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REGULAR ARTICLE

DETECTION AND ANALYSIS OF LYSOZYME ACTIVITY IN SOME TUBEROUS PLANTS AND *CALOTROPIS PROCERA*'S LATEX

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SUMMARY

Tuber extract of all the plant species used in the study exhibited lysozyme activity confirming the ubiquitous presence of lysozyme in plants. Among the different plants screened for protein content the tuber extract of *Solanum tuberosum* showed highest buffer soluble protein while tuber extract of *Raphanus sativus* showed the lowest protein content in sodium acetate buffer (50 Mm; pH 5.0). Tuber extract of *Raphanus sativus* showed highest lysozyme activity among all the plant species tested in this study and the activity was increased when the tuber was extracted with sodium phosphate buffer (50mM; pH 7.0). The lowest lysozyme was observed with tuber extract of *Daucus carota* in phosphate buffer (50mM; pH 7.0). The latex of the tropical species *Calotropis procera* is well known for being a rich source of the lysozyme. Lysozyme of Calotropis procera latex is not thermo labile. It did not lose much of its activity when the latex was incubated at different temperatures for 24 hours. A positive pointer for purification of this enzyme in future. Calotropis procera lysozyme can be was specifically isolated and purified from the whole latex with ammonium sulphate precipitation with 95% saturation. *Calotropis procera* lysozyme retained its activity even after precipitation with ammonium sulphate and dialysis and could hydrolyse the cell wall of *Micrococcus lysodeikticus*.

Key words: Micrococcus lysodeikticus, Tuberous Plants, Latex, Ammonium sulphate precipitation, Polyacrylamide gel electrophoresis

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1. Introduction

Soon after his discovery of 'lysozyme' (EC 3.2.1.17). Fleming detected the presence of this enzyme in many vegetable tissues, especially in the flowers (Fleming, 1922). A number of roots and tubers were also examined (Fleming, 1922). Since Fleming's observations, only a very small number of studies have been devoted to plant lysozymes, and these all deal with the enzyme contained in latex. First reported lysozyme activity in crude proteolytic enzyme preparations from papaya and fig latex (Meyer, 1946). Lysozymes have also been isolated and characterized from the latex of fig, *Hevea brasiliensis* (Glazer, 1969; Tata, 1976) and *C. procera* (Shukla, 1985). Apart from latex containing plants, lysozymes have been studied from other plant species also. Lysozymes are defined as 1, 4-β-N-acetylmuramidases cleaving the glycosidic bond between the C-1 of N-acetylmumaric acid and the C-4 of N-acetylglycosamine in the bacterial pepticoglycan (During, 1994). Some lysozymes are displayed a more or less pronounced chitinase (EC 3.2.1.14) activity corresponding to a random hydrolysis of 1, 4-β—acetylglucosamine linkage in chitin. Lysozyme is found in egg white, tears and other secretion. It is responsible for breaking down the polysaccharide walls of many kinds of bacteria and thus it provides some protection against infection. Lysozyme activity has also been found in bacteria,

bacteriophage and plants and in human leukocytes, nasal secretions, saliva and tears. Audy *et al.* (1988) have screened 410 plant species representing 116 families for lysozyme activity. A total number of 168 species belonging to 71 families have been shown to contain lysozymes. According to them even gymnosperms and bryophytes do contain lysozyme.

In the present note, to detect the occurrence of lysozymes in tuberous plants such as *Raphanus sativus*, *Zingiber officinale*, *Daucus carota*, *Solanum tuberosum*, *Ipomea batatas*, *Arisaema triphyllum*, and latex from *Calotropis procera* using colorimetric and gel activity staining procedures on SDS-PAGE. Effort has also been made to partially purify the lysozyme from the latex of *Calotropis procera* with different saturations of ammonium sulphate and the results obtained have been discussed with available literature.

2. Material and Methods

Methods for the preparation of buffers and phytopathological techniques were generally followed from Biochemistry Handbook by Long (1961) and Plant Pathologist's Pocket book by Johnston and Booth (1983) respectively.

Plant sources

Plants such as, *Raphanus sativus* (Brassicaceae), *Zingiber officinale* (Zingiberaceae),*Daucus carota* (Apiaceae), *Solanum tuberosum* (Solanaceae), *Ipomea batatas* (Solanaceae), *Arisaema triphyllum* (Araceae) and *Calotropis procera* (Asclepiadaceae) latex were collected in and around Chennai.

Preparation of crude enzyme extract from the tubers

Apparently healthy tubers were washed several times with distilled water, blotted and ground in a pre-cooled mortar and pestle with acid washed sand at 4°C. Tuber homogenates (1 g/3 mL; w/v) were obtained using Tris-HCl buffer (50 mM; pH 7.0), Tris-HCl buffer (50 mM; pH 8.5), Sodium acetate buffer (50mM; pH 5.0) and Phosphate buffer (50 mM; pH 7.0) with equivalent amount of acid wash sand. Extraction buffer included 0.1% ascorbic acid and 0.001% polyvinyl polypyrrolidone. The homogenate was filtered through 4 layers of cheese cloth and the filtrate was centrifuged at 10,000 g (Beckman J2-21 centrifuge, USA) for 20 min. at 4°C. The clear supernatant was used as the enzyme source.

Latex preparation

The crude latex of non-cultivated and healthy plants was collected in distilled water (ratio 1:1) in plastic tubes that were shaken gently, closed and maintained at environmental temperature (25-28°C) until handled in the laboratory. The samples were initially submitted to centrifugation at 25 °C during 10 min in a bench centrifuge.

Estimation of protein

A known volume cell free homogenate of tubers and the latex was used for protein estimation. The volume of the extract and the latex was made up to 1mL with distilled water and to which 5mL of Bradford's reagent was added. It was mixed well and immediately read at 595nm using a spectrophotometer (Milton Roy). The protein content was calculated using a standard graph constructed with bovine serum albumin (Sigma Chemical Co., USA)

Substrate Preparation

A known amount (5mg) of the lyophilized cell walls of *Micrococcus lysodeikticus* was dissolved in 1 mL of sodium acetate buffer (50 mM. pH-5.0). From this stock, 60 µl of the suspension was used to arrive at 300 µg/3mL reaction mixture.

Lysozyme Assay

Lysozyme activity was measured as the rate of lysis of *M. lysodeikticus* cell walls. To a known amount of protein (250 µg), 60 micro litre of the substrate stock as prepared above was added and the volume was made up to 3.0 mL with sodium acetate buffer (50mM pH-5.2). The reaction mixture was incubated at 37°C and decrease in absorbance was recorded. The enzyme activity was monitored at 570 nm in Milton Roy Spectrophotometer and the enzyme activity was calculated as the amount of protein

required to decrease the absorbance value by 0.01 units.

Protein on Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in polyacrylamide slab gel (10% (w/v) separating and 5% (w/v) stacking) according to the method of Laemmli (1970). The SDS-PAGE solutions were prepared, adjusted the pH wherever necessary, and stored in amber colored bottles at 4°C until further use. SDS-PAGE was performed using the crude supernatants obtained from tuberous plant species described above and with latex of *C. procera*. Electrophoresis was performed at room temperature for 1-2 hours at a constant current (150V).

Detection of lysozyme activity on SDS-PAGE under non-reducing condition

The lysozyme activity on SDS-PAGE was analyzed following the method of Audy et al, (1988). The separating gel was incorporated with lyophilized cell walls of M. lysodeikticus (0.2% w/v). After protein separation the gel was incubated in sodium phosphate buffer, (50mM; pH5.0) with 1% (v/v) Triton X-100 for 16 hours at 37°C under gentle shaking. The lytic activity of lysozyme was visualized as a clear transparent zone against the greyish background. The gel was stored in 7% (v/v) acetic acid and then photographed.

Fractionation of *C. procera* latex proteins with ammonium sulphate

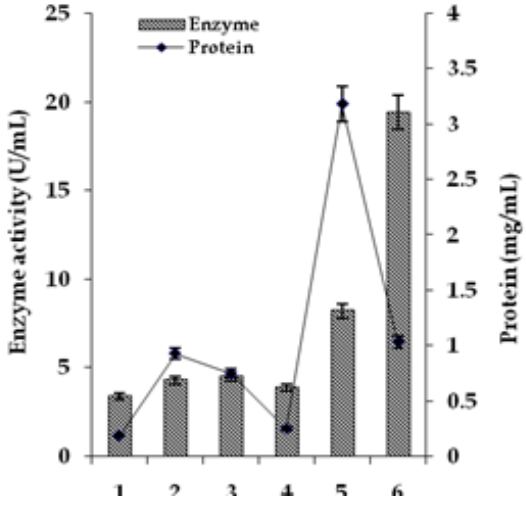
The latex was collected in a clean glass beaker by breaking tender parts of the plant. This latex was diluted with equal volume of phosphate buffer (10 Mm; pH 7.0) and kept overnight at 4°C. The supernatant was decanted and centrifuged at 12,000g for 20 min at 4°C. The clear supernatant was decanted and dialyzed against 10 mM phosphate buffer (pH 7.0). The supernatant was subjected to sequential precipitation with ammonium sulphate 20%, 40%, 60%, 80% and 95% saturations. The precipitated protein pellet was dissolved in 10 mM phosphate buffer (pH 7.0) and dialyzed against the same buffer to remove the salts sulphate. The protein concentration in the supernatant was estimated according to the method of Bradford method described below and used as the enzyme source

3. Result and Discussion

Protein content and lysozyme activity in sodium acetate buffer pH 5.0

The buffer soluble protein content of different tubers extracted with sodium acetate buffer 50mM (pH 5.0). Among the tubers *Solanum tuberosum (*3.18mg/mL) was the highest buffer soluble protein content and *Arisaema triphyllum* (19.4 U/mL) observed highest enzyme activity (Fig. 1). The protein content of various tuber extracts could be arranged in following orders, *Solanum tuberosum* (3.18mg/mL), *Arisaema triphyllum* (1.035 mg/mL), *Daucus carota* (0.93 mg/mL), *Ipomea batatas* (0.75 mg/mL), *Zingiber officinale* (0.252 mg/mL), *Raphanus sativu* (0.192mg/mL). The tuber of *Arisaema triphyllum* (19.4 U/mL) observed the highest lysozyme activity. The lysozyme activity in the extracts of various tuber could be arranged in following orders *Arisaema triphyllum* (19.4 U/mL), *Solanum tuberosum* (8.2 U/mL), *Ipomea batata* (4.5 U/mL, *Daucus carota* (4.3 U/mL), *Zingiber officinale* (3.9 U/mL), *Raphanus sativus* (3.4 U/mL) (Fig. 1).

Figure: 1. Protein content and lysozyme activity of plant samples using Sodium acetate buffer pH-5.0

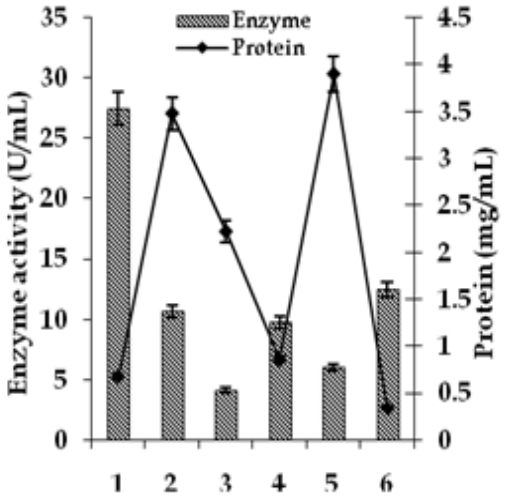


1. *Raphanus sativus*; 2. *Daucus carota*; 3.*Ipomea batatas*; 4. *Zingiber officinale*; 5. *Solanum tuberosum*; 6. *Arisaema triphyllum*

Protein content and lysozyme activity in phosphate buffer pH 7.0

The buffer soluble protein content of different tubers extracted with phosphate buffer 50 mM (pH 7.0). Among the tubers *Solanum tuberosum* (3.9 mg/mL) was the highest buffer soluble protein content and *Arisaema triphyllum* (0.334 mg/mL) was the least amount of protein (Fig. 2). The protein content of various tuber extracts could be arranged in following orders, *Solanum* tuberosum (3.9 mg/mL), Daucus *carota* (3.48 mg/mL), *Ipomea batatas* (2.22 mg/mL), *Zingiber officinale* (0.855 mg/mL), *Raphanus sativus* (0.675 mg/mL), *Arisaema triphyllum* (0.334 mg/mL). The tuber extract of *Raphanus sativus* (27.5 U/mL) was the highest lysozyme activity while *Ipomea batatas* (4.1 U/mL) showed the least lysozyme activity. The lysozyme activity of various tuber extracts could be arranged in following orders *Raphanus sativus* (27.5 U/mL), *Arisaema triphyllum* (12.5 U/mL), *Daucus carota* (10.6 U/mL), *Gingiber officinale* (9.7 U/mL),*Solanum tuberosum* (6.0 U/mL), *Ipomea batatas* (4.1 U/mL)(Fig. 2).

Figure: 2. Protein content and lysozyme activity in phosphate buffer pH 7.0

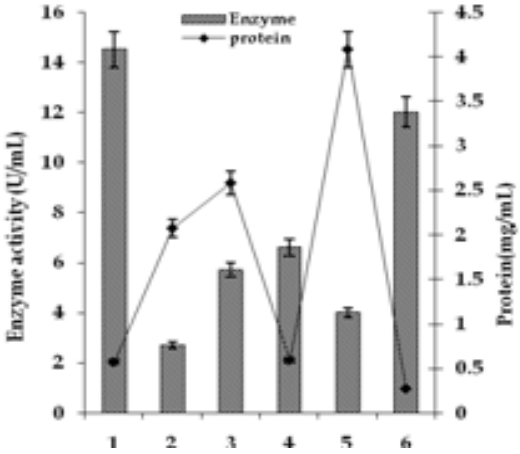


1. *Raphanus sativus*; 2. *Daucus carota*; 3.*Ipomea batatas*; 4. *Zingiber officinale*; 5. *Solanum tuberosum*; 6. *Arisaema triphyllum*

Protein content and lysozyme activity in Tris- HCL buffer pH 7.0

The buffer soluble protein content of different tubers extracted with Tris- HCL buffer 50 mM (pH 7.0). Among the tubers *Solanum tuberosum* (4.08 mg/mL) was the highest buffer soluble protein content and the extract of *Arisaema triphyllum* (0.27 mg/mL) was the least amount of protein (Fig. 3). The protein content of various tuber extracts could be arranged in following orders, *Solanum tuberosum* (4.08 mg/mL), *Ipomea batatas* (2.58 mg/mL), *Daucus carota* (2.07 mg/mL), *Zingiber officinale* (0.588 mg/mL), *Raphanus sativus* (0.57 mg/mL), *Arisaema triphyllum* (0.27 mg/mL). The tuber extract of *Raphanus sativus* (14.5 U/mL) was the highest enzyme activity whereas the lowest lysozyme activity was observed with the extract of *Daucus carota* (2.7 U/mL). And the lysozyme activity of various tuber extracts could be arranged in following orders *Raphanus sativus* (14.5 U/mL), *Arisaema triphyllum* (12.0 U/mL), *Gingiber officinale* (6.6 U/mL), *Ipomea batatas* (5.7 U/mL), *Solanum tuberosum* (4.0 U/mL), *Daucus carota* (2.7 U/mL) (Fig. 3).

Figure: 3. Protein content and lysozyme activity in Tris- HCL buffer pH 7.0

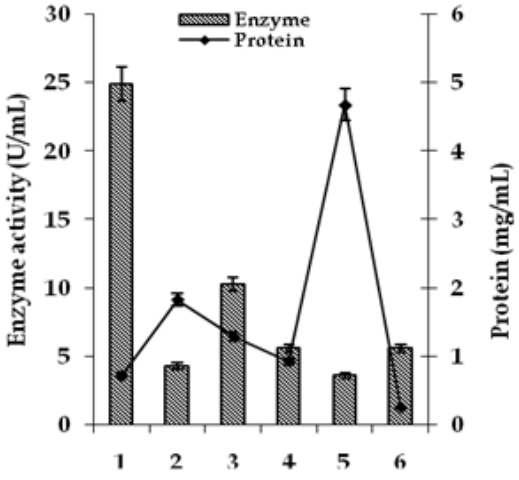


1. *Raphanus sativus*; 2. *Daucus carota*; 3.*Ipomea batatas*; 4. *Zingiber officinale*; 5. *Solanum tuberosum*; 6. *Arisaema triphyllum*

Protein content and Lysozyme activity in Tris -HCL buffer pH 8.5

The buffer soluble protein content of different tubers extracted with Tris-HCL buffer 50 mM (pH 8.5). Among the tubers of *Solanum tuberosum* (4.68 mg/mL) showed highest buffer soluble protein content and *Raphanus sativus* (24.9 U/mL), showed highest enzyme activity (Fig. 4). The protein content of various tuber extracts could be arranged in following orders, *Daucus carota* (1.83 mg/mL) > *Ipomea batatas*(1.29 mg/mL), *Zingiber officinale*(0.924 mg/mL), *Raphanus sativus* (0.72 mg/mL), *Arisaema triphyllum*(0.252 mg/mL). And the lysozyme activity of various tuber extracts could be arranged in following orders *Raphanus sativus* (24.9 U/mL), *Ipomea batatas* (10.3 U/mL), *Arisaema triphyllum* (5.6 U/mL), *Daucus carota* (4.3 U/mL), *Gingiber officinale* (5.6 U/mL), *Solanum tuberosum* (3.6 U/mL) (Fig. 4)

Figure: 4. Protein content and Lysozyme activity in Tris -HCL buffer pH 8.5

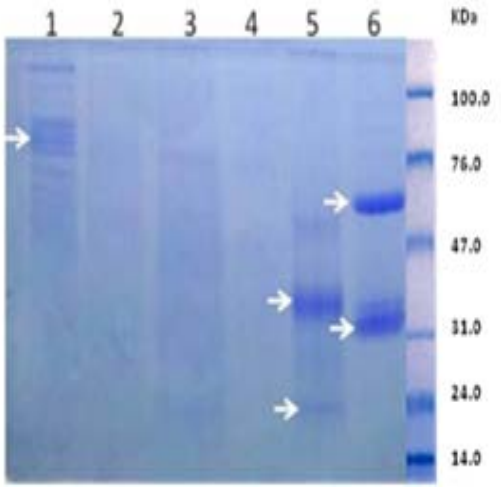


1. *Raphanus sativus*; 2. *Daucus carota*; 3.*Ipomea batatas*; 4. *Zingiber officinale*; 5. *Solanum tuberosum*; 6. *Arisaema triphyllum*

Protein profile of extracts of different tubers on SDS PAGE

The protein profile of extracts of tubers extracted with appropriate buffer. The extract of *Raphanus sativus* showed several high molecular weight proteins (lane 1, arrowhead) aggregated close to each other (Fig. 5). The extract of *Solanum tuberosum* showed two prominent proteins one of which around 35 kDa and the other one approximately 24 kDa (lane 5, arrowheads). There were two very prominent proteins, approximately 60 and 30 kDa respectively, observed with the extracts of *Arisaema triphyllum* (lane 6, arrow heads). Although same amounts of proteins were loaded in each of the wells, the extracts of *Daucus carota, Ipomea batatas* and *Zingiber officinale* did not show clear proteins bands on the gel (Fig. 5;Lanes 2,3,&4).

Figure: 5. Protein profile of extracts of different tubers on SDS PAGE



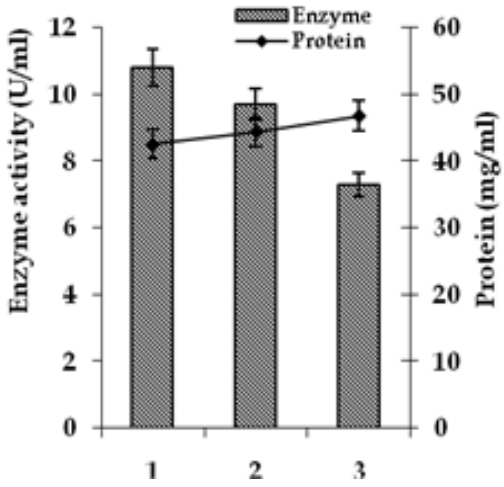
Lanes: 1. *Raphanus sativus*; 2. *Daucus carota*; 3.*Ipomea batatas*; 4. *Zingiber officinale*; 5. *Solanum tuberosum*; 6. *Arisaema triphyllum*

***Calotropis procera* latex protein content and lysozyme activity at different temperature**

The latex was incubated at three different temperatures like 4°C, room temperature and 37°C for 24 hours and the lysozyme assay was carried out as described in materials and methods. The latex incubated at room temperature showed highest lysozyme activity closely followed by the samples incubated at 4°C and 37°C (Fig. 6). The samples incubated at 4°C and 37°C lost enzyme activity to 11 and 32% respectively after 24 hours when compared to the sample incubated at room temperature. Therefore, it

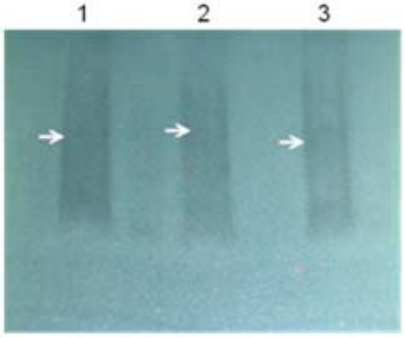
was decided to partially purify the latex lysozyme at this temperature (Fig. 7).

Figure: 6. *Calotropis procera* latex protein content and lysozyme activity at different temperature



1. 30ºC; 2. 4 ºC; 3. 37 ºC

Figure: 7. Gel activity staining for lysozyme of latex of *calotropis procera* incubated at different temperature for 24 h

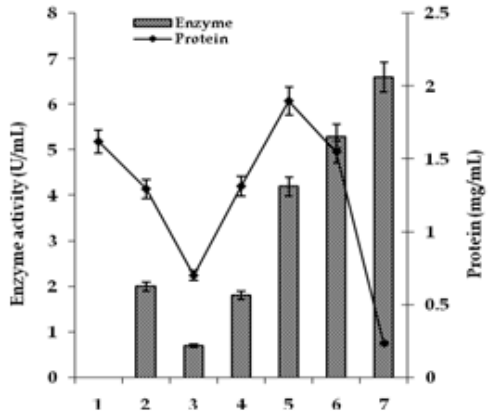


Lanes: 1. 30ºC; 2. 4 ºC; 3. 37 ºC (Each lane received 50 µg of protein)

Protein content and Lysozyme activity of crude extract and ammonium sulphate precipitated proteins of *C. procera* Latex

The protein content and lysozyme activity of whole latex and ammonium sulphate precipitated fractions. The fraction obtained after 60% saturation yielded the highest amount of protein (1.899 mg/mL) than the other saturations used. The amount of protein yield could be arranged in the following order 60% (1.89 mg/mL), 80% (1.548 mg/mL), 40% (1.314 mg/mL), 20% (0.702 mg/mL), 95% (0.234 mg/mL). As evident from the figure 8, the proteins obtained after 95% saturation exhibited the highest lysozyme activity (6.6 U/mL) followed by 80% (5.3 U/mL), 60% (4.2 U/mL). The lowest lysozyme activity was observed with proteins precipitated with 20% ammonium sulphate saturation (0.7 units/mL; Fig. 8).

Figure: 8. Protein content and lysozyme activity of whole latex and ammonium sulphate precipitated fractions



1. Wholelatex; 2. Supernatant after centrifugation of whole latex; 3. 20% Saturation; 4. 40% Saturation; 5. 60% Saturation
6. 80% Saturation; 7. 95% Saturation

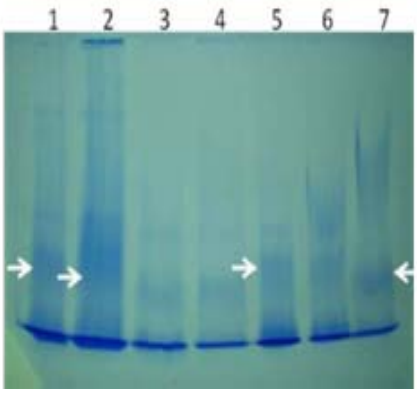
The proteins from the latex of *C. procera* were fractionated with ammonium sulphate at different saturations ie. 20,40,60,80 and 95% in an effort to partially purify and characterize the physico chemical properties. The precipitated proteins were collected by centrifugation and the proteins were dialysed against phosphate buffer, 10 mM; pH 6.0 to remove the salts. After dialysis, protein estimation (Fig. 9) and lysozyme activity were determined by colorimetrical and on sds-page.

Protein profile of whole latex and ammonium sulphate precipitated proteins on SDS – PAGE

The proteins from the whole latex and from the ammonium suphate precipitated fractions were electrophoresed on 10% SDS-

polyacrylamide gel and the resolved proteins were stained with coomassie blue R-250. Both the whole latex and ammonium sulphate fractionated proteins at 60, 80 and 95% saturations all exhibited prominent protein bands of approximate molecular mass 20-40 kDa (Fig. 9, lanes 1, 2, 5, 6 and 7, arrowheads).

Figure: 9. Protein profile of whole latex and ammonium precipitated protein on SDS-PAGE

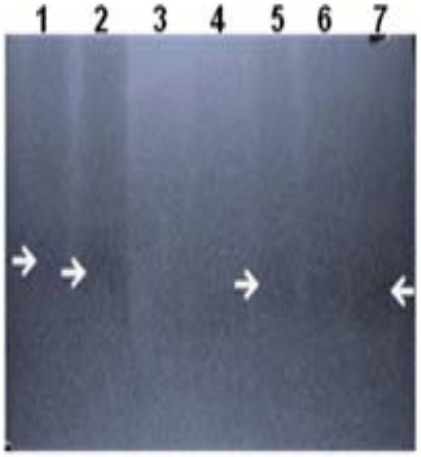


Lanes:1. Wholelatex; 2. Supernatant after centrifugation of whole latex; 3. 20% Saturation; 4. 40% Saturation; 5. 60% Saturation
6. 80% Saturation; 7. 95% Saturation

Lysozyme activity of whole latex and ammonium sulphate precipitated on SDS – PAGE

The hydrolytic activity of *C. procera* latex lysozyme on SDS-PAGE incorporated with lyophilized cell walls of *M. lysodeikticus* (Fig. 10). The whole latex, supernatant after centrifugation and all the proteins precipitated with different saturations of ammonium sulphate hydrolysed the cell wall and the lysed areas appeared transparent against the greyish background. The lytic activity was more prominent in latex after centrifugation (lane 2) and the protein precipitated with ammonium sulphate at 95% saturation (lane 7).

Figure: 10. Lysozyme activity of whole latex and ammonium sulphate precipitated on SDS – PAGE



Lanes:1. Wholelatex; 2. Supernatant after centrifugation of whole latex; 3. 20% Saturation; 4. 40% Saturation; 5. 60% Saturation
6. 80% Saturation; 7. 95% Saturation

4. Conclusion

In this present study to detect the presence and quantify the lysozyme in several tubers of plant species such as *R. sativus*, *Z. officinale*, *D. carota*, *S. tuberosum*, *I. batatas*, *A. triphyllum*, and from the latex of *C. procera.* We wanted to see if the *C. procera* lysozyme is stable over a period of time at different temperatures. The whole latex was incubated at three different temperatures such as 4°C, room temperature and 37°C for 24 hours. They were then assayed for lysozyme activity calorimetrically as well as by enzyme activity staining method. As evident from figure 7 the enzyme remained active in all the three temperatures after 24 hours. It was concluded that *C. procera* lysozyme is not thermo labile.

Having verified the thermal stability of *C. procera* lysozyme, it was aimed to partially purify the enzyme and study the physic-chemical properties. Therefore the proteins from the latex were sequentially precipitated with ammonium sulphate. As is evident in Figure 10, the proteins precipitated with 95% saturation showed relatively more lytic activity than the proteins precipitated with 20, 40, 60 and 80% saturations. Evidently the proteins precipitated with 95% saturation had higher level of protein of approximate

molecular weight of 15 kDA (Fig. 10; lane 7, arrowhead). It is evident from the results that the *C. procera* lysozyme is specifically precipitated with 95% saturation of ammonium sulphate and can be used for further purification. Experiments are on to completely purify and characterize the enzyme.

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