). Meat tenderisation by the endogenous proteases in the muscle after slaughter is a complex process which varies with the nutritional, physiological and even psychological (i.e. frightened or not) state of the animal at the time of slaughter. Meat of older animals remains tough but can be tenderised by injecting inactive papain into the jugular vein of the live animals shortly before slaughter. Proteases are also used in the baking industry. Where appropriate, dough may be prepared more quickly if its gluten is partially hydrolysed. A heat-labile fungal protease is used

so that it is inactivated early in the subsequent baking. Weak-gluten flour is required for biscuits in order that the dough can be spread thinly and retain decorative impressions (Rizzello *et al.*, 2007).

3.10.2 Detergent industry

Subtilisins are defined by their catalytic mechanism as serine proteases. Subtilisins are used in all types of laundry detergents and in automatic dishwashing detergents. Their function is to degrade proteinaceous stains typical stains include blood, milk, egg, grass and sauces (Maurer, 2004).

3.10.3 Leather industry

Leather processing is one of the important industries closely related to everyday life. Alkaline proteases are used to remove hair from hides. Alkaline proteases can be used which enables the swelling of hair roots, and the subsequent attack of protease on the hair follicle protein allowing easy removal of the hair (Arunachalam and Saritha, 2009). Proteases have been used, in the past, to 'shrinkproof' wool. Wool fibres are covered in overlapping scales pointing towards the fibre tip. A successful method involves the partial hydrolysis of the scale tips with the protease papain. This method also gave the wool a silky luster and added to its value.

3.10.4 Cosmetics

Nagase Biochemicals, Japan is the major supplier for producing many skin care products. Powder type skin care cosmetic is contains protease obtained from *Bacillus subtilitis*. Papain is very active in the hydrolysis of peptides as well as proteins. Papain is most commonly used in cosmetics (Nagase catalog, 2009).

3.11 Use of enzymes in treating skin diseases

In the past, a large number of proteolytic enzymes of plant and bacterial origin have been studied as a replacement for the mechanical debridement (removal of dead skin) of burns (Klasen, 2000). Debrase gel dressing, comprising a mixture of enzymes extracted from pineapple is used for the treatment of partial-thickness and full-thickness burns. Vibrilase (recombinant vibriolysin), a proteolytic enzyme from the marine microorganism Vibrio proteolyticus, has been shown to have efficacy against denatured proteins such as those found in burned skin. Some encouraging results have been obtained recently from a study with the collagenase clostridiopeptidase in children with partial-thickness burns (Ozcan *et al.,* 2002). Chondroitinases could be used for the treatment of spinal injuries where they have been demonstrated to promote regeneration of injured spinal cord. The enzyme acts by removing, in the glial scar, the accumulated chondroitin sulfate that inhibits axon growth (Bradbury *et al.*, 2002). Hyaluronidase has a similar hydrolytic activity on chondroitin sulfate and may also help in the regeneration of damaged nerve tissue (Moon *et al.,* 2003). The latex of Araujia hortorum fruit has been used in folk medicine as a local application to warts (Watt and Breyer-Brandwijk, 1962). Papaya enzymes can be used to treat ulcers, papain has also been used to treat cold sores. Papain also helps to clean away dead tissues from the body. Papain enzymes have been found to be extremely effective in dissolving fats to purge and soften the skin. Papain can also work wonders for sports injuries such as bruising and swelling because of its ability to reduce swelling and thin mucous. Modern medicine has found uses for bloodroot as an anti-cancer agent, particularly for the treatment of skin cancer, and as a dissolving agent for skin growths such as warts (Greenfield and Davis, 2004). Bromelain, protease obtained from the stem of the pineapple is used in debridement of burns (Tochi *et al.,* 2008).

3.12 Advantages of enzyme treatment

Enzymatic hydrolysis is strongly preferred over chemical methods because it yields hydrolyzates containing well-defined peptide mixtures and avoids the destruction of L-amino acids and the formation of toxic substances like lysino-alanine (Lahl and Brown, 1994; Mahmoud, 1994).

3.13 Banana

Musa sapientum which is commonly called banana is a herbaceous plant of the family *Musaceae*. It is known to have originated from the tropical region of Southern Asia. The *Musa sapientum* grows up to a height of about 2-8m with leaves of about 3.5m in length. The stem which is also called pseudostem produces a single bunch of banana before dying and replaced by new pseudostem. The fruit grows in hanging cluster, with twenty fruits to a tier and 3 – 20 tiers to a bunch. The pulp of a ripe banana is essentially a sugarrich, easily-digested food. It contains about 70% water; solid material is mostly carbohydrate (27%); fat (0.3%) and protein (1.2%) contents are generally low. In energy terms, each gram provides one calorie. Eleven vitamins have been recorded and the fruit is considered a good source of vitamins A, B1, B2 and C. The fruit is protected by its peel which is discarded as waste after the inner fleshy portion is eaten (Anhwange *etal.,* 2009). *Musa sapientum* fruits have been reported to prevent anaemia by stimulating the production of haemoglobin in the blood. Its role to regulate blood pressure has been associated with the high content of potassium. Banana helps in solving the problem of constipation without necessary resorting to laxatives (Akinyosoye, 1991). Banana a tropical plant may protect itself from the oxidative stress caused by strong sunshine and high temperature by producing large amounts of antioxidant (Kanazawa and Sakakibara, 2000). Banana should be considered to be a good source of natural antioxidant for foods and functional food source against cancer and heart disease (Someya *et al.,* 2002). Bananas are one of the most popular fruits on the world and it well be known that fruits contain various antioxidants compounds such as gallocatechin and dopamine. Since the banana fruits are widely available, they been used as food without apparent toxic effect (Mokbel and Hashinaga, 2005). Ripe bananas, being one of the most easily digested foods are widely used in the nutrition of infants and of people suffering from various intestinal disorders. Bananas also have a special place in diets low in fats, cholesterol and salt. Sodium is present in trace amounts while the potassium level is around 400 mg/100g pulp. Because of the low lipid and high energy value, bananas are recommended for obese and geriatric patients. Bananas are useful for the treatment of peptic ulcers, infant diarrhoea, in coeliac disease (sufferers of this disease normally have a marked intolerance to carbohydrates, but are able to digest bananas readily) and in colitis. The high carbohydrate and low fat content of the banana makes it suitable for low fat diets (Sharrock and Lusty, 2000).

Certain compounds in banana behave like angiotensin-converting enzyme (ACE) inhibitors. These ACE inhibitors prevent the release of angiotensin and they prevent the rise of blood pressure (Anon, 1999).

3.13.1 Banana peel

Banana peel is rich in dietary fibre (50% on a dry matter (DW) basis), proteins (7% DW), essential amino acids, polyunsaturated fatty acids and potassium (Emaga *et al.,* 2007). Banana peel is most commonly used as a feed for the livestock. Banana peel is also used as an adsorbent for water purification (Annadurai *et al.,* 2002). Banana peel is rich in phytochemical compounds, mainly antioxidants. Banana peel contains lutein an antioxidant from the carotenoid family, which provides nutritional support to the eyes. Ethyl acetate and water soluble fractions of banana peel exhibits high antimicrobial and antioxidant activity. Most of the compounds isolated from green peel ß-sitosterol, malic acid, 12-hydroxystrearic acid and succinic acid, showed significant antibacterial activities and low antioxidant activities. While, those compounds isolated from water soluble extracts glycoside and monosaccharide components displayed significant antioxidant and low antimicrobial activity (Mokbel and Hashinaga, 2005). Dietary fibre has shown beneficial effects in the prevention of several diseases, such as cardiovascular diseases, diverticulosis, constipation, irritable colon, colon cancer, and diabetes (Rodriguez *et al.*, 2006). The fruit fibre has a better quality than other fibre sources due to its high total and soluble fibre content, water and oil holding capacities, and colonic fermentability, as well as a lower phytic acid and caloric value content (Figuerola *et al.*, 2005). A high dietary fibre content of banana peel (about 50 g/ 100 g) is indicative of a good source of dietary fibre (Happi Emaga *et al.*, 2007). Cellulose, lignin, and hemicelluloses contents of banana peels, the components of the insoluble dietary fibre fractions, varied from 7 to 12 g/100 g, 6.4 to 9.6 g/100 g and 6.4 to 8.4 g/100 g, respectively, whereas pectin contents, a component of the soluble dietary fibre ranged from 13.0 to 21.7 g/100 g (Wachirasiri *et al.*, 2009).

Various nutrients, amino acid composition, mineral content and the anti-nutritional compounds present in the banana peels are tabulated;

Table 3.1: Nutrient composition of banana peels

Nutrient composition	Type of banana peels		
	Green	Almost ripe	Ripe
Dry matter (%)	91.62	92.38	95.66
Crude protein (%)	5.19	6.61	4.77
Ether extract (%)	10.66	14.20	14.56
Crude fiber (%)	11.58	11.10	11.95
Ash (%)	16.30	14.27	14.58
Calcium (%)	0.37	0.38	0.36
Phosphorus (%)	0.28	0.29	0.23
Gross Energy (Kcal/Kg)	4383	4692	4592
Tannin (%)	6.84	4.97	4.69

Table 3.3: Amino acid composition of banana peels

Amino acid composition	Type of banana peels		
	Green	Almost ripe	Ripe
Aspartic acid (%)	0.299	0.409	0.331
Threonine (%)	0.140	0.189	0.153
Serine (%)	0.156	0.211	0.169
Glutamic acid (%)	0.382	0.539	0.484
Proline (%)	0.129	0.173	0.171

Glycine (%)	0.196	0.273	0.228
Alanine (%)	0.250	0.286	0.255
Cysteine (%)	0.059	0.080	0.061
Valine (%)	0.193	0.260	0.223
Methionine (%)	0.051	0.063	0.060
Isoleucine (%)	0.122	0.155	0.127
Leucine (%)	0.225	0.297	0.292
Phenylalanine (%)	0.061	0.080	0.064
Lysine (%)	0.119	0.136	0.104
Arginine (%)	0.078	0.102	0.084

Table 3.3: Minerals composition of *Musa sapientum* peel

Element	Concentration (mg/g)
Potassium	78.10±6.58
Calcium	19.20±0.00
Sodium	24.30±0.12
Iron	0.61±0.22
Manganese	76020±0.00
Bromine	0.04±0.00
Rubidium	0.21±0.05
Strontium	0.03±0.01
Zirconium	0.02±0.00
Niobium	0.02±0.00

Table 3.4: Proximate composition and anti - nutritional content of *Musa sapientum* peel

Parameter	Concentration (mg/g)
Moisture (%)	6.70±02.22
Ash (%)	8.50±1.52
Organic matter (%)	91.50±0.05
Protein (%)	0.90±0.25
Crude Lipid (%)	1.70±0.10
Carbohydrate (%)	59.00±1.36
Crude Fibre (%)	31.70±0.25
Hydrogen cyanide (mg/g)	1.33±0.10
Oxalate (mg/g)	0.51±0.14
Phytate (mg/g)	0.28±0.06
Saponins (mg/g)	24.00±0.27

3.13.2 Uses of banana peels

Banana peels are used in adsorption of toxic heavy metals like Lead and Cadmium from water (Anwar *et al.,* 2009). Banana peels are also used in the treatment of nipple fissures in women (Novak *et al.,* 2003). It is suggested that banana peel contains certain enzymes that is used to dissolve the warts and thus it is used in the folk medicine in the treatment of planar and genital warts.

Chapter 4
Materials and Methods

4. MATERIALS AND METHODS

4.1 Banana Varieties

The varieties of Banana used for screening protease activity are Banana cv. Karpooravalli (*Musa spp* –Karpooravalli –ABB), Banana cv. Nendran (*Musa spp* – French Plaintain – AAB), Banana cv. Mondhan (*Musa spp* – Bluggoe – ABB), Banana cv. Poovan (*Musa spp* – Mysore – AAB), Banana . Red Banana (*Musa spp* – Red Banana – AAA), Banana cv. Rasthali (*Musa spp* – Rasthali – AAB) and Banana cv. Nadan (*Musa spp* – Nadan – AAB) authenticated by Dr. T.N. Balamohan, Professor and Head, Department of Fruit and Crops, TNAU, Coimbatore.

4.2. Enzyme Extraction

The banana fruit was purchased from the market and the enzyme from the banana peel was isolated by using Tris HCl (pH 7.0) using mortar and pestle at 4°C. The extract was centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatant that serves as enzyme solution was used for enzyme assays.

4.3. Enzyme assay

Protease activity was assayed by spectrophotometric measurement of the release of tyrosine by casein digestion at 660nm by the method of Cupp-Enyard (2008). One unit will hydrolyze casein to produce color equivalent to 1.0 mmole (181 mg) of tyrosine per minute at pH 7.5 at 37 °C (colour by Folin & Ciocalteu's reagent).

Materials required:

1) 50 mM Potassium Phosphate buffer, pH 7.5 at 37°C.

(Prepare 200 ml in deionized water using Potassium phosphate, Dibasic, Trihydrate. Adjust to pH 7.5 at 37°C with 1 M HCl.)

2) 0.65% (w/v) Casein Solution (Casein)

(Prepare 125 ml in Potassium phosphate buffer using Casein. Heat gently (do not boil) to 80-90 °C for 10 minutes with stirring. Adjust the pH to 7.5 at 37 °C, if necessary, with either 1 M NaOH or 1 M HCl.)

3) **110 mM Trichloroacetic Acid Reagent (TCA)**

(Dilute 9 ml of Trichloroacetic Acid, 6.1 N, approximately 100% (w/v), to 500 ml with deionized water.)

4) **Folin & Ciocalteu's Phenol Reagent (F-C)**

(Dilute 10 ml of Folin & Ciocalteu's Phenol Reagent, to 40 ml with deionized water.)

5) **500 mM Sodium Carbonate Solution**

(Prepare 500 ml in deionized water using Sodium Carbonate Anhydrous.)

6) **1.1 mM L-Tyrosine Standard (Std Soln)**

(Prepare 100 ml in deionized water using L-Tyrosine, Free Base, Heat gently (do not boil) until tyrosine dissolves and cool to room temperature.)

Table 4.1: Assay for protease

Pipette the following reagents into suitable vials (in milliliters):

REAGENTS	BLANK	TEST
Casein	2.50	2.50
Equilibrate to 37°C		
Enzyme solution	---	1.00
Mix by swirling and incubate at 37°C for exactly 10 minutes.		
Trichloroacetic acid	2.50	2.50
Enzyme solution	1.00	---
Mix by swirling and incubate at 37 °7C for about 30 minutes.		
Filter through Whatman #50 filter paper and use the filtrate in color development.		

Colour development:

Pipette the following reagents into 4 dram vials (in milliliters):

Table 4.2: Colour development for protease assay

REAGENTS	BLANK	TEST
Test filtrate	---	1.00
Blank filtrate	1.00	---
Sodium carbonate	2.50	2.50
Folin-Ciocalteau reagent	0.50	0.50
Mix by swirling and incubate at 37 °C for 30 minutes.		
Remove the vials and allow them to cool to room temperature.		
Filter through a 0.45 mm filter immediately prior to reading.		
Read the absorbance at 660nm for each of the vials in suitable cuvettes.		

Standard Curve:

Prepare a standard curve by pipetting the following reagents into suitable vials (in milliliters):

Table 4.3: Assay for L-tyrosine standard

REAGENTS	BLANK	STANDARD 1	STANDARD 2	STANDARD 3	STANDARD 4
Standard solution	---	0.05	0.10	0.20	0.40
Deionised water	2.00	1.95	1.90	1.80	1.60
Sodium carbonate	2.50	2.50	2.50	2.50	2.50
Folin-Ciocalteau reagent	0.50	0.50	0.50	0.50	0.50

4.4. Protein estimation

4.4.1. Alkaline copper reagent

- Reagent A was prepared by dissolving 2% sodium carbonate in 0.1 N sodium hydroxide.
- Reagent B was prepared by dissolving 0.5% copper sulphate in 1% sodium potassium tartarate.
- Reagent C was prepared by mixing 50 ml of reagent A and 1ml of reagent B just before use.

4.4.2. Folin-Ciocalteau's reagent

2N Folin Ciocalteau reagent commercially available was diluted with distilled water in the ration 1:1 (v/v) just before use.

Procedure:

A standard protein curve was constructed by the method described by Lowry *et al* (1951). Bovine serum albumin (BSA) 1 mg/ml was used as standard protein. Different concentrations of BSA solutions were pipette out in the test tube and the total volume was made up to 1 ml with distilled water. 5 ml of alkaline copper reagent was added to each tube, mixed thoroughly and allowed to stand at room temperature for 10 minutes. Then to each tube 0.5 ml of 1N Folin-Ciocalteau's reagent was added with immediate mixing. The extinction was read after 20 minutes incubation at 660nm in ELICO spectrophotometer. A standard graph was constructed with optical density at 660nm vs concentration of protein in mg/ml.

4.5 Characterization of protease

4.5.1 Effect of temperature on protease activity

For temperature studies, the reaction mixture contained 1.00 ml of the enzyme solution, 2.5 ml of casein were incubated at different temperature range from 10°C to 100°C for 10 minutes. The reaction was stopped by addition of Trichloroacetic acid and filtered. Blue color was developed by addition of sodium carbonate and Folin-Ciocalteau reagent to the filtrate. The blue color was spectrophotometrically read at 660nm using ELICO spectrophotometer.

4.5.2 Effect of pH on protease activity

The effect of pH on the enzyme activity was done at varying pH of the reaction mixture at 3.0, 5.0, 7.0, 9.0 and 11.0 using 0.1 M NaOH or 1.0 M HCl to adjust the pH of the buffer. The protease assay was carried out by the method given by Cupp-Enyard (2008) to determine the protease activity.

4.5.3 Effect of substrate concentration on protease activity

The effect of substrate concentration on enzyme activity was studied by incubating 1.0 ml of enzyme with 2.5 ml of casein at different concentrations (2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml and 1.0 mg/ml) and by following the assay procedure given by Cupp-Enyard (2008) to determine the protease activity. The apparent K_m and V_{max} values were determined.

4.5.4 Effect of enzyme concentration on protease activity

The effect of enzyme concentration on protease activity was determined by preparing reaction mixtures of varying enzyme concentrations from 10 mg/ml-100 mg/ml with an interval of 10 mg/ml, adding 2.5 ml of casein and following the assay procedure given by Cupp-Enyard (2008) to determine the protease activity.

4.5.5 Effect of inhibitors on protease activity

For inhibitor studies the reaction mixture contained 1.0 ml of the enzyme solution, 200 μl of inhibitors (1,10 Phenanthroline, EDTA, Iodoacetate, Iodoacetimide and N-bromosuccinylamide) at concentrations 20mM, 40mM, 60mM, 80 mM and 100mM were incubated for 15 minutes at 37°C and enzyme activity was determined by adding 2.5 ml of casein and following the assay procedure given by Cupp-Enyard (2008) to determine the protease activity.

4.5.6 Effect of metal ions on protease activity

For studying the effect of metal ions, the reaction mixture contained 1.0 ml of the enzyme solution, 200 μl of metal ions (Hg^{2+}, Fe^{2+}, Na^{+}, Cu^{2+}, Ag^{+}) at concentrations 20mM, 40mM, 60mM, 80mM and 100mM were incubated for 15 minutes at 37°C and enzyme activity was determined by adding 2.5 ml of casein and following the assay procedure given by Cupp-Enyard (2008) to determine the protease activity.

4.6 Partial purification of protease

4.6.1 Ammonium sulphate fractionation

After the extraction of enzyme, it was purified by ammonium sulphate precipitation. The quantity of ammonium sulphate required to attain a particular saturation was calculated using the following formula:

Salt required= [533(final concentration – intial concentration)] / [100-(0.3* final concentration)]

The enzyme solution was brought to 0-50 percent saturation of ammonium sulphate by the addition of required quantity of salt and stirring continuously on ice for three hours. The solution was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was saved and brought from 50 to 70 percent saturation (13.49g ammonium sulphate/ 100 ml). The insoluble proteins were collected by centrifugation at 10,000 rpm for 10 minutes at 4°C and dissolved in minimum volume of Tris HCl buffer at pH 7.0.

4.6.2 Dialysis

The precipitated fractions were dialyzed against the same buffer (Tris HCl, pH 7.0) at lower concentration of 10 mM with three changes. Dialysis was carried out at 4°C overnight.

4.7 Native-PAGE zymography

For native-PAGE zymograpy, the enzyme extract was loaded onto the 12% resolving gel copolymerized with 0.5% gelatin without heating followed by electrophoresis with 100 V at 4°C. After electrophoresis, the gel was incubated in developing buffer (50mM Tris, 0.2mM NaCl and 5mM CaCl, pH 8.0). The gel was equilibrated for 30 minutes at room temperature with gentle agitation, then was decanted and replaved with developing buffer and incubated at 37°C for atleast 14 hours. The gel was stained with 0.5% (w/v) Coomasssie Brilliant Blue R-250 for 30 minutes and destained with acetic acid:methanol:distilled water (1:4:5) solution to reveal clear areas where the gelatin had been degraded .

4.8 SDS PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 4% stacking gel and 15% resolving gel according to the method of Laemmli (1970) under reducing and non-reducing conditions. 20μL of the banana peel extract and 10μL sample buffer , containing β-mercaptoethanol for reducing condition and without β-mercaptoethanol for non-reducing condition, denatured at 100°C for 5 min. 20 μL of the sample was loaded into the gels. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue G250 and destained with acetic acid:methanol:distilled water (1:2:7) solution.

4.9 Assay for collagenase

Collagenolytic activity was measure by the method of Moore and Stein (1954) with a slight modification. A reaction mixture, which contains 5 mg of collagen type I, 1 ml of 50 mM Tris-HCl (pH 7.5) that contained 5 mM $CaCl_2$ and0.1 ml of the enzyme solution, was typically incubated at 37°C for 1 hour. The reaction was stopped by adding 0.2 ml of 50% trichloroacetic acid. After 10 minutes at room temperature, the solution was centrifuged at 1800×g for 20 minutes. The supernatant (0.2 ml) was mixed with 1.0 ml of a ninhydrin solution, incubated at 100°C for 20 minutes, then cooled to room temperature. Subsequently, the mixture was diluted with 5 ml of 50% 1-propanol for an absorption measurement at 570 nm. A buffer (50 mM Tris-HCl, pH 7.5) that contained 5 mM $CaCl_2$ was used instead of an enzyme solution as the reference. The concentration of hydrolyzed- amino acid was determined by a standard curve that was based on a solution of L-leucine. One unit (U) of enzyme activity is defined as the amount of enzyme that is required for the hydrolysis of 1 mmol of substrate per hour.

Chapter 5
Results and Discussion

5. RESULTS AND DISCUSSION

1 Enzyme source

The enzyme source was the filtrate obtained from the banana peel using Tris HCl (pH .0) as the extraction buffer.

.2 Enzyme assay

Enzyme assay was performed as mentioned in Chapter 4 (Materials and Methods). The rude filtrates of different varieties of banana peel were assayed for proteolytic activity.

.3 Screening for high protease activity in different varieties of banana peel

Eight varieties of banana peel (Poovan, Rasthali, Moris, Mondhan, Nadan, Karpooravalli, evvaazhi, Nendran) were assayed for protease activity by following the method suggested by 'upp Enyard (2008). High protease activity was observed in Sevvaazhi (*Musa spp* – Red anana- AAA) variety of banana peel with specific activity 16 U/mg (Fig 5.1). Thus, the Red anana variety peel was used to characterize the protease enzyme.

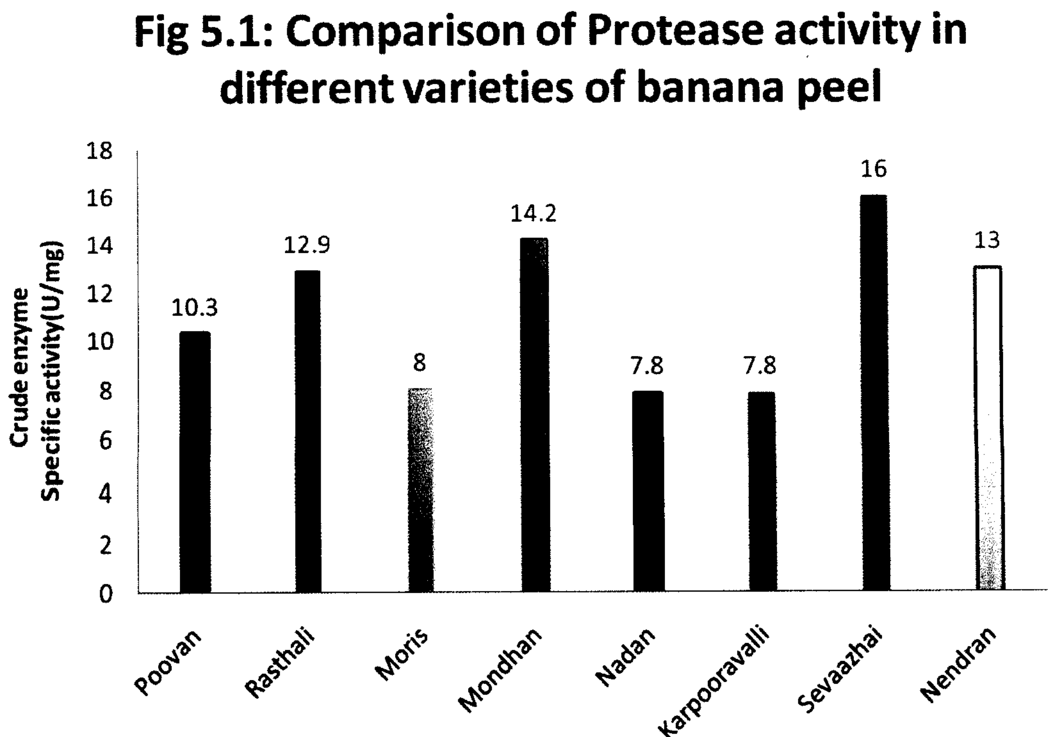
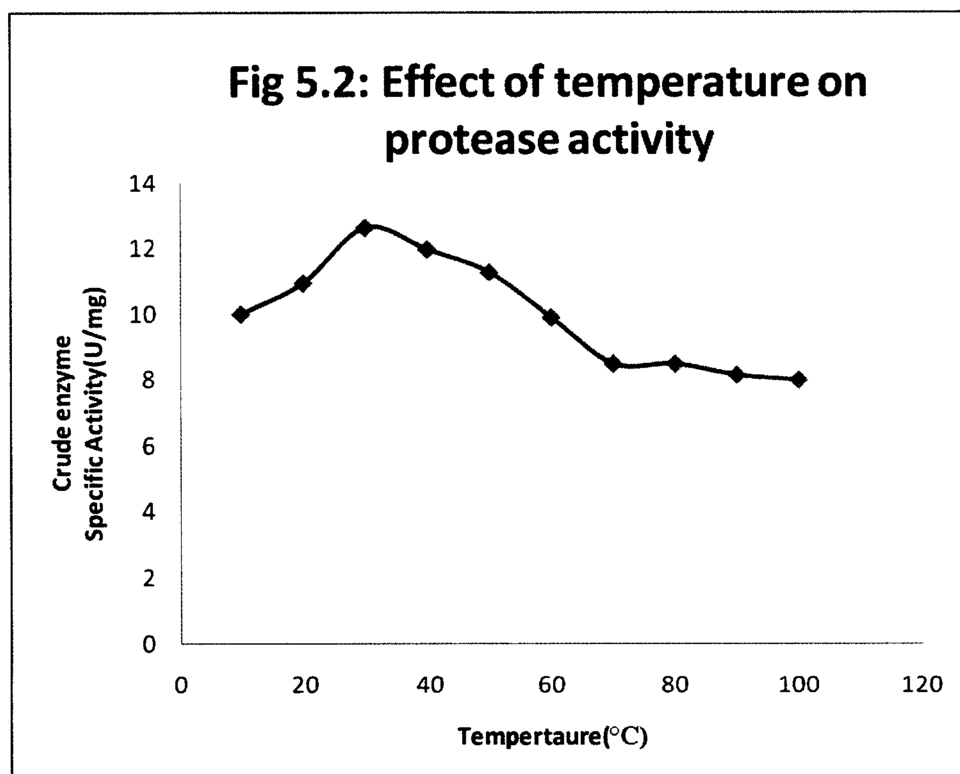


Fig 5.1: Comparison of Protease activity in different varieties of banana peel

.4 Characterization of protease

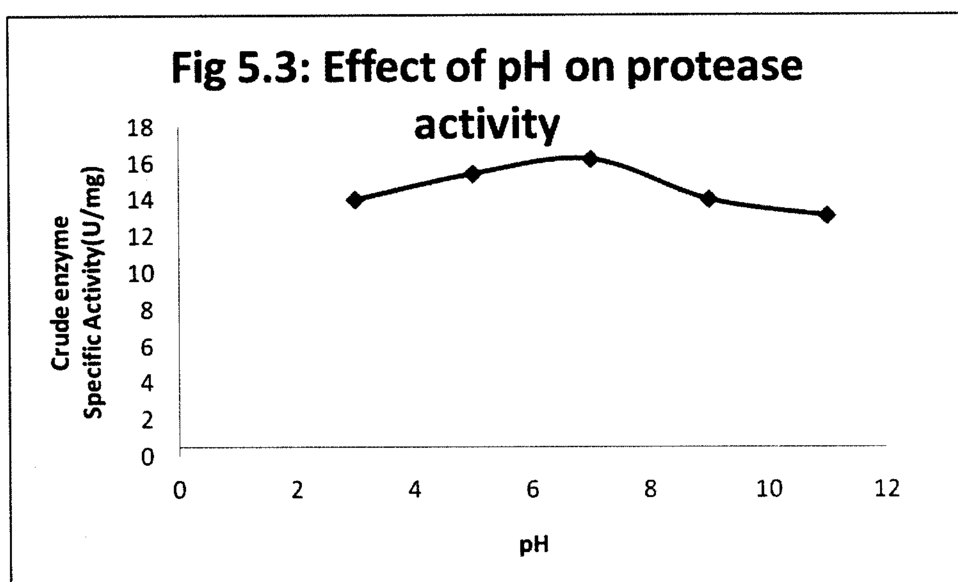
.4.1 Effect of temperature on protease activity

The protease enzyme activity assays was carried out at a temperature range from 10°C - 00°C, using casein as the substrate. The optimum temperature for protease activity was found ɔ be at 30°C (Fig 5.2). Cysteine protease isolated from the germinating cotyledons of horse ram seeds had an optimum activity at 40°C (Jinka *et al.*, 2009). The alkaline protease isolated ·om the maggots had a maximum activity at 45°C (Raimi *et al.*, 2010). *Choreospondias xillaris* (Lapsi) fruit protease had an optimum enzyme activity at 30°C (Prajapati *et al.*, 2009). 'he proteolytic enzymes obtained from the fermented seeds of locust bean and melon seeds had n optimum activity at 40°C (Evans *et al.*, 2009). Crude extract obtained from latex of *Araujia orototorum* fruits had an optimum activity at 70°C (Priolo *et al.*, 2000).



.4.2 Effect of pH on protease activity

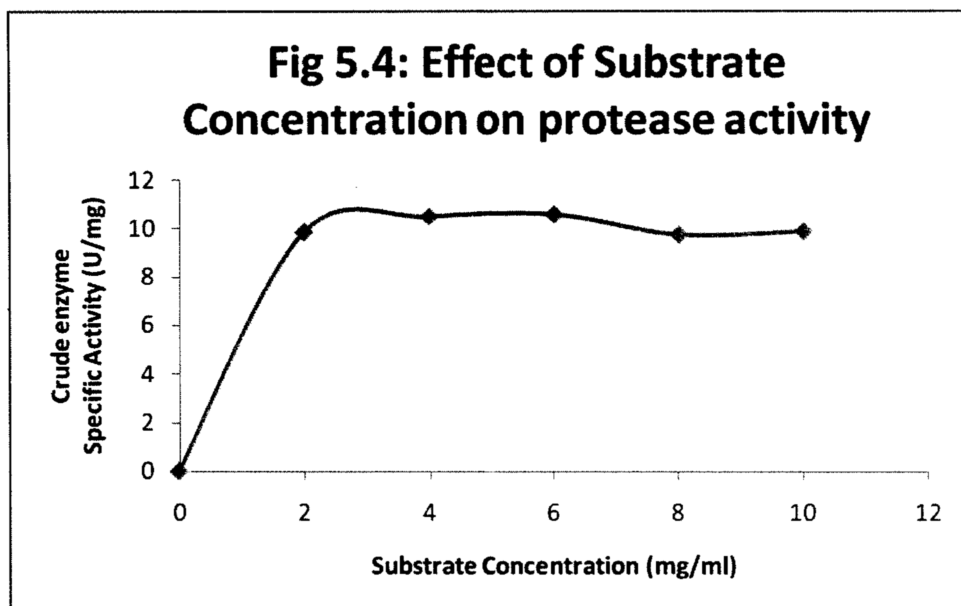
The protease activity assays was carried out at pH 3.0, 5.0, 7.0, 9.0 and 11.0, using casein s the substrate. The optimum pH for protease activity was found to be at pH 7.0 (Fig 5.3). roteolytic activity was found to be optimum at pH 8.0 for the *Choreospondias axillaris* (Lapsi) uit (Prajapati *et al.,* 2009). Optimum pH for the protease obtained from the latex of the *Araujia orotorum* fruits was 7.5-8.5 for the crude preparation (Priolo *et al.,* 2000). Low molecular veight protease obtained from the *Pseudomonas sp.,* had an optimum proteolytic activity at pH .0 (Dutta *et al.*, 2005). Maximum proteolytic activity was obtained at a pH range 6-7.2 from the ermented melon seeds (Evans *et al.,* 2009). Collagenolytic protease obtained from Filefish, *Iovoden modestrus* had an optimum activity at pH 7.0-8.0 (Kim *et al.,* 2002).



5.4.3 Effect of substrate concentration on protease activity

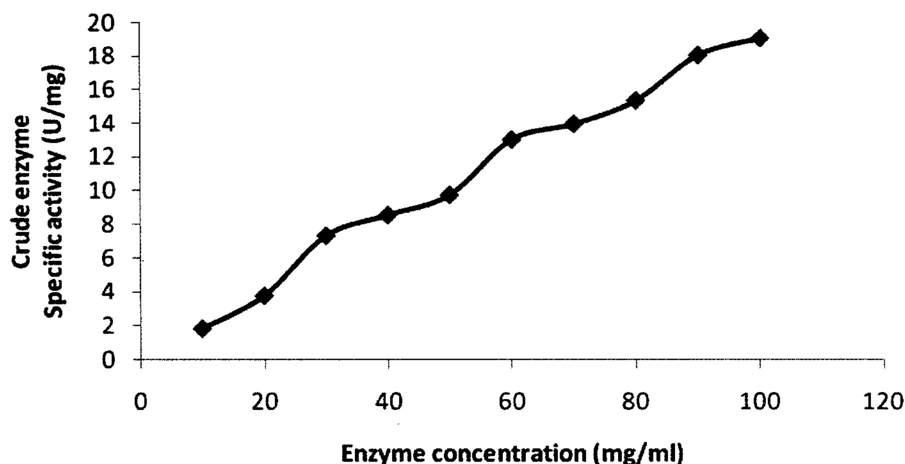
The effect of substrate on protease activity was carried out using casein at different concentrations (0 mg/ml,2 mg/ml, 4mg/ml, 6mg/ml, 6mg/ml, 8mg/ml and 10mg/ml). Protease activity was found to be maximum at substrate concentration 6mg/ml. V_{max}, K_m value was found to be 10.59 U/mg and 43 μM respectively. K_m and V_{max} values for lapsi fruit protease were found to be 13.09 μM and 15.87 pmoles/min respectively (Prajapati *et al.,* 2009). The enzyme kinetic studies on alkaline protease obtained from maggots revealed a V_{max} 1.21 mmol/min and a K_m of 0.11mM which indicates that the enzyme has affinity for casein as a

substrate (Raimi *et al.,* 2010). The K_m for the crude protease from fermenting locust bean and melon seeds were 5.8×10^{-2} M and 4.8×10^{-2} M, while the V_{max} were 5.1×10^{-2} s^{-1} and 3.43×10^{-2} s^{-1} respectively, which shows that the enzyme exhibited considerable proteolytic activity on the substrate (Evans *et al.,* 2009).



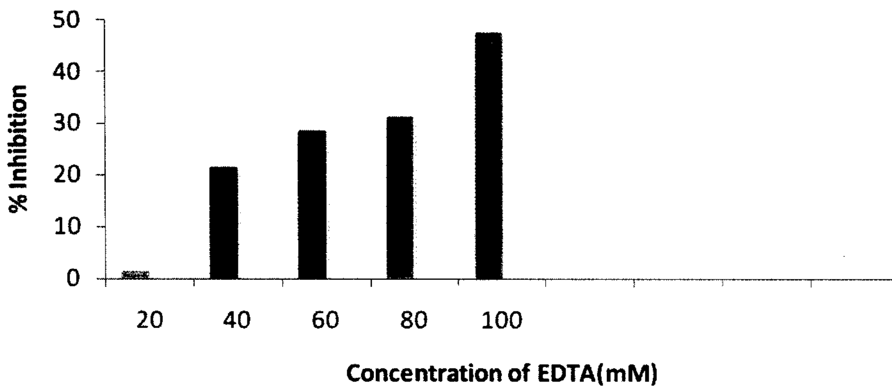
5.4.4 Effect of Enzyme concentration on protease activity

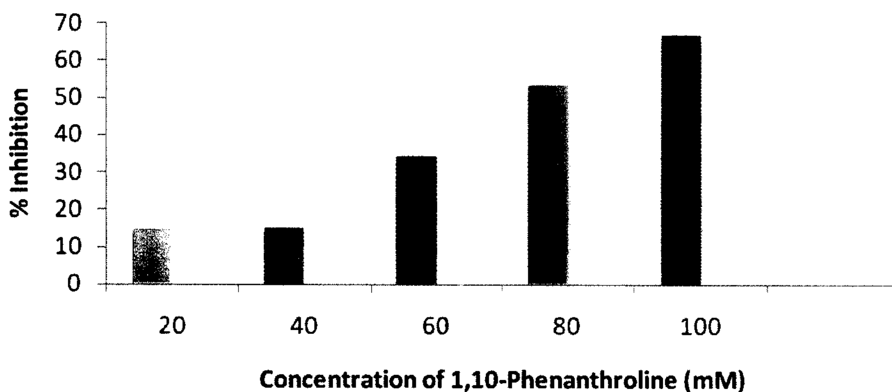
The effect of enzyme concentration on protease activity was carried out at different enzyme concentrations (10 mg/ml-100mg/ml), using casein as the substrate. With an increase in the concentration of the enzyme, there was an increase in the specific activity of the enzyme (Fig 5.5). The study of the effect of initial enzyme concentration for the enzyme isolated from *Pseudomonas* sp. showed that at low concentration, the rate of hydrolysis increased linearly and finally the curve became asymptotic at higher enzyme concentration, therey indicating a substrate-limiting condition (Dutta *et al.,* 2005).



5.4.5 Effect of inhibitors on protease activity

Inhibitors like EDTA (Fig 5.6) and 1,10-Phenanthroline were tested to find out their effect on protease activity. Out of these inhibitors used, maximum inhibition of 67% was found in 100mM concentration of 1,10-Phenanthroline (Fig 5.7). The purified collagenase enzyme (member of serine protease) obtained from Mackerer, *Scomber japonicas* showed that the enzyme was not inhibited by EDTA and 1,10-Phenanthroline (Park *et al.,* 2002). The metalloprotease obtained from Dry grass pea (*Lathyrus sativus* L.) seeds were strongly inhibited by 1,10-Phenanthroline, but the enzyme was not inhibited by EDTA . To identify the type of metal cofactor required for the protease activity, it was tested by using different metal salts to overcome inhibitory effect of 1,10-Phenanthroline. The inhibition of 1,10-Phenanthroline was overcome by subsequent addition of Zn^{2+} ions, which indicate that the metalloprotease is a Zn^{2+}-dependent proteases in the dry seeds (Ramakrishna *et al.,* 2010).

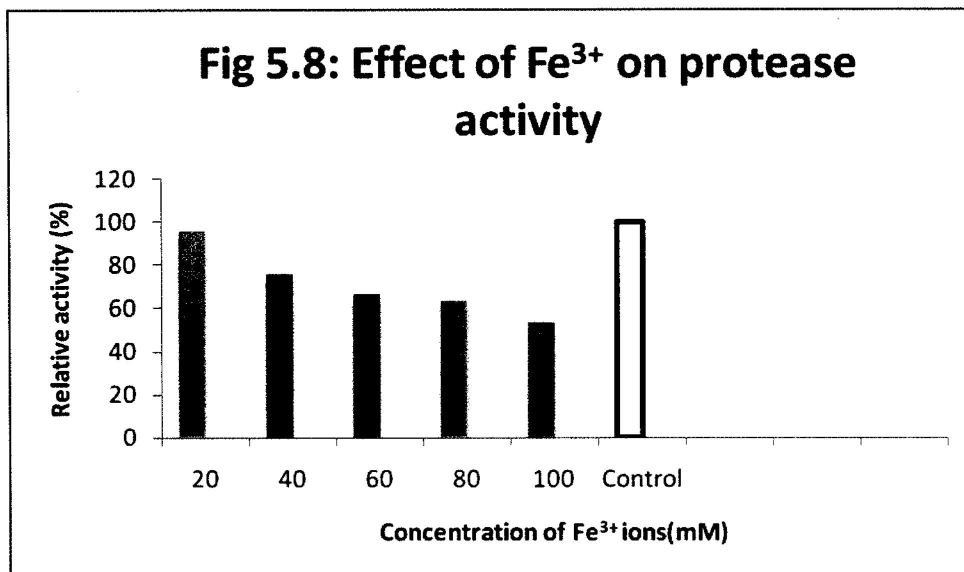




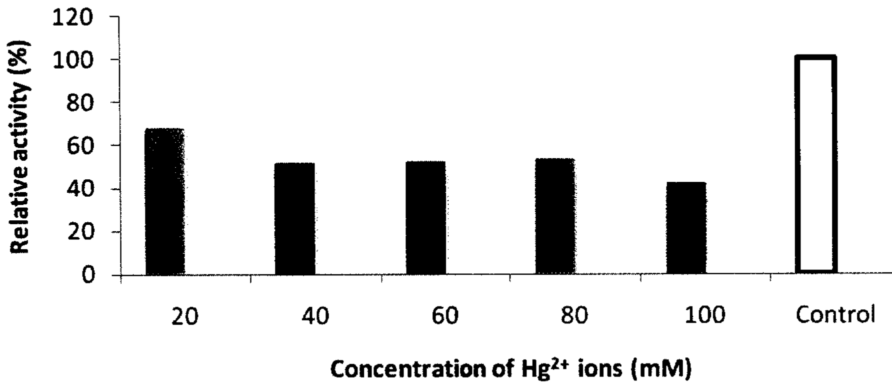
5.4.6 Effect of metal ions on protease activity

Several metal ions like Fe^{3+}, Hg^{2+}, Cu^{2+}, Ca^{2+} and Na+ were tested to find out their effect on protease activity. The protease enzyme isolated from the *Araujia horotorum* was inhibited by the presence of Hg^{2+} ions, but the proteolytic activity was almost completely reverted by adding cysteine to the incubation mixture (Priolo *et al.,* 2000). The collagenase obtained from Mackerel was inhibited by Mn^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and Hg^{2+} (Park *et al.,* 2002).

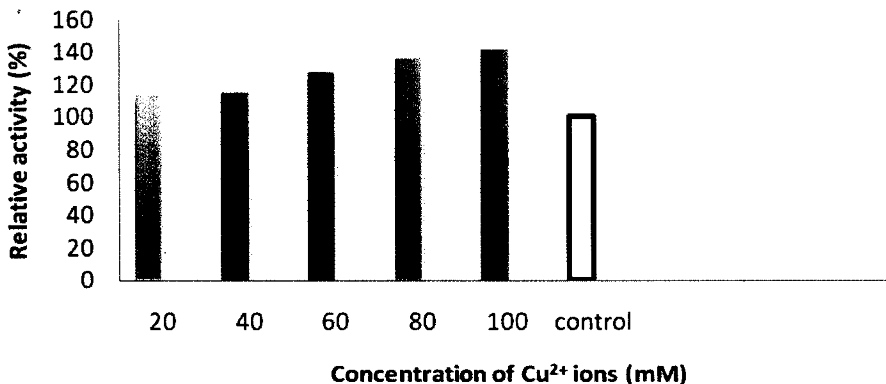
- There was a decrease in the protease activity with an increase in the concentration of Fe^{3+} ions. Maximum inhibition of protease activity was found at 100mM concentration of Fe^{3+} ions. The relative activity was only 53% at 100mM concentration of Fe^{3+} ions (Fig 5.8).



- Hg^{2+} ions affected protease activity to a greater extent. With an increase in the concentration of the Hg^{2+} ions there was a gradual decrease in the protease activity. The relative activity was only 41.92% at 100mM concentration of Hg^{2+} ions (Fig 5.9).

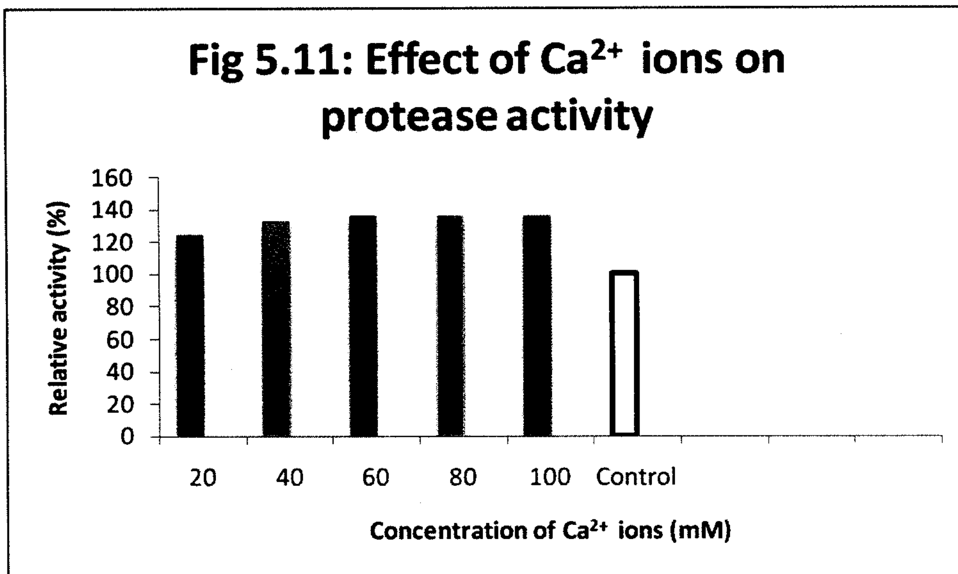


- With an increase in the concentration of Cu^{2+} ions there was an increase in the protease activity. At 100mM concentration of Cu^{2+} ions, the relative activity was found to be 141% which indicates that the Copper ions act as an enhancer for the protease enzyme (Fig 5.10).

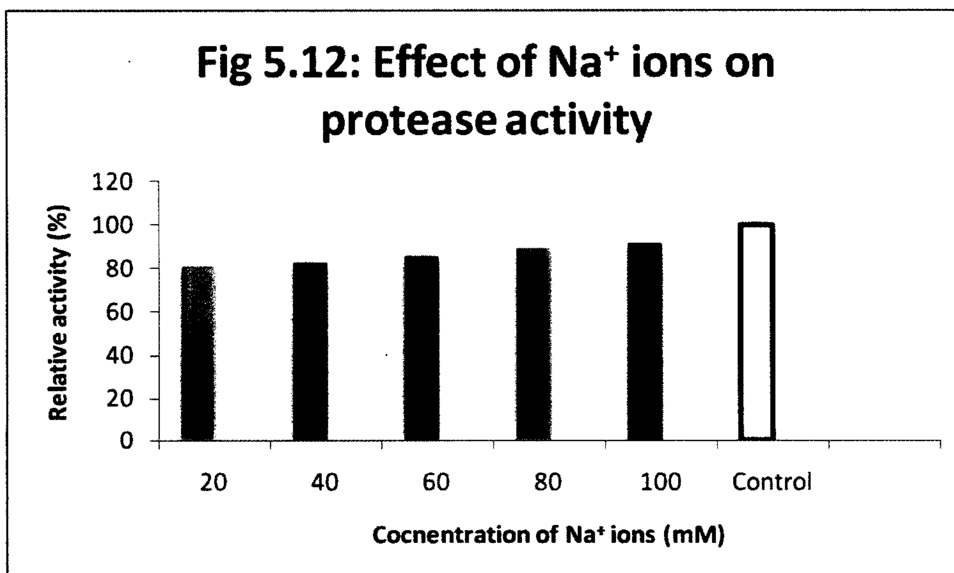


- Ca^{2+} ions are the enhancers of protease enzyme. With an increase in the concentration of Ca^{2+} ions there was a gradual increase in the protease actvity and there was a saturation in

the protease activity from 60mM concentration of Ca^{2+} ions. Maximum relative activity was 135% observed at 60, 80, 100mM concentration of Ca^{2+} ions (Fig 5.11).

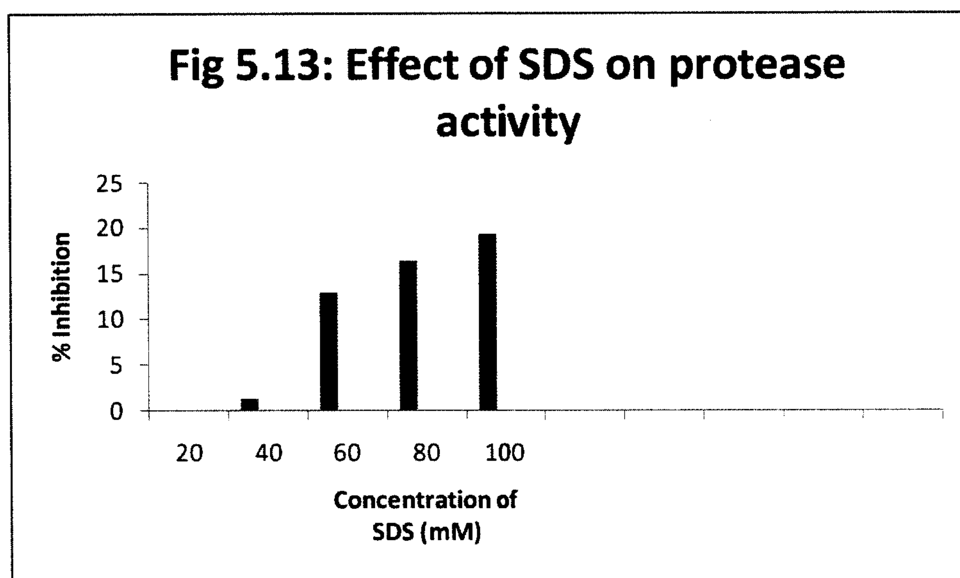


- Na^+ ions enhances the activity of protease enzyme. With an increase in the concentration of Na^+ ions there is an increase in protease activity (Fig 5.12).



5.4.7 Effect of SDS on protease activity

SDS (Sodium dodecyl sulphate) which denatures the protein, had a great effect on protease activity. With an increase in the concentration of SDS, there was a decrease in the protease activity (Fig 5.13). This is because incubation of SDS at different concentrations with the enzyme for 15 minutes denatures the enzyme and thus the enzyme is not free to react with casein thereby resulting in the decrease in protease activity. There was a decrease in 19.25% of the enzyme activity at 100mM concentration of SDS. Increase in the protease activity for the protease isolated from Lapsi fruit was observed on increasing the concentration of SDS in the reaction mixture (Prajapti *et al.*,2009).



5.5 Partial purification of protease

Protease was partially purified from the banana peel extract filtrate. A purification of upto 1.6 fold was obtained through ammonium sulphate fractionation and dialysis steps. Protease isolated from *Pseudomonas* sp., revealed a fold purification of 4.0 after acetone precipitation and 5.0 after purifying the enzyme by CM-Sephadex column (Dutta *et al.,* 2005). Metalloprotease obtained from the dry seeds of grass pea exhibited a purification fold of 5,110 for ammonium sulphate precipitated enzyme and for the enzyme purified by casein-alginate gel filtration respectively (Ramakrishna *et al.,*

2010). *Araujian h I* is a protease obtained from the latex of *Araujia hortorum* fruits. The purification of the main proteolytic component was carried out by cation exchange chromatography and the purification fold was found to be 6 (Priolo *et al.,* 2000).

5.5.1 Crude extract

The crude enzyme extract from the Red banana peel had a specific activity of 7.9 U/mg (Table 5.1).

5.5.2 Ammonium sulphate fractionation

The crude extract was subjected to ammonium sulphate fractionation. Differential fractionation of protein in the crude extract was done by a range of ammonium sulphate fractionations (10 to 100%). The supernatant and precipitate of each fraction were tested for protease activity. Protease activity in the supernatants started decreasing after removing 50% of saturated ammonium sulphate fraction. In the precipitate, protease activity reached a maximum in the 60% saturation. Ammonium sulphate fractionation yielded a protease preparation having 1.5 fold higher specific activity over the crude extract (Table 5.1). The enzyme yield after ammonium sulphate precipitation was 15%.

5.5.3 Dialysis

The ammonium sulphate fractionated samples were dissolved by using 100mM Tris HCl (pH 7.0). The dissolved pellets were then dialysed using 10mM Tris HCl (pH 7.0) buffer at 4°C. The dialysed sample was then assayed for protease activity. The purification fold after dialysis was found to be 1.606 times higher than the specific activity of the crude extract. The enzyme yield after dialysis was 12.85% (Table 5.1).

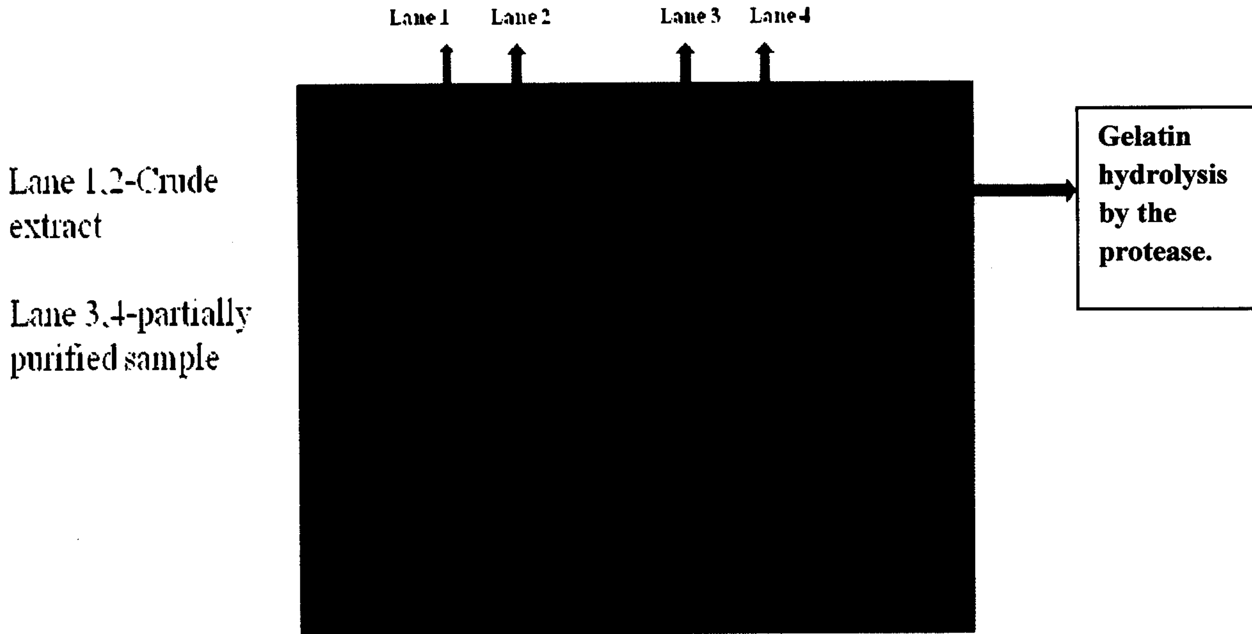
Table 5.1: Protease activity and recoveries in the stages of purification

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
Crude extract	0.940	790	7.9	100	1.0
Ammonium sulphate precipitation	0.451	118.6	11.86	15	1.5
Dialysis	0.394	101.52	12.69	12.85	1.606

5.6 Native PAGE zymography

Native PAGE zymography was performed as mentioned in Chapter 4 (Materials and Methods). The protease activity was charcterized by zymogram analysis which allowed visualization of the enzyme activity *in situ*. The light bands indicating the location of proteases and their activities against the dark background are shown in Fig 5.14. The light bands were clearly observed that indicated the presence of proteases in banana peel. SDS-PAGE zymography was performed for the protease enzyme obtained from the kiwifruit extracts, as the SDS zymography failed to show the protease activity, native PAGE zymogram was performed to visualize the enzyme activity. Two light bands were clearly observed which indicated the presence of at least two proteases in kiwifruit (Mohammadian *et al.,* 2010). Native electrophoresis was perfromed for the crude protease enzyme isolated from the latex of *Araujia hortorum* fruits that showed two fractions, one of them with higher proteolytic activity in the zymogram (Priolo *et al.,* 2000). Gelatin PAGE zymogram was carried out for the protease obtained from the dry seeds of grass pea and three different bands on gelatin PAGE was observed that indicated the presence of protease in the seed extract (Ramakrishna *et al.,* 2010).

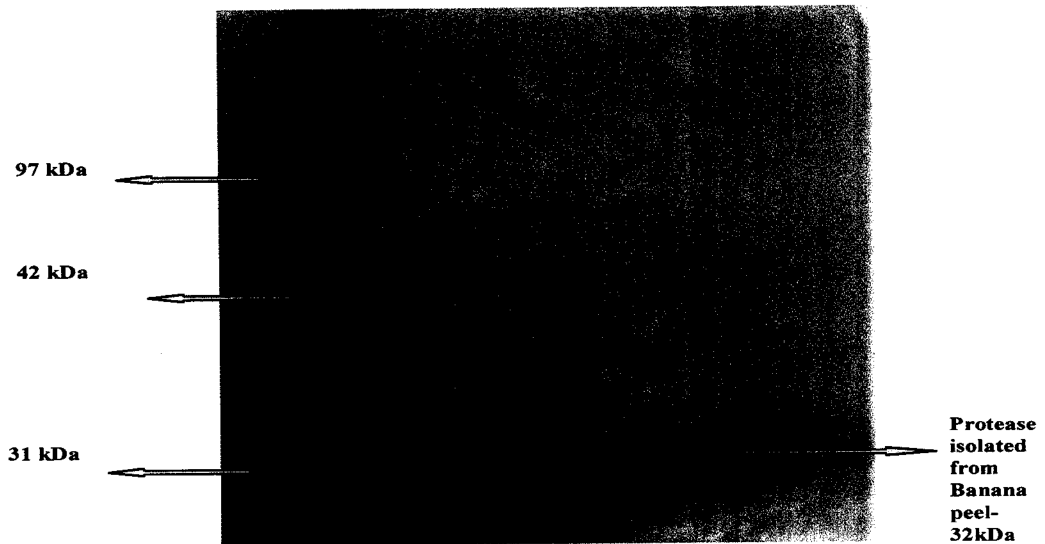
Fig 5.14: Native PAGE zymogram



5.7 SDS PAGE

SDS PAGE was carried out for the crude extracts of kiwifruit extracts and it showed two bands with molecular weight of 29 and 24 kDa, in reducing conditions, were more significant with different intensities (Mohammadian *et al.*, 2010). SDS PAGE performed for the purified protease *Araujiain h I* revealed the presence of single unique band with relative mass of about 25.5 kDa (Priolo *et al.*, 2000). Three different isoforms of protease obtained from the dry seeds of grass pea showed three distinct bands in SDS PAGE and the isoforms exhibited relative mass 25, 18, 14 kDa respectively (Ramakrishna *et al.*, 2010). Similarly, SDS PAGE was carried out for the crude extracts of protease obtained from the red banana peel with the SDS marker in 4% stacking gel and 15% separating gel. A single band was obtained in the SDS PAGE gel. The molecular weight of the protease was found to be 32 kDa (Fig 5.15).

Fig 5.15 SDS PAGE



5.8 Collagenase assay

Collagenase assay was performed for the crude extract obtained from the red banana peel according to the method given by Moore and Stein (1954) by using type I collagen obtained from the rat tail as the substrate. The specific activity for the collagenase enzyme was found to be 17.17 ± 0.26 U/mg for the crude extract obtained from the banana peels. Collagenase obtained from the internal organs of the Mackerel, *Scomber japonicus* was purified by Acetone precipitation and using DEAE-Sephadex column. The specific activity of the purified collagenase was higher than those of the collagenase obtained from the crude extract. The specific activity for the crude collagenase obtained from Mackerel was found to be 16.5 U/mg where as the specific activity for the collagenase after acetone precipitation was found to be 42.3 U/mg and the specific activity for collagenase purified by DEAE Sephadex column was 189.1 U/mg (Park *et al.,* 2002). Two cysteine proteases GP2 and GP3 isolated from the rhizomes of the ginger also exhibited collagenolytic activity. This suggests that ginger proteases can

be used as an alternative to papain, in commercial applications such as meat tenderization, where collagen is the target substrate (Kim *et al.,* 2007).

Similarly, the banana peel extracts were found to exhibit collagenolytic activity, thus it is evident that banana peel extracts can be used for the treatment of warts since the warts consists of collagen as the major connective tissue fibre.

Chapter 6
Summary and Conclusion

6. SUMMARY AND CONCLUSION

Eight different varieties of banana (Poovan, Rasthali, Moris, Mondhan, Nadan, Karpooravalli, Sevvaazhi, Nendran) obtained from the local market were screened for the proteolytic activity. High protease activity was observed in Sevvazhi (*Musa spp* – Red Banana-AAA) variety of banana peel that had a specific activity of 16 U/mg. The protease enzyme isolated from the peel of Red banana had an optimum activity at temperature 30°C and at pH 7.0. The K_m and V_{max} value for the protease enzyme was found to be 43 µM and 10.59 U/mg. Protease enzyme was found to be inhibited by 1,10-Phenanthroline and EDTA. Maximum inhibition of 67% of the enzyme was made by 1,10-Phenathroline at a concentration of 100mM. Protease enzyme was found to be inhibited by Hg^{2+} (41.92% inhibition) and Fe^{3+} (53% inhibition) ions at 100mM concentrations of the metal ions. Cu^{2+}, Ca^{2+} and Na^{+} ions were found to enhance the protease activity.

The enzyme was partially purified by ammonium sulphate fractionation and dialysis and the purification fold was found to be 1.5 and 1.606 respectively. The yield of the protease enzyme obtained by ammonium sulphate fractionation and dialysis was found to be 15% and 12.85% respectively. The native PAGE zymography was performed for the crude enzyme and partially purified enzyme by using gelatin as the substrate in the resolving gel. Clear bands were obtained after electrophoresis which proved that protease enzyme is present in the peel extract. SDS PAGE was performed for the enzyme extract and the molecular weight was found to be kDa.

Collagenase assay was performed for the enzyme extract by using Type I Rat tail collagen as the substrate. The specific activity was found to be 17.17 ± 0.26 U/mg. Thus it is evident that banana peel extract can be used to treat genital warts as the warts are mainly composed of collagen as the major connective fibre. Thus, by treating the warts with banana peel extract helps in dissolving the warts.

Appendices

APPENDICES

Appendix I

Phosphate Buffer

Stock solutions:

A: 0.1M solution of monobasic sodium phosphate

B: 0.1M solution of dibasic sodium hydrogen phosphate

X ml of A, Y ml of B diluted to a total of 200 ml.

A (ml)	B (ml)	pH
92.0	8.0	5.8
87.7	12.3	6.0
81.5	18.5	6.2
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0
33	67	7.1
28	72	7.2
23	77	7.3
19	81	7.4
16	84	7.5
8.5	91.5	7.8
5.3	94.7	8.0

Citrate buffer

Stock solutions:

A: 0.1 M solution of citric acid

B: 0.1 M solution of sodium citrate

X ml of A and Y ml of B diluted to a total of 100ml.

A (ml)	**B (ml)**	**pH**
46.5	3.5	3.0
43.7	6.3	3.2
40.0	10	3.4
37.0	13	3.6
35.0	15	3.8
33.0	17	4.0
31.5	18.5	4.2
28.0	22	4.4
25.5	24.5	4.6
23.0	27	4.8
20.5	28.5	5.0
18.0	32	5.2
16.0	34	5.4
13.7	36.3	5.6
11.8	38.2	5.8
9.5	41.5	6.0
7.2	42.8	6.2

Appendix II

Estimation of protein by Lowry's method:

Stock standard Bovine serum albumin

Dissolve 100 mg of BSA and make up to 100 ml with distilled water.

Working standard solution

Take 10 ml of the stock solution and dilute to 100 ml with distilled water.

References

REFERENCES

1) Abdullah, A.N., Walzman, M. and Wade, A. (1993). Treatment of external genital warts comparing cryotherapy (liquid nitrogen) and trichloracetic acid', Sex Transm Dis Vol.20, pp.344–345.
2) Akinyosoye, V.O. (1991) Tropical Agriculture, Macmillan Publishers Limited, Ibadan, pp.65 – 68.
3) Anhwange, B.A., Ugye, T.J. and Nyiaatagher, T.D. (2009) 'Chemical Composition Of Musa Sapientum (Banana) Peels', Electronic journal of environmental, agricultural and food chemistry Vol.8, No.6, pp. 437-442.
4) Annadurai, G., Juang, R. S. and Lee, D. J. (2002) 'Use of cellulose-based wastes for adsorption of dyes from aqueous solutions', Journal of Hazardous Materials Vol92, No.3, pp.263–274.
5) Anon. (1999) 'Two is enough - really. Sweet tidings on treating high blood pressure', Asiaweek.
6) Anwar, J., Shafique, U., Zaman, W., Salman, M., Dar, A. and Anwar, S. (2010) 'Removal of Pb(II) and Cd(II) from water by adsorption on peels of banana', Bioresource Technology Vol.101, pp.1752–1755.
7) Arunachalam, C. and Saritha, K. (2009) 'Protease enzyme: an eco-friendly alternative for leather industry', Indian Journal of Science and Technology Vol.2, No.12, pp. 29-32.
8) Banerjee, S., Halder, B., Barman, N.R. and Ghosh, A.K. (2011) 'An overview on different variety of *Musa* species: Importance and its enormous pharmacological action', IJPI'S Journal of Pharmacognosy and Herbal Formulations Vol.1, No.2, pp.1-11.
9) Barrett,A.J. (1994) 'Classification of peptitades', MethodsEnzymol, Vol. 244, pp.1- 15.
10) Beutner, K.R., Ferenczy, A. (1997) 'Therapeutic approaches to genital warts', Am J Med Vol.102, No.5A, pp.28–37.

11) Beutner, K.R., Reitano, M.V., Richwald, G.A. and Wiley, D.J.(1998) 'External genital warts: report of the American Medical Association Consensus Conference, AMA Expert Panel on External Genital Warts', Clin Infect Dis Vol.27, pp.796-806.

12) Bharti, A.C., Shukla, S., Mahata, S., Hedau, S. and Das, B.C. (2009) 'Anti-human papillomavirus therapeutics: Facts & future' Indian J Med Res Vol.130, pp. 296-310.

13) Bonnez, W., Oakes, D., Bailey-Farchione, A. (1995) 'A randomised, doubleblind, placebo-controlled trial of systemically administered interferon-α, β, γ or in combination with cryotherapy for treatment of condylomata acuminata', J Infect Dis Vol.171, pp.1081–1089.

14) Bradbury, E., Moon, L., Popat, R., King, V.R., Bennett, G.S., Patel, P.N., Fawcett, J.W. and McMahon, S.B. (2002) 'Chondroitinase ABC promotes functional recovery after spinal cord injury', Nature Vol.416, pp.636-640.

15) Chiplankar. J.M., Gangodkar, S.V., Wagh, U.V., Ghadh, G.D., Rele, M.V. and Srinivasan, M.C. (1985) 'Application of Alkaline protease from Conidiobolus in animal cell culture', Biotechnolo Lett. Vol.7, pp.665-668.

16) Conley, L.J., Ellerbrock, T.V., Bush, T.J., Chiasson, M.A., Sawo, D., Wright, T.C. (2002) 'HIV-1 infection and risk of vulvovaginal and perianal condylomata acuminata and intraepithelial neoplasia: a prospective cohort study', Lancet, Vol.359, pp.108-113.

17) Cupp-Enyard, C. (2008) 'Sigma's Non-specific Protease Activity Assay - Casein as a Substrate', Journal of Visualized Experiments Vol.19, pp.899.

18) Dutta. J.R., Dutta. P.k. and Banerjee. R. (2005) 'Kinetic study of low molecular weight protease isolated from the newly isolated *Pseudomonas* sp. Using artificial neural network', Indian Journal of Biotechnology Vol.4 , pp.127-133.

19) Emaga, T. H., Andrianaivo, R. H., Wathelet, B., Tchango, J. T. and Paquot, M. (2007) 'Effects of the stage of maturation and varieties on the chemical composition of banana and plantain peels', Food Chemistry Vol.103, No.2, pp.590–600.

20) Evans, C.E., Yisa, J. and Egwim, P.O. (2009) 'Kinetics studies of protease in fermenting locust beans (Parkia biglobosa) and melon seed (Citrullus vulgaris)', African Journal of Biochemistry Research Vol.3, No. 4, pp. 145-149.

21) Fabbrocini, G., Costanzo, M.P.D., Riccardo, A.M.,, Quarto, M., Colasanti, A., Roberti, G.and Monfrecola, G. (2001) 'Photodynamic therapy with topical d-aminolaevulinic acid for the treatment of plantar warts', Journal of Photochemistry and Photobiology B: Biology Vol. 61, pp. 30–34.

22) Fehrmann, F. and Laimins, L.A. (2003) 'Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation', Oncogene Vol. 22, pp.5201–5207.

23) Ferenczy, A. (1984) 'Treating genital condyloma in pregnancy with the carbon dioxide laser', Am J Obstet Gynecol Vol.148, pp.9–12.

24) Ferguson, L.R. and Pearson, A. (1991) 'Chromosomal changes in Chines hamsterAA8 cells caused by podophyllin, a common treatment for genital warts', Mutation Research Vol.266, pp.231-239.

25) Figuerola, F., Hurtado, M. L., Estevez, A. M., Chiffelle, I. and Asenjo, F. (2005) 'Fibre concentrates from apple pomace and citrus peel as potential fibre sources for food enrichment', Food Chemistry Vol.9, pp.395-401.

26) Fischer, G. and Rogers, M. (1997) 'Cimetidine therapy for warts in Children', J Am Acad Dermatol Vol.37, pp.289-302.

27) Fraser, P.A., Lacey, C.J.N. and Maw, R.D. (1993) 'Motion: podophyllotoxin is superior to podophyllin in the treatment of genital warts', J Eur Acad Dermatol Venereol Vol.2, pp.328–334.

28) Georgala, S., Danopolou, I. and Katsarou, A. (1989) 'Dinitrochlorobenzene treatment of condylomata acuminata', Australas J Dermatol Vol.30, pp.103–105.

29) Godley, M.J., Bradbeer, C.S., Gellan, M. and Thin, R.N.T. (1987) 'Cryotherapy compared with trichloracetic acid in treating genital warts', Genitourin Med Vol.63, pp.390–392.

30) Greenfield, J. and Davis, J.M. (2004) 'Bloodroot (*Sanguinaria canadensis* L.): Medicinal Herb Production Guide', Grower's Guides, pp. 1-6.

31) Gruis, D., Selinger, D.A., Curran, J.M. and Jung, R. (2002) 'Redundant proteolytic mechanisms process seed storage proteins in the absence of seed-type members of the

vacuolar processing enzyme family of cysteine proteases', Plant Cell Vol.14, pp.2863–2882.

32) Gunter, J. (2003) 'Genital and perianal warts: New treatment opportunities for human papillomavirus infection', Am J Obstet Gynecol Vol.189, pp.3-11.

33) Gupta, R., Beg, Q.K. and Lorenz, P. (2002) 'Bacterial alkaline proteases: molecular approaches and industrial applications', Appl Microbiol Biotechnol Vol.59, pp.15–32.

34) Happi Emaga, T., Andrianaivo, R. H., Wathelet, B., Tchango, J. T. and Paquot, M. (2007) 'Effects of the stage of maturation and varieties on the chemical composition of banana and plantain peels', Food Chemistry Vol.103, pp.590- 600.

35) Hengge, U.R. and Ruzicka, T. (2004) 'Topical immunomodulation in dermatology: potential of toll-like receptor agonists', Dermatol Surg Vol.30, pp.1101–1112.

36) Holzhausen, M., Spolidorio, L.C. and vergndle, N. (2005) 'Role of protease activated receptor-2 in inflammation and its possible implications as putative mediator of periodontitis', Mem. Inst. Oswaldo Cruz, Rio de Janciso Vol.100, No.1, pp. 177 – 180.

37) Jablonska, S. (1984) 'Wart viruses: human papilloma viruses', Semin Dermatol Vol.3, pp.120-129.

38) Jaivoot, P., Palivanich, M., Theppakorn, T., Nitsawang, S. and Kanasawud, P. (2002) 'Separation of proteases from papaya peel', In: 14th Annual Meeting Thai Soc. Biotechnol: Biotechnology for better living in the new economy, Khon Kaen, Thailand.

39) Jinka, R., Ramakrishna, V., Rao, S.K. and Rao, R.P (2009) 'Purification and characterization of cysteine protease from germinating cotyledons of horse gram', BMC Biochemistry Vol.10, pp. 1-11.

40) Kanazawa, K. and Sakakibara, H. (2000) 'High content of dopamine, a strong antioxidant, in Cavendish banana', J.Agricul. and Food Chem Vol.48, pp. 844-848.

41) Ketnawa, S., Sai-Ut, S., Theppakorn, T., Chaiwut, P. and Rawdkuen, S. (2009) 'Partitioning of bromelain from pineapple peel (Nang Lae cultv.) by aqueous two phase system', As. J. Food Ag-Ind Vol.2, No.04, pp.457-468.

42) Khiljee, S., Rehman, N.U., Sarfraz, M.K., Montazeri .H., Khiljee, T. and Lobenberg, R. (2010) 'In Vitro Release of Indian Penny Wort, Walnut, and Turmeric from Topical

Preparations Using Two Different Types of Membranes', Diss Tech vol.17, No.4, pp.27-32.

43) Kim, M., Hamilton, S.E., Guddat, L.W. and Overall, C.M. (2007) 'Plant collagenase: Unique collagenolytic activity of cysteine proteases from ginger', Biochimica et Biophysica Acta Vol.1770, pp.1627–1635.

44) Kim, S.K., Park, P.J., Kim, J.B. and Shahidi, F. (2002) 'Purification and Characterization of a Collagenolytic Protease from the Filefish, *Novoden modestrus',* Journal of Biochemistry and Molecular Biology, Vol. 35, No. 2, pp. 165-171.

45) Klasen, H.J. (2000) 'A review on the nonoperative removal of necrotic tissue from burn wounds', Burns Vol.26, pp.207-222.

46) Lacey, C.J.N., Thompson, H.S.G., Monteiro, E.F., O'Neill, T., Davies, M.L.and Holding, F.P. (1999) 'Phase IIa safety and immunogenicity of a therapeutic vaccine, TA-GW, in patients with genital warts', J Infect Dis Vol.179, pp.612–618.

47) Laemmli, U. K. (1970) 'Cleavage of structural proteins during the assembly of the head of bacteriophage T4', Nature Vol.227, pp.680–685.

48) Lahl, W. J. and Brown, S. D. (1994) 'Enzymatic production of protein hydrolyzates for food use', Food Technol Vol.48, pp. 68–71.

49) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) 'Protein measurement with the folin reagent', The Journal of Biological ChemistryVol. 193, pp.265–275.

50) Mahmoud, M. I. (1994) 'Physicochemical and functional properties of protein hydrolysates in nutritional products', Food Technol. Vol.48, pp.89–95.

51) Mala, B.R., Aparna, M.T.,Mohinis, G. and Vasanti, V.D. (1998) 'Molecular and biotechnological aspects of microbial proteases', Microbiol Molbiol Rev Vol.62, pp.597-635.

52) Mammas, I., Sourvinos, G., Giannoudis, A. and Spandidos, D.A. (2008) 'Human papilloma virus (HPV) and host cellular interactions', Pathol Oncol Res Vol.14, pp.345–354.

53) Mansell, P.W.A., Litwin, M.S., Ichinose, H. and Krementz, E.T. (1975) 'Delayed hypersensitivity to 5-fluorouracil following topical chemotherapy of cutaneous Cancers', Cancer Res Vol.35 pp.1288–1294.

54) Maurer, K.H. (2004) 'Detergent proteases', Current Opinion in Biotechnology Vol.15, pp.330–334.

55) Meltzer, S.M., Bradley, J., Monk, M.D. and Tewari, K.S. (2009) 'Green tea catechins for treatment of external genital warts', Am J Obstet Gynecol Vol.200, pp.233.e1-233.e7.

56) Mohammadian, M.A., Koldeh, J.R. and Sajedi, R.H. (2010) 'The comparison of protease activity and total protein in three cultivars of kiwifruit of Northern Iran during fruit development', Acta Physiol Plant Vol.33, No. 3, pp. 343-348.

57) Mokbel, M.S. and Hashinaga, F. (2005) 'Effect of heat, calcium chloride and modified atmosphere on the shelf life of banana fruits', Food Preserv. Sci Vol.30, pp.179-184.

58) Moon, L., Asher, R. and Fawcett, J. (2003) 'Limited growth of severed CNS axons after treatment of adult rat brain with hyaluronidase', J Neurosci Res Vol.71, pp.21-37.

59) Moore, S. and Stein, W. (1954) A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. **211**, 907-913.

60) Mostafaie, A., Bidmeshkipour, A., Shirvani, Z., Mansouri, K. and Chalabi, M (2008) 'Kiwifruit actinidin: a proper new collagenase for isolation of cells from different tissue', Appl Biochem Biotechnol Vol.144, pp.123–131.

61) Nagase (2009) 'Ingrediants for cosmetics and personal care products', Catalog, pp.1-8.

62) Novak,F.R., Almeida, J.A.G. and Silva, R.S. (2003) 'Banana peel: a possible source of infection in the treatment of nipple fissures', Jornal de Pediatria Vol.7, No.3,pp.221-226.

63) Ozcan, C., Ergun, O., Celik, A., Corduk, N. and Ozok, G. (2002) 'Enzymatic debridement of burn wound with collagenase in children with partial-thickness burns', Burns Vol.28, pp.791-794.

64) Park, P.J., Lee, S.H., Byun, H.G., Kim, S.H. and Kim, S.K. (2002) 'Purification and Characterization of a Collagenase from the Mackerel, *Scomber japonicus*', Journal of Biochemistry and Molecular Biology, Vol. 35, No. 6, pp. 576-582.

65) Prajapati. S., Sharma. S. and Agrawal. V.P. (2009) 'Characterization of *Choreospondias axillaris* (Lapsi) fruit protease', Int J Life Sci Vol.3 , pp. 24-31.

66) Priolo, N., Valle, S.M., Arribere, C., Lopez, L. and Caffini, N. (2000) 'Isolation and Characterization of a Cysteine Protease from the Latex of Araujia hortorum Fruits', Journal of Protein Chemistry Vol. 19, No. 1,pp.39-48.

67) Raimi, O. G., Kappo, M.A., Fajana, O. O., Oku, S. N. and Adeniji, M. A. (2010). 'Alkaline Protease From Maggots: A Likely Source of Industrial Enzyme', Journal of Cell And Tissue Research, Vol. 10, No.3, pp. 2419-2422.

68) Ramakrishna, V., Rajasekhar, S. and Reddy, L.S. (2010) 'Identification and Purification of Metalloprotease from Dry Grass Pea (Lathyrus sativus L.) Seeds', Appl Biochem Biotechnol Vol.160, pp.63–71.

69) Rao, M. B., Tanksale, A.M., Ghalge, M.S. and Deshpande, W. (1998) 'Molecular and Biotechnological aspects of microbial proteases', Microbiol. Mol. Biol. Rev. Vol.62, pp.597 – 635.

70) Rees, B. (1985) 'The Treatment of Warts', Clinics in Dermatology Vol.3, No.4, pp.179-184.

71) Rizzello, C.G., Angelis,M.D., Cagno,R.D., Camarca, A., Silan, M., Losito,I., Vincenzi, M.D.,Bari, M.D.D., Palmisan, F., Maurano, F., Gianfrani, C. and Gobbett, M. (2007)' Highly Efficient Gluten Degradation by Lactobacilli and Fungal Proteases during Food Processing: New Perspectives for Celiac Disease' Applied and Environmental Microbiology, Vol. 73, No. 14, pp. 4499-4507.

72) Rodriguez, R., Jimenez, A., Fernandez-Bolanos, J., Guillen, R. and Heredia, A. (2006) 'Dietary fibre from vegetable products as source of functional ingredients', Trends in Food Science and Technology Vol.17, pp.3-15.

73) Rudenskaya, G. N., Bogacheva, A. M., Preusser, A., Kuznetsova, A. V., Dunaevsky, Ya. E., Golovkin, B. N., and Stepanov,V. M. (1998) 'Taraxalisin—A serine proteinase from dandelion', Taraxacum officinale Webb s.l., FEBS Lett.Vol.437, pp. 237–240.

74) Sabni, B.S. (2005) 'Genital Warts', Case report, pp.1-14.

75) Saini, R., Khim, T.P., Rahman, S.A., Ismail, M. and Tang, H. (2010) 'High-risk human papillomavirus in the oral cavity of women with cervical cancer, and their children', Virology Journal Vol.7, pp.131.

76) Sharrock, S. and Lusty, C. (2000) 'Nutritive value of banana', INIBAP annual report 1999, pp.28-31.

77) Shimomura, T., Miyazawa, K., Komiyama, Y., Hiraoka, H., Naka, D., Marimoto, Y. and Kitamura, N. (1995) 'Activation of hepatocyte growth factor activator', Eur. J. Biochem Vol.229, pp. 257 -261.

78) Shiohara, T., Hayakawa, J. and Nagashima, M.(1989) ' Interferongamma as adjuvant therapy for resistant warts', J Am Acad Dermatol Vol.21, pp. 387-394.

79) Sims, G.K. (2006) 'Nitrogen Starvation Promotes Biodegradation of N-Heterocyclic Compounds in Soil', Soil Biology & Biochemistry Vol.38, pp.2478-2480.

80) Snoeck, R., Bossens, M. and Parent, D. (2001) 'Phase II double-blind, placebocontrolled study of the safety and efficacy of cidofovir topical gel for the treatment of patients with human papillomavirus infection', Clin Infect Dis Vol.33, pp.597–602.

81) Someya, S., Yoshiki, Y. and Okubo, K. (2002) 'Antioxidant compounds from bananas (*Musa* Cavendish)', Food Chem Vol.79, pp.351-354.

82) Steinberg, B.M., Topp, W.C., Schneider, P.S. and Abramson, A.L. (1983) 'Laryngeal papillomavirus infection during clinical remission', N Engl f Med Vol.308, pp.1261-1264.

83) Stone, K., Becker, T., Hagdu, A. and Kraus, S. (1990) 'Treatment of external genital warts: a randomised clinical trial comparing podophyllin, cryotherapy,and electrodessication', Genitourin Med Vol.66, pp.16–19.

84) Storck, S. (2010) 'Condylomata acuminata; Penile warts; Human papilloma virus (HPV); Venereal warts; Condyloma; HPV DNA test; Sexually transmitted disease (STD)', Medicine Plus Medicine Encyclopedia, Link:http://www.nlm.nih.gov/medlineplus/ency/article/000886.html.

85) Svanberg, K., Anderson, T., Killander,D., Wang, I., Stenram, U., Andersson-Engels, S., Berg, R., Johansson, J. and Svanberg, S. (1994) 'Photodynamic therapy of non-melanoma malignant tumors of the skin using topical d-aminolaevulinic acid sensitization and laser irradiation', Br. J. Dermatol. Vol.130, pp.743–751.

86) Tartrakoon, T., Chalearmsan, N., Vearasilp, T. and Meulen, U. (1999) 'The Nutritive Value of Banana Peel (*Musa sapieutum* L.) in Growing Pigs', In: Proceedings of the Deutscher Tropentag, Berlin, 1999.

87) Tochi, B.N., Wang, Z., Xu, S.Y. and Zhang, W. (2008) 'Therapeutic Application of Pineapple Protease (Bromelain): A Review' Pakistan Journal of Nutrition Vol.7, No.4, pp. 513-520.

88) Tyring, S.K., Arany, I. and Stanley, M.A. (1998) 'A randomised, controlled, molecular study of condylomata acuminata clearance during treatment with Imiquimod', J Infect Dis Vol.78, pp.551–555.

89) Wachirasiri, P., Julakarangka, S. and Wanlapa, S. (2009) 'The effects of banana peel preparations on the properties of banana peel dietary fibre concentrate', J. Sci. Technol Vol.31, No.6,pp.605-611, Nov. - Dec. 2009.

90) Walter, S.D. (1974) 'On the detection of household aggregation of disease', Biometrics Vol.30, pp.525-538.

91) Watt, J. M. and Breyer-Brandwijk, M. G. (1962) 'The Medicinal and Poisonous Plants of Southern and Eastern Africa. Being an Account of Their Medicinal and Other Uses, Chemical Composition, Pharmacological Effects and Toxicology in Man and Animal', 2nd ed., E. & S. Livingstone, Edinburgh, U.K.

92) Zahedi, R. (2000) 'Analysis of a protease invaded in mammary development', Lawrence Berkeley National Laboratory. www.Cbcrp.org/research/page Grant.asp? grant_ id = 2137.

93) Zur Hausen, H. (1996) 'Papillomavirus infections--a major cause of human cancers', Biochim Biophys Acta Vol.1288, pp.55-78.