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RESEARCH ARTICLE

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF GENETICALLY ENGINEERED PAPAIN FROM CARICA PAPAYA

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Abstract

Background: Commercially available papain is prepared by performing a tedious and costly purification method that yields papain at different degrees of purity. **Methods:** The expressed ~43 kDa active papain was purified by single-step affinity chromatography and confirmed by SDS-PAGE analysis and Western blotting. A 3.7-fold lab-scale purification and 64 % recovery yield of the enzyme were achieved. **Results:** In this research work, the recombinant stem papain has been expressed in *E. coli* BL21. The purified enzyme exhibited maximum activity at a pH range of 5-8 on synthetic substrates studied with an optimum temperature of 45 °C. It was inhibited by E-64 (10μM) but only slightly inhibited by non-cysteine protease inhibitors and activated by all the Sulphur-containing reducing reagents studied. Kinetic studies on the enzyme yielded lower values of K_i and K_m coupled with a higher k_{cat}/K_m ratio for recombinant papain; implying that it had more affinities towards the inhibitor used and all the substrates than commercial papain. **Conclusion:** This study successfully developed expression, characterization, and cultivation conditions for better production of recombinant papain from *Carica papaya* in *E.coli* (BL21-AI). Drying technologies such as spray drying and freeze-drying could be explored to establish the best means of recombinant papain technology

Keywords*: Papain, Escherichia coli BL21-A, Expression, Purification*

INTRODUCTION

Background

The numerous industrial and therapeutic applications of enzymes necessitated their production. For commodities enzymes, large-scale processes are involved (Hodgson, 1994). On the other hand, for specialties enzymes that are utilized in advanced applications and research, small-scale processes are employed (Thomas *et al*., 2002). Enzymes derived from plants and animals are normally extracted or recovered from the corresponding tissues and fluids. Conversely, fermentation is used for the production of microbial enzymes. In this

case, the enzymes are recovered from either cell paste (intracellular) or spent fermentation medium (extracellular).

For over a long period of time, microbial enzymes have been steadily and increasingly substituting other forms of enzymes and might now stand for about 90% of the entire enzymes world market (Illanes, 2008). This is attributable to the fact that microorganisms are exceptional cell systems for enzyme production. In fact, they are fairly versatile, metabolically active and very simple to produce on a large scale by fermentation. In addition, their genetic and environmental manipulations are quite simple. Furthermore, their microbial nutritional requirements are uncomplicated and supply is not affected by recurring fluctuations (Blanch and Clark, 1997). These qualities have made the microbial enzymes production to be more consistent, easier and cheaper. This reality has significantly increased the quantity and quality of microbial enzymes products through excellent fermentation processes. As the approval of insulin in 1982, more than 120 recombinant drugs have been approved and become available as very valuable therapeutic options (Theo, 2007).

Papain is a name given to protease enzymes present in carica plant (*papaya*). Papain (EC 3.4.22.2) is the most important protease present in extracts of papaya and is the chief enzyme fraction present in the juice of the carica papaya fruit (Kelly, 1996). Papain also contains some other small cysteine endopeptidases (ananain, comosain) and other enzymes such as peroxidases and glucosidases (Maurer, 2001). More detailed characterization of bromalain has been conducted as compared to fruit papaya, although the later enzyme was known much earlier than the former one (Harrach *et al.*, 1998). Papain has broader industrial and therapeutic uses in contrast to bromalain that is commercially unavailable (Larocca *et al.*, 2010).

Study Aim:

This study aimed at developing expression, characterization and cultivation conditions for better production of recombinant papain from Carica papaya.

Materials and Methods:

Chemicals

L-Cysteine, L-arabinose and casein were purchased from Sigma Chemicals Company (USA). Luria Bertani (LB) growth media used was a product of Merck, Germany. All other chemicals used were of analytical grade.

Cloning of the papain gene

A complete mRNA sequence of the papain gene (MEROO647) was accessed from the National Centre of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). A pair of primers Papain-F (3'-AAA TAA AAC ATC TCA- 5') and Papain-R (5'-GCC GTA GAA AAG CCC TAT TAA A- 5), were designed using Primer3 software (version 4.0) (http://frodo.wi.mit.edu) and synthesized by Eurogentec AIT (Singapore) to be used for reverse transcription polymerase chain reaction (PCR) experiments.

The ACAC bases were added to the forward primer in order to make it compatible with the TOPO vector (Invitrogen, USA). The reverse transcription PCR was performed using the SuperScript III Reverse Transcriptase Kit (Invitrogen, USA) following the manufacturer's protocol. A 30 cycle's reaction was performed at 94 ◦C (2 min) for denaturing, 58 ◦C (30 s) for annealing, and 72 ◦C (2 min) for extension. The PCR product was sequenced (Solegen, Korea), and the results were blasted against the NCBI database to confirm that the correct gene was amplified. Subsequently, this PCR product was cloned into a pENTR/TEV/DTOPO cloning vector (Invitrogen, USA) following the manufacturer's instructions.

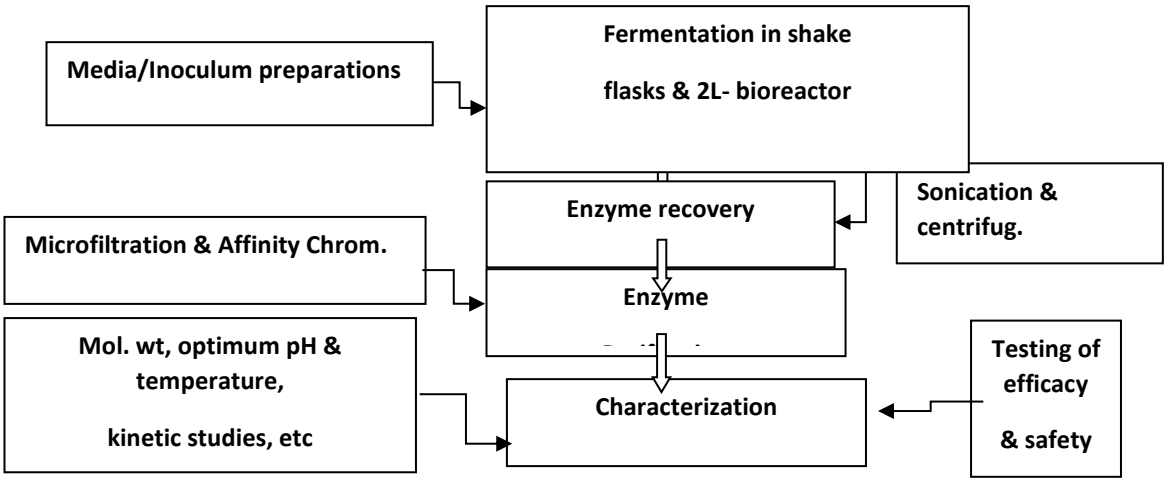


Fig. 1. Flowchart showing the experimental Methodology.

Expression and Transformation of recombinant papain

The recombinant papain molecule was sub-cloned into a pDEST17 (Invitrogen, USA) expression vector by a LR recombination reaction using the LR clonase II enzyme mixture (Invitrogen, USA) following the manufacturer's instructions. The antibiotic resistance gene(s) on the plasmid only allows the transformed cells to grow on the plate. The colonies that grow on the plates are analyzed to confirm the presence of the recombinant plasmid and the papain gene. Positive transformants were selected by colony PCR and restriction enzyme digestion and were grown in 100 mL LB broth at 37 ◦C with medium agitation (200 rpm) (Rabelo *et al*., 2024.)

Enzyme Extraction and Recovery

The harvested frozen cells were suspended in 50 mM potassium phosphate, pH 7.8 and then subjected to sonication (sonicator, 150v/t model, Biologics, Inc. USA) on ice for 3 minutes (Cycles: 0.5, Amplitude: 20). The sonication was conducted using 6-10 sec burst, with 10 sec interval. This was followed by centrifugation (8000×*g*) at 4 ºC for 30 min and the supernatant was collected for further analysis (Xu *et al*., 2006).

Purification of Papain

Recombinant papain was purified using the Fast Protein Liquid Chromatography (FPLC) technique. The cell lysate was centrifuged at 10,000 rpm for 30 min at 4 ◦C to collect the supernatant. The supernatant (15 mL) was loaded onto a pre-equilibrated Ni-NTA column according to the manufacturer's protocol (Qiagen, Germany). After a wash step, the bound recombinant papain was recovered by adding 30 mL of elution buffer containing 250 mM imidazole to the column prior to centrifugation.

Determination of Protein Concentration

The protein concentration of purified papain was determined by Bradford's method (Bradford, 1976). A protein standard curve was generated by initially preparing serial dilutions of bovine serum albumin (BSA) of different concentrations and the absorbance readings were measured at 595 nm wavelength. The average of triplicate absorbance readings were then plotted against the different BSA concentrations to produce the protein standard curve Extrapolation of the curve or using the linear regression equation generated, allows the estimation of the concentration of unknown protein samples.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

After each step of enzyme recovery and purification (in section 3.2.4), the protein fractions were tested by SDS-PAGE in 12.5 % polyacrylamide (Laemli, 1970) using a Mini-PROTEAN® system. Protein (four volumes) was added to one volume of 5X SDS sample buffer and the mixture was heated at 95 °C for 5 minutes. The sample was then loaded on SDS-polyacrylamide gel consisting of 12 % resolving gel and 5 % stacking gel. The electrophoresis was carried out at 150 V for 1 hour in a mini tank (Bio-Rad, USA) that was filled with 1X running buffer. The gels were then stained with Coomassie staining solution for 30 minutes and then destained with destaining solution. The protein bands were eventually visualized using an imager (AlphaImager, USA).

Western Blot Analysis

The protein bands on the SDS-polyacrylamide gel were transferred to polyvinylidene fluoride (PVDF) membrane using Mini-Trans-Blot system (Bio-Rad Laboratory, Inc., USA). The membrane was subsequently incubated at 4 ºC (for one hour) with 5% dried milk powder in TBST buffer (0.1 M Tris–HCl, pH 7.4, 0.9% NaCl, and 0.1% Tween 20) so as to block non-specific binding. This was then followed by incubation with primary antibody (Anti-His Tag, clone HIS.H8, Millipore) in a ratio of 1:1000. The membrane was later incubated with secondary antibody (goat anti-rabbit IgG (H+L)-HRP) conjugate, Bio-Rad Laboratory, Inc., USA) at the same ratio as described above. The membrane was then incubated with BCIP (5-bromo-4-chloro-3-indolyl phosphate) color development solution for 4 hrs. Finally, the PVDF membrane was air dried and the images of the Western blot were visualized and analyzed using AlphaImager software (Alpha Innotech, USA).

Characterization of the Recombinant Papain

The effects of diverse factors influencing the activity and stability of the purified recombinant papain were studied. These include pH, temperature, activators and inhibitors that can improve or repress the activity of the enzyme. Besides, kinetic studies were undertaken on recombinant papain using four synthetic substrates: N-α-carbobenzoxy-L-alanyl-*p*-nitrophenyl ester (ZANPE), N-α-carbobenzoxy-L-arginyl-L-arginine-*p*-nitroanilide (ZAANA), N-α-carbobenzoxy-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide (ZPVANA) and L-pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide (PFLNA). Furthermore, kinetic of papain inhibition by E-64 against synthetic substrates was also examined. Conducting kinetic studies on recombinant papain under optimum conditions of pH and temperature is highly desirable for enhancement of its standardization as an enzymatic preparation for therapeutic, industrial and analytical applications.

Effects of pH

Effect of pH on synthetic substrates was determined over a pH range of 2-12 in the following 0.1 M buffer systems: Glycine/HCl (pH 2−4.), sodium phosphate (pH 5−8), Tris−HCl (pH 8–10) and sodium bicarbonate (pH 10−12). Prior to the addition of the substrate, 0.3 ml of Papain preparations were pre-incubated with 1.4 ml of each of the buffer solution at 45 ^{o}C for 5 mins. This was followed by the addition of 0.3 ml substrate solution into the mixture and incubated for further 5 mins. 1974). For ZAANA changes in the absorbance were measured at 410 nm for the *p*-nitroaniline generated (Filippova *et al*., 1984; Rowan and Buttle, 1994).

Effects of Temperature

The optimum temperature of recombinant Papain (recPPN) and commercial Papain (cmPPN) was determined by using ZAANA as the substrate under its established optimum pH condition. The assay was carried out in 0.1 M sodium phosphate buffer (pH 4.0) as described above for this substrate (section 3.2.8.1) at various temperature range of 4-75 ^{o}C.

Effects of Inhibitors of Papain Activity

The effects of specific cysteine protease inhibitors (Salvesen and Nagase, 2001) on amidolytic activity of Papain were determined by measuring the residual enzyme activity on ZAANA substrate. This was conducted after pre-incubation of the enzyme preparation in 0.1 M phosphate buffer (pH 8.0) at 45 ºC for 30 mins in the presence of inhibitor. The inhibitors evaluated were 1, 10-phenantroline (10 mM), E-64 (10 µM), Phenyl methyl sulfonyl fluoride (PMSF) (1 mM), leupeptin (100 µM), chymostatin (100 µM) and pepstatin A (1 µM). The concentration of each inhibitor was selected based on supplier's information and inhibitor's mode of action. Controls were prepared by pre-incubating the Papain solution with the appropriate solvent used to dissolve the inhibitors.

Effects of Activators on Papain Activity

The effect of Papain activity enhancers was determined by measuring the enzyme activity on ZAANA substrate after pre-incubating the enzyme sample with the activator in 0.1 M phosphate buffer (pH 8.0) at 45 ºC for 30 mins. The activators assayed were Dithiothreitol (DTT) (15mM), cysteine (15 mM) and 2-mercaptoethanol (30 mM). The concentration used for each activator was chosen on the basis of supplier's information and activator's mode of action. The activity was compared with that of the enzyme control without the addition of inhibitors or activators.

Titration of the Papain Active Site with E-64 (inhibitor)

Active site titration was conducted in order to study a dose–response relationship between the purified papain and increasing concentrations of E-64 (cysteine protease inhibitor). The method was adapted from Barrett *et al*., (1982) with minor modification. The enzyme was pre-incubated with the activation buffer (0.1 M phosphate buffer, pH 4.0, containing 4 mMEDTA and 8 mM DTT). Fractions of the enzyme mixture (150 µl) were incubated with 50 µl of different concentrations (0–10 µM) of E-64 solutions for 30 mins at 45 ºC. Then, the residual activity of Papain was measured on ZAANA (Rowan and Buttle, 1994). Enzymatic activity was expressed in percent of residual activity on the substrate compared to the control.

Determination of Kinetic Parameters on Synthetic Substrates

Kinetic parameters (k_{cat}, K_m and V_{max}) were determined on N-α-carbobenzoxy-L-arginyl-L-arginine-*p*-nitroanilide (ZAANA). The concentrations of substrates used ranged from 0.1 to 0.6 mM in the reaction mixture. From the product concentrations as a function of time curves, instantaneous rates were calculated at several different substrate concentrations under described conditions. Kinetic parameters of hydrolysis by papain were calculated using linear regression analysis by means of Lineweaver-Burk plots.

Inhibition Kinetics of E-64 on Papain using Synthetic Substrates

The determinations of K_i were carried out by lowering the enzyme and inhibitor concentrations to obtain a nonlinearity of dose-response curves. Papain solution was pre-incubated in an activation buffer (as described in section 3.2.8.1) with increasing concentrations of E-64 inhibitor (1-5µM) for 10 min at 45 ºC. Residual activity of the enzyme was measured on the four different synthetic substrates as described above (section 3.2.8.1) under optimum conditions of pH and temperature. Control experiments were also carried out in the absence of inhibitor. Inhibitory constants (K_i) were determined as the intersection on the x-axis of the secondary plots of the slopes (K_m/V_{max}) obtained from the Lineweaver–Burk lines against E-64 concentrations.

***In vitro* Testing of Recombinant papain**

In order to assess the therapeutic potentials of the recombinant bromelain, *in vitro* studies were conducted using different cell lines. The use of cell culture is highly advantageous, as it creates systems for ready, direct access and evaluation of tissues. The ready access to the cells provides the option for easy studies of cellular mechanisms that may suggest new potential drug targets. Besides, in the case of pathologically-derived tissue, it can be applied in the

evaluation of therapeutic agents that potentially may treat the dysfunction. In this study, *in vitro* cytotoxicity assays were conducted to evaluate the effect of recombinant bromelain against tumor cell lines (Murine melanoma, Tm5) and normal cell lines (Chinese hamster ovary, CHO). The efficacy of the enzyme was compared to that of cisplatin, a metal based drug that is administered in clinical treatment. Furthermore, anti-inflammatory activity of the enzyme was investigated on murine macrophage cell lines (Arnau *et al.*, 2020).

***Cytotoxicity Assays of Recombinant papain*:** The application of *in vitro* cytotoxicity assays on cultured tumor cell lines continues to represent the standard method for the initial screening of antitumor therapeutic agents (Heinrich *et al.*, 2011). Hence, two different cell lines Tm5 and CHO were used in evaluating the activity of bromelain. The CHO cell line was cultured using a Dulbecco's Modified Eagle's Medium (DMEM) adjusted to contain 3.7 g/L sodium bicarbonate, pH 7.2, supplemented with 10% fetal bovine serum, streptomycin (100 µg/ml) and penicillin (100 U/ml) as antibiotics (Heinrich, *et al.*, 2011). On the other hand, The Tm5 cells were cultured as described by Oba-Shinjo (2006) using the Roswell Park Memorial Institute (RPMI) 1640 medium (Cult lab), adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/L glucose, 2.38 g/L HEPES, at a pH of 6.9., supplemented with 10% fetal bovine serum and gentimycin (10 µg/ml). All the cells were kept in a humidified atmosphere with 5% CO_2 at 37 °C. When the cells reached confluence, they were removed from the T-flasks using 10 mM EDTA in phosphate buffered saline (PBS) and then counted for the experiments.

Anti-inflammatory Assay for Recombinant Bromelain

The murine macrophage cell lines RAW 264.7 were cultured and incubated using the same conditions as described above for CHO cell line (Heinrich *et al.*, 2011).

Measurements of Nitrite Production

The cells were cultured in triplicate in 96-well, micro culture plates at 37°C in humidified 5% (v/v) CO_2 for 24 hrs preceding the harvest of culture supernatants for analysis. Cell culture (100 µl) was then pipetted into each well of the plate at a concentration of 1.0×10^5 cells/well. DMEM containing bromelain (50 µl) was then added into each well and the mixtures were incubated for 2 hrs at 5% CO_2 and 37°C. This was followed by the addition of 50 µl of DMEM containing lipo polysaccharide (LPS) into to the wells (to make final LPS concentration of 1 µg/ml) and incubated for 24 hrs. The absorbance was measured in microplates at 540 nm against a calibration curve with sodium nitrite standard (Fana *et al.*, 2012).

Preparation of Nitrite Standard Curve

Nitric oxide (NO) plays a significant role in immune response, vascular regulation and apoptosis. It is readily oxidized to nitrite and nitrate which are used to quantify NO production. Griess assay was used to prepare the standard curve. Briefly, a serial dilution of nitrate standard was prepared to appropriate concentrations and pipetted into microplate wells. Then 50 µl of griess reagent 1 were added into the wells and followed by 50 µl of Griess reagent 2. The plate was allowed to stand for 10 min at room temperature for color development and then absorbance was measured at 540nm wavelength (Raghunath *et al.*, 2020)

Annual Effective Dose $\boldsymbol{A_{eff.}}\ \boldsymbol{(mSvy^{-1})}$ and External Dose $\boldsymbol{ED}\ \boldsymbol{(nGyhr^{-1})}$

In this study, the annual effective dose is calculated by employing the outdoor external dose, occupancy factor, or proportion of the total outdoor time that an individual is exposed to the radiation $\mu = 0.2$ of $8760\ hr$ within a year, and the conversion factor $(CF) = 0.7\ (SvGy^{-1})$ for converting the absorbed dose in air to an effective dose.

The equations given by UNSCEAR, 2000 and Qureshi et al. 2014 are as follows:

$$A_{eff.}\ (mSvy^{-1}) = ED\ (nGyhr^{-1}) \times 0.2 \times 8760\ hr \times 0.7\ SvGy^{-1} \times 10^{-3} \dots\dots\dots\dots (3)$$

Where

$$ED\ (nGyhr^{-1}) = \frac{DR\ (\mu Svhr^{-1})}{Q} \times 10^{-3} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (4)$$

Where Q is equal to 1

Estimation of Excess Lifetime Cancer Risk (ELCR)

In order to assess the cancer risk of the workers resulting from BIR exposure, excess lifetime cancer risk (ELCR) was estimated by using equation (5) Abdulkareem *et al.* 2023.

$$ELCR = A_{equi.} \times \mathrm{DL} \times \mathrm{RF} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (5)$$

A_{equi}. Is the Annual Equivalent Dose, DL is the average Duration of Life and is approximately equal to70 $years$ RF, is the Risk Factor or fatal cancer risk measured in per sievert(Sv^{-1}). For stochastic effects from low-dose background radiation, ICRP 2007. A value of 0.05 for public exposure.

Results:

Recombinant papain expression and purification

In this study, the recombinant papain was expressed as soluble (active) and insoluble (inactive) enzyme in *E. coli* BL21-AI. The amplified fragments were successfully cloned into the pBR322 vector. A double digestion of the plasmid DNA by BamHI and EcoRI identified a positive clone, which was observed to have fragments of 2841 and 862 bp in size. The restriction BamHI cut the inserted DNA fragment once at 820 bp and EcoRI cut the TOPO vector DNA once at 673 bp.

Recombinant *E. coli* harboring papain gene was grown in shake flasks for the expression study. The enzyme expression was induced at OD_{600} of 0.4 with 0.2% L-arabinose. Harvesting of the culture was attained at 4-hr post induction period. The harvested cells were subjected to sonication for cell lysis and then followed by centrifugation to afford a clarified crude cell lysate.

The enzyme fractions were further purified by affinity chromatography using either Nickel-NTA spin column or Ni-NTA His•Bind resin via an automated fast protein liquid chromatography (FPLC) system. Moreover, microfiltration was also partly employed to concentrate the papain lysate before purification by chromatographic technique. The activity of recombinant papain was determined using ZAANA substrate as described by Silverstein (1974) with minor modifications.

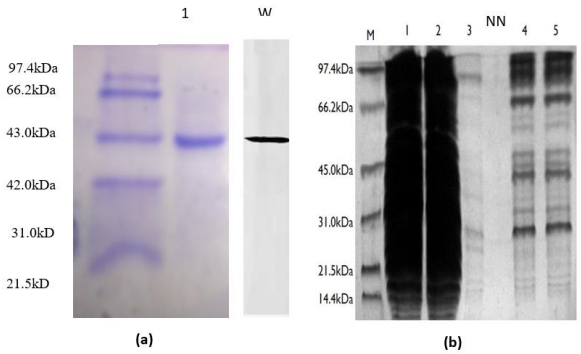


Figure 1: Showing the SDS-PAGE analysis of the purified recombinant Papain expressed in *E.coli* BL21-AI.

(a) SDS-PAGE showing purified soluble papain.
Lane M: protein molecular weight marker;
lane 1: purified soluble recombinant papain:
lane W: western blot analysis.

(b) SDS-PAGE showing purified insoluble (denatured) papain.
Lane M: protein molecular weight marker;
lane 1 & 2: crude enzyme lysate;
lane 3: washing step;
lanes 4&5: eluted purified insoluble recombinant papain

Table 1: Purification scheme for recombinant Papain (Ni-NTA chromatography)

Protein fraction		[a]Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
SOLUBLE						
	Crude	2572±12	41.26± 0.46	62.40	1.00	100
	Purified	1646±08	7.2± 0.08	228.60	3.70	64.0
INSOLUBLE	Crude	476±03	26.8± 0.28	17.80	1.00	100
	Purified	288±02	5.9± 0.03	48.80	2.70	60.50

[a]ZGNA substrate was used for the activity measurement.

Table 2: Purification scheme for recombinant papain (Microfiltartion & Ni-NTA chromatography)

Protein fraction	[a]Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude lysate	31604± 21	418.60 ±05	75.50	1.00	100
Microfiltration	24743 ± 13	140.05 ±03	176.67	2.34	78.29
Ni-NTA	17964 ± 10	50.41 ± 0.54	356.36	4.72	56.84

[a]ZGNA ester was used for the activity measurement

Characterization of recombinant papain

Papain at various pH Ranges

The results are shown in the Table below. It can be observed that the pH optimum ranges for recPPN and cmPPN were found to be 5-8 and 6-8, respectively. This implied that the recPPN has a slightly broader pH range than cPPN (Table 4.4).

Table 3: Specific activities of Papain towards synthetic substrates measured at different pH values

	Activity (U/mg	
pH	RecPPN	CmPPN
4.0	2.46 ±0.08	2.03 ± 0.06
6.0	1.874 ±0.05	1.621±0.07
8.0	1.524±0.07	1.158±0.03
12	1.168±0.06	0.896±0.04

Glycine/HCl (pH 2−4.), Sodium phosphate (pH 5−8)„ Tris−HCl (pH 8–10) and sodium bicarbonate (pH 10−12).

Papain at Various Temperature Ranges

The results obtained for the effect of temperature on both papain activities are shown in Figure 4.2. It can be inferred that both enzymes have maximum activities at optimum temperature of 45 °C. Nevertheless, recPPN had higher specific activity over cPPN at the optimum temperature. Both enzymes tend to lose complete activity at a temperature of 75 °C. The optimum temperature of 45 °C obtained using ZAANA substrate is similar to the value reported by Amid *et al.*, (2011).

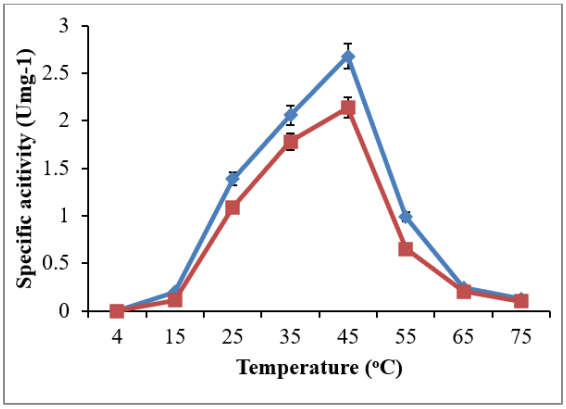


Figure 2: Activity of papain at different temperature values: recPPN (♦) and cmPPN (■) activity vs. temperature measured on ZPVANA

Effect of Inhibitors and Activators on Papain Activity

The effects of inhibitors and activators on papain activity are presented in Table 4.5. The results show the residual activity of the purified enzyme after its activation and inhibition with the following class-specific inhibitors: PMSF, chymostatin, leupeptin (inhibitor of serine proteases), pepstatine A (inhibitor of aspartic proteinases), 1, 10-phenanthroline (metalloproteinase inhibitor) and E-64 (inhibitor of cysteine proteases) on ZAANA.

Table 4: Effects of specific inhibitors/activators on papain activity

Inhibitor/activator	Concentration	Residual activity	
		recPPN	CmPPN
Control		100	100
Inhibitor			
1, 10 phenanthroline	10 Mm	93.21	95.63
PMSF	1 mM	91.80	89.52
Leupeptin	100 µM	84.33	87.10
Chymostatin	100 µM	85.24	83.82
bE-64	10 µM	0	0
aPepstatine A	1 µM	97.21	95.74
Activator			
aCysteine	15 mM	187.11	133.83
aDTT	15 mM	148.54	136.30
2-mercaptoetahnol	30 mM	143.80	151.72

Note. Concentrations of activators/inhibitors used were selected based on literature reports and supplier's instructions

Among the various thiol activators tested, cysteine was found to be the most potent activator of this sulfhydryl protease. This property of thiol-dependence confirmed that they belong to the family of cysteine proteases. The cysteine residue involved in the catalytic site of the purified enzyme was manifested by the inhibition with 10 µM E-64 and by activation with reducing agents such as 15 mM cysteine and 15 mM DTT.

Titration of Active Site of papain with E-64

The results obtained in the active site titration assays are shown in Figure 3

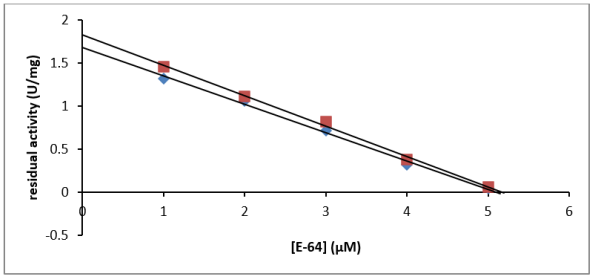


Figure 3: Active site titration of papain with E-64: Plots for recPPN (♦) and cmPPN (■). Residual activity was measured on ZAANA.

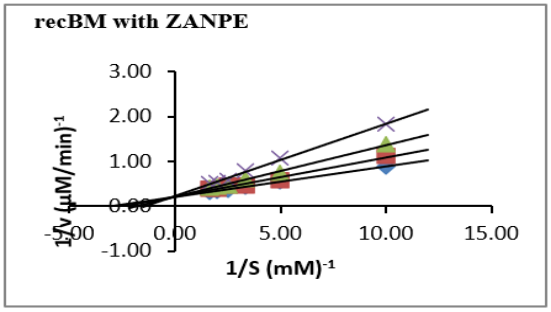
Estimations of Kinetic Parameters for Papain

The Michaelis–Menten constant (K_m), maximum reaction velocity (V_{max}), turnover number (k_{cat}) and k_{cat}/K_m for the synthetic substrates were determined by plotting the activity data obtained under pH and temperature optima as a function of substrate concentration in Line weaver–Burk plots. The results obtained from the plots in absence of E-64 (control) are presented in Figures 4.4, 4.5. It can be deduced that the values of K_m varied significantly for both papain with different substrates.

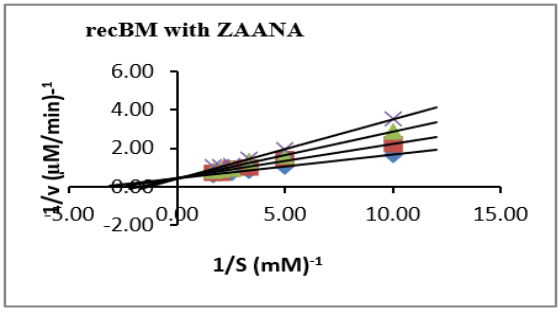
Table 5: Kinetic parameters obtained for papain towards synthetic substrates and E-64

Enzyme	K_m (mM)	V_{max} (µM/min)$^{-1}$	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_i for E-64 (10^{-3} mM)
recPPN	0.29	2.32	8.24	28.41	4.40
cmPPN	0.42	2.25	7.50	17.86	6.22

At pH 4.0 and temperature 45°



(a)



(b)

Figure 4: Lineweaver–Burk plots of cBM in the absence and presence of E-64. Four different synthetic substrates were used: (a) ZANPE (b) ZAANA Measurement was made at different E-64 concentrations (0-5 µM): control, 0 µM (♦), 1 µM (■), 3 µM (▲) and 5 µM (X)

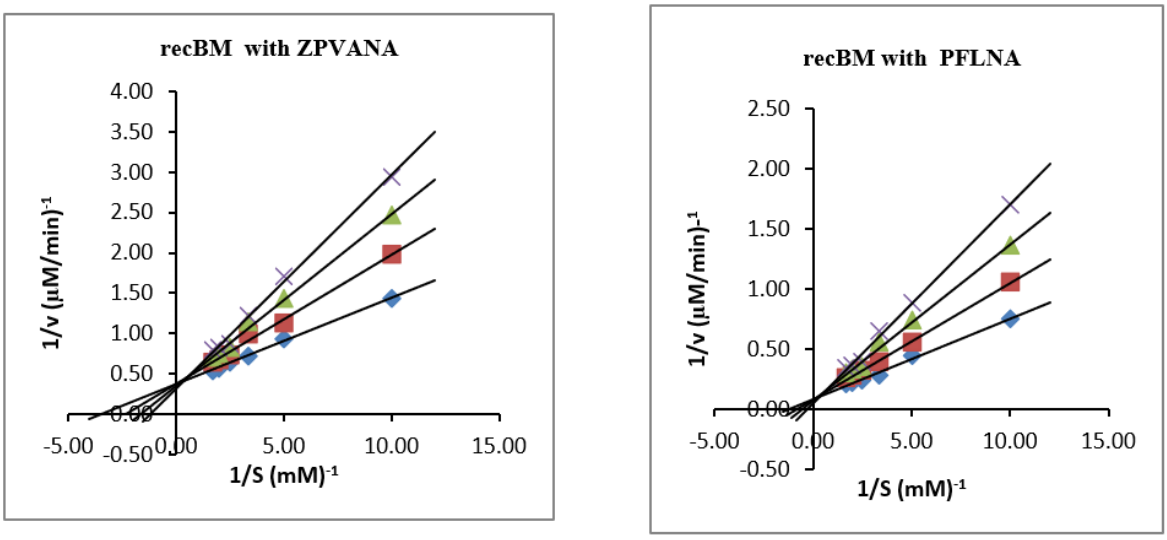


Figure 5: Lineweaver–Burk plots of recBM in the absence and presence of E-64. Four different synthetic substrates were used: (a) ZPVANA and (b) PFLNA. Measurement was made at different E-64 concentrations (0-5µM): control, 0 µM (♦), 1 µM (■), 3 µM (▲) and 5 µM (X).

Discussion:

The results obtained from this study revealed that the enzyme was effectively expressed in *E. coli* BL21-AI as native (soluble) and denatured (insoluble) enzyme as shown in Figure 4.1. This agreed with the work of Amid et al., 2012. The samples from each step of the purification technique were analyzed by SDS-PAGE as exhibited in Figures 3.1a & 3.1b for soluble and insoluble bromelain, respectively. It could be seen clearly from these figures that the recombinant enzyme was purified to near homogeneity in one affinity purification step. The molecular weight of the purified soluble enzyme (containing six-His tag and signal peptide) was found to be approximately 43 kDa on SDS–PAGE (Figure 3.1a).

The presence of recPPN is further confirmed by Western blot analysis as anti-His IgG antibody in Western blot binds specifically to the His_{6x} tag in recPPN gene (Figure 3.1a). The results depicted the size of the enzyme to be around ~43 kDa, a value which is very close to the predicted size of ~39 kDa size of the natural papain gene (AT3G5470) in the absence of His_{6x} tag.

Papain is known to have broad substrate specificity and hydrolyses a large number of natural and synthetic substrates (Maurer, 2001). Thus, papain activity can be assayed using synthetic substrates. The activities of recombinant papain (rec PPN) were compared with those of commercial papain (cmPPN) using synthetic substrates at different pH values (2-12).

The activities of recPPN and cmPPN started to decline drastically beyond pH 4.0 and the enzymes were almost completely inactivated at pH 12. Moreover, papain activity-pH profile obtained is consistent with that of most cysteine proteases belonging to the papain family. In pH range of 5–8, the enzymes have hydrogen bond between the thiol and imidazole functional group (Cys-His) that is critical for catalytic activity (Valles *et al.*, 2008).

Study of proteinase inhibition is vital in obtaining information on the nature of catalytic site, the identification of individual proteinases and quantification through active site titration. It also enhances investigation of the biological functions of the proteinases. The results obtained indicated that cmPPN is slightly more sensitive to the most of inhibitors as compared to recPPN (Spok 2023).

This demonstrated that E-64 reacted specifically with the active site of the protease (Barret *et al.*, 1982) and inhibited it even in the presence of reducing agents used to activate cysteine proteases

(Salvesen and Nagase, 2001). It has been established that cysteine proteases and E-64 react on an equimolecular basis, thus, the inhibitor is very suitable for the enzyme active site titration (Barrett *et al.*, 1982).

The plot of residual recPPN activity as a function of E-64 concentration generated a straight line which intersected the abscissa at 5.13 µM E-64. This value (5.13 µM) is considered to be the molar concentration of the enzyme active site which corresponds to 50.9% of the original molar concentration of active recPPN (calculated from protein concentration and molecular mass of the enzyme). On the other hand, for cmPPN, the intersection was at 5.25 µM E-64. Thus, the molar concentration of the cmPPN active site is 5.25 µM and this corresponds to 49.3% of the original active enzyme. This signifies that almost half of both the enzymes are in inactive form. Similar effects had been previously reported for other cysteine protease (Hieronymain I and II) by Bruno *et al.*, (2003 & 2006).

Determination of kinetic parameters of an enzyme is an essential tool in elucidating the entire catalytic process. The lowest K_m values for both cmPPN and recPPN are recorded (0.34 and 0.29 mM), while higher values were seen (0.60 and 0.80 mM). Besides, recPPN has the lowest K_m values (0.29 - 0.8 mM) in same substrates studied as compared to cmPPN (0.34 - 0.68 mM). The k_{cat}/K_m ratio sometimes called the specificity constant is the best way to compare the catalytic efficiency of enzyme (Morcelle *et al.*, 2004). ZAANA was found to have the highest k_{cat}/K_m (49.57 and 52.53 $mM^{-1}s^{-1}$) as such this substrate is highly recommended for the determination of the papain activity at optimal experimental conditions. ZAANA has been a classic substrate for cysteine endopeptidases (Filippova *et al.,* 1984).

Conclusion

This research work explored the expression, purification and characterization of recombinant papain from *E.coli* BL21-AI. Using various techniques of enzyme recovery and purification, the study had successfully expressed and purified the enzyme. The results indicated that both the soluble and insoluble forms of papain were purified to near homogeneity using a single step of Ni-NTA affinity chromatographic technique as established by the SDS-PAGE and western blot analysis.

The results obtained revealed that recombinant papain clearly showed higher activities than commercial papain against all the substrates tested at various pH and temperature ranges. Furthermore, the *in vitro* study showed that both recombinant Papain (recPPN) and commercial Papain (recPPN) exhibited cytotoxic activity against Tm5 tumor cell lines almost comparable to that of cisplatin.

The study had sucessfuly improved on the existing laboratory-scale strategies for the production of highly purified and characterized recombinant papain expressed in *E. coli* BL21-AI. It had also been discovered that eventhough the recPPN used in this study lacks glycosylation and contains His6x tag and signal peptide, its catalytic properties are almost indistinguishable from those of mature cPPN. It is hoped that the study will pave way for better utilzation of the enzyme in its several application fields.

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Authors' Contributions

This work was carried out in collaboration among all authors. Authors MSM, JOE and MMAD Designed the study. Authors MSM and VKP performed the Bioinformatics' analysis. Authors MSM, APD, MU wrote the protocol and wrote the first draft of the manuscript. Authors MSM and HRA manage the literature search of the work. All authors read and approved the final manuscript

Competing Interests

The author declares that there are no conflicts of interest related to this study.

Reference

Amid, A., Ismail, N., Yusof, F., Salleh, H.M. (2020). Expression, purification and characterization of a recombinant stem bromelain from *Ananas comosus*. *Process Biochem.,* 46, 2232-2239.

Anwar, T. Ahmad, B. & Younus, H. (2021). Cross-linked stem bromelain: A more stabilized active preparation. *Biocatalysis and Biotransformation* 25(6), 453-458.

Arnau, J., Lauritzen, C., Petersen, G.E. & Pedersen, J. (2020). Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins. *Protein Expr. Purif.,* 48, 1–13.

Aspmo, S. I., Horn, S. J., & Eijsink, V. G. H. (2023). Enzymatic hydrolysis of Atlantic cod (*Gadus morhua L.*) viscera. *Proc. Biochem.,* 40(5), 1957–1966.

Babu, B. R., Rastogi, N. K. & Raghavarao, K. S. M. S. (2020). Liquid–liquid extraction of bromelain and polyphenol oxidase using aqueous two-phase system. *Chemical Engineering and Processing,* 47, 83–89.

Baez, R., Lopes, M.T., Salas, C.E. & Hernandez, M. (2020). In vivo antitumoral activity of stem

Rabelo, A.P.B., Tambourgi, E.B. & Pessoa, A. (2024). Bromelain partitioning in two-phase aqueous systems containing PEO–PPO–PEO block copolymers. *J. Chromat B.*, 807, 61–68.

Raghunath, T. Mahajan and Shamkant B. Badgujar (2020). Biological aspects of proteolytic enzymes: A Review. *J. Pharm. Res.,* 3(9), 2048-2068.

Raja, B.S., Sucheta, P.D., Vijayan, E. and Suresh, K.G.(2024). Roles of proteases in pathology of cardiac diseases. *Molecular and Cellular Biochemistry,* 263, 241-256.

Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.,* 62(3), 597–635.

Singer, A.J., McClain, S.A. & Taira, B.R. (2020). Rapid and selective enzymatic debridement of porcine comb burns with bromelainderived Debrase: Acute-phase preservation of noninjured tissue and zone of stasis. *J. Burn Care Res.* 31(2), 304-9.

Snowden, H.M., Renfrew, M.J. & Woolridge, M.W. (2021). Treatments for breast engorgement during lactation. *Cochrane Database Syst. Rev,* (2), CD000046.

Song, M., Nie, H., Zhou, Y., Zhu, L. & Bao, J. (2021). Affinity Adsorption of Bromelain on Reactive Red 120 Immobilized Magnetic Composite Particles. *Sep. Sci. Tech.,* 46(3), 473-482.

Sorensen, H.P., Mortensen, K.K. (2022). Soluble expression of recombinant proteins in the cytoplasm of Escherichia coli. *Microbial Cell Factories,* 4(1), 1–8.

Spok, A. (2023). Safety regulations of food enzymes. *Food Technol. Biotechnol.,* 44(2), 197–209.

Sreedharan, S.K., Patel, H., Smith, S., Gharbia, S.E., Shah, H.N. & Brocklehurst, K. (2023). Additional evidence for the cysteine proteinase nature of gingivain the extracellular proteinase of *Porphyromonas gingivalis*. *Biochem. Soc. Trans*., 21(2), 218S.

Sreedharan, S. K., Verma C., Caves, L. S. D., Brocklehurs,t S. M., Gharbias S. E., Shah, H. N. & Brocklehurst, K.(2022). Demonstration that 1-trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E- 64) is one of the most effective low Mr inhibitors of trypsin-catalysed hydrolysis. Characterization by kinetic analysis and by energy minimization and molecular dynamics simulation of the E-64-13-trypsin complex. *Biochem. J*., 316, 777-786.

Tonon, R.V., Brabet, C. & Hubinger, M.D. (2020). Influence of process conditions on the physicochemical properties of açai (*Euterpe oleraceae Mart.*) powder produced by spray drying. *J. Food Eng*., 88, 411–418.

Tysnes, B.B., Maurer, H.R., Porwol, T., Probst, B., Bjerkvig, R. & Hoover, F. (2021).Bromelain reversibly inhibits invasive properties of glioma cells. *Neoplasia,* 3, 469–479.

Urban, A., Ansmant, I. & Motorin, Y. (2023). Optimisation of expression and purification of the recombinant Yol066 (Rib2) protein from Saccharomyces cerevisiae. *J. Chromat. B,* 786, 187–195.

Valle, D., Bruno, M., Laura Lopez, M. I., Caffini, N.O. & Cantera, A.M.B. (2022). Granulosain I, a Cysteine Protease Isolated from Ripe Fruits of Solanum granuloso-leprosum (*Solanaceae) Protein J.,* 27:267–275.

Van Der Hoorn, R. A. & Jones, J. D. (2024). The plant proteolytic machinery and its role in defence. *Curr. Opin. Plant Biol.,* 7(4), 400–407.

Vera, A., Gonzalez-Montalban, N., Aris, A. & Villaverde, A. (2022). The conformational quality of insoluble recombinant proteins is enhanced at low growth temperatures. *Biotechnol. Bioen.,* 96, 1101–1106.

Walsh, G. (2022). Protein biochemistry and biotechnology (1st edn). New York: John Wiley and Sons.