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Tarikh : 1 Disember 2011

Prof. Madya Rosma Ahmad
Pusat Pengajian Teknologi Industri
Universiti Sains Malaysia

Puan,

LAPORAN AKHIR SKIM GERAN PENYELIDIKAN FUNDAMENTAL (FRGS)
Tajuk Projek : Understanding The Structure Function of Cempedak Lead Protease
No. Akaun : 203/PTEKIND/671043

Dengan hormatnya perkara di atas dirujuk.

2. Terlebih dahulu saya ucapkan ribuan terima kasih di atas satu salinan laporan akhir untuk projek penyelidikan seperti tajuk di atas.

3. Adalah dimaklumkan walaupun projek ini telah selesai, kerjasama Jabatan Bendahari dipohon untuk menguruskan penutupan akaun projek pada selewat-lewatnya **31 Disember 2011**. Tempoh ini bertujuan untuk menyelesaikan semua urusan tuntutan dan bayaran yang telah dibelanjakan di dalam tempoh projek. Walau bagaimanapun, puan dinasihatkan supaya tidak mengeluarkan borang-borang pesanan baru di dalam tempoh ini.

4. Selanjutnya sila ambil perhatian terhadap perkara-perkara berikut sekiranya berkaitan:

(i) Semua penerbitan harus merakamkan penghargaan kepada **Skim Geran Penyelidikan Fundamental (FRGS)** dan puan dipohon mengemukakan satu salinan ke Pejabat ini.

(ii) Bahagian Penyelidikan & Inovasi boleh/akan mengagihkan semula peralatan yang telah dibeli menggunakan peruntukan geran ini seandainya terdapat penyelidik lain yang memerlukan peralatan tersebut.

5. Akhir sekali, tahniah di atas usaha dan kejayaan pihak puan dapat menyelesaikan projek ini dengan jayanya.

Sekian, terima kasih.

"BERKHIDMAT UNTUK NEGARA"
'Memastikan Kelestarian Hari Esok'

Yang menjalankan tugas,

(AMRA OTHMAN)
Penolong Pendaftar
Unit Pengurusan Geran & Kontrak

HAH, HAR, SM

LAPORAN AKHIR SKIM GERAN PENYELIDIKAN FUNDAMENTAL (FRGS)
Tajuk Projek : Understanding The Structure Function of Cempedak Lead Protease
No. Akaun : 203/PTEKIND/671043

s.k. Dekan Penyelidikan
Pelantar Sains Fundamental
Pejabat Pelantar Penyelidikan
Universiti Sains Malaysia

Dekan
Pusat Pengajian Teknologi Industri
Universiti Sains Malaysia

Timbalan Dekan
(Pengajian Siswazah & Penyelidikan)
Pusat Pengajian Teknologi Industri
Universiti Sains Malaysia

Ketua Pustakawan
Perpustakaan Hamzah Sendut
Universiti Sains Malaysia

} Disampaikan satu salinan laporan akhir projek untuk simpanan Perpustakaan

Penolong Bendahari Kanan
Unit Kumpulan Wang Penyelidikan
Jabatan Bendahari
Universiti Sains Malaysia

Pegawai Sains
Pelantar Sains Fundamental
Pejabat Pelantar Penyelidikan
Universiti Sains Malaysia

} Mohon kerjasama pihak puan untuk menguruskan penutupan akaun projek selewat-lewatnya pada **31 Disember 2011** dan mohon kemukakan satu salinan penyata kewangan terakhir ke Pejabat ini untuk tujuan rekod

FINAL REPORT
FUNDAMENTAL RESEARCH GRANT SCHEME (FRGS)
Laporan Akhir Skim Geran Penyelidikan Asas (FRGS) IPT
Pindaan 1/2010

RESEARCH TITLE : **Understanding The Structure Function of Cempedak Leaf Protease**
Tajuk Penyelidikan

PROJECT LEADER : DR ROSMA AHMAD
Ketua Projek

PROJECT MEMBERS (including GRA) : 1. Pn Wan Nadiah Wan Abdullah
2. Wong Shen Siung
Ahli Projek

PROJECT ACHIEVEMENT *(Prestasi Projek)*

B

ACHIEVEMENT PERCENTAGE			
Project progress according to milestones achieved up to this period	**0 - 50%**	**51 - 75%**	**76 - 100%**
Percentage			/

RESEARCH OUTPUT		
Number of articles/ manuscripts/ books *(Please attach the First Page of Publication)*	**Indexed Journal**	***Non-Indexed Journal***
	1 (Food Chemistry – under review)	
Conference Proceeding *(Please attach the First Page of Publication)*	**International**	***National***
	2	
Intellectual Property *(Please specify)*		

HUMAN CAPITAL DEVELOPMENT

Human Capital	**Number**				**Others** (please specify)
	On-going		**Graduated**		
Citizen	Malaysian	Non Malaysian	Malaysian	Non Malaysian	
PhD Student					
Master Student	1 (awaiting viva)				
Undergraduate Student			3		
Total	1		3		

EXPENDITURE *(Perbelanjaan)*

C

Budget Approved *(Peruntukan diluluskan)*	:	RM30,000.00
Amount Spent *(Jumlah Perbelanjaan)*	:	RM28,685.22
Balance *(Baki)*	:	RM1,314.78
Percentage of Amount Spent *(Peratusan Belanja)*	:	95.6%

ADDITIONAL RESEARCH ACTIVITIES THAT CONTRIBUTE TOWARDS DEVELOPING SOFT AND HARD SKILLS
(Aktiviti Penyelidikan Sampingan yang menyumbang kepada pembangunan kemahiran insaniah)

D

International

Activity	Date (Month, Year)	Organizer
(e.g : Course/ Seminar/ Symposium/ Conference/ Workshop/ Site Visit) 1. International Conference on Environmental Research and Technology 2. *2nd USM-UNAIR Collaborative Conference*	1. May, 2008 2. Feb, 2009	1. USM 2. Universitas of Airlangga, Surabaya

National

Activity	Date (Month, Year)	Organizer
(e.g : Course/ Seminar/ Symposium/ Conference/ Workshop/ Site Visit)		

PROBLEMS / CONSTRAINTS IF ANY *(Masalah/ Kekangan sekiranya ada)*

E

RECOMMENDATION (*Cadangan Penambahbaikan*)

F

RESEARCH ABSTRACT – Not More Than 200 Words (*Abstrak Penyelidikan – Tidak Melebihi 200 patah perkataan*)

The presence of protease in *Artocarpus integer* leaves which were traditionally used as meat tenderizer was verified through this project. Purification protocols and experiments to characterize the partial purified protease were carried out. Purification procedures of temperature phase partitioning with Triton X-114, ammonium sulphate precipitation and gel filtration chromatography resulted in a 12-fold purity with the specific activity of 76.67 U/mg. The cysteinic nature of the enzyme was confirmed through the inhibition by E-64 and iodoacetamide and was supported by the enhancement of activity with cysteine and 2-mercaptoethanol. The protease retained almost 70% of its activity over a broad spectrum of pH (pH 6 to 12) with optimum activity recorded at pH 10 and the enzyme was stable up to 70 °C with 80 % of its activity recovered. Addition of 5 mM Ca^{2+} stimulates its activity and the kinetic study of the enzyme gives a K_m value of 0.304 mg/ml. The molecular weight of the protease is 69 kDa.

Date : 8 Feb 2011
Tarikh

Project Leader's Signature:
Tandatangan Ketua Projek

COMMENTS, IF ANY/ ENDORSEMENT BY RESEARCH MANAGEMENT CENTER (RMC)
(Komen, sekiranya ada/ Pengesahan oleh Pusat Pengurusan Penyelidikan)

H .....

.....

Name:
Nama:

Signature:
Tandatangan:

Date:
Tarikh:

Cempedak *(Artocarpus integer)* leaf as a new source of proteolytic enzyme for meat tenderization

Rosma, A.*, Cheong, M.W., Liong, M. T., Wan Nadiah, W. A. and Azhar, M.E
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ABSTRACT
Plants (cempedak leaf, jackfruit leaf, papaya leaf, unripe papaya fruit, ginger, galangal, turmeric and lime grass) were screened for protease activity. Unused and wasted cempedak leaf was found to have the highest enzyme activity of 1.8 U/g leaf. The effect of aqueous and crude cempedak leaf extract on meat tenderization was studied. Beef meat samples were immersed in distilled water (as control), crude leaf extract and solution containing commercial protease enzyme for 1 h at room temperature (25 °C) and subjected to various evaluation methods. An increase in tenderness, pH, water-holding capacity and protein solubility was observed in meat treated with cempedak leaf extract. Electrophoretic (SDS-PAGE) patterns also showed a reduction in the number of high molecular weight bands in meat samples after enzyme treatments. Remarkable deformation and disruption of muscle fibre and connective tissue in enzyme-treated samples were observed with scanning electron microscope. In conclusion, crude cempedak leaf enzyme can be an alternative source of plant protease enzyme with high commercial potential.

Keyword: Cempedak leaves, electrophoresis, meat tenderization, proteolytic enzyme.

1. INTRODUCTION

Among those attributes of eating quality of meat, tenderness has been identified as the most important factor affecting taste perception and satisfaction of consumer. Meat tenderization can be divided into physical or chemical methods. Among these methods, treatment with protease enzymes is one of the popular methods applied for meat tenderization purpose (Naveena *et al*. 2004; Gerelt *et al*., 2000). Since early generation, papain was used to tender the beef and produced steak as soft as fresh meat. At present, most enzymes used for this purpose are plant protease such as bromelain and papain which have been widely used in Europe and United States of America (Chen *et al*., 2006).

Besides these popular protease enzymes, tenderizing efficacy of cucumin from the melon variety fruits of *Cucumis trigonus Roxb* plant and zingibain which was isolated from ginger rhizome (*Zingiber officinale roscoe*) was studied by Naveena *et al*. (2004). Dried and coarsely ground fruits of *C. trigonus Roxb* (locally known as kachri) are traditionally used as meat tenderizer in some part of India. This research reported on screening of selected Malaysian plants and herbs for protease activity which were cempedak leaves (*Artocarpus integer*), jack-fruit leaves (*Artocarpus heterophyllus*); papaya (*Carica papaya*); lemon grass (*Cymbopogon citrates*); galangal (*Alpinia galangal*); ginger (*Zingiber officinale*) and turmeric (*Curcuma domestica*). The crude extract which contained the highest protease activity was then applied to fresh meat for tenderization study.

2. MATERIALS AND METHODS

Source of plants

Unripe papaya fruit, lemon grass, galangal, ginger and turmeric rhizomes were obtained from wet market of Taman Tun Sardon. Cempedak and jack-fruit leaves were freshly plucked from trees that were located beside Desasiswa Tekun, Universiti Sains Malaysia. Each of the plants was blended with 2 parts of distilled water and filtered using cheese cloth. Extracts were then further filtered with filter paper (Whatman No 1) prior to centrifugation (3500 rpm for 15 min). Extracts were immediately assayed for protease activity.

Protease activity assay

Proteolytic enzyme activity was determined by the modified method of Wang *et al*. (1974) with bovine serum albumin (BSA) as substrate. Duplicated samples were prepared prior to analysis. The enzyme reaction mixture was incubated at 38 °C in a heating block (TECHNE, DB-3A, England) for 20 min. Subsequently, the reaction was stopped by the addition of 4.0 ml trichloroacetic acid (5% w/v) and the sample was centrifuged at 5970 ×g (High Speed Centrifuge HETTICH, Germany) for 30 min. The absorbance of the supernatant was

NEUTRAL PROTEASE ACTIVITY OF *ARTOCARPUS INTEGER*'S LEAF

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Protease from *Artocarpus integer* plants or better known as *Cempedak* tree by the locals were extracted from its leaves by homogenizing with 4 parts of sodium phosphate buffer (pH 7, 0.05M) containing 1% (w/v) ascorbic acid and 0.02% (w/v) PVPP. The homogenized mixtures were centrifuged at 10,000×*g* (4 °C, 15 mins) and were later added with further 0.01 % (w/v) PVPP. The crude enzyme extract was subjected to series of ammonium sulphate concentrations ranging from 0% up to 90% (w/v). The precipitated proteins were later removed through centrifugation at 5000×*g* (4 °C, 20 mins) and recovered with a minimum volume of buffer. The protein suspension was dialyzed against sodium phosphate (pH7, 0.05M) with two changes of buffer (100 times volume of sample) for 24 hours. The selection of ion exchange matrix involved both the anion (Sephadex DEAE and DEAE Cellulose) and cation (SP Sephadex and CM Sephadex) exchangers. The effectiveness of the exchangers was evaluated based on their ability to retain most of the targeted protein and was eluted with 0.4M NaCl.

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Title: Artocarpus integer Leaf Protease as Meat Tenderizer: Purification and Characterization

Article Type: Research Article (max 7,500 words)

Keywords: Artocarpus integer; Cysteine protease; Characteristic; Proteolytic

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Corresponding Author's Institution: Universiti Sains Malaysia

First Author: Zulfigar Siti Balqis

Order of Authors: Zulfigar Siti Balqis; Wan Abdullah Wan Nadiah, MSc; Ahmad Rosma, PhD

Abstract: The presence of protease in Artocarpus integer leaves which were traditionally used as meat tenderizer was verified through the formation of a band at 69 kDa with caseinolytic zymography. Purification procedures of temperature phase partitioning with Triton X-114, ammonium sulphate precipitation and gel filtration chromatography resulted in a 12-fold purity with the specific activity of 76.67 U/mg. The cysteinic nature of the enzyme was confirmed through the inhibition by E-64 and iodoacetamide and was supported by the enhancement of activity with cysteine and 2-mercaptoethanol. The protease retained almost 70% of its activity over a broad spectrum of pH (pH 6 to 12) with optimum activity recorded at pH 10. The enzyme was stable up to 70 oC with 80 % of its activity recovered. Addition of 5 mM Ca2+ stimulates its activity and the kinetic study of the enzyme gives a Km value of 0.304 mg/ml.

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Artocarpus integer leaf protease: Purification and characterisation

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ABSTRACT

The presence of a protease in *Artocarpus integer* leaves, which are traditionally used as a meat tenderiser, was verified by the presence of a band at 69 kDa, using caseinolytic zymography. Purification by temperature phase partitioning with Triton X-114, ammonium sulphate precipitation and gel filtration chromatography yielded a preparation with a 12-fold increase in enzyme purity and a final specific activity of 76.67 U/mg. The cysteinic nature of this enzyme was confirmed through inhibition of enzyme activity by E-64 and iodoacetamide and enhancement of activity by cysteine and 2-mercaptoethanol. The protease retained 70% of its activity over a broad pH range (pH 6–12), with optimal activity recorded at pH 10 and 40 °C. The enzyme was stable at temperatures up to 70 °C, with 80% of its activity intact. Addition of 5 mM Ca^{2+} stimulated enzyme activity and a kinetic study of the enzyme yielded K_m and V_{max} values of 0.304 mg/mL and 0.735 mg/mL/min, respectively.

1. Introduction

Current market demand in the food industry has contributed to an ever-increasing interest in discovering new enzymes with distinct substrate specificities. The quest for new enzyme sources often involves consideration of the ease of enzyme availability and the possible economic benefits that may arise from developing the potential enzyme source. The genus *Artocarpus*, which covers a vast area of South and Southeast Asia, New Guinea, the southern Pacific, Sri Lanka, India, Pakistan and Indo-China towards the Malaysian archipelago (Lemmens, Soerianagara, & Wong, 1995), has been used for various applications. *Artocarpus integer* (*Thunb. Merr*) syn. *A. chempeden* (*Lour.*) Stokes syn. *Polyphema champeden*, commonly known as *chempedak*, is known for its edible fruit, and previous studies of this species have focused on the characterisation of bioactive compounds and lectins derived from various parts of the plant (Abdul Rahman, Karsani, Othman, Abdul Rahman, & Hashim, 2002; Boonlaksiri et al., 2000; Lim, Chua, & Hashim, 1997).

Consumers judge meat quality using several considerations, including tenderness, juiciness and meat flavour. Enzymatic treatment is one method of meat tenderisation, and the proteases most widely used for tenderisation originate from plants. Proteases act on muscle fibres and connective tissues, and cooking temperatures act as a means of controlling enzyme activity. Although others may prefer methods such as mechanical tenderisation or natural aging, commercial proteases such as papain and bromelain, which are available in powdered form, are suitable for domestic use and can be made commercially available.

Leaves of *A. integer* are used as a meat tenderiser by rural Malay communities, suggesting that a strong proteolytic enzyme is present in this plant. This hypothesis was verified by a previous work of Rosma, Cheong, Liong, Wan Nadiah, and Azhar (2008), who studied the capability of the crude extract to act as a meat tenderiser and compared the efficacy of the extract to a commercial enzyme. Positive results, such as improved tenderness, augmented water-holding capacity, increased protein solubility and remarkable deformation of muscle fibres and connective tissues, were observed.

Besides the discovery of *A. integer*'s tenderising capabilities, the commercial development of this protease is also supported by the availability of leaves from trimming and pruning methods used during industrial fruit cultivation. This agricultural activity removes excess leaves, twigs and small branches to avoid excessive tree growth and to divert nutrients more effectively to developing fruits (Yaacob, 1980), hence providing a continuous enzyme source. Given the ease of availability and abundant supply of the enzyme source, *A. integer* offers a new alternative for the profitable production of a beneficial enzyme that might replace existing commercial proteases.

However, a fundamental knowledge of the properties of an enzyme, such as its stability, optimal working conditions and mechanistic class, is essential before considering its potential uses in the food industry. In this study, we therefore continued the previous work by Rosma et al. (2008) to isolate and characterise *A. integer* leaf protease.

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2. Materials and methods

2.1. Reagents

Ethylenediaminetetraacetic acid disodium salt (EDTA), iodoacetamide, phenylmethylsulfonyl fluoride (PMSF), *trans*-epoxysuccinyl-l-leucylamido-(4-guanidino)butane (E-64), sodium dodecylsulphate (SDS) and Sephadex G-75 were purchased from Fluka (Buchs, Switzerland). Casein, bovine serum albumin (BSA), acrylamide, *N,N*-methylbisacrylamide, polyvinylpolypyrrolidone (PVPP) and low range molecular weight (LMW) marker (6500–66,000 Da) were purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals were obtained from other commercial sources and were of the highest purity available.

2.2. Extraction of protease

The protease used in the present study was extracted from *A. integer* leaves, which were freshly plucked from a tree located in Lunas, Kedah. Fresh *A. integer* leaves were cut and homogenised with 5 volumes (w/v) of cold 0.05 M sodium phosphate buffer, pH 7, containing 100 mM NaCl, 10 mM ascorbic acid, and PVPP. The ratio of fresh leaves to PVPP was 1:1 (w/w). The homogenate was filtered through a muslin cloth before centrifugation at 10,000*g* for 20 min. The supernatant was subjected to temperature phase partitioning by the addition of Triton X-114, 6% (v/v) at 4 °C. The mixture was stirred at 4 °C for 15 min and warmed to 37 °C in a thermostatic bath until the solution became spontaneously turbid, then centrifuged at 2000*g* for 10 min at room temperature. The upper clear phase was carefully pipetted out and subjected to ammonium sulphate precipitation. This fraction is known as the crude extract.

2.3. Purification

Ammonium sulphate precipitation was performed by the addition of saturated ammonium sulphate up to a saturation of 60% (v/v) and left to stand for 30 min. The precipitate was recovered by centrifugation at 7000*g* for 15 min and was reconstituted with a minimum volume of 0.05 M sodium phosphate buffer, pH 7, containing 10 mM ascorbic acid and dialysed against the same buffer for 24 h. The dialysed enzyme (15 mL) was loaded onto a Sephadex G-75 column (2.5 × 50 cm) equilibrated with 0.05 M sodium phosphate buffer, pH 7, and eluted with the same buffer at a flow rate of 48 mL/h. Active fractions from gel filtration chromatography were pooled for further characterisation and will henceforth be referred to as 'purified enzyme' in this manuscript. All purification procedures were carried out at 4 °C.

2.4. Assay of protease activity

Protease activity was determined using casein as the enzyme substrate, as previously described by Kaneda and Uchikoba (1994). An enzyme solution of 0.1 mL was added to 0.9 mL of 1% (w/v) casein in 0.2 M sodium phosphate buffer solution, pH 7.0, and incubated at 38 °C in a heating block for 20 min. The reaction was stopped by the addition of 3 mL of trichloroacetic acid (5%, w/v) and then centrifuged at 6000*g* for 20 min. The absorbance of the supernatant was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme catalysing the production of 1 μmol of tyrosine per minute at 38 °C. An L-tyrosine standard curve was constructed for a concentration range of 0–180 μg/mL.

2.5. Determination of protein concentration

Protein concentration was determined by the dye binding method (Bradford, 1976). BSA was used as the standard protein and a standard curve was constructed for a concentration range of 0–1 mg/mL.

2.6. Caseinolytic zymography

Zymographic analysis was performed using SDS–PAGE through a 12% separating gel and a 4% stacking gel under non-reducing conditions (Bah, Paulsen, Diallo, & Johansen, 2006) with the incorporation of 1% (w/v) casein as the substrate for the enzyme instead of gelatine. Samples were prepared by mixing with loading buffer in a 2:1 ratio. The loading buffer consisted of 0.125 M Tris–HCl, pH 6.8, 20% (v/v) glycerol and 2% (w/v) SDS. The gels were carefully rinsed with distilled water before incubating in a 0.2 M sodium phosphate buffer, pH 7, at 40 °C for 24 h following electrophoresis. The gels were stained with 0.1% (w/v) Coomassie Blue R250 dye in 5:1:4 (v/v/v) methanol:acetic acid:distilled water and destained in the same solution without the dye. Caseinolytic activity led to the appearance on the gels of clear bands on a blue background. The molecular weight of the protease was estimated using UVI band Advance Software (UVITEC, Cambridge, UK), and the standard used was LMW (6.5–66 kDa).

2.7. Optimal temperature

The effects of temperature on enzymatic activity were determined using a standard protease assay (Section 2.4). The incubation temperature was varied to 4 °C, 25 °C, 40 °C, 50 °C and 60 °C. A plot of relative enzyme activity versus temperature was constructed to obtain the optimal temperature for maximum enzyme activity.

2.8. Optimal pH

The optimal pH was determined by measuring the hydrolysing activity of the purified enzyme on 1% (w/v) substrate dissolved in buffers of different pH. BSA was used as a substrate in buffers ranging from pH 4 to 12, and casein was used as a substrate in buffers ranging from pH 6 to 12 because of the insolubility of casein in acidic buffers. The buffers used were sodium citrate (pH 4–6), sodium phosphate (pH 6–8), glycine–NaOH (pH 9–10), $NaHCO_3$–NaOH (pH 11) and KCl–NaOH (pH 12–13). The molarity for all buffers used was 0.2 M. A plot of relative enzyme activity versus pH was constructed, to obtain the optimal pH for maximum enzyme activity.

2.9. Temperature stability studies

The temperature stability of the enzyme was investigated by incubating the enzyme in heating blocks set at temperatures ranging from 40 °C to 80 °C for 15 min. Aliquots of enzyme were removed at the end of the incubation period and cooled in an ice bath. The aliquots were assayed (Section 2.4) for relative enzyme activity, which was expressed as a percentage of the initial activity.

2.10. Effects of inhibitors, activators and metal ions

The effects of various compounds on the catalytic activity of *A. integer* leaf protease were studied using group-specific protease inhibitors. The enzyme was pre-incubated with different inhibitors at a ratio of 1:1 (v/v) at designated concentrations for 10 min at 30 °C. An assay performed without any inhibitors served as a reference for the initial enzyme activity and was indicated as 100%

activity. Similar experiments were performed using thiol-specific reagents, cysteine and 2-mercaptoethanol, to study the effects of activators on the proteolytic activity of *A. integer* leaf protease. The enzyme was pre-incubated with activators at final concentrations ranging from 0 to 30 mM for 15 min at 30 °C at a 1:1 ratio. The effect of metal ions on proteolytic activity was studied by pre-incubating the enzyme with different metal ion solutions (1:1) (v/v) at final concentrations of 1, 5 and 10 mM for 15 min at 30 °C.

2.11. Effect of substrate concentration on reaction velocity

The effect of substrate concentration on the reaction velocity of the enzyme was investigated using casein prepared in the optimal pH (pH 10) buffer. The concentration of casein was varied from 0.5 to 50 mg/mL, and casein was incubated with the enzyme for 20 min at 38 °C. The protease activity was assayed as described in Section 2.4. The Michaelis–Menten constants, K_m and V_{max}, were calculated from a Lineweaver–Burk plot.

2.12. Effect of the protease on natural substrates

The relative activity of *A. integer* protease was tested against four different natural substrates (casein, BSA, gelatine and haemoglobin). Enzyme activity was measured by incubating 0.1 mL of purified enzyme with 0.9 mL of 1% (w/v) substrate prepared in a 0.2 M sodium phosphate buffer, pH 7, and enzyme activity was assayed as described in Section 2.4. Relative enzyme activity was calculated by comparing the protease activity on each substrate with casein as the reference.

3. Results and discussion

3.1. Extraction and purification of protease

The extraction of enzyme from *A. integer* leaves required careful buffer formulation, as high concentrations of phenolic compounds in the leaves hamper its efficient extraction. *Artocarpus* plants are rich in phenolic compounds (Jagtap & Bapat, 2010), and these compounds are able to bind and precipitate enzymes, resulting in a loss of enzymatic activity. The use of Triton X-114 in the extraction medium (Bordier, 1981) trapped phenolics in the detergent phase, permitting the removal of these compounds and subsequently increasing the purity of the enzyme preparation by 1.2-fold (Table 1). The sudden onset of turbidity signified that membrane proteins, phospholipids, phenolic compounds and chlorophylls were trapped in a detergent-rich phase, while soluble and loosely associated membrane proteins and enzymes were recovered in the aqueous phase (Bru, Sanchez-Ferrer, Perez-Bilabert, Lopez-Nicolas, & Garcia Carmona, 1995). This method of temperature phase partitioning with Triton X-114 served both as an extraction method and as a purification procedure. The conventional method of ammonium sulphate precipitation at 60% saturation was employed to remove non-protein components that interfered with spectroscopic absorbance at 280 nm. The ammonium sulphate precipitate step increased the concentration of enzyme in the bulk extract, increasing the specific activity of the preparation to 15.32 U/mg. A final gel filtration step using Sephadex G-75 columns resolved an active peak (Fig. 1) with a specific activity of 74.67 U/mg, thus increasing the enzyme purity of the preparation by 12.6-fold. Fractions 19–26 were pooled for further characterisation.

3.2. Optimal pH

The optimal pH was determined using both BSA and casein as enzyme substrates. The use of casein is limited to a neutral to basic pH range because of the low solubility of casein under acidic conditions. For this reason, BSA was also adapted as an enzyme substrate to obtain a wider pH profile for protease activity. Sarath, de la Motte, and Wagner (1989) reported that variations in assay buffer pH can manipulate ionisable groups on protein surfaces and affect their susceptibility to enzymatic digestion. Therefore, the optimal pH obtained from an analysis may reflect more on a substrate's susceptibility to the enzyme at a given pH rather than on the actual activity of the enzyme. However, our analysis showed variability in the degree of hydrolysis but showed no effect on the pH profile. Both casein and BSA showed maximal enzymatic activity at pH 10 (Fig. 2a).

The enzyme was relatively inactive in the acidic pH range, with no activity detected below pH 4, similar to other cysteine proteases, such as papain (Glazer & Smith, 1971) and ervatamin A (Nallamsetty, Kundu, & Jagannadham, 2003). The enzymatic activity accelerated as the pH increased up to pH 10. However, a sharp decline in activity was observed beyond this maximal point, and the half maximal activity of *A. integer* leaf protease with casein occurred between approximately pH 9.2 and 11.5. Unlike serine proteases, which are generally more active in the alkaline pH range (Antao & Malcata, 2005), the optimal pH of cysteine proteases is commonly distributed within the near neutral region (Salleh, Razak, Rahman, & Basri, 2006). However, a few studies of cysteine proteases extracted from legume leaves, such as FLCP-1 from *Phaseolus vulgaris* (Popovic, Kidric, Puizdar, & Brzin, 1998) and *Glycine max* (Huangpu & Graham, 1995), showed maximal activities at pH 9.5 when these enzymes were tested against synthetic substrates. The maximal performance of *A. integer* leaf protease at pH 10 was therefore relatively high compared with other plant cysteine proteases.

3.3. Optimal temperature and temperature stability

The effect of temperature on the degree of hydrolysis was tested by varying the incubation temperature to a refrigerated condition (4 °C), to room temperature (25 °C) and through a range of temperatures to 40 °C, 50 °C and 60 °C. The protease activity increased steadily as the temperature increased up to 40 °C. However, a decrease in activity was observed with further increases in temperature. However, almost 45% of enzyme activity was still detectable at 60 °C (Fig. 2b).

The temperature stability of the enzyme was investigated from a temperature range of 40–80 °C. No significant difference was found in residual enzyme activity from 40 °C to 70 °C, with almost 80% of enzyme activity retained. However, a sharp decline was

Table 1
Purification of protease from *A. integer* leaves. Protease activities were determined using casein as a substrate.

Stage	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude	44.7	265	5.94	1.0	100
Triton X-114	33.8	247	7.32	1.2	93
$(NH_4)_2SO_4$ dialysate	2.61	40.0	15.3	2.6	15
Sephadex G-75	0.07	4.48	74.7	12.6	2

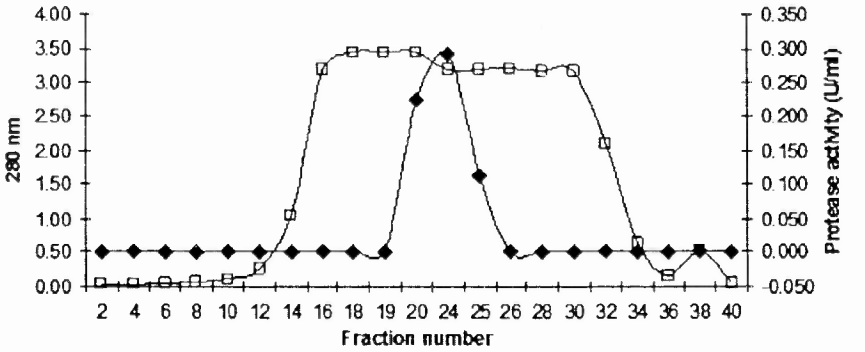

Fig. 1. Elution profiles of protein (□) and protease activity (♦) on sample eluted from Sephadex G-75 column. The column was equilibrated with 50 mM sodium phosphate buffer at pH 7 with a flow rate of 48 mL/h.

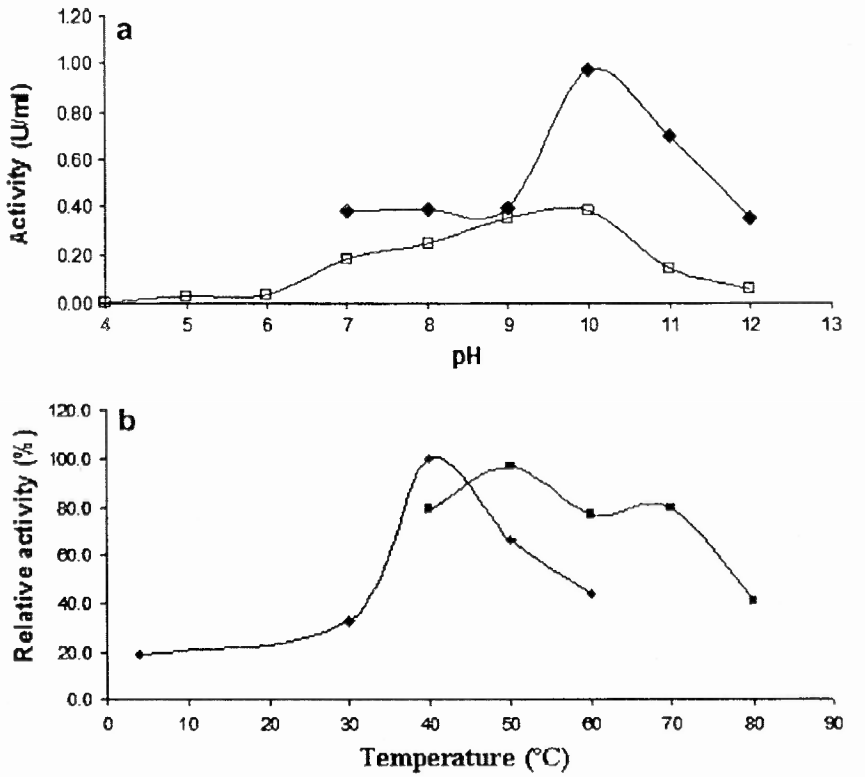

Fig. 2. (a) Effects of pH on protease activity. Protease activity was determined using BSA (□) and casein (♦) within a pH range of 4–12. (b) Effects of temperature on protease activity (♦) and stability (■).

observed after incubation at 80 °C, at which point more than half of enzyme activity was lost (Fig. 2b). However, prolonged incubation of the crude *A. integer* enzyme was impossible at elevated temperatures because of a rapidly occurring browning reaction that occurred following the oxidation of the remaining phenolic compounds in the solution.

It is notable that *A. integer* leaf protease exhibited a wide range of high temperature stability. Similar responses were also observed for procerain, a cysteine protease extracted from the latex of *Calotropis procera* (Dubey & Jagannadham, 2003), which retained its full activity during incubation for 15 min at temperatures up to 70 °C. Such stability was also observed for ervatamin A extracted from *Ervatamia coronaria* (Nallamsetty et al., 2003). The wide range of high temperature stability explains the traditional used of the leaf by rural populations whereby the leaves and meat are cooked together for about 15–30 min to tenderise the meat (personal communication).

3.4. Effect of inhibitors, activators and metal ions

Various inhibitors specific to different classes of proteases were used to investigate the amino acid residues that might define the mechanistic class of this enzyme. The inhibitors used were PMSF (serine), E-64 and iodoacetamide (cysteine), EDTA (metallo) and pepstatin A (aspartic). The enzyme was markedly inhibited by both thiol-specific inhibitors, with only 30% and 17% of residual enzyme activity remaining following treatment with E-64 and iodoacetamide, respectively (Table 2). E-64 is a highly selective cysteine protease inhibitor and does not react with serine proteases, except for trypsin. The high degree of residual activity following treatment

Table 2
Effects of various inhibitors on the activity of *A. integer* leaf protease.

Inhibitor	Mechanistic class	Concentration (mM)	Relative activity (%)
Iodoacetamide	Cysteine	1.0	17
E-64	Cysteine	1.0	30
EDTA	Metallo	10	88
Pepstatin A	Aspartic	0.01	93
PMSF	Serine	1.0	100

with pepstatin A (93%) ruled out the possibility of this enzyme being an acidic protease, which was also supported by the fact that *A. integer* leaf protease was relatively inactive under acidic conditions (Section 3.2). As for PMSF and EDTA, both inhibitors were unable to effectively inhibit enzyme activity, although the highest effective concentrations for these inhibitors were tested. Addition of the chelating agent EDTA had no marked effect on protease activity, suggesting that the enzyme was able to hydrolyse proteins in the absence of metal ions. This result ruled out the possibility of this enzyme being a metalloprotease, which would require metal ions to function.

Two thiol-specific activators, cysteine and 2-mercaptoethanol, were incubated with the enzyme at different concentrations to study the effect of activators on *A. integer* leaf protease activity. These results (Table 3) revealed that 5 mM cysteine was needed to produce the highest protease activation level (200%). However, the addition of cysteine beyond this maximal point led to a loss of activity, likely because of the reduction of disulphide bridges (Antao & Malcata, 2005). In the case of 2-mercaptoethanol, enzyme activity was stimulated with increasing activator concentrations up to 30 mM, producing an almost 200% increase in activity. The activating effect of these thiol-specific activators and inhibition by the cysteine-specific inhibitors strongly supports the hypothesis that the enzyme extracted belongs to a family of cysteine proteases.

Table 3
Effects of various reagents on the protease activity of *A. integer* leaf.

Reagent	Concentration (mM)	Relative activity (%)
Cu^{2+}	1	26.6
	5	7.5
	10	5.6
Mn^{2+}	1	53.8
	5	53.8
	10	66.4
Zn^{2+}	1	108.4
	5	74.8
	10	64.3
Mg^{2+}	1	97.0
	5	90.9
	10	76.2
Ca^{2+}	1	58.0
	5	114.0
	10	112.6
Cysteine	0.5	171.8
	1.0	195.8
	2.0	162.5
	5.0	212.5
	10.0	147.9
	20.0	134.3
	30.0	71.9
2-Mercaptoethanol	0.5	127.1
	1.0	130.8
	2.0	159.4
	5.0	135.4
	10.0	160.1
	20.0	162.5
	30.0	187.5

A. integer protease was inhibited by all metal ions tested except Ca^{2+} (Table 3). The activating effect of Ca^{2+} on *A. integer* protease activity was detected at a concentration of 5 mM, unlike a serine protease isolated from the latex of jackfruit (*A. heterophyllus*), which was slightly inhibited by the presence of this cation (Prasad & Virupaksha, 1989). Ca^{2+} is commonly known to have a stabilising effect on enzymes, where it is hypothesised to provide protection from protein autolysis (Mihalyi, 1978, chap. 5). However, other metal ions used inhibited the activity of *A. integer* protease. Cu^{2+} greatly reduced the proteolytic capability of the enzyme even at the low concentration of 1 mM, with only 27% of relative enzymatic activity retained.

3.5. Effects of substrate concentration on reaction velocity

The kinetics of the purified protease were studied by varying casein concentrations from 0 to 50 mg/mL at the optimal pH. Protease activity was increased with increasing substrate concentrations only up to a concentration of 20 mg/mL. Subsequent addition of substrate resulted in a sharp decrease of activity and complete inhibition at a concentration of 50 mg/mL. This phenomenon is known as substrate inhibition, where the reaction velocity increases with the addition of substrate until a point where further substrate addition causes a decline in enzyme activity (Reed, Lieb, & Nijhout, 2010). Substrate inhibition occurred in almost 20% of tested enzymes of different enzymatic classes (Chaplin & Bucke, 1990, chap. 1). Reed et al. (2010) listed serine proteases among the enzymes that exhibited such characteristics. One of the hypotheses proposed to explain this phenomenon was the existence of a second subsite where additional substrates could bind and act as allosteric inhibitors (Reed et al., 2010). A Lineweaver–Burk reciprocal curve was plotted with casein as a substrate at concentrations of up to 20 mg/mL, and the K_m and V_{max} values obtained from this curve were 0.304 mg/mL and 0.735 mg/mL/min, respectively.

3.6. Effects of natural substrates on protease activity

Natural substrates are commonly used in assays that involve less-characterised or newly characterised enzymes. Four natural substrates (casein, gelatine, BSA and haemoglobin) were assayed against *A. integer* protease to study the possible industrial applications of the protease on different proteins. Enzyme activities were compared to casein, which was set as 100% activity and decreased in activity from haemoglobin (138%), followed by BSA (81%) and gelatine (24%). The high degree of casein hydrolysis suggests that *A. integer* leaf protease may have useful applications in the milk clotting industry. However, this outcome is not conclusive, and a more detailed study on milk clotting properties is required. Besides being relatively susceptible to hydrolysis by *A. integer* leaf protease, both haemoglobin and casein are extensively used in protease assays, since they are inexpensive and readily available in highly purified forms (Sarath et al., 1989).

3.7. Caseinolytic zymography

Caseinolytic zymography was used specifically to detect the presence of protease in *A. integer* leaves and was performed on the Triton X-114 treated extract, the ammonium sulphate dialysate, and the pooled gel filtration fraction. Because of the lack of scientific evidence establishing the presence of this enzyme in *A. integer* leaves, this analysis was essential to detect the protease in the crude extract. A single distinct band at 69 kDa was

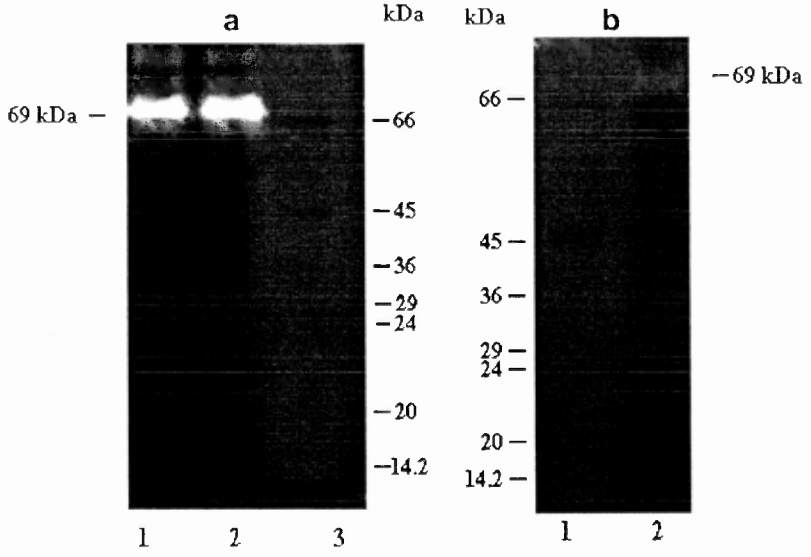

Fig. 3. Zymography analysis of the fractions from each step in the purification of protease from *A. integer* leaves. (a) Lane 1 – crude enzyme, Lane 2 – dialysed enzyme, Lane 3 – molecular weight markers. (b) Lane 1 – molecular weight markers, Lane 2 – gel filtration fraction.

detected at all three stages of purification (Fig. 3a and b). These results showed no significant changes in the bands apparent in the sample treated with Triton X-114 compared with the ammonium sulphate dialysate sample (Fig. 3a), suggesting that no noticeable denaturation occurred following ammonium sulphate fractionation. The pooled gel filtration fraction also showed a band with the same molecular weight, providing further evidence of the presence of protease in the isolated fraction (Fig. 3b). The other plant cysteine proteases, papain, bromelain (Bruno, Pardo, Caffini, & Lopez, 2002), ananain (Lee, Albee, Bernascomi, & Edmunds, 1997), araujiain (Priolo, Marcelle del Valle, Arribere, Lopez, & Caffini, 2000) and ervatamin (Nallamsetty et al., 2003), have smaller molecular weight molecules between 16 and 45 kDa, making the *A. integer* leaf protease a unique cysteine protease. Cysteine proteases are represented by 70 families belonging to 12 different clans (Salas, Gomes, Hernandez, & Lopes, 2008). A structural analysis and amino acid sequence study are necessary to determine the relationship of this *A. integer* protease to other cysteine protease families and clans.

4. Conclusions

This study has successfully isolated a 69 kDa cysteine protease from the leaves of *A. integer*. The purification strategy used adapted the temperature phase partitioning method using Triton X-114 to isolate the active enzyme from interfering phenolic compounds. The data presented indicate that the purified enzyme was stable in the alkaline region with an optimal pH recorded at pH 10. The purified enzyme was also able to retain its activity after 15 min of pre-incubation at temperatures up to 70 °C. In addition, enzyme activity was not affected by inhibitors of serine protease (PMSF), aspartyl protease (Pepstatin A) or metalloprotease (EDTA). Stimulation of enzyme activity was observed following the addition of 5 mM Ca^{2+}, and a kinetic study of the enzyme gave K_m and V_{max} values of 0.304 mg/mL and 0.735 mg/mL/min, respectively. The general characteristics reported in this work are the first steps towards acquiring a fundamental understanding of this enzyme. The findings presented in this work will hopefully establish a potential source of protease that can be further developed and characterised.

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