Serine protease from *Artocarpus altilis* (breadfruit) latex

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Abstract. Breadfruit is recognized as a plant with the potential to be cultivated as an alternative staple food due to its high starch content and envisioned as a solution to mitigate hunger. The plant bears abundant fruits throughout the year with its productivity is comparable to other staple crops and it thrives well in countries where the poor population is high. Breadfruit however, exudes copious amount of latex upon harvesting and the stained epicarp often perceived as low in quality. Standardized methods of harvesting in plantations include a stage of latex draining by inverting the fruit for several hours. The latex will be drained to the ground and considered as an agricultural waste with no current commercial application. Despite being considered as a nuisance, plant latexes is a rich source of proteases functioned as a defensive mechanism against pathogenic attacks. In the effort to identify its potential, the breadfruit latex protease was purified and its characteristics were determined. The stability of the protease was investigated and its kinetics of inactivation was estimated in this research. Based on the analyses, breadfruit latex was discovered to consist of a serine protease with highly stable properties, potentially developed as an alternative commercial protease.

1. Introduction

Breadfruit (*Artocarpus altilis* (Forsberg) Parkinson) has the potential to be grown as staple food where Graham & Bravo (1980) reported that the high starch content is comparable with other main staple crops. A mature tree produces abundant of fruits (Ragone, 2011), with 70 - 75% of its pulp is edible (Jones et al., 2015) and they are productive for throughout its lifetime (Ragone, 2006). The tree uses less land usage compared to other crops, yielding almost 6 tonne per hectare of dry weight (edible portion) (Sauerborn, 2002).

Breadfruit however, exudes copious amount of latex upon harvesting which adheres to the surface of the fruit where stained epicarp is often seemed as low in quality by the consumers. The Breadfruit Institute had outlined a compilation of best practices of breadfruit cultivation which elaborated on latex draining process by inverting the fruits after harvesting until the flow of the sap reduces (Elevitch, 2014). The latex however, will be drained to the ground and have no current commercial applications. However, latexes are rich in proteases that regulate various functionalities of a plant where their concentrations are several fold higher compared to those from other plant parts (Konno et al., 2004). Researches on plant proteases are of interest as mainly because of their pH and temperatures stabilities (Oliver-Salvador et al., 2011; Sharma et al., 2009; Tomar et al., 2008) where highly stable proteases are desired in industries that involve harsh processing conditions. Proteases with distinct specificities are appreciated in food industries such as in cheese and wine making to generate signature tastes, as well as meat tenderizers or as milk clotting agent.
Therefore in this study, we had focused on the purification and characterizations of *A. altilis* latex protease in the effort to identify its potential applications which could be developed as alternative commercial enzyme.

2. Materials and Methods

2.1. Reagents

Bovine serum albumin and casein (Sigma-Aldrich, USA). Ethylenediaminetetraacetic acid disodium salt (EDTA), iodoacetamide and phenylmethylsulfonyl fluoride (PMSF) from Fluka (Switzerland). HiTrap SP Sepharose Fast Flow (SP FF) column from GE Healthcare (Sweden). Vivaspin 15R 5000 MWCO concentrator was from Sartorius Stedim (Germany).

2.2. Methods

2.2.1. Latex extraction and crude protease preparation. Latex from breadfruit was extracted from the stems and epicarp of fruits at the approximate maturity of 16 – 18 weeks. The latex was extracted through incisions of the epicarp and stem of the fruit. The latex was immediately diluted in 1:1 (v/v) ratio into ice-cold sodium citrate buffer (0.2 M, pH 5) containing 0.1 M of sodium chloride (NaCl), 1% (w/v) ascorbate, 5 mM EDTA and 1% PVPP and subsequently centrifuged (10000 × g, 4°C) for 15 minutes (Kubota, Japan). The supernatant was filtered with a filter paper. The centrifugation was carried out in two cycles. The resulting supernatant was lyophilized and stored for subsequent analysis.

2.2.2. Purification. The lyophilized protease was added with sodium citrate buffer (0.05 M, pH 4.5) and filtered using 0.45 μm syringe filter prior to purification through HiTrap SP FF column (5 × 5 cm) (GE Healthcare, Sweden). The sample was injected into column using a syringe fixed to an adaptor. Unbound proteins were washed out until no proteins could be detected with spectrophotometric analysis at 280 nm. Target proteins were eluted using stepwise elution with sodium citrate buffer (0.05 M, pH 4.5) containing 0.0 to 0.5 M NaCl. Fractions of 1 mL were collected and the protein content was monitored through spectrophotometric analysis read at 280 nm. Fractions containing active proteins were pooled and concentrated with Vivaspin concentrator (Sartorius Stedim Biotech, Germany).

2.2.3. Protease assay. The protease (0.025 mL) was mixed with 0.225 mL of casein at the concentration of 1% (w/v) in glycine-NaOH (0.2 M, pH 10) and was left to stand for 20 minutes at 37°C. The reaction was stopped by adding 0.75 mL of 5% (w/v) TCA and subsequently centrifuged for 10 minute at 9000 × g. The supernatant was analysed with by reading the absorbance at 280 nm and referred to a standard curve of L-tyrosine (0 to 180 μg/mL). Samples were tested in triplicate and averaged. One unit of proteolytic activity was described as the amount of protease catalysing the formation of 1 μmol of L-tyrosine per minute under the described assay condition.

2.2.4. Soluble protein content assay. The protein concentration was measured according to the Bradford method (1976). Protein sample solution (0.050 mL) was added with 2.5 mL of Bradford reagent and was left to stand at 25°C for 20 minutes. The resulting colour was read at 595 nm with a UV 1280 spectrophotometer (Shimadzu, Japan) and bovine serum albumin (BSA) at a range of 0 – 0.1 mg/mL was used as standard reference. Samples were tested in triplicate and averaged.

2.2.5. Classification of protease. Determination of *A. altilis* latex protease group was carried out as described by Garcia-Carreno (1992). The protease was pre-incubated with different concentrations of inhibitors at 37°C for 60 minutes. Reference samples were prepared by excluding the respective inhibitors and substituted with the diluent only. The reference samples for each inhibitor were indicated as 100% and the activity of the samples were expressed as the percentage relative to the respective reference samples. The inhibitors used were iodoacetamide for cysteine protease,
phenylmethanesulfonyl fluoride (PMSF) for serine protease, pepstatin A for aspartic acid and EDTA for metalloprotease.

2.2.6. Effect of temperature on protease activity. Investigation on the effect of temperature on *A. altilis* protease activity was carried out by varying the incubation temperature in the range of 25 to 95°C. The progress curve of product formations for each incubation temperature was recorded every 5 minute intervals for 30 minutes and the rate of reactions (*k*) were determined from the slope of the curves. The reaction rates (*k*) were plotted against incubation temperatures to obtain the optimum temperature for the described assay condition.

2.2.7. Temperature stability and kinetics of thermal inactivation. The thermal stability study was carried out by incubating the protease without its substrate at different temperatures (37 to 75°C) for 160 minutes. Aliquots of the protease solution (100 μL) were removed periodically and cooled to 4°C before being subjected to the standard protease assay (Section 2.2.3). The residual activity (%) was described as the percentage of the initial activity of the protease.

The inactivation rate constant for each temperature (*k₆*) were obtained from the slopes of the semi-log plot of residual activity against the incubation time. Half-life of *A. altilis* protease (*t₁/₂*) at each temperature was calculated based on the equation:

\[ t_{1/2} = \frac{\ln 2}{k_d} \]

where *k₆* is constant for the inactivation rate.

2.2.6. Effect of pH on protease activity and pH stability. The effect of pH on *A. altilis* protease was investigated by measuring its hydrolysing activity through the standard protease assay on 1% (w/v) substrates solubilized in buffers of different pH. Casein was used as substrate in the pH range of 6 - 11 due to its insolubility in acidic solutions and bovine serum albumin (BSA) was used in buffers of pH 3 - 11. The buffers used were sodium citrate (pH 3 - 6), sodium phosphate (pH 7 - 8), Tris-HCl (pH 8 - 9) and glycine-NaOH (pH 9 - 11). The pH stability of *A. altilis* protease was investigated by incubating the protease without the substrate in the ratio of 1:1 (v/v) with buffers of different pH. The mixtures were incubated at 37°C and the residual activities (%) were determined after 24 h. The initial activity of the enzyme at 0 h was indicated as 100%. The buffers used were sodium citrate (pH 3 - 6), sodium phosphate (pH 7 - 8), Tris-HCl (pH 8 - 9) and glycine-NaOH (pH 9 - 11). All buffers used were at 0.2 M concentration.

3. Results and Discussion
3.1. Extraction and Purification of protease from *A. altilis* latex
The latex of *A. altilis* was extracted from fruits at the phase of ‘mature but not ripe’ (16-18 weeks), a stage suitable for harvesting due to the balance between its palatability and longer shelf life (Elevitch et al., 2014). The lyophilized crude latex containing approximately 108.7 mg of protein was purified with a single step cation exchange chromatography by 3.1-fold with the specific activity of 4.89 U/mg (Table 1).
3.2. Classification of protease

Determination of the protease’s mechanistic class using specific inhibitors indicated that *A. altilis* is a serine protease. The protease was inhibited by an inhibitor of serine protease, PMSF even at the low concentration of 1 mM resulting to a residual activity of 89.0 ± 7% and 27.3 ± 5% at the concentrations of 1 and 10 mM respectively. No inhibitory activities however, were detected with the utilizations of other class specific inhibitors. Serine proteases have the advantage of being relatively stable against oxidations compared to cysteine proteases. Serine proteases are common in plant latex and they usually share similar biochemical properties. Plant latex serine proteases were reported to exhibit outstanding stabilities in wide pH range and demonstrated resistance against high temperatures (Antao & Malcata, 2005).

3.3. Effect of temperature on protease activity

The effect of temperature on the activity of *A. altilis* protease was studied by incubating the reaction mixture in different temperatures from 25 - 90°C. The progress of product formations were measured at various points throughout the incubation period and the rates of reaction were estimated from the slope of the curves (Figure 1). The product formations were discovered to increase over time throughout the 30 minutes of incubation period when incubated at 25 - 80°C. The activity of *A. altilis* protease assayed at different temperatures were obtained from the slopes of the curves in Figure 1 and plotted against their respective temperatures (Figure 2).

![Figure 1. Progress of product formations by *A. altilis* protease at different incubation temperatures.](image1)

![Figure 2. Effect of temperature on *A. altilis* protease activity.](image2)
The results showed that the *A. altilis* protease exerted high maximal activity at 80°C in the described assay condition. The activity however plunged at 90°C indicating the occurrence of enzyme denaturation. High optimal temperatures were also reported in other plant latex proteases such as *Wrightia tinctoria* (70°C) (Tomar et al., 2008), *Ficus racemosa* (60°C) (Devaraj et al., 2006) and *Cryptolepsis buchani* (70 - 75°C) (Pande et al., 2006). Despite the high optimal temperatures, one has to bear in mind that these values actually represents the temperature where the enzyme exerts its maximal activity only in the described assay condition and it is not a fixed property of an enzyme.

3.4. Thermal stability

The *A. altilis* protease had displayed outstanding thermal stability when it was able to retain almost 100% of its activity after 160 minutes of incubation at 37°C (Figure 3). The protease was even able to retain almost 30% of its activity when incubated at the high temperature of 70°C after 160 minutes of incubation period. This analysis indicated that the thermal stability of *A. altilis* protease is generally superior compared to other proteases from plant latexes such as of *Ficus racemosa* (Devaraj et al., 2008) and *Ficus benghalensis* (Sharma et al., 2015).

Given the outstanding thermal stability of this protease, the kinetics of thermal inactivation was investigated by plotting the log of residual activities against the incubation time (Figure 4) and the specific rate of inactivation constants ($k_d$) for each temperature were obtained from the slopes of the curves. The thermal stability of *A. altilis* protease was also evaluated through its half-life values ($t_{1/2}$). The half-life of *A. altilis* protease at 55°C ($t_{1/2} = 182$ min) is comparable to a heat-stable protease from *Bacillus* sp. EMB9 ($t_{1/2} = 190$ min; T= 60°C) (Sinha and Khare, 2013). Furthermore, the *A. altilis* with $t_{1/2}$ of 146.5 min at 65°C was discovered to be more superior in comparison to a thermostable protease isolated from *Aspergillus fumigatus* ($t_{1/2} = 65$ min; T = 50°C) (Hernandez-Martinez et al., 2011).

3.5. Optimum pH and pH stability

The optimum pH was studied by using casein and BSA at the concentration of 1% (w/v) dissolved in different pH solutions. The experiment demonstrated that both substrates were maximally hydrolysed at pH 10 (Figure 5) and retained almost 90% of its activity at pH 11 (Figure 6). This outcome is similar to other serine proteases which showed maximal activity in the alkaline pH range (Dubey & Jagannadham, 2003; Morcelle et al., 2004; Yadav et al., 2011). The *A. altilis* protease was also discovered to be stable over a wide pH range (pH 4 – 11) with no significant reductions were detected when the protease was incubated in buffers of pH 4 -11. A significant decrease however was detected when the protease was incubated at pH 3 with only 34% of its activity.
remained after 24 h. This is due to the protein unfolding which occurs at extreme pH environments, attributed to the intramolecular charge repulsions (Fink et al., 1994).

4. Conclusions
In conclusion, the purification procedure through a single step cation exchange chromatography had isolated a serine protease from the latex of *A. altilis*. From the characterizations of the enzyme, it was evident that the protease was able to withstand high temperatures and it is stable in a wide pH range of 4 – 11. The protease was also discovered to be maximally active in alkaline conditions, a characteristic commonly discovered in serine proteases from plant latexes. Given the ease of availability of the enzyme source, the *A. altilis* latex protease is potentially developed to be applied in industries which involved harsh processing conditions and high temperature procedures.

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