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Characterization of a Partially Purified Protease from the Seeds of *Mangifera indica* Cv. Bangalora

Mary Dorothy Anitha Sebastian*[1] and D. Sudarsanam[2]

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ABSTRACT

A protease was isolated from *Mangifera indica* cv. Bangalora, partially purified and characterized. The presence of the protease was confirmed by the zones of clearance that formed when the crude and partially purified enzyme were loaded on casein agar plates. The protease shows broad substrate specificity with a greater affinity for casein as a substrate. The partially purified protease has a specific activity of 16.8 U/ml which corresponds to 134.4 U per gram of the mango seed. The protease was found to be an alkaline protease showing higher enzyme activities at alkaline pH with an optimum pH of 8.0 and showed good activity in the temperature range of 30 – 50°C with an optimum temperature of 40°C. The enzyme is stable when kept for 1 h at an alkaline pH range between pH 7 and 10 and at temperatures between 30 – 50°C. The K_m *and* V_{max} values of the protease were determined to be 2.3 mg/ml and 136.99 μmol/min, respectively. The enzyme is activated in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+} and it is inhibited in the presence of Zn^{2+} and Fe^{2+}. The protease was strongly inhibited by PMSF showing that it is a serine protease. This protease may be of therapeutic and industrial value.

***Key words*:** *Mangifera indica*, Seeds, Alkaline serine protease, Partial purification, Enzyme activity

Several proteases are present in plant tissues where they play important roles in cellular signalling, defense and cellular metabolism. Proteases obtained from plant sources have been found to have medicinal and industrial value [1]. For example, bromelain obtained from pineapple has been found to have anti-microbial, anti-inflammatory, anticancer, antithrombotic etc. [2]. Proteases are therefore of great value to the pharmaceutical industry. It is also used in the food processing industry as well as in the textile and cosmetic industry [3-4]. Proteases such as papain from papaya and ficin from fig have also been shown to have medical and industrial applications [5-6].

In contrast to human encoded enzymes, many plant proteases possess unique properties such as higher pH stability and thermostability and ability to work at broad pH ranges and temperatures [7]. The seeds of several leguminous plants such as *Vicia faba* possess high amounts of protease. Plant proteases can be obtained from the plant tissues and can be used in partially purified form although appreciable yields may not be obtained. The yield of plant proteases can be increased by the use of tissue culture and recombinant DNA techniques.

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The seeds of many fruits go to waste after the use of the fruit for food purposes. Seeds can act as good bioreactors for the production of enzymes including proteases, amylases and lipases. In this study, an attempt has been made to isolate and characterize a protease from *Mangifera indica* (cv. Bangalora) seeds with the aim of investigating whether it could be used for therapeutic or industrial purposes.

MATERIALS AND METHODS

Preparation of the crude seed extract

For preparing the crude extract of the mango seed, 25 g of fresh seed sample was homogenized in 100 ml of 0.05 M Tris HCl buffer (pH 7.5). The homogenate was centrifuged at 8000 rpm for 15 min and the supernatant was collected.

Partial purification of the protease

The protease was partially purified from the crude extract by ammonium sulphate fractionation at 80% ammonium sulphate saturation. The white precipitate obtained was collected by centrifugation at 8000 rpm for 15 min, dissolved in 10 ml of 0.05 M Tris HCl buffer (pH 7.5) and dialyzed overnight against the same buffer.

Casein agar plate assay

The casein agar plates were prepared by dissolving 250 mg of skimmed milk powder in 50 ml of 0.05 M Tris HCl buffer (pH 7.5) and then adding 500 mg of agar to this solution. The solution was heated till the agar dissolved

completely. 15 ml of the casein agar solution was poured into a Petri plate and allowed to cool and solidify. Six wells were punched in the solidified agar and 20 µl of the crude extract was added to the top three wells and the partially purified protease was added to each of the three bottom wells. The Petri plate was incubated overnight at room temperature. The zones of clearance that formed around the wells were measured.

Substrate specificity of the protease

The substrate specificity of the protease enzyme was determined by measuring the percentage of protease activities when using 1% concentrations of casein, bovine serum albumin (BSA) and gelatin as substrates. The highest protease activity shown among the three substrates was assumed to be 100% and the percentage of residual activities for the substrates was calculated based on this.

Determination of the enzyme activity and protein content of the partially purified protease

The activity of the protease was determined according to the modified procedure of Tsuchida *et al*. [8]. 1% casein in Tris HCl buffer (pH 7.5) was used as the substrate for the protease. 0.5 ml of the casein solution was incubated with 0.5 ml enzyme extract at 37°C for 10 min after which the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. The reaction mixture was subjected to centrifugation at 8000 rpm for 15 min and the supernatant was mixed with 5 ml of 0.44 M sodium carbonate and 1 ml of two-fold diluted Folin Ciocalteau reagent. After 30 min of incubation at room temperature, the blue color that developed was read at 660 nm against a reagent blank. Tyrosine was used as the reference standard. One unit (U) of protease activity is defined as the amount of enzyme required to catalyze the formation of 1 µmol of tyrosine per minute. The protein concentration of the partially purified protease sample was determined by the Bradford method using bovine serum albumin (BSA) as the standard [9]. The enzyme activity was calculated as follows:

$$\text{Enzyme activity} = \frac{\text{µmols of Tyrosine released} \times \text{Reaction volume}}{\text{Sample volume} \times \text{Reaction time} \times \text{Volume assayed}}$$

Determination of the optimum pH of the protease

The activity of the protease was determined at different pH ranging from 6 to 11. The buffers used were 0.05 M sodium phosphate (pH 6.0 - 7.0), 0.05 M Tris-HCl (pH 7.5 - 9.0) and glycine-NaOH (pH 10.0 - 11.0) buffers. 0.5 ml of the casein solution was incubated with 0.5 ml enzyme extract and 1 ml of each of the buffers at 37°C for 10 min after which the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. The reaction mixture was subjected to centrifugation at 8000 rpm for 15 min and the supernatant was mixed with 5 ml of 0.44 M sodium carbonate and 1 ml of two-fold diluted Folin Ciocalteau reagent. After 30 min of incubation at room temperature, the blue color that developed was read at 660 nm against a reagent blank. The enzyme activity at the optimum pH was assumed to be 100% and the percentage relative enzyme activity was calculated for the other pH based on this.

pH stability of the protease

The pH stability was determined by calculating the enzyme activities after incubating the protease enzyme in buffers of different pH ranging from pH 7.0 to 10.0 for 1 h at 40°C. The enzyme activity at this different pH when incubated for 10 min for the standard protease assay was assumed to be 100%.

Determination of the optimum temperature of the protease

The activity of the protease was determined at different temperatures ranging from 20 to 80°C by providing the optimum pH (pH 8.0) for the enzyme reaction. 0.5 ml of the casein solution was incubated with 0.5 ml enzyme extract at different temperatures ranging from 20 to 80°C for 10 min after which the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. The reaction mixture was subjected to centrifugation at 8000 rpm for 15 min and the supernatant was mixed with 5 ml of 0.44 M sodium carbonate and 1 ml of two-fold diluted Folin Ciocalteau reagent. After 30 min of incubation at room temperature, the blue color that developed was read at 660 nm against a reagent blank. The enzyme activity at the optimum temperature was assumed to be 100% and the percentage relative enzyme activity was calculated for the other temperatures based on this.

Thermal stability of the protease

The thermal stability by calculating the enzyme activities after incubating the enzyme at different temperatures ranging from 30 to 70°C for 1 h. The enzyme activity at these different temperatures when incubated for 10 min for the standard protease assay was assumed to be 100%.

Determination of the K_m and V_{max} for the protease

The effect of varying substrate concentration on the activity of the protease was studied to determine the K_m *and* V_{max} values of the protease. The concentration of the casein substrate was varied between 2 – 20 mg and the protease activity was assayed at these different substrate concentrations. The K_m *and* V_{max} values were obtained from the Lineweaver-Burk plot of the values.

Effect of metal ions on the activity of the protease

The effect of certain metal ions on the activity of the protease was studied by measuring the percentage residual enzyme activity after incubating the enzyme at its optimum pH (pH 8.0) and temperature (40°C) with 1mM of the metal ions K^+, Na^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+} (as their chloride salts) for 20 min and then measuring the percentage residual enzyme activity. The enzyme activity in the absence of any metal ions at the optimum pH and temperature was assumed to be 100%.

Effect of inhibitors on the activity of the protease

The effect of different protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF), Iodoacetic acid (sodium iodoacetate) and Ethylenediaminetetraacetic acid (EDTA) on the activity of the protease was determined by incubating the enzyme at its optimum pH (pH 8.0) and temperature (40°C) with 5mM of these inhibitors for 20 min and then measuring the percentage residual enzyme activity. The enzyme activity in the absence of any inhibitors at the optimum pH and temperature was assumed to be 100%.

RESULTS AND DISCUSSION

As shown in (Fig 1), zones of clearance measuring 0.8 cm were formed around the top three wells with crude extract and zones of clearance measuring 1.3 cm in diameter were

formed around the bottom three wells with the partially purified protease on the casein agar plate, showing that there was appreciable protease activity in the crude and partially purified protease samples and that the activity of the enzyme is greater in the partially purified sample compared to the crude extract.

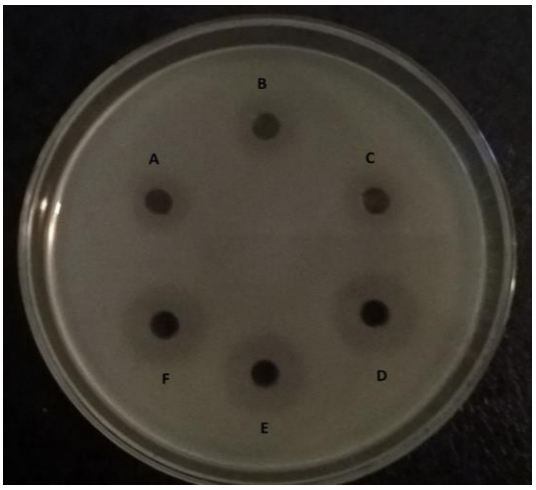

Fig 1 Casein agar plate assay: wells A, B and C contain crude extract whereas wells D, E and F contain the partially purified protease sample

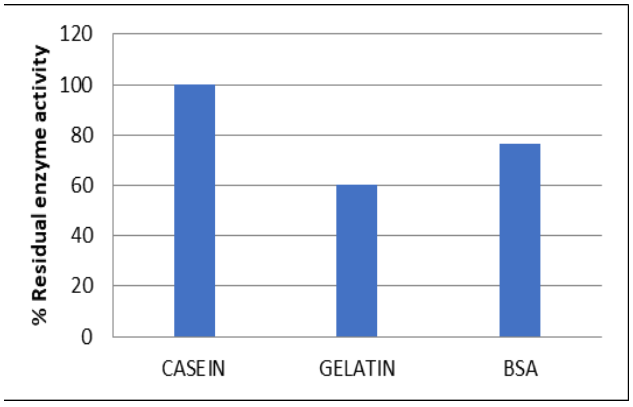

Fig 2 Substrate specificity of the protease

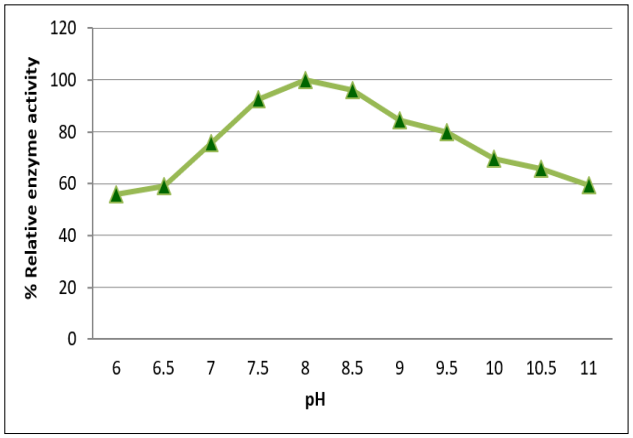

Fig 3 Determination of the optimum pH of the protease

As shown in (Fig 2), the protease shows broad substrate specificity with a greater affinity for casein as a substrate compared to gelatin and BSA. With BSA and gelatin as substrates the enzyme shows 76 and 60% of residual enzyme activity. Proteases with a high affinity for casein possess good milk-clotting properties and are often very useful in the dairy industry especially in cheese production where the quality and yield of cheese is influenced by the extent of hydrolysis of milk by a protease enzyme [10].

The specific activity of the protease sample after ammonium sulphate fractionation and dialysis was determined to be 16.8 U/mg which corresponds to 134.4 U/mg per gram of the mango seed. The protein content of the partially purified protease sample was 2 mg/ml. Partially purified bromelain isolated from pineapple core has also shown similar specific activity after ammonium sulphate fractionation and dialysis [11].

The optimum pH of the partially purified protease was determined to be pH 8.0 at which it showed maximum enzyme activity. The protease was found to be an alkaline protease showing higher enzyme activities at alkaline pH ranges between pH 7 – 10 (Fig 3) and even at pH 10 the enzyme retained 70% of its activity. This shows that the enzyme works at a broad pH range of 7 – 10. The enzyme activities at pH 7 – 10 did not change even when incubated at this pH for 1 h, showing that the enzyme is stable over this pH range. Proteases which show high activity and are stable at alkaline pH are very useful for the textile industry, detergent industry and leather processing industry [12].

The optimum temperature of the partially purified protease was determined to be 40°C (Fig 4). The protease showed good activity in the temperature range of 30 – 50°C. The enzyme retained 100% of its activity at temperatures of 30 and 40°C after incubation for 1 h. During incubation for 1 h at 50°C the activity decreased by 20% and at 60 and 70°C the activity decreased to below 50% showing that the enzyme is thermostable in the range of 30 - 50°C and most plant proteases are reported to work in the temperature range of 20 - 50°C [13]. The capacity of the enzyme to work at alkaline pH and at temperatures between 30 - 50°C as well as its pH stability and thermostability indicates that it can be of value as a therapeutic and industrial enzyme [14].

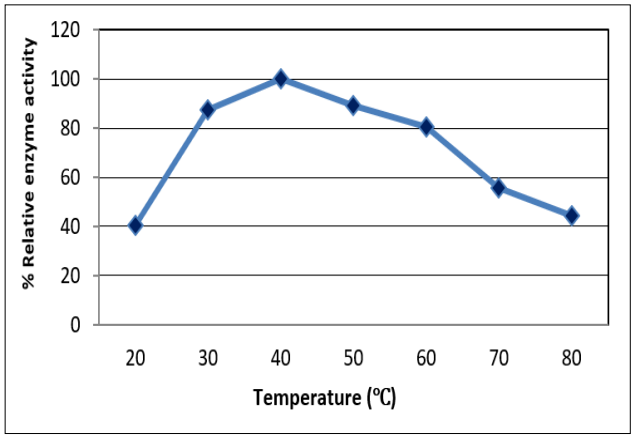

Fig 4 Determination of the optimum temperature of the protease

The K_m *and* V_{max} values of the protease were determined to be 2.3 mg/ml and 136.99 μmol/min, respectively (Fig 5). The K_m value indicates that the enzyme has a good affinity for casein as a substrate. As mentioned earlier, this high affinity for casein may aid the increased milk-clotting properties of the enzyme for use in the dairy industry. Such proteases are also of great value in the food processing industry for meat tenderization, dough softening in baking, fruit juice clarification etc. [15- 16].

The enzyme activity is influenced by metal ions. The residual enzyme activity in the presence of Mg^{2+}, Ca^{2+} and $Mn^{2+}$was 130, 115 and 108% respectively, showing that the

enzyme was activated by these metal ions. In the presence of K^+ and Na^+ also the enzyme activity remains appreciably high with the percentage residual activity decreasing only by 15 and 30%, respectively. However, in the presence of Zn^{2+} and Fe^{2+} the activity decreased by 75 and 80% respectively showing that the enzyme is inhibited by these metal ions (Fig 6). Several plant proteases have been known to be activated by Mg^{2+} and Ca^{2+} [17, 18] and by Mn^{2+} [19]. The protease was strongly inhibited by PMSF retaining only 10% of its activity in the presence of PMSF. This indicates that the protease is a serine protease possessing a serine residue in its active site. Iodoacetic acid and EDTA did not inhibit the enzyme which retained 90% of its activity in the presence of Iodoacetic acid and EDTA (Fig 7).

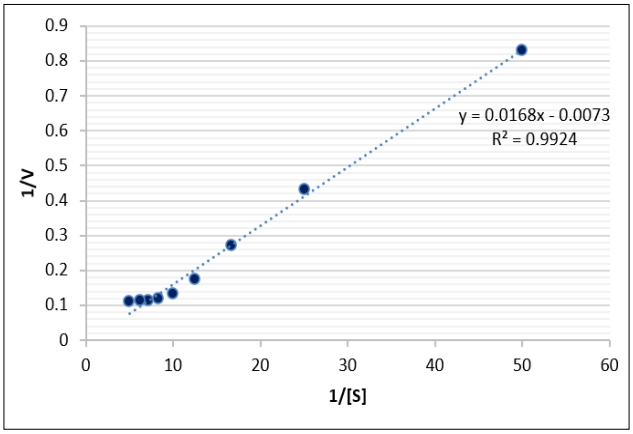

Fig 5 Determination of the K_m and V_{max} of the protease

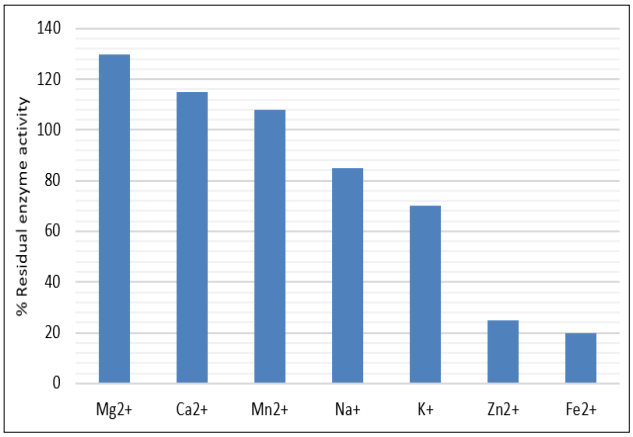

Fig 6 Effect of metal ions on the activity of the protease

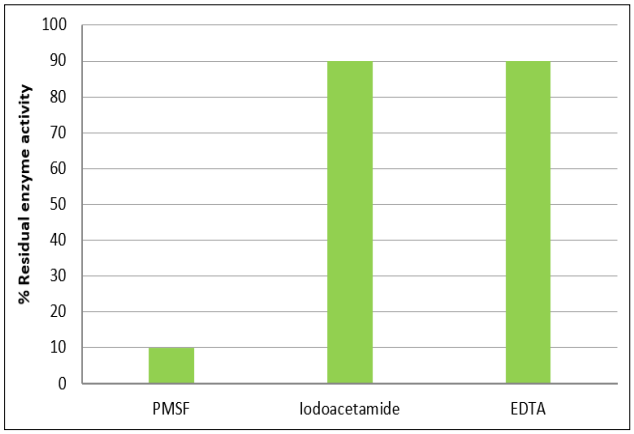

Fig 7 Effect of inhibitors on the activity of the protease

Serine proteases are widespread in nature and several plant serine proteases from seeds have been isolated and studied [20-22]. Many plant serine proteases have been shown to possess similar characteristics as the enzyme in this study. An alkaline serine protease purified from mango peel has also been reported to show similar properties to the protease studied here with an optimum pH of 8.0, PMSF inhibition and activation by Mg^{2+} and Ca^{2+} [23]. Similar results have also been obtained in the case of a serine protease isolated from the seeds of *Caesalpinia bonducella* [24-25]. Many plant alkaline serine proteases such as Cucumisin have been reported to possess fibrinolytic, thrombolytic and anticancer activities and have been used in the food processing industry, textile industry, leather industry etc. [17, 26]. The enzyme reported in this study has to be further investigated for therapeutical and industrial applications. Mango is a tropical fruit and *Mangifera indica* cv. Bangalora is available almost throughout the year. The seeds of this fruit are almost always thrown away as waste. In view of the findings of this study, mango seed can now be used as source of protease.

CONCLUSION

Plant proteases have been used over the years as therapeutic agents and cosmetic agents while also being used in various industries such as the food, textile and detergent industry. Discovery and isolation of potent plant proteases with unique substrate specificities, from readily available sources such as mango seeds, will help in the use of safe plant-derived enzymes in therapeutic, cosmetic and industrial products which are meant for human use.

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