Characterization of Bromelain from *Morinda citrifolia* (Noni)

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Abstract

Morinda citrifolia from the Rubiaceae family, collected in parish of St. Andrew, Jamaica, West Indies, was investigated for the presence of the Bromelain-like protein enzyme. In the process Bromelain from *Morinda citrifolia* was partially purified and characterized. Fresh plant material was extracted in buffer (sodium acetate-acetic acid (10mM), L-cysteine (1mM), and sodium chloride (0.1M), freeze dried and analyzed using column chromatography and assayed using spectrophotometry in a five step procedure. The major purification steps involved were ammonium sulphate precipitation, gel-filtration (Sephadex G200), ion exchange chromatography (CM sephadex C25) and (DEAE sephadex A25). The protein content was determined using the Bradford method. The enzyme displayed an optimum activity at pH 7.1 and a temperature optimum at 35 ℃. A purification fold of 70.9 and percentage recovery of 3.3 was obtained. Inhibition studies using several different inhibitors of the enzyme revealed that the enzyme was susceptible to copper sulphate (0.1mM), mercury chloride (0.1mM), cobalt sulphate (0.1mM), zinc sulphate (0.1mM) and phenylmercury acetate (0.1mM). Both casein and p-Nitrophenylbenzyloxycarbonyl-L-lysinate (CLN) were used as substrates for the enzyme, with the enzyme displaying greater activity when using casein as substrate. Bromelain-like protein enzyme showed high affinity for the substrate casein with a Km of 48.5 µM.

Key words: Morinda citrifolia; Bbromelain; Proteolytic enzyme.

1. Introduction

Among the medicinal plants discovered by the ancestors of Polynesians, *Morinda citrifolia* L (Noni) is one of the traditional folk medicinal plants that have been used for over 2000 years in Polynesia [1]. Noni has been reported to have a broad range of therapeutic and nutritional value [2, 3].

Bromelain has been known since 1867 and was first observed in the pineapple plant, *Ananas comosus*. Bromelain is found in several members of the family Rubiaceae. The fruit of the pineapple, *Ananas comosus (L),* is a rich source of a mixture of cysteine

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proteinases the most abundant among them being Bromelain which hydrolytically cleaves the internal peptide bonds in protein with relatively broad specificity [4, 5]. The pineapple proteinases find uses in various industrial and medicinal applications including brewing, meat tenderizing, prevention of diarrhea, digestive aids and treatment of edema [6-8]. Bromelain is reported to have anti-tumor activity [9, 10] and can proteolytically remove certain cell surface molecules that affect lymphocyte migration and activation [11]. However, the pharmacologically active component of noni is believed to be due to the alkaloid xeronine [12]. Oral bromelain was also anecdotally reported to induce clinical and endoscopic remission of ulcerative colitis in twenty patients; whose disease was refactory to multi-agent conventional medical therapy [13,14].

Bromelain is a general name for a family of sulfhydryl proteolytic enzymes first identified in -*Ananas comosus*- the pineapple plant [15,16]. It is a standardized complex of proteases from pineapple plant [17]. Bromelain contains a peroxidase, acid phosphatase, several protease inhibitors and organically bound calcium [18]. Bromelain is derived from a natural source and hence it exhibits variability in its physiological activity. Various sources of Bromelain display similar proteolytic activity [18, 19].

Bromelain is usually distinguished as either fruit or stem Bromelain depending on its source [15]. Unlike stem Bromelain the fruit enzyme is an acidic protein [20, 21]. Stem Bromelain is the most abundant proteinase within bromelain preparations derived from pineapple stem. Other proteinases that are present in smaller quantities include fruit bromelain (major proteinase present in pineapple fruit) and ananain. Several model peptide substrates of the B (P3)-P2-P1-indicator (where B = a blocking group such as Z-benzyloxycarbonyl or Bz-benzoyl, and P1, P2, and P3 represent specific amino acids) have been used to characterize the proteolytic activity of purified bromelain enzymes. Aminolytic cleavage of the substrate results in the release of a free indicator that can be detected either fluorescently or colorimetrically. Stem Bromelain preferentially cleaves the Z-Arg-Arg model substrate, whereas fruit Bromelain and ananain show minimal activity against this substrate. In contrast, fruit Bromelain and ananain but not stem bromelain efficiently cleave the Bz-Phe-Val-Arg substrate [15, 22]. These enzymes also differ in their susceptibility to inactivation. Ananain is reported to be rapidly inactivated by the chicken egg white proteinase inhibitor cystatin and by the suicide substrate, E-64 [Trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane], but these inhibitors either very slowly or only minimally inactivate stem and fruit bromelain [15, 23].

2. Materials and Methods

2.1. *Plant material*

Noni (*Morinda citrifolia*) fruit was obtained from trees located on the grounds of the University of the West Indies, Mona, St Andrew.

2.2. *Reagents*

L-Cysteine, polydextran (PDX G.F-25:50-150 μm), Sephadex G200, CM Sephadex C25, DEAE Sephadex A25, polyethylene glycol (PEG), trichloroacetic acid (TCA), casein and

p-Nitrophenyl-benzyloxycarbonyl-L-lysinate (CLN) were obtained from Sigma (St Louis, MO, USA). Mercury chloride, phenylmercury acetate, copper sulphate, cobalt sulphate, zinc sulphate, ferrous sulphate and hydrogen peroxide were obtained from BDH Chemicals (Poole, UK). All other reagents were analar in grade.

2.3. *Extraction of bromelain-like protein enzyme from Morinda citrifolia*

Freshly picked mature (ripe and green) noni fruits were obtained from the botanical gardens of the Life Science Department (UWI, Mona). These were washed, dried and chopped into small pieces. They were then weighed (900 g) and homogenized in an equal volume of cold buffer (900 ml) made from sodium acetate-acetic acid (10mM), L-cysteine (1mM), and sodium chloride (0.1M). This homogenate was then filtered through double layers of cheesecloth and centrifuged using a bench top centrifuge at 3000 rpm. The supernatant was then brought to 40% saturation with ammonium sulphate and was then centrifuged at 10,000 x g for 15 minutes in a Beckman J21 ultra centrifuge (Beckman Coulter Fullerton, California USA). The pellet was then re-suspended in 60 ml of the extraction buffer and freeze dried on a Labconco (77550 Lyph-lock 18L) freeze dry system at –25 ℃ for 3-4 days.

2.4. *Gel-Filtration using casein as substrate to assay fractions*

A polydextran gel-filtration (PDX G.F-25: 50 –150µm) column was prepared by washing the gel approximately 10 times in distilled water and soaking in 80 mL of a sodium dihydrogen phosphate (NaH_2PO_4) buffer (0.03M; pH 7.1). After washing, the gel was made into a slurry and carefully placed in a glass column (2.5 cm x 32.1 cm). This was left to pack overnight. The freeze dried pellet (5 g) was weighed out and dissolved in extraction buffer (10 ml) and filtered. The filtrate was carefully added on to the PDX G.F-25 (50 –150 µm) column. After adding the filtrate to the PDX G.F. 25 (50-150µm) column approximately 22 fractions (3 ml each) were collected in 10 ml test tubes and stored at 4 ℃.

From these stored fractions, alternate fractions were assayed for enzyme activity by a modification of the method outlined in [24]. This was done by placing 0.7 mL of the collected fractions into test tubes containing 5 ml of 0.012 mg/ml casein in sodium dihydrogen phosphate (NaH_2PO_4) buffer (0.03 M; pH 7.1). A solution of L-cysteine (0.15 M, 0.2 ml) and water (0.1ml) were added to the reaction mixture. This was then left to incubate in a water bath for 10 minutes at 35 ℃. The fractions were removed from the water bath, and the reaction arrested by the addition of a 5 ml mixture of trichloroacetic acid (TCA, 1.8 mg/ml), glacial acetic acid (3.9 %) and sodium acetate (30 mg/ml). The fractions were further incubated for 30 minutes at 35 ℃, and were then removed and filtered through Whatman filter paper (number 2). The fractions were assayed for enzyme activity at an absorbance of 275nm using a Spectrophotometer (Ultrospec 3300 *pro*). Protein determination of each fraction was done using the Bradford method [25].

Fractions with the highest activities were pooled, assayed for enzyme activity then dialysed against polyethylene glycol (10%) dissolved in sodium dihydrogen phosphate (NaH$_2$PO$_4$) buffer (0.03 M; pH 7.1). From this pooled concentrated fraction 5 ml was removed and carefully added to a column (2.5 cm x 32.1 cm) packed with Sephadex G 200 gel.

2.5. *Gel-filtration using p-nitrophenyl benzyloxycarbonyl-L-lysinate (CLN) as substrate to assay fractions*

The fractions (from PDX GF-25**:** 50 –150 µm column) were also assayed for enzyme activity using *p*-nitrophenyl benzyloxycarbonyl-L-lysinate (CLN) as substrate. From alternate fractions collected from PDX GF-25 (50-150 µm) column, 100µl volumes were removed and 50µl of CLN (15 mM) in acetonitrile:water (90:20% v/v) added. To these fractions 3 ml of a buffer containing sodium acetate-acetic acid (10 mM), L-cysteine (1 mM) and sodium chloride (0.1M) with a pH of 4.6 was also added.   These fractions were incubated for 10 minutes at ambient temperature then 3 ml of a sodium bicarbonate (0.03 M) solution added and left at room temperature for 5 min for development of colour (yellow). Enzyme activity was measured at 405nm and protein was determined at 280 nm using a Ultraspec 3300 *pro* spectrophotometer.

Assayed fractions with activity using both casein and CLN as substrates were pooled together, enzyme activity was measured and protein determined using the Bradford method [25]. These pooled fractions were then dialysed against 10% polyethylene glycol (PEG), and dissolved in sodium dihydrogen phosphate buffer (0.03 M; pH 7.1).

2.6. *Gel filtration using sephadex G200*

These pooled fractions were dialysed for at least 18 h at 4 ℃ or until they were significantly concentrated.   From the dialysed fraction, a concentrated volume (5 ml) was removed and added to a column packed with Sephadex G 200 gel prepared using a similar procedure as that for the PDX G.F-25 (50-150 µm) column. Alternate fractions were then assayed for enzyme activity using the same procedure as in the PDX G.F-25 (50-150 µm) column. Both substrates (casein and CLN) were employed where alternate fractions were assayed for enzyme activity and protein respectively. Fractions with the highest activities were pooled, dialysed (as mentioned above) and stored for use in ion- exchange chromatography.

2.7. *Ion exchange chromatography*

A similar procedure was carried out as in gel-filtration using the pooled concentrated fractions from the Sephadex G 200 column. The concentrated G 200 fractions (5 ml) were added to a column packed with carboxy methyl (CM) Sephadex- C25 and 20 fractions were collected and assayed for enzyme activity using both casein and CLN as

substrates. The fractions with the highest activities were pooled and dialysed against polyethylene glycol (10%). From the pooled concentrated fractions, 5 ml was carefully added to a column packed with diethylaminoethyl (DEAE) Sephadex-A25 and 20 fractions were collected. The same procedures were carried out to assay these fractions as in the CM Sephadex C25 assay. The pooled concentrated fractions were stored at 0ºC until needed for further enzyme studies.

3. Results and Discussion

Bromelain-like protein enzyme was eluted early from the PDX GF-25 (50 – 150 µm) column and displayed a single peak when assayed with either casein or CLN as substrate (Figs. 1a and 1b). This gel material trapped the small molecules (small polysaccharides and small polypeptides) while allowing the larger molecules ($M_r > 15000$ kDa) to pass through the column, thus paving the way for further purification.
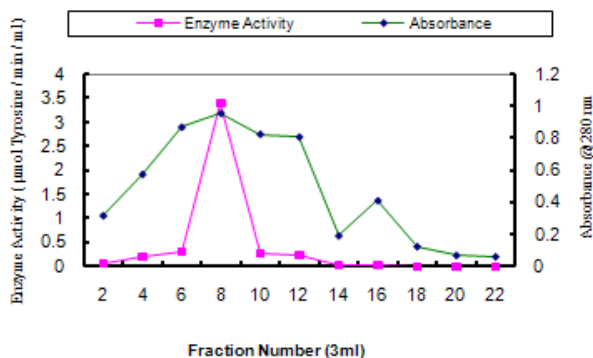
Fig. 1a. Elution profile of bromelain-like protein enzyme (*M. citrifolia*) on PDX.G.F 25 (50 – 150 µm) using casein as substrate.
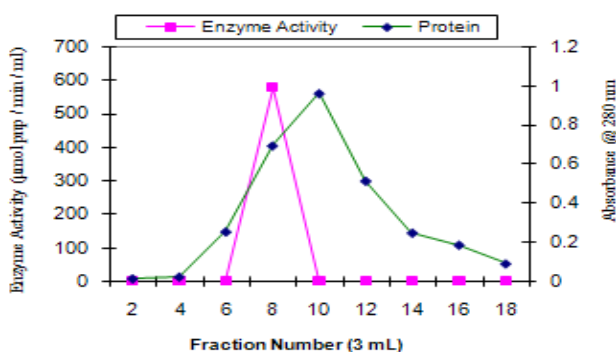
Fig. 1b. Elution profile of bromelain-like protein enzyme (*M. citrifolia*) on PDX.G.F 25 (50 – 150 µm) using CLN as substrate

Gel-filtration chromatography using sephadex G200 indicated the possibility of isoenzymes, as three protein peaks were eluted from the column, all displaying activity when assayed with either casein or CLN as substrate (Figs. 2a and 2b). This is not unusual as Ota *et. al* [20] suggested that procedures of fractionation of crude fruit and stem Bromelain involving successive use of gel filtration resulted in the production of one major and one minor active component designated as fruit Bromelain A (FBA) and fruit Bromelain B (FBB) respectively. The presence of more than one proteolytically active component was also observed by Heinicke and Gortner [26], where electrophoretic separation of crude bromelain at pH 6.5, gave four distinct components with proteolytic activity.   Fractions numbered 10-17(Fig. 2a) were pooled and designated as fraction 1 (G200: 10-17); this fraction was applied to a column packed with CM sephadex C25 (Fig. 3a).   Fractions 18 – 30 (Fig. 2a) were also pooled and designated as Fraction 2 (G200: 18-30) and was also applied to a column packed with CM sephadex C25 (Fig 3b).
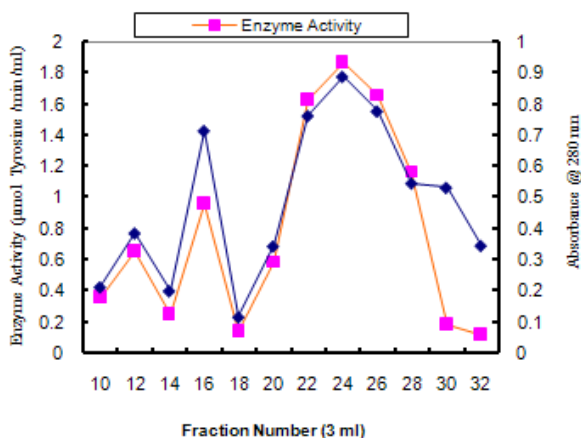

Fig. 2a. Elution profile of bromelain-like protein enzyme (*M. citrifolia*) on sephadex G 200 using casein as substrate.
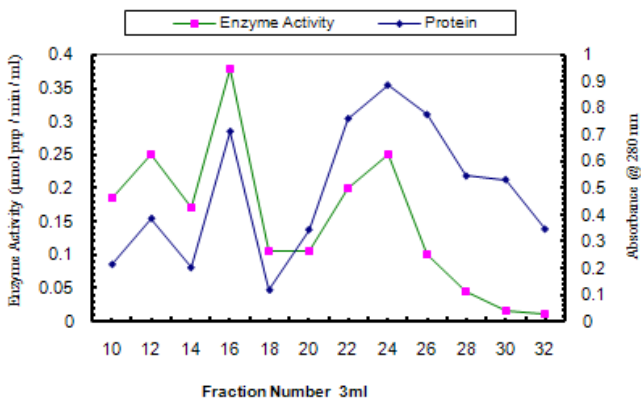

Fig. 2b. Elution profile of bromelain-like protein enzyme (*M. citrifolia*) on sephadex G 200 using CLN as substrate.

Figs 3a and 3b shows the elution profile obtained from the cation exchanger CM Sephadex C25. When the column was eluted with buffer a single peak was obtained for Fraction1 (G200:10-17) and Fraction 2 (G200: 18-30), as is shown in Figs. 3a and 3b, respectively.
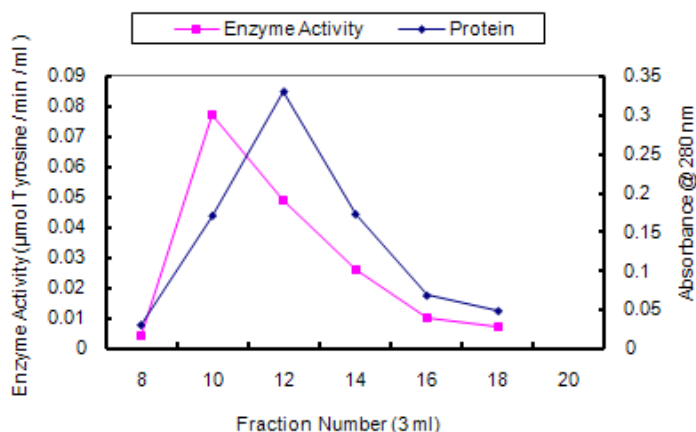

Fig. 3a. Elution profile of bromelain – like protein enzyme (*M. citrifolia*) on CM Sephadex C25 (obtained from fraction 1of G-200:10 – 17) using casein as substrate.
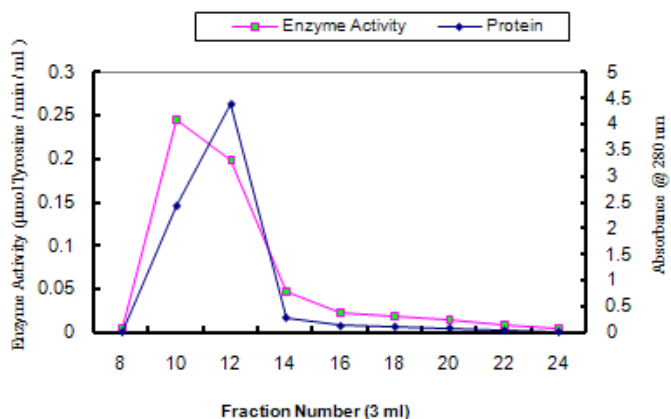

Fig. 3b. Elution profile of bromelain – like protein enzyme (*M. citrifolia*) on CM Sephadex C25 (obtained from fraction 2 of G-200:18-30) using casein as substrate.

The final step in the purification procedure employed the use of an anion exchanger DEAE sephadex A25. Bromelain-like protein enzyme was eluted in fractions 6 –12 of 14 fractions (Fig. 4a) and in fractions10- 14 of 24 fractions (Fig. 4b).

DEAE sephadex A25 (Figs. 4a and 4b) is a positively charged resin, therefore at pH 7.1 the negatively charged enzyme is bound to the anion exchanger. Increasing the ionic strength of the buffer (sodium dihydrogen phosphate) from 30 mM to 300 mM was sufficient to dislodge the enzyme from the resin. This step afforded the purification of Bromelain-like protein enzyme which showed a specific activity of 375 ± 1.73 μmol tyrosine/min/mg protein, a recovery of 3.3 % and a purification fold of 70.9 (Table 1).
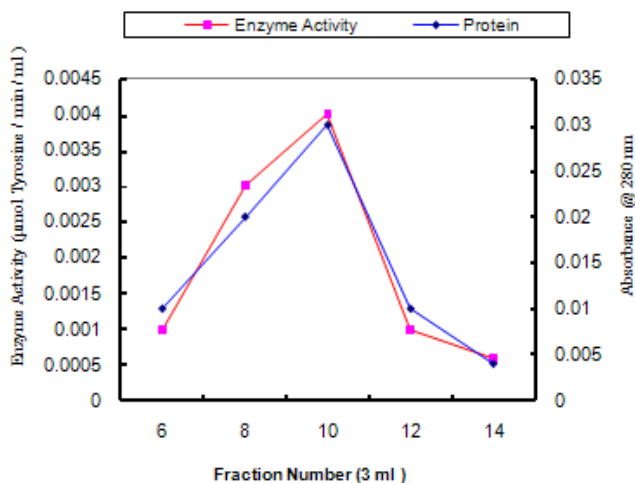

Fig. 4a. Elution profile of bromelain – like protein enzyme (*M. citrifolia*) on DEAE Sephadex A25 (fraction 1 C25A) using casein as substrate.
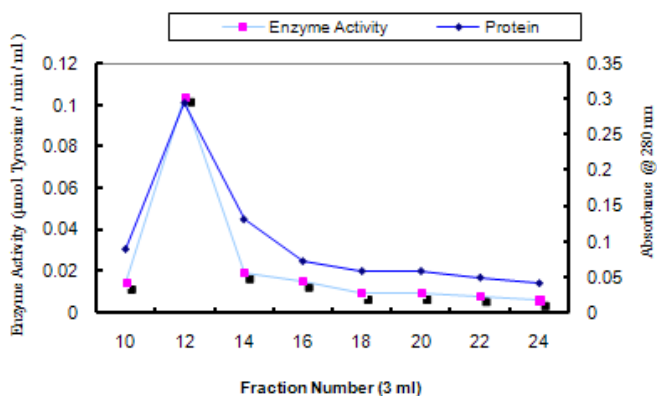

Fig. 4b. Elution profile of bromelain – like protein enzyme (*M. citrifolia*) on DEAE Sephadex A25 (fraction 2 C25A) using casein as substrate.

Table 1. Bromelain-like protein enzyme purification from *Morinda citrifol.*

Fractions	Total protein mg	Total activity µg/min	Specific activity µg/min/mg	Purification fold	% Recovery
Homogenate	2182.4±1.43	11532±8.44	5.3±0.02	1	100
Supernatant from 1st spin	504±1.01	11174±3.32	22.2±0.03	4.2	96.9
Pellet from $(NH_4)_2SO_4$ ppt*	143.5±0.19	8008 ±2.17	56±0.87	10.6	69.4
P.D.X. GF 25	72 ±1.58	4943.1±1.12	68.7±0.26	12.9	42.9
Sephadex G200	25.6±0.10	4439.4±9.02	173.3 ±0.19	32.8	38.5
CM Sephadex C25	18.9±0.24	3360.2±2.01	177.8 ±0.41	33.6	29.1
DEAE Sephadex A25	6.8±0.11	2550±3.23	375±1.73	70.9	3.3

*ppt = precipitation

The optimum pH of the enzyme was observed at pH 7.1 with activity of 274.77±37.29 µmol/min/ml (Fig. 5). However, as pH increased there was a decrease in enzyme activity; this lost of activity is due to an increase in the alkalinity of the reaction mixture. Fig. 6 shows that as the temperature increased from 0^0C to approximately 37^0C there was   an increase in enzyme activity. At approximately 35^0C the highest activity was recorded at 600.55 ± 34.68 µmol/min/ml, indicating optimum activity. Activity was still obvious as the temperature was increased even further but there was a significant fall observed between 35^0C and 40^0C. At 40^0C the activity obtained was 159.8±15.61 µmol/ml/min as compared to 600.6±34.74 µmol/ml/min at 35^0C (Fig. 6).
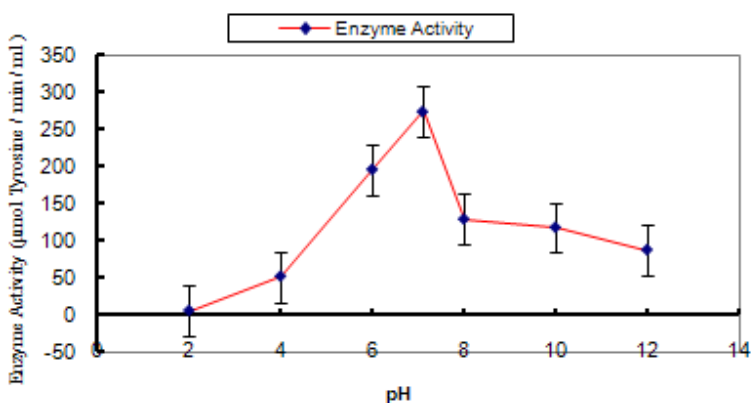

Fig. 5. Graph showing effects of pH on enzyme activity in *M. citrifolia* using casein as substrate for enzyme assay.
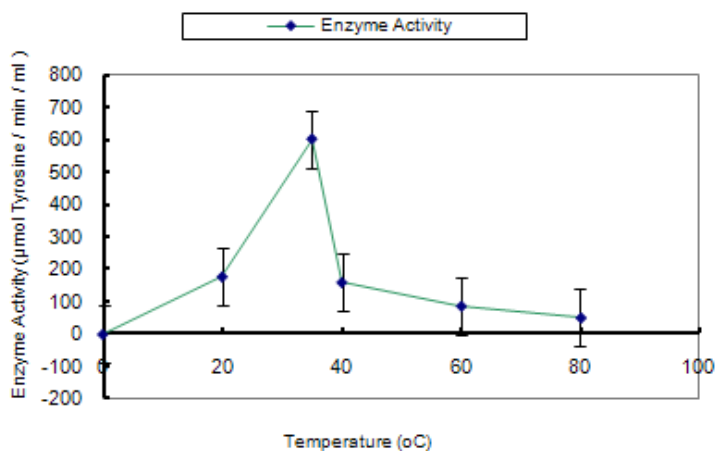
Fig. 6. Graph showing effects of temperature on enzyme activity in *M. citrifolia* using casein as substrate for enzyme assay.

Bromelian can be inhibited by a whole range of compounds including oxidizing agents like hydrogen peroxide and certain metal ions. Cobalt, copper and zinc ions at a concentration of 0.1mM have been shown to inhibit enzyme activity in avocado by 98%, 96%, and 96% respectively [27]. Several inhibitors were used to inhibit Bromelain, and the types of inhibition were determined accordingly. In this study, mercury chloride (HgCl) inhibited Bromelain activity by 44.6 % (Table 2). A Lineweaver–Burke plot of the inhibition of Bromelain by mercury chloride revealed that HgCl acted in a non–competitive manner with a Km of 48.5μM (Fig. 7). Copper (Cu^{2+}) and cobalt (Co^{2+}) also showed percentage inhibitions of Bromelain at 3 % and 26 %, respectively (Table 2).

Table 2.   The effect of inhibitors on Bromelain-like protein enzyme activity.

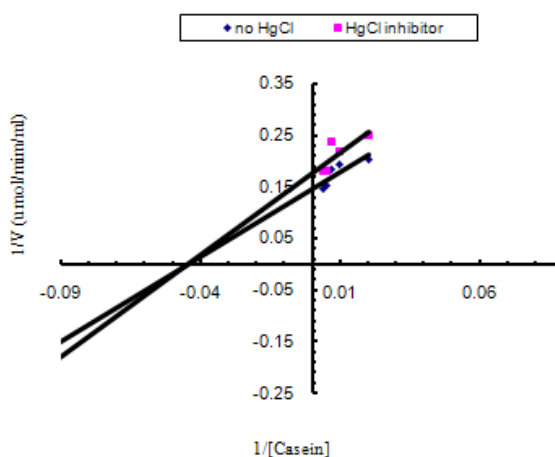| Inhibitor	Conc. (mM)	Enzyme activity (μmol tyrosine/min/ml) without inhibitor	Enzyme activity (μmol tyrosine/min/ml) with inhibitor	% Inhibition
Phenylmercuric acetate	0.1	180.22 ± 1.28	32.53 ± 3.09	82
Mercury chloride	0.1	212.82 ± 4.17	118.07 ± 1.52	44.6
Co^{2+}	0.1	212.79 ± 4.21	157.4 ± 3.04	26
H_2O_2	0.8	162.38 ± 3.42	56.13 ± 14.08	65.4
Fe^{2+}	0.1	1852.32 ± 1.67	222.18 ± 1.74	88
Cu^{2+}	0.1	212.79 ± 4.21	205.84 ± 4.36	3
Zn^{2+}	0.1	200.03 ± 1.23	112.25 ± 1.75	44

Fig. 7. Lineweaver-Burke plot showing non-competitive inhibition.

Phenylmercury acetate, iron (Fe^{2+}) and hydrogen peroxide (H_2O_2) showed high percentage inhibitions of 82 %, 88 % and 65.4 % respectively (Table 2). Similar results were obtained by McGarvey *et al.* [28] for inhibition by copper and cobalt at similar concentrations for avocado fruit. In a study done by Shukor *et al.* [29], bromelain (obtained from Sigma) was also inhibited by Hg^{2+} and Cu^{2+} at concentrations similar to those used in this study. Recent inhibition studies on bromelain have been reported by Masdor and Said [30].

Two substrates (casein and CLN) were used to assay for Bromelain from *M. citrifolia* At all stages of the purification procedure casein gave higher protein concentration and higher activity than CLN (data not shown). Table 1 shows the purification of Bromelain from Morinda citrifolia, there was an increase in specific activity through the purification procedure, culminating in a 70.9 fold purification.

Acknowledgement

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