Biochemical Characterization of Protease Isolated from Different Parts of 
Choerospondias axillaris (Lapsi)

Shrawan Kumar Upadhyay*, Rubin Thapa Magar and Chandan Jung Thapa
Department of Biochemistry, Universal Science College, Chakupat, Lalitpur, Nepal

Abstract
Proteases were isolated from leaf, bark and root of Choerospondias axillaris, locally called Lapsi. Choerospondias axillaris is a dioecious, deciduous fruit bearing tree of the family Anacardiaceae. A native of the Nepal hills (850-1900 m). 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 bark 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 dioecious, but it is difficult to distinguish male and female plants at the seedling stage [3].

Agrawal et al. [4] were first to observe strong proteolytic activity in Lapsi’s leaves. 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 trichloroacetic acid TCA [6]. The present study was conducted to isolate, purify and characterize the proteases from different parts of the lapsi plant.

Keywords: Protease; Lapsi; Purification; Characterization

Introduction
Choerospondias axillaris (locally called “lapsi”) is a large, deciduous fruit-bearing dioecious tree of the family Anacardiaceae. A native of the Nepal hills (850-1900 m). 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 bark 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 dioecious, but it is difficult to distinguish male and female plants at the seedling stage [3].

Experimental Procedure
Materials
Leaf, bark and root of Choerospondias axillaris were collected from Sirutaar area Bhaktapur, Nepal. All other reagents used were of analytical grade.

Preparation of the crude extracts
Samples were shed dried and ground to fine powder. 24, 23 and 10 g of leaf, bark and root powder respectively were washed with 100 ml of acetone. Then sample was dried to remove remained acetone. The acetone washed sample was heated at 70°C on water bath with 0.1 M phosphate buffer of pH 7; 200 ml for leaf and bark samples and 100 ml for root samples. After 30 min of heat treatment samples were filtered and the filtrates were used as crude extracts. Thus obtained extracts were used for protein estimation and enzyme assay.

TCA precipitation of the crude extracts
Filtrates i.e., crude extracts were then subjected to TCA precipitation [6]. 2.45 M of TCA solution was used to precipitate the crude extract so as to make final concentration of TCA 0.2 M. Then samples were subjected to centrifugation at 10000 rpm for 15 min at 4°C. After centrifugation, the pellets obtained were dissolved on chilled acetone and again centrifuged at 14000 rpm for 5 min at 4°C and repeated twice to remove remaining TCA. Thus obtained pellets were dissolved in 0.1 M phosphate buffer of pH 7 and were used for protein estimation and enzyme assay.

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

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 leaf, bark and root [8]. Using the standard calibration curve of BSA the amount of BSA in different fractions was calculated. (Note: molecular weight of BSA 69323.4 Da [9]).

*Corresponding author: Shrawan Kumar Upadhyay, Department of Biochemistry, Universal Science College, Chakupat, Lalitpur, Nepal, E-mail: shrawankumar_44@yahoo.com

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Protease assay of different fractions of the samples

The protease activity of crude, TCA precipitated and ammonium sulfate precipitated extracts of leaf, bark and root were calculated with BSA as a substrate by direct method. A reaction mixture of 200 µl was made with BSA (1 mg/ml), extract and 0.1M phosphate buffer of pH 7. The reaction mixture was then incubated. The reaction mixture was then quenched with 2.3 ml of Bradford reagent and the absorbance was measured at 595 nm. 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.

Characterization of protease of 20-80% ammonium sulfate precipitated extracts

The 20-80% ammonium sulfate precipitated extract of each sample-leaf, bark and root were 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.

Assessment of kinetic constants of enzymes of 20-80% ammonium sulfate precipitated extracts

The reciprocal of reaction velocity was plotted against the reciprocal substrate concentration on protease activity were measured. The Michaelis-Menten constant (Km) and maximum reaction velocity (V_max) of enzyme were determined from the Lineweaver-Burk plot [10].

Statistical Analysis

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

Results

The protein concentration, purification steps and specific activity all the fractions of each samples can be summarized in the tables 1-3. The activity of each fraction of the samples is shown in the figure 1.

Optimum pH

Optimal proteolytic activity for leaf was obtained at pH 9 and comparable activities were also seen at pH 3, and pH 6 (Figure 2A), while for bark the optimal activity was seen at pH 5 and comparable activity was also seen at pH 2 (Figure 2B). In case of root the maximum activity was seen at pH 10 and comparable activities were seen too at pH 2, pH 6 and pH 8 (Figure 2C).

Optimum temperature

The optimum temperature of leaf extract was found to be 20°C (room temperature). The activity gradually decreased with increase in temperature upto 80°C and then activity got rised slightly (Figure 3A). The optimal temperature for bark was found to be 60°C (Figure 3B) and for root the optimal temperature was 35°C (Figure 3C).

Optimum time of Incubation

The proteolytic activity of leaf, bark as well as root were maximum at 15 min and then decreased gradually (Figure 4A –4C).

Protease assay of different fractions of the samples

The protease activity of crude, TCA precipitated and ammonium sulfate precipitated extracts of leaf, bark and root were calculated with BSA as a substrate by direct method. A reaction mixture of 200 µl was made with BSA (1 mg/ml), extract and 0.1M phosphate buffer of pH 7. The reaction mixture was then incubated. The reaction mixture was then quenched with 2.3 ml of Bradford reagent and the absorbance was measured at 595 nm. 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.

Characterization of protease of 20-80% ammonium sulfate precipitated extracts

The 20-80% ammonium sulfate precipitated extract of each sample-leaf, bark and root were 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.

Assessment of kinetic constants of enzymes of 20-80% ammonium sulfate precipitated extracts

The reciprocal of reaction velocity was plotted against the reciprocal 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].

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Optimum amount of enzyme

The effect of enzyme on protease activity of leaf extract was found to increasing linearly upto 50 µg of enzyme and then decreased (Figure 5A), while in case of bark the optimal enzyme concentration was approx. 25µg (Figure 5B). The optimal concentration of enzyme for root was found to be 39 µg (Figure 5C).

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 5.61 nM and 185.16 pmol/min respectively for leaf, while for bark $K_m$ and $V_{max}$ values were 2.36 nM and 82.64 pmol/min respectively. The $K_m$ and $V_{max}$ values for root were 1.53 nM and 52.91 pmol/min respectively (Figure 6).

Discussions

In leaf and bark, the decrease in specific activity of TCA precipitated and again increase in specific activity during $(\text{NH}_4)_2\text{SO}_4$ salt precipitation indicates significant loss of protease activity during TCA precipitation. However in root, there is successive increase in specific activity from crude extract to $(\text{NH}_4)_2\text{SO}_4$ salt precipitation indicates successive purification of protease. The high optimal temperature of bark protease indicates that the bark protease is quite more thermostable than other proteases this may be due to presence of higher amount of cysteine residues. Beyond these optimal temperatures the activities were decreased due to denaturation of proteases due to heat.
(NH$_4$)$_2$SO$_4$ salt and characterizes. All proteases were actively working. Lapsi has been successively purified to homogeneity by TCA and

Acknowledgement

Conclusion

The bell shaped curve obtained for effect of substrate concentration on protease activity indicated that the enzyme concentration enhances the rate of reaction only up to optimal concentration and then started to inhibit the rate of reaction due to over saturation of enzyme. The low $K_m$ value of the enzyme indicated that the substrate is tightly bound to enzyme. 

**Table 1:** Summary of protease purification of leaf.

| Purification step       | Volume (ml) | Protein concentration (mg/ml) | Total protein (mg) | Activity (nmol min$^{-1}$ ml$^{-1}$) | Total activity (nmol min$^{-1}$) | Specific activity (nmol min$^{-1}$ mg$^{-1}$) | Purification factor | Overall yield (%) |
|-------------------------|-------------|-------------------------------|--------------------|--------------------------------------|----------------------------------|---------------------------------------------|-------------------|------------------|
| Crude extract           | 152         | 2.06 ± 0.13                   | 313.79 ± 1.33      | 1.494 ± 0.03                         | 227.15 ± 4.67                   | 0.74 ± 0.01                                 | 1                 | 100              |
| TCA ppt. extract        | 8           | 2.66 ± 0.56                   | 21.29 ± 0.46       | 1.74 ± 0.07                          | 13.92 ± 0.56                    | 0.654 ± 0.03                                | 0.88              | 6.13             |
| 20-80% ammonium         | 9           | 2.1 ± 1.24                    | 17.494 ± 1.63      | 1.707 ± 0.01                         | 15.36 ± 0.14                    | 0.883 ± 0.07                                | 1.19              | 6.76             |

**Table 2:** Summary of protease purification of bark.

| Purification step       | Volume (ml) | Protein concentration (mg/ml) | Total protein (mg) | Activity (nmol min$^{-1}$ ml$^{-1}$) | Total activity (nmol min$^{-1}$) | Specific activity (nmol min$^{-1}$ mg$^{-1}$) | Purification factor | Overall yield (%) |
|-------------------------|-------------|-------------------------------|--------------------|--------------------------------------|----------------------------------|---------------------------------------------|-------------------|------------------|
| Crude extract           | 148         | 1.02 ± 0.66                   | 150.38 ± 3.26      | 0.663 ± 0.04                         | 98.062 ± 6.49                   | 0.653 ± 0.05                                | 1                 | 100              |
| TCA ppt. extract        | 3           | 1.93 ± 1.28                   | 5.784 ± 0.19       | 0.883 ± 0.005                        | 2.643 ± 0.01                    | 0.458 ± 0.01                                | 0.7               | 2.7              |
| 20-80% ammonium         | 3.7         | 0.61 ± 0.56                   | 2.257 ± 0.04       | 0.469 ± 0.002                        | 1.336 ± 0.008                   | 0.769 ± 0.01                                | 1.18              | 1.8              |

**Table 3:** Summary of protease purification of root.

| Purification step       | Volume (ml) | Protein concentration (mg/ml) | Total protein (mg) | Activity (nmol min$^{-1}$ ml$^{-1}$) | Total activity (nmol min$^{-1}$) | Specific activity (nmol min$^{-1}$ mg$^{-1}$) | Purification factor | Overall yield (%) |
|-------------------------|-------------|-------------------------------|--------------------|--------------------------------------|----------------------------------|---------------------------------------------|-------------------|------------------|
| Crude extract           | 43          | 1.94 ± 1.2                    | 83.33 ± 1.72       | 1.096 ± 0.01                         | 47.12 ± 0.49                    | 0.666 ± 0.01                                | 1                 | 100              |
| TCA ppt. extract        | 3           | 1.35 ± 0.58                   | 4.05 ± 0.09        | 0.986 ± 0.01                         | 2.957 ± 0.04                    | 0.731 ± 0.01                                | 1.29              | 6.28             |
| 20-80% ammonium         | 6.2         | 0.57 ± 0.59                   | 3.52 ± 0.07        | 0.695 ± 0.01                         | 4.213 ± 0.04                    | 1.225 ± 0.04                                | 2.16              | 8.94             |

Recommendations

Biochemical determination of type of protease present in different parts of Lapsi plant can be done. Phytochemical screening, Antioxidant and Antimicrobial Activity can be checked in different part of the Lapsi plant.

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