Biochemistry & Analytical Biochemistry
ISSN: 2161-1009

Upadhyay et al., Biochem Anal Biochem 2013, 2:3
DOI: 10.4172/2161-1009.1000135

Research Article
Open Access

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 dioecous, deciduous fruit bearing plant with multiple daily uses. The protease was extracted with 0.1 M phosphate buffer of pH 7 and then precipitated successively with TCA and ammonium sulfate. The protease from leaf showed maximum activity at pH 9, temperature at 20°C. While, bark protease showed optimum pH 5 and temperature 60°C. In case of root the optimum pH was 10 and the optimum temperature 35°C. The optimum time of incubation leaf, bark and root was 15 minute. The bell shaped curve was obtained for the effect of enzyme conc. with optimum enzyme conc. 50 µg for leaf, 30 µg for bark and 50 µg for root. The K_m and V_{max} value of leaf were 5.61 µM and 185.18 pmol/min, K_m and V_{max} value of bark were 2.36 µM and 82.64 pmol/min. While for root, the K_m and V_{max} values were 1.53 µM and 52.91 pmol/min.

Keywords: Protease; Lapsi; Purification; Characterization

Introduction

Choerospondias axillaris (locally called "lapsi") is a large, deciduous fruit-bearing dioecous 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 dioecous, 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 tricloroacetic acid TCA [6]. The present study was conducted to isolate, purify and characterize the proteases from different parts of the lapsi plant.

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 grinded 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

Received April 18, 2013; **Accepted** May 22, 2013; **Published** May 25, 2013

Citation: Upadhyay SK, Magar RT, Thapa CJ (2013) Biochemical Characterization of Protease Isolated from Different Parts of *Choerospondias axillaris* (Lapsi). Biochem Anal Biochem 2:135. doi:10.4172/2161-1009.1000135

Copyright: © 2013 Upadhyay SK, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Citation: Upadhyay SK, Magar RT, Thapa CJ (2013) Biochemical Characterization of Protease Isolated from Different Parts of Choerospondias axillaris (Lapsi). Biochem Anal Biochem 2:135. doi:10.4172/2161-1009.1000135

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 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].

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 80C 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 (Figue 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).

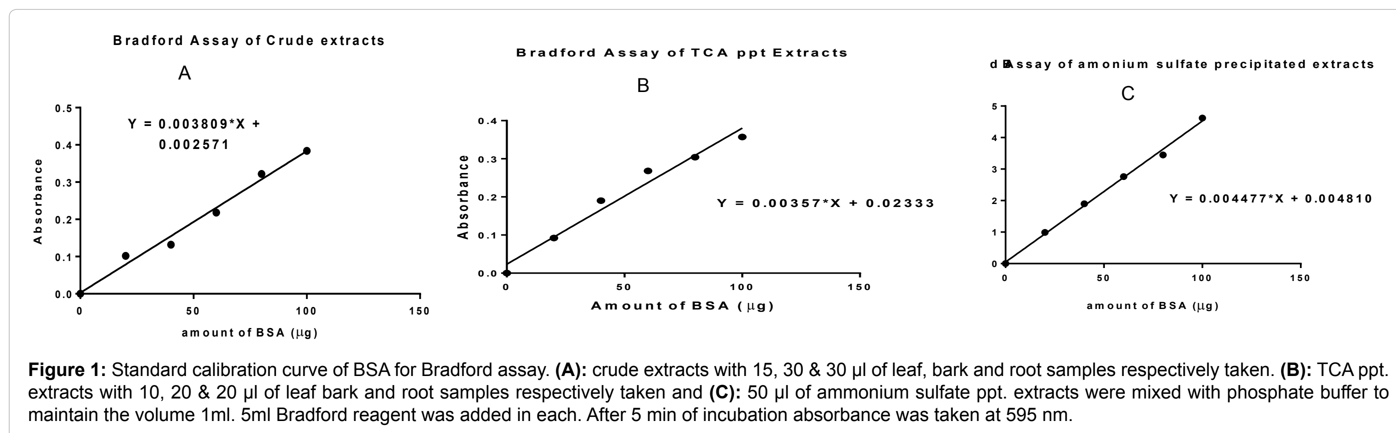

Figure 1: Standard calibration curve of BSA for Bradford assay. **(A):** crude extracts with 15, 30 & 30 µl of leaf, bark and root samples respectively taken. **(B):** TCA ppt. extracts with 10, 20 & 20 µl of leaf bark and root samples respectively taken and **(C):** 50 µl of ammonium sulfate ppt. extracts 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 595 nm.

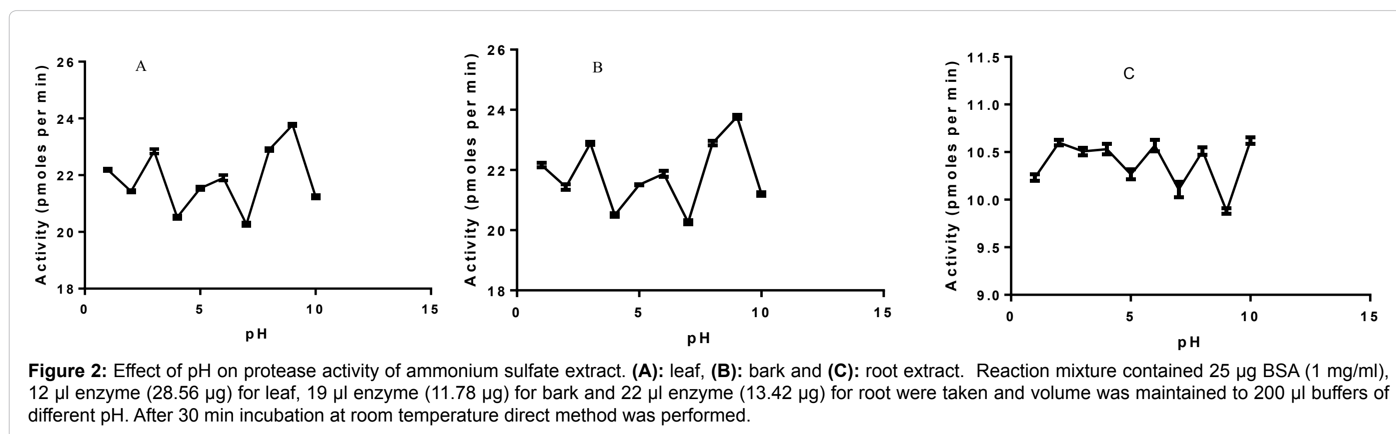

Figure 2: Effect of pH on protease activity of ammonium sulfate extract. **(A):** leaf, **(B):** bark and **(C):** root extract. Reaction mixture contained 25 µg BSA (1 mg/ml), 12 µl enzyme (28.56 µg) for leaf, 19 µl enzyme (11.78 µg) for bark and 22 µl enzyme (13.42 µg) for root were taken and volume was maintained to 200 µl buffers of different pH. After 30 min incubation at room temperature direct method was performed.

Citation: Upadhyay SK, Magar RT, Thapa CJ (2013) Biochemical Characterization of Protease Isolated from Different Parts of Choerospondias axillaris (Lapsi). Biochem Anal Biochem 2:135. doi:10.4172/2161-1009.1000135

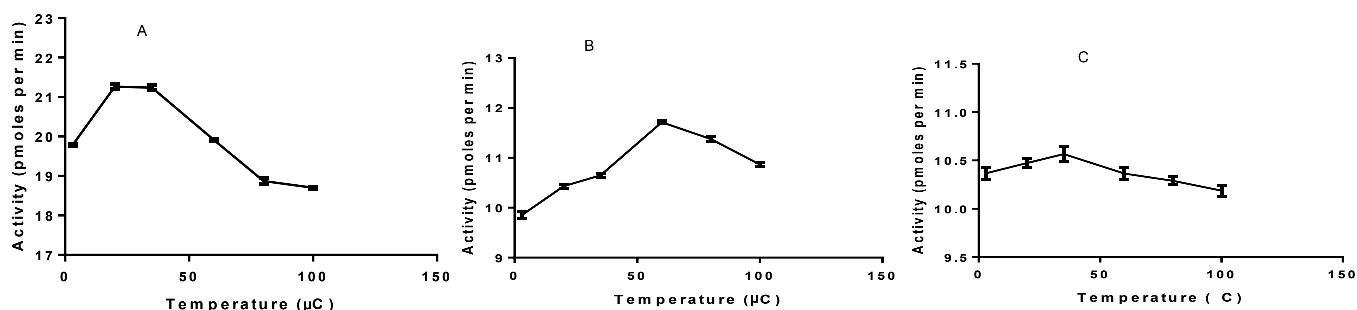

Figure 3: Effect of temperature on protease activity. **(A)** leaf extract, **(B)** bark extracts and **(C)** root extracts. The enzymes were incubated at different temperature. 200 µl reaction system was made with 12 µl of enzyme, 50 µg of BSA for leaf, 19 µl of enzyme, 25 µg of BSA for bark and 22 µl of enzyme, 25 µg of BSA for root and appropriate amount of buffer. Direct method was performed after 30 min of incubation.

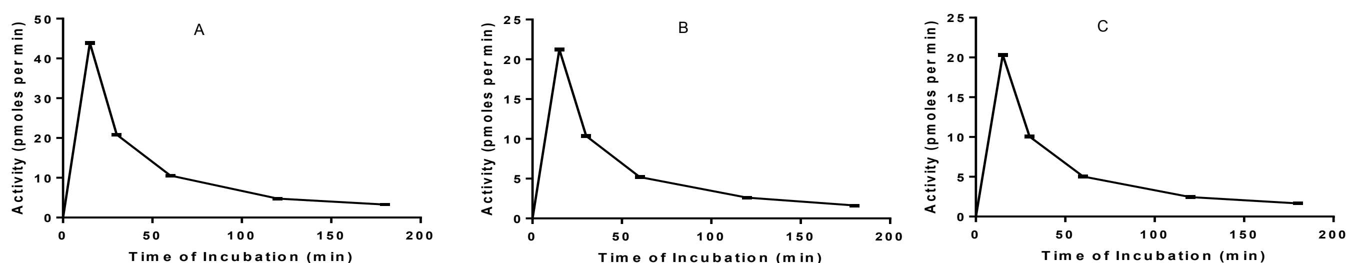

Figure 4: Effect of time of incubation on protease activity. **(A)** leaf extracts, **(B)** bark extracts,and **(C)** root extracts. 200 µl of reaction system containing 50 µg of BSA (1 mg/ml) 12 µl of enzyme for leaf, 25 µg BSA and 19 µl enzyme for bark and 25 µg of BSA and 22 µ enzyme for root extracts were incubated for different time intervals and direct method was performed.

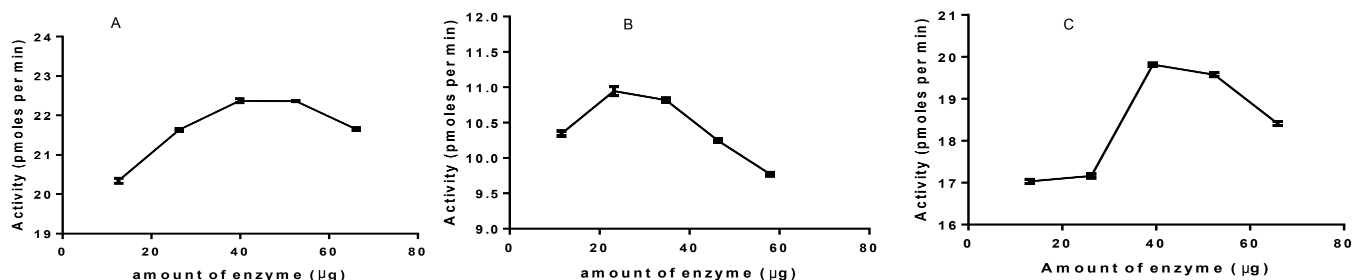

Figure 5: Effect of enzyme concentration on protease activity. **(**A): leaf, (B): bark and (C): root. 50 µg of BSA for leaf and 25 µg of BSA for bark and root (1 mg/ml) with varying amount of protease was added. The final volume was made 200 µl by adding phosphate buffer. It was the incubated for 30 min at room temperature and direct method was performed.

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 $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ 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 ammount of cysteine residues. Beyound these optimal temperatures the activities were decreased due to denaturation of proteases due to heat.

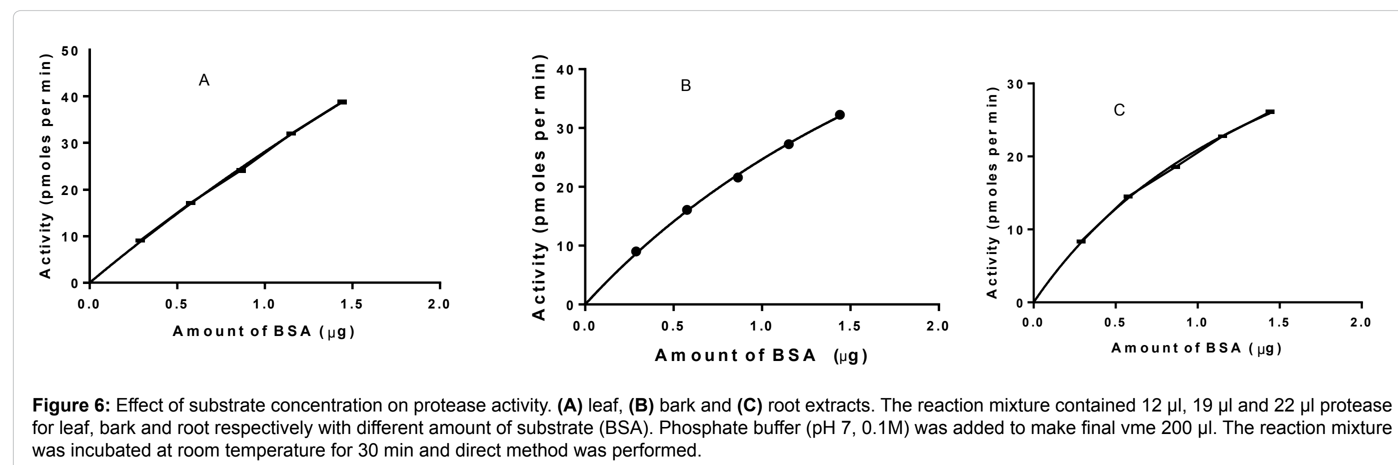

Figure 6: Effect of substrate concentration on protease activity. **(A)** leaf, **(B)** bark and **(C)** root extracts. The reaction mixture contained 12 µl, 19 µl and 22 µl protease for leaf, bark and root respectively with different amount of substrate (BSA). Phosphate buffer (pH 7, 0.1M) was added to make final vme 200 µl. The reaction mixture was incubated at room temperature for 30 min and direct method was performed.

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[a]	Overall yield (%)[b]
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 sulfate ppt. extract	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 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[a]	Overall yield (%)[b]
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 sulfate ppt. extract	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 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[a]	Overall yield (%)[b]
Crude extract	43	1.94 ± 1.2	83.33 ± 1.72	1.096 ± 0.01	4712 ± 0.49	0.566 ± 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 sulfate ppt. extract	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

Note: [a]Defined as: purification factor=(specific activity of fraction/specific activity of homogenate).

[b]Defined as: overall yield=total activity of fraction/total activity of homogenate)×100.

Table 3: Summary of protease purification of root.

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

Conclusion

The protease from leaf, bark and root of *Choerospondias axillaries* (Lapsi) have been successively purified to homogeneity by TCA and $(NH_4)_2SO_4$ salt and characterizes. All proteases were actively working for up to 15 minutes. The bark protease is quite more thermostable than that of leaf and root. Leaf and root proteases were alkaline while bark protease was acidic. The root has more affinity for substrate than other indicated by lowest K_m value.

Acknowledgement

The authors are thankful to the authority of Universal Science College Chakupat, Lalitpur, Nepal for providing the required facilities to carry out this research work.

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.

References

1. Nguyen DD, Nguyen NH, Nguyen TT, Phan TS, Nguyen VD, et al. (1996) The use of a water extract from the bark of Choerospondias axillaris in the treatment of second degree burns. Scand J Plast Reconstr Surg Hand Surg 30: 139-144.

2. Shah DJ (1978) Ascorbic acid (Vitamin C) content of Lapsi-pulp and peel at different stage of maturation. Research Bulletin 2035 B. S. Food Research Section, HMGN, Department of Food and Agricultural Marketing Services, Kathmandu.

3. Agrawal GK, Khandka DK, Jha B, Thapa A, Tamrakar S, et al. (1991) Studies on methods for early sex determination of *Choreospondias axillaris* a fruit bearing Himalayan tree. Biotechnology Letters (Nepal) 1: 23-28.

4. Agrawal VP, Keshari A, Singh D (1992) Study of Lapsi (*Choreospondias axillaris*) protease. Unpublished result.
5. Dekhang RN, Sharma G (2006): Study of Protease from the leaves of *Choreospondias axillaris* (Lapsi). Submitted to Universal Science College, Biochemistry Department for the fulfillment of degree requirement of B.Sc. Biochemistry.
6. Singh R (2007) Purification and Characterization of leaves protease from *Choerospondias axillaris* (Lapsi). Submitted to Universal Science College, Biochemistry Department for the fulfillment of degree requirement of B.Sc. Biochemistry.
7. McPhie P (1971) Dialysis. Meth Enzymol 22: 23-33.
8. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.
9. Hilger C, Grigioni F, De Beaufort C, Michel G, Freilinger J, et al. (2001) Differential binding of IgG and IgA antibodies to antigenic determinants of bovine serum albumin. Clin Exp Immunol 123: 387-394.
10. Lineweaver H, Burk D (1934) The determination of enzyme dissociation constants. J Am Chem Soc 56: 658-666.

Citation: Upadhyay SK, Magar RT, Thapa CJ (2013) Biochemical Characterization of Protease Isolated from Different Parts of *Choerospondias axillaris* (Lapsi). Biochem Anal Biochem 2:135. doi:10.4172/2161-1009.1000135