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Characterization of protease Isolated from root of *Choreospondias axillaries* (Lapsi)

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Abstract: *Choreospondias axillaries,* locally called Lapsi is a dioecous, deciduous fruit bearing plant, with multiple daily uses. The protease was extracted with 0.1M phosphate buffer of pH 7 and then precipitated successively with TCA and ammonium sulfate. Thus obtained ammonium sulfate precipitated fraction of protease was characterized. The protease from root showed maximum activity at pH 10, temperature at 35°C. The optimum time of incubation was 15 minute. A bell shaped curve was obtained for the effect of enzyme concentration with optimum enzyme 50µg. The K_m and V_{max} value of root were 1.53nM and 52.91pmol/min.

Keywords: Protease, Lapsi, Purification, Characterization, Isolation

1. Introduction

Choreospondias axillaries: (Locally called "lapsi"), is a large, deciduous fruit-bearing dioecous tree of the family Anacardiaceae. A native of the Nepal hills (850-1900m). The tree has also been reported from India, China, Thailand, Japan and Vietnam. Lapsi wood is used as light construction timber and fuel wood; seed stones are used as fuel in brick kilns and the root has medicinal value for treating secondary burns [1]. Nepal is unique in processing and utilizing lapsi fruits. Lapsi fruit is consumed fresh, pickled or processed into a variety of sweet and sour fruit products locally called "Titaura" and Candy. The fruits are rich in vitamin C content [2]. Though the tree is dioecous, but it is difficult to distinguish male and female plants at the seedling stage [3].

Agrawal and Kesari were first to observe strong proteolytic activity in Lapsi leaves [4]. Dekhang and Sharma reported optimum pH of 7 for the protease [5]. The protease is not inhibited by phenyl-methane-sulfonyl-fluoride (PMSF) and somewhat about 20-30% inhibited by sodium iodoacetic acid, thus revealing that protease is not a serine protease [5]. No smaller proteolytic products of BSA could be seen in SDS-PAGE using silver staining indicating that the protease is not exopeptidase, Protease activity can be repeatedly precipitated by 0.2 M tricloroacetic acid TCA [6].

The present study was conducted to isolate, purify and characterize the proteases from the root of lapsi plant.

2. Experimental

2.1. Materials

Roots of *Choreospondias axillaries* were collected from Sirutaar area Bhaktapur, Nepal. All other reagents used were of analytical grade.

2.2. Preparation of the Crude Extracts

Roots were shed dried and grinded to fine powder.10g of root powder was washed with 50ml of acetone. Then sample was dried to remove remained acetone. The acetone washed sample was heated with 100ml phosphate buffer (pH 7, 0.1M) at 70ºC for 30 min on water bath. After 30min of heat treatment sample was filtered and the filtrate was used as crude extract. Thus obtained extract was used for protein estimation and enzyme assay.

2.3. TCA Precipitation of the Crude Extracts

The filtrate i.e. crude extract was then subjected to TCA precipitation [6]. 2.45M of TCA solution was used to precipitate the crude extract so as to make final concentration of TCA 0.2M. Then sample was subjected to centrifugation at 10000rpm for 15 min at 4ºC. After

centrifugation the pellet obtained was dissolved on chilled acetone and again centrifuged at 14000rpm for 5 min at 4°C and repeated twice to remove remaining TCA. Thus obtained pellet was dissolved in 0.1M phosphate buffer of pH 7 and was used for protein estimation and enzyme assay.

2.4. Ammonium Sulfate Salt Precipitation of TCA Precipitated Extracts

The TCA precipitated extract was precipitated by ammonium sulfate (20-80% w/v). The solution were kept on for an hour in cold condition (4°C) and then centrifuged at 10000rpm for 15 min at 4°C. The precipitate was dialyzed against 0.1M phosphate buffer of pH 7 [7]. Following dialysis the fractions of sample was mixed and this extract (ammonium sulfate precipitated) was used for protein estimation and enzyme assay.

2.5. Protein Estimation of Different Fractions of the Sample by Bradford Assay

Bradford assay was carried out to determine the protein concentration of crude, TCA precipitated and ammonium sulfate precipitated extracts of root [8]. Using the standard calibration curve of BSA (Figure 1) the amount of BSA in different fractions was calculated. (**Note:** molecular weight of BSA 69323.4 Da [9].)

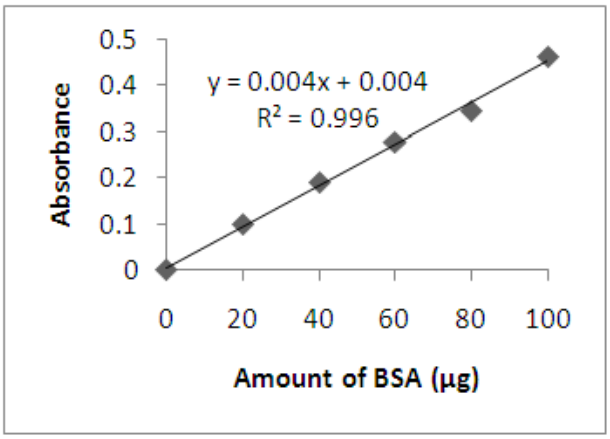

Figure 1. *Standard calibration curve of BSA for Bradford assay. 30, 20 & 50µl of crude, TCA precipitated and ammonium sulfate precipitated extracts respectively were mixed with phosphate buffer to maintain the volume 1ml. 5ml Bradford reagent was added in each. After 5 min of incubation absorbance was taken at 595nm.*

2.6. Protease Assay of Different Fractions of the Samples

The protease activity of crude, TCA precipitated and ammonium sulfate precipitated extracts of root were calculated with BSA as a substrate by direct method. A reaction mixture of 200µl was made with BSA (1mg/ml), extract and 0.1M phosphate buffer of pH 7. The reaction mixture was then incubated. The reaction mixture was then quenched with 2.3ml of Bradford reagent and the absorbance was measured at 595nm. The amount of BSA, enzymes, buffer, along with incubation time and temperature varied with different experiments that were done. However 2 controls a) enzymes only and b) BSA only, both without incubation was taken in each case. The calculation performed henceforth is control dependent.

2.7. Characterization of Protease of 20-80% Ammonium Sulfate Precipitated Extracts

The 20-80% ammonium sulfate precipitated extract of root was characterized with BSA as a substrate. The effect of pH, temperature, time of incubation, enzyme concentration and the substrate concentration on protease activity were measured.

2.8. Assessment of Kinetic Constants of Enzymes of 20-80% Ammonium Sulfate Precipitated Extracts

The reciprocal of reaction velocity was plotted against the reciprocal of the corresponding substrate concentration, giving the Lineweaver-Burk plot for the enzyme with the substrate BSA. The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) of enzyme were determined from the Lineweaver-Burk plot [10].

2.9. Statistical Analysis

All data were computes from the mean of three independent experiments and expressed as mean ± SD. Statistical analysis was carried out using GraphPad Prism 6 software.

3. Results

The crude extract contained 1.65mg/ml of protein with specific activity 659.58pmol min^{-1} mg^{-1}. The TCA precipitated extracts contained 1.26mg/ml of protein with specific activity 783.33pmol min^{-1} mg^{-1} and the ammonium sulfate precipitated extract contained 0.61mg/ml of protein with specific activity 2235.7pmol min^{-1} mg^{-1}. The specific activity decreased from crude extract to TCA precipitation and then increased to ammonium sulfate precipitation.

3.1 Optimum pH and Temperature of the Protease

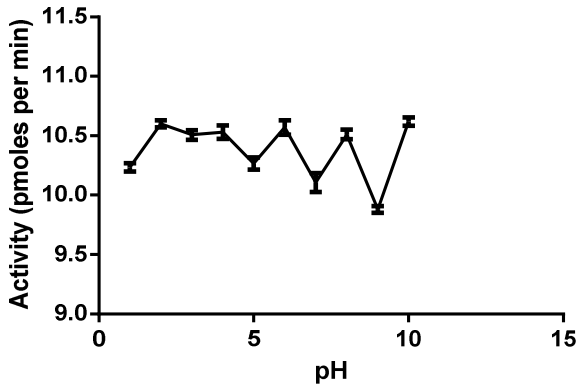

Figure 2. *Effect of pH on Protease Activity of Ammonium Sulfate Extract. Reaction mixture contained 25µg BSA (1mg/ml), 22µl enzyme (13.42µg) and 153µl of buffers with difference in pH. After 30 min incubation at room temperature direct method was performed.*

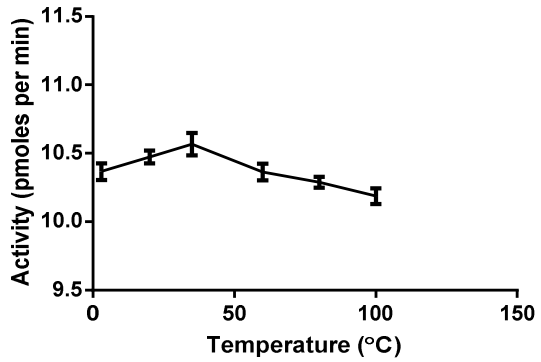

Figure 3. *Effect of Temperature on Protease Activity. The enzyme was incubated at different temperature. 200µl reaction system was mixed with 22µl of enzyme, 25µg of BSA and 153µl of phosphate buffer. Direct method was performed after 30 min of incubation.*

The protease showed a pH optimum at pH 10 (Figure 2) and maximum activity at 35ºC (Figure 3) with BSA as a substrate. Above the optimum temperature proteolytic activity decreased gradually with increase in temperature.

3.2. Optimum Time of Incubation and Enzyme Concentration of the Protease

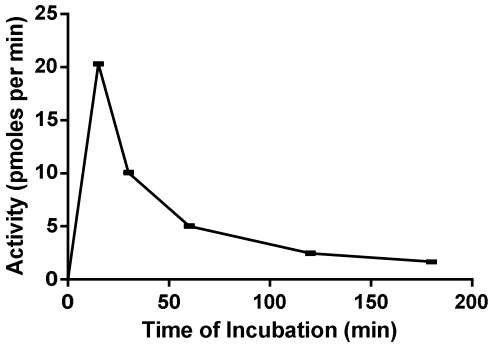

Figure 4. *Effect of Time of Incubation on Protease Activity. 200µl of reaction system containing 25µg of BSA (1mg/ml, 22µl of enzyme,) and 153µl phosphate buffer was incubated for different time intervals and direct method was performed.*

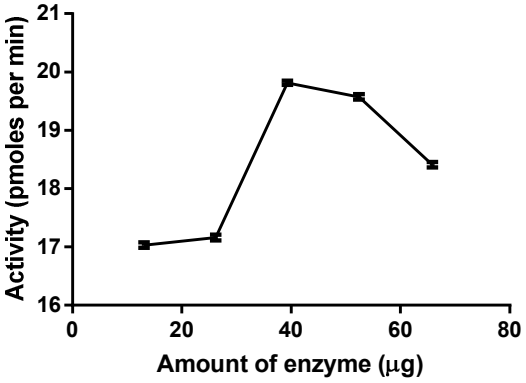

Figure 5. *Effect of Enzyme Concentration on Protease Activity. 25µg of BSA (1mg/ml) and varying amount of protease was added. The final volume was made 200µl by adding phosphate buffer. It was the incubated for 30 min at room temperature and direct method was performed.*

The optimum time of incubation of root protease was found to be 15 min (Figure 4). The protease activity was higher in the range of 45 - 60µg of enzyme with optimum amount of enzyme 50µg (Figure 5). Above the optimum amount of enzyme the activity was decreased.

3.3. Effect of Substrate Concentration on Protease Activity and Kinetic Constants

Lineweaver-Burk plot was used to determine the K_m and V_{max} values of lapsi root protease. The K_m and V_{max} values were determined to be 1.53nM and 52.91pmol/min respectively.

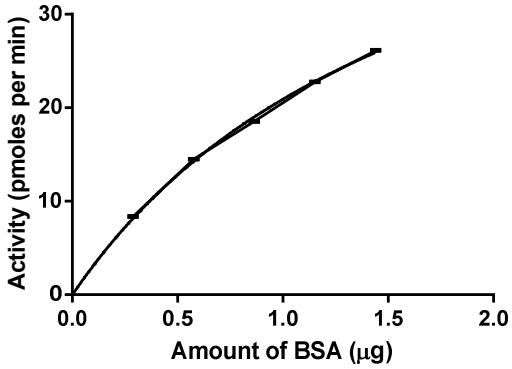

Figure 6. *Effect of Substrate Concentration on Protease Activity. The reaction mixture contained 22µl protease (13.42µg), different amount of substrate (BSA) and phosphate buffer (pH 7, 0.1M) was added to make final volume 200µl. The reaction mixture was incubated at room temperature for 30 min and direct method was performed.*

4. Discussions

The specific activity of lapsi root protease increased from crude extract to TCA precipitated extracts and from TCA precipitated extract to ammonium sulfate precipitated extracts indicates that the successive purification with good recovery of protease.

The optimal pH 10 of root protease suggests that it is of acidic protease.

The initial increase in protease activity with temperature up-to optimal temperature is may be due to collision between substrate and the protease, however after optimal temperature the decrease in proteolytic activity is due to inactivation of protease by heat.

The proteolytic activity decreases after optimal incubation time (15min) is due to inactivation of enzyme with time. The bell shaped curve for the effect of enzyme concentration on protease activity indicates that the protease activity increases with increase in enzyme concentration only up-to optimal concentration and then begins to decrease due to oversaturation of enzyme.

The linear line obtained for the effect of substrate concentration on protease activity indicates that the rate of reaction increases with increase in substrate concentration. The low Km value of root protease indicates that the substrate is tightly bound to enzyme.

5. Conclusions

The protease from root of lapsi has been successfully purified to homogeneity by TCA and ammonium sulfate and characterized.

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