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Isolation and purification of anticoagulant enzymes from plant latex

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Abstract
The plant latex is a complex mixture of organic, inorganic and hydrolytic enzymes such as proteases which acts as anticoagulant and finds its uses in surgeries, heart diseases and blood clot treatment. In the present study, latex was collected from *Euphorbia milii, Jatropha malacophylla, Thevetia peruviana, Euphorbia pulcherimma* and *Artocarpus altilis* to evaluate the best source of anticoagulant enzyme though four other plants were originally screened as well but failed to show the enzyme. Among the five, *A. altilis* and *J. malacophylla* showed the highest anticoagulant activity by milk clotting, blood clotting and APTT assay. The samples were subjected to three-step purification, i.e., salt precipitation, dialysis, ion exchange and gel filtration. The fold purification of *A. altilis* was found to be 5.37 and 2.31 for *J. malacophylla* respectively after the gel filtration. The percentage of yield of *A. altilis* was found to be 63.23% and 26.4% for *J. malacophylla*. Molecular weight of *J. malacophylla* sample and *A. altilis* was found to be ~80kDa and ~105kDa respectively, determined by SDS PAGE. Both enzymes showed optimum activity at pH 7. *A. altilis* showed optimum activity at 35°C, incubation time of 40 minutes, substrate concentration of 60mM, with $MgCl_2$ as activator the activity increased at 600µL and with EDTA as inhibitor the activity increased at 400µL. *J. malacophylla* showed optimum activity at 45°C, incubation time of 10 minutes, substrate concentration of 80mM and stable at 35 °C which is the human body temperature. *A. altilis* showed optimum conditions for human administration making it therapeutically viable.

Keywords: Anticoagulant, APTT, Gel filtration, Ion exchange, SDS PAGE

INTRODUCTION

Latex is an aqueous emulsion found in the vacuoles of special secretory cells called laticifers, which contain lipids, rubbers, resins, sugars, many proteins and enzymes (Fonseca *et al.*, 2010). The latices of several plants are medicinally important and are exclusively used as a common remedy for immediate blood clotting in fresh cuts and assist in wound healing (Shivaprasad *et al.,* 2010).

Anticoagulants are the group of drugs whose role is to prevent coagulation, unlike fibrinolytic drugs whose role is to stimulate the breakdown of a formed clot. (Yentis *et al.,* 2004). Clinically, anticoagulants are used for a variety of conditions, but always for the same reason – to prevent aberrant clot formation, a result of the presence of a hypercoagulable state. The cause for this may be varied and the context will therefore direct the choice of drugs used. (David and Carl, 2009). Thrombin synthesis and fibrin formation occur rapidly at vascular injury sites. Regulation of coagulation is exerted at multiple levels, either by enzyme inhibition or by cofactor modulation activity. The most important regulators of coagulation are Antithrombin, protein C and protein S. Along with TFPI and the fibrinolytic system, the main natural anticoagulant and antithrombotic mechanisms is constituted by these in organisms. Serine-protease inhibitor antithrombin (AT) inhibits most of the enzymes generated during activation of coagulation. AT preferentially inhibits free enzymes, whereas enzymes that are part of the intrinsic tenase or prothrombinase complexes are less accessible for inhibition (De Caterina *et al.,* 2013).

Plant latex is used as an effective treatment for would healing in developing countries. *Carica papaya* latex has been used for wound healing in mice burn. In addition, ethanolic and dichloromethane extracts of *Mammea americana* latex have been found to possess excellent antisecretory and gastroprotective effects in all gastric models. Fig tree latex has been used to treat warts in short duration therapy with no reports of any side effect . Plant latex has clot inducing and dissolving properties in human hemostasis. (Jaruwan and Sampomg, 2011). Oral anticoagulants are effective for primary and secondary prevention of venous thromboembolism, for prevention of systemic embolism in patients with tissue or mechanical prosthetic heart valve or AF, for prevention of AMI in patients with peripheral arterial disease, for prevention of stroke, recurrent infarction, or death in patients with AMI, and for prevention of myocardial infarction (MI) in men at high risk. (Jack *et al*., 2001).

Latices from manguruungy plant species like *Calotropis procera and C.gigantean* (Ascelpiadaceae), *Jatropa curcus* and *Euphorbia ligularia* (Euphorbiaceae), *Argemone mexicana* (Papaveraceae), *Abutilon indicum* (Malvaceae), *Litsea glutinosa* (Lauraceae), *Ficus hispida* (Moraceae), *Chromolaena odorata* (Asteraceae), *Cryptolepis buchanani* (Periplocaceae), and *Hyptis suaveolens* (Lamiaceae) are applied over contemporary cuts to prevent haemorrhage and later applied over wounds to boost healing (Ashwani *et al.,* 1999). Based on the review, proteolytic enzyme has lot of application and commercial value for this reason finding out the novel source for the protease enzyme is always arsenal. Small attempt has been made to evaluate the best source of proteolytic activity from the latex.

MATERIALS AND METHODS

Extraction of latex from the plant source for the proteolytic activity/anticoagulant enzyme

Plant material and collection of latex: The plant latex sample of *E. pulcherimma, C. procera, F. bengalensis, F. benjamina, S.podophyllum, T. peruviana, E. milii, J. malacophylla* and *A. altilis* was collected early in the morning by superficial incision of stem or leaves of healthy plants and allowing the milky latex to drain in clean glass vials separately, brought to the laboratory and refrigerated till the experiment started. (Raghunath *et al.,* 2015).

Milk coagulation and blood coagulation method: A drop of latex sample was added to a thin layer of milk taken in a petri dish. The formation of clear zone by the breakage of milk protein by the latex showed the presence of anticoagulant enzymes. The selected latex samples were *Euphorbia pulcherimma, Thevetia peruviana, Euphorbia milii, Jatropha malacophylla* and *Artocarpus altilis*, which were tested with 1ml of blood. The blood clotting time was noted for all samples by using 50µL of whole latex in 100µL of blood to determine the anticoagulant property, while for control 50 µL of phosphate buffer was used in blood. The samples were selected depending on the longest coagulation time. Finally, the samples were subjected to APTT (Activated partial thromboplastin time) Assay and the selected latex samples were subjected to protease enzyme isolation.

Enzyme assay: Proteolytic activity was determined by the enzyme assay using 0.65% casein as a substrate. The protease activity was expressed as amount of enzyme required to produce peptide equivalent to µg of tyrosine/min/mg protein at 37°C and protein content was determined according to Lowry's method using Bovine serum albumin as the standard protein (Lowry *et al.,* 1951).

Purification

Ammonium sulphate precipitation: The crude enzyme of *Artocarpus altilis* and *Jatropha malacophylla* was precipitated by addition of 44% ammonium sulphate to 70% saturation. The enzyme was incubated overnight at 4°C. The enzyme was then centrifuged at 6000RPM for 10 minutes and the supernatant was discarded. The pellet was dissolved in 10ml of 10mM Tris HCl.

Dialysis: Cellulose acetate membrane with a molecular weight cut off 8kDa was used for the dialysis. (Mishra *et al.*, 2017). The membrane was activated by adding 100ml of boiling water taken in a beaker to which 2% Sodium Bicarbonate was added and boiled for 20 minutes. The membrane was then transferred to a beaker of 100ml of boiling water and boiled for 20 minutes. The membrane was cooled and one end of the membrane was tied making sure there is no leakage. The precipitated sample was added of the membrane and the open end was tied. The membrane bags are suspended in a beaker containing water and incubated overnight at 4°C. The water in the beaker was changed and stirred using magnetic stirrer for 30 minutes. The water was again changed and stirred three times.

Ion exchange: Pre-processing of column was done by washing with methanol and drying it. The chromatography column packed with DEAE (Diethyl Amino Ethyl) cellulose was washed using distilled water one to two times and sonicated for 15 minutes. The matrix was activated using activation buffer of concentration of 1M of NaCl and Tris HCl. (Moidutty *et al*., 2015). The dialyzed enzyme was poured in the column and collected till the mark. The buffers of concentrations of 25mM, 50mM, 75mM, 100mM and 125mM were added and the elusions were collected in 5 test tubes and incubated overnight at 4°C.

Gel filtration: The elusion with highest protein activity determined by the method of Lowry *et al.*

(1951) was used for Gel Filtration. SEPHADEX G75 resin was loaded in the column and left to settle. (Mishra *et al.*,2017). The elusion was added followed by 20ml phosphate buffer of pH 7.5 of 0.1M concentration. 20 elusions were collected with 1ml per eppendorf at the rate of 1ml per 2 minutes. The highest protease activity was determined at 280nm using Spectrophotometer and the specific activity was calculated.

Characterization: The enzymes of *A. altilis* and *J. malacophylla* were assayed to find their stability under different parameters. This was done by subjecting the enzyme to the pH ranging from 4-10 using 0.1M sodium acetate buffer (pH 4, 5, 6), 0.1M Phosphate Buffer (pH 7, 8) and 0.2M Glycine Buffer (pH 9, 10). The enzymes were checked for maximum activity under the temperature ranges of 25°C, 35°C, 45°C, 55°and 65°C along with incubating the enzyme for different time durations of 10-60 minutes. By the addition concentrations 0.2-1.2% of Casein as substrate the enzyme activity was checked. The above methods were verified by Photospectrometry. (El-Hofi *et al.,* 2014; Das *et al.,* 2010 and El-Bendary *et al.,* 2007).

Determination of molecular weight: Molecular weight of the protein was determined by the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (**Sunita** *et* al., 2013). The gels (12% separating gel and 4% stacking gel) were loaded with a mixture of denatured 50µl enzyme and 50µl SDS loading dye. After run down, the gel was stained with Coomassie Brilliant Blue R-250 and then decolorized with a solution containing methanol and acetic acid (Yang *et al.*, 2017).

RESULTS AND DISCUSSION

Screening for proteolytic activity: From the many plant latex samples collected, latex of 5 different plants such as *Euphorbia pulcherimma, Thevatia peruviana, Euphorbia milii, Jatropha malacophylla* and *Artocarpus altilis* showed caseinolytic activity and milk clotting activity. All the five latex showed a clear zone formation when they were added to a thin layer of milk taken in petri dishes signifying the ability of caseinolysis(Fig. 1). *Euphorbia pulcherimma, Thevatia peruviana* and *Euphorbia milii* showed positive results while *Jatropha malacophylla* and *Artocarpus altilis* showed high proteolytic activity where blood did not coagulate (Fig. 2). The plants *Euphorbia pulcherrima*, *Jatropha malacophylla* and *Artocarpus altilis* are plants on which research work is rarely done. When APTT Assay was done using the plants with high proteolytic activity, *Jatropha malacophylla* and *Artocarpus altilis* did not show gel formation affirming the presence of coagulation inhibitors.

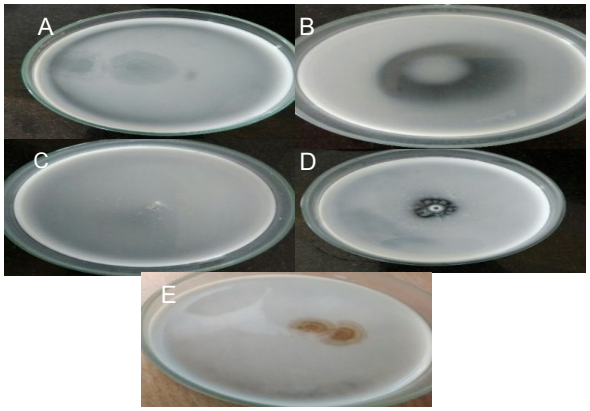

Fig. 1. *Milk clotting activity seen in, A. Euphorbia pulcherimma B. Thevatia peruviana C. Euphorbia milii D. Jatropha malacophylla E. Artocarpus altilis.*

Purification: Latex of *Artocarpus altilis* and *Jatropha malacophylla* was taken for purification, which was done by four-step process of Ammonium sulphate precipitation, Dialysis, Ion Exchange and Gel filtration in order to obtain the pure enzyme with the most efficient activity. After dialysis, first elution solution of *Artocarpus altilis* and second elution solution of *Jatropha malacophylla* showed highest enzyme activity which was purified by DEAE cellulose column for SEPHADEX gel filtration. The obtained elutions were checked for enzyme activity spectrometrically. Twenty elusions were collected out of which the sixteenth elusion of *Artocarpus altilis* and *Jatropha malacophylla* showed highest protease activity. Table 1 and Table 2 summarize the results of the purification of protease from 5 ml latex of the plant.

A. altilis shows enzyme activity of 1.72mg/ml, percentage yield of 63.23% and fold purification of 5.37 after gel filtration(Table 1), while *J. malacophylla* showed enzyme activity of 0.56mg/ml, percentage yield 26.41% and fold purification 2.312 (Table 2). When compared, *A. altilis* showed significant higher values than *J. malacophylla* in terms of both protease activity and purified enzyme. *A. altilis* and *J. malacophylla* showed higher percentage yield than *Plumeria rubra* Linn which gave 7.95% (Chanda *et al.,* 2011) and enzyme obtained from *Bacillus* spp. showed percentage yield of 7.5% (Vengadaramana *et al.,* 2011) also in enzyme purified from *Bacillus sphaericus,* the per-

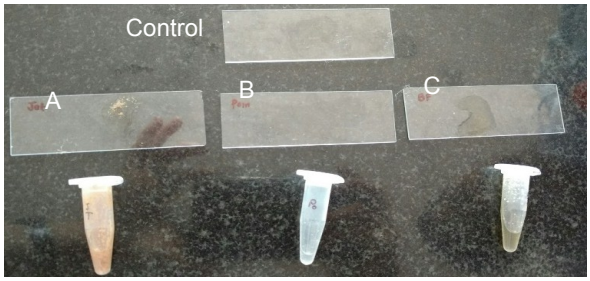

Fig. 2: APTT Assay on A. *Jatropha malacophylla,* B. *Euphorbia pulcherrima* and, C. *Artocarpus altilis*

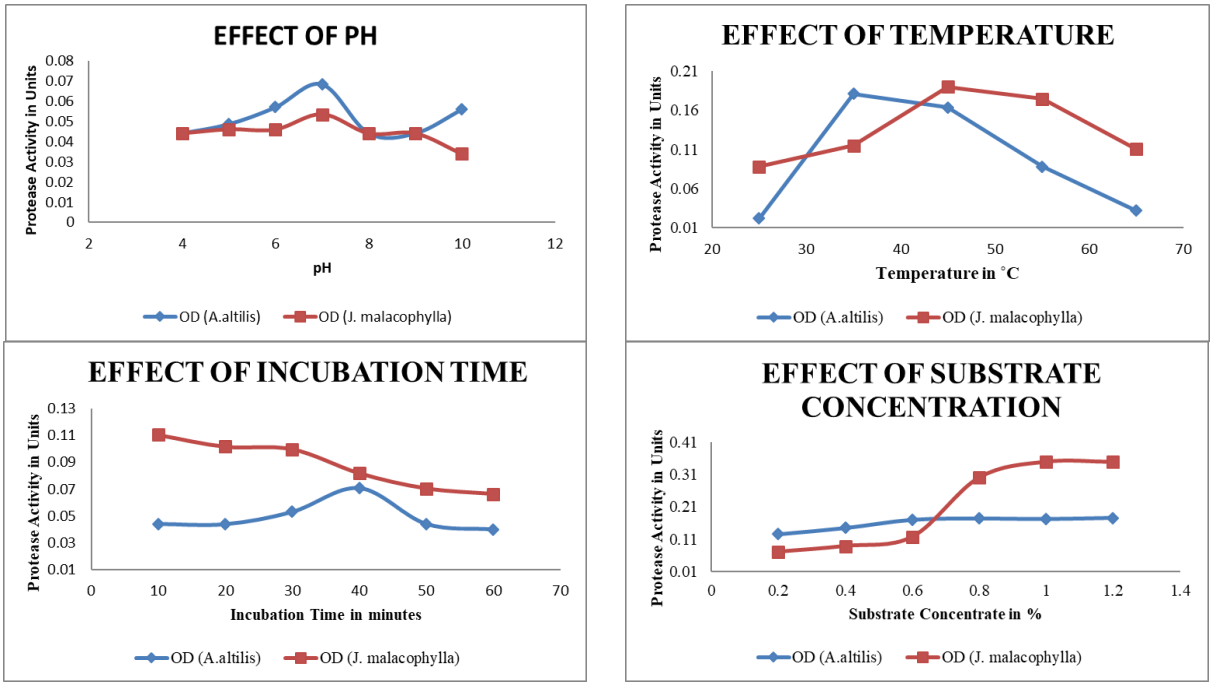

Fig. 3. *Graphical representation of the characterized enzyme activity of A.altilis and M.malacophylla*

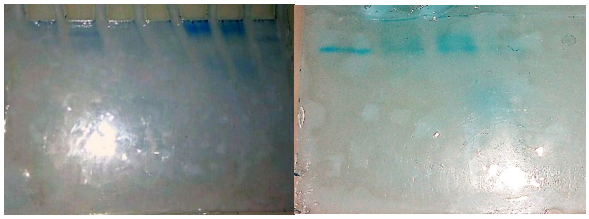

Fig. 4. *SDS-Polyacrylamide Gel Electrophoresis of the Purified latex of A. altilis and J. malacophylla.*

centage yield was found to be 25% (Balaraman *et al.,* 2007) proving *A.altilis* being a better source than any of the above mentioned.

Characterization: The optimum pH for proteolytic activity of both the plant latex was around 7.0. The enzyme obtained from *J. malacophylla* was very stable in the range of 5 to 6 while *A. altilis* was stable in the range 4 to 5. Above pH 7.5, enzyme activity abruptly decreased. (Dubey *et al.,* 2011). This signifies that the enzyme works well in the body conditions of mammals at neutral pH in stable state. *Plumeria rubra* showed similar results which is reported by Chanda *et al.* (2011) while protease obtained by Chickpeas was stable at pH 8.0 (Shamsi *et al.,* 2016). The effect of temperature on specific protease activity was found out by varying the temperature at 25°C, 35°C, 45°C, 55° C and 65°C. *A altilis* showed optimum activity at 35°C and *J. malacophylla* showed optimum activity at 45°C. Normal human body temperature is 37°C, which is close to the optimum temperature of *A. altilis,* inferring that the enzyme will show its full potential when put for clinical use. Whereas the protease of *J. malacophylla* is thermally stable at 45°C unlike other found enzymes, which change their conformation when exposed to high temperature. Bengal gram, Lentil and Groundnut showed similar result, which was reported by Akhtaruzzaman *et al.,* (2012) inferring that *A.altilis* can be a possible substitute considering high demand and price of these plant products but Chickpea was stable in the range 4°C to 37°C reported by Shamsi *et al.,* (2016) which is easily not acceptable to be used in a mammalian system. The enzymatic activity was tested at different incubation time of 10minutes, 20minutes, 30minutes, 40minutes, 50 minutes and 60minutes. *A. altilis* showed maximum activity for 10minutes of incubation and then with increase in the incubation time the activity decreased. While in *J. malacophylla,* there was steady activity from 10 to 30 minutes of incubation and the maximum activity was shown at 40 minutes of incubation.

Different protein substrate concentrations of 0.2%, 0.4%, 0.6%, 0.8%, 1.0% and 1.2% were used and it was found that maximum protease activity was observed in 0.6% concentration in *A. altilis* while in *J. malacophylla* in was observed in 0.8%. The least activity was observed in 0.8% in *A. altilis* and in *J. malacophylla* the least activity was in 0.2% (Racheal *et al.,* 2015). It was seen that the enzyme obtained from *Bacillus subtillis* showed highest activity at 4% substrate concentration reported by Moidutty *et al.* (2015) showing that both *J. malacophylla* and *A. altilis* have a better action mode in spite of high concentration of substrate (Fig. 3).

Molecular weight determination: The apparent molecular weight of the purified protease is ~80kDa and ~105kDa for *A. altilis* and *J. malacophylla* respectively based on SDS-PAGE using

Table 1. Protease enzyme purification yield for *A. altilis.*

Sample	Protease	Activity	Specific Activity	Fold Purification	Percentage Yield
Crude	0.06186 U/ml	2.72 mg/ml	0.022 U/mg	1	100%
Salt Precipitation	0.1281 U/ml	2.54 mg/ml	0.050 U/mg	2.27	93.38%
Dialysis	0.132 U/ml	2.34 mg/ml	0.0564 U/mg	2.56	86.02%
Ion Exchange	0.1962 U/ml	2.24 mg/ml	0.0875 U/mg	3.977	82.35%
Gel Filtration	0.203 U/ml	1.72 mg/ml	0.1182 U/mg	5.37	63.23%

Table 2. Protease enzyme purification yield for *J. malacophylla.*

Sample	Protease	Activity	Specific Activity	Fold Purification	Percentage Yield
Crude	0.2474 U/ml	2.12 mg/ml	0.116 U/mg	1	100%
Salt Precipitation	0.268 U/ml	1.50 mg/ml	0.117 U/mg	1.0086	70.75%
Dialysis	0.244 U/ml	1.4mg/ml	0.174 U/mg	1.5	66.03%
Ion Exchange	0.251 U/ml	1.02mg/ml	0.246 U/mg	2.1206	60.37%
Gel Filtration	0.1502 U/ml	0.56mg/ml	0.2682 U/mg	2.312	26.41%

Novex Sharp Prestained Protein Marker (Fig. 4). While the molecular weight of protease enzyme obtained from Chickpeas was found to be 23kDa by Shamsi *et al* (2016). This difference in the molecular weight are expected due to the difference in the features of the plant itself and also influenced by the methods of purification used.

Conclusion

In the present study, *A. altilis* and *J. malacophylla* were purified to obtain pure protease enzyme. Among the two, *A. altilis* proved itself by showing higher activity (63.23% percentage yield), stability (optimum temperature of 35° and pH between 4 and 5) and effectiveness. Naturally obtained enzymes tend to be more effective even in small amounts and show eminent results. Henceforth, when brought to industry level it may prove to be significantly influential and medically noteworthy. In addition, with the characters such as stability at pH 7 and 35ºC, it is fit for treatment in mammals as it matches the body conditions. Further exploitation of these enzymes can lead to a necessary transformation in the field of medicine.

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