Characterization of *Choreospondias axillaris* (Lapsi) fruit protease

Sabin Prajapati¹, Samanta Sharma¹ and Vishwanath P. Agrawal¹”²

¹Universal Science College, Pokhara University  and ²Research Laboratory for Biotechnology and Biochemistry (RLABB), Kathmandu, Nepal.

**Abstract**

A catalytically powerful protease from *Choreospondias axillaris* (Lapsi) fruit has been reported. *C. axillaris* (Lapsi) is dioecious, deciduous fruit bearing tree. The protease extracted from the pulp of the fruit is highly thermo stable, autoclavable and extreme acidic and basic pH resistant. Its activity was retained even after multiple trichloro acetic acid (TCA) precipitation. The proteolytic activity of the protease increased linearly up to protein concentration of 62.28µg. It possesses a Km value of 13.09 µM and Vmax 15.87 pmoles/min for bovine serum albumin (BSA) as a substrate. Sodium Dodecyl Sulphate (SDS) activated the proteolytic activity.

**Introduction**

*Choreospondias axillaris* (Lapsi) belongs to the family Anacardiaceae. It is a large, fruit bearing dioecious, deciduous tree native to the hills of Nepal (865-1900m). Lapsi fruit is consumed fresh, pickled or processed into a variety of sweet and sour fruit products locally called “Mada” or “Titaura”. It is a rich source of vitamin C. It is also believed to aid in digestion and is often consumed after a protein rich meal. Seed stones are used as fuel in brick kilns in the factories and the trunk of the tree is used as fuel wood and timber.

Agrawal and Kesari (1992) first observed very strong proteolytic activity in the Lapsi leaves protease. Later, Dekhang and Sharma (2006) reported optimum pH of this protease to be 7. Also, no smaller proteolytic products of BSA could be seen in SDS- PAGE using silver staining indicating that the protease is not an exopeptidase. The protease was not inhibited by phenyl methane sulfonyl fluoride (PMSF) and only 20-30% inhibited by sodium iodoacetic acid which shows the protease is not a serine protease (Dekhang and Sharma, 2006). Its activity was retained even after multiple TCA precipitation (Singh and Giri, 2007). Karki et al. (2009) noted the Km of the protease to be 29.1µM and Vmax 52.63pmoles/ min for BSA as a substrate. They also showed that the protease had a specificity towards four major amino acyl residues namely alanine, tyrosine, phenylalanine and aspartate / threonine.

No work on Lapsi fruit protease has been reported so far, which motivated us to work on Lapsi fruit. Based upon the knowledge about the Lapsi leaf protease, the present research is carried out on the fruit.

The research was done for the fulfillment of degree requirement for B. Sc. Biochemistry (SP and SS)
Materials and Methods

Isolation of crude cell free extract from Lapsi fruits:
The fruits used for the research were obtained from the RLABB which had been stored at -20°C for over a year.
The steps for the isolation of crude cell free extract:

- Ripe fruit was taken (Each measuring roughly 10-16 g).
- Removal of skin from the Lapsi fruit.
- Separation of fleshy pulp from the seed by scraping.
- Preparation of slurry in pH 7 phosphate buffer (0.1M), 50ml phosphate buffer for 5 gm fresh pulp weighed.
- Filtration of slurry using Whatman’s no1 filter paper.
- Heat treatment of the filtrate at 70°C for 30 min.
- Double TCA precipitation of protein:-
  - Addition of 2.45M TCA to the heat treated filtrate to make the final concentration 0.2M.
  - Incubation at -20°C for 15 min and centrifugation at 10000 rpm for 15 min.
  - Dissolving of pellet in 500µl pH 7 phosphate buffer (0.1 M).
  - The process was repeated twice.
- Addition of equal volume of ethanol: ether (1:1) followed by incubation at -20°C for 1 hour and centrifugation at 10000 rpm for 15 min.
- Re-suspension of the pellet in minimal volume of ethanol: ether (1:1) followed by overnight incubation at -20°C and centrifugation at 10000 rpm for 15 min.
- Suspension of pellet in minimal volume of pH 7 phosphate 0.1M buffer to obtain cell free extract (CFE).

Determination of protein:
Standard Bradford curve was prepared by adding different concentration of BSA (stock solution 1mg/ml) to phosphate buffer (pH 7, 0.1M) to make the final volume 200µl. 2.3ml Bradford reagent was added to it. Absorbance was taken at 595nm after 2 min incubation.
Using the standard calibration curve of BSA (Fig. 1), the amount of protein was determined to be 5.49µg in 20µl CFE. Since the pellet obtained from 5 gm of pulp (average amount of pulp obtained from single fruit) was suspended in 250µl Phosphate buffer (pH7, 0.1M), we could conclude that 1.37 mg of protein could be obtained from 100 gm of fresh lapsi fruit pulp.
To 20µl crude CFE, 180µl phosphate buffer (pH7,0.1M) was added to make the volume 200µl. 2.3ml Bradford reagent was added to it. This 2.5ml system was made to stand for 2 min and then absorbance was taken at 595nm.

**Determination of proteolytic activity:**

- **Direct Method:** A reaction mixture of 200µl was made with BSA(1mg/ml), double TCA(2.45M) precipitated extract and phosphate buffer pH7 (0.1M). The reaction mixture was then incubated. It was then quenched with 2.3ml Bradford reagent and the absorbance measured at 595nm. (Bradford MM 1976, Saleemudin et al.1980). The amount of BSA, CFE, buffer, along with incubation time and temperature varied with different experiments that were done. However, 2 controls a) CFE only and b) BSA only, both without incubation was taken in each case. The calculation performed henceforth is control dependent.

- **Indirect Method:** The reaction was quenched by adding 2.45M TCA so as to make the final concentration 0.2M. It was then incubated in freeze for 15 min and then centrifuged at 10000rpm for 15 min. The pellet was suspended in 200µl pH 7 phosphate buffer (0.1M) and the amount of protein determined by Bradford method (2.5ml system). (Singh and Giri 2007)

**Determination of effect of SDS on proteolytic activity**

Since SDS interferes in dye binding protein quantization methods like Bradford assay, it was essential that we remove SDS from the reaction mixture. The SDS was removed using acidified acetone/methanol method. Methanol aids in dissociation of SDS/protein complex whereas acetone helps in protein precipitation.

*Acidified acetone/methanol method* (Lebendiker 2002).: Precipitating reagent (50% methanol, 50% acetone and 0.5mM HCl) was stored at -20°C. To 1 volume of reaction mixture 4 volume of precipitating reagent was added to quench the reaction and left overnight at -20°C. It was then centrifuged at 13000 rpm for 15 min. The pellet was dried and suspended in pH 7 phosphate buffer (0.1M) and Bradford assay (2.5ml system) was performed. This method removed almost 80% SDS.
Results

1. **Effect of protein on proteolytic activity:**
   The proteolytic activity of Lapsi fruit protease was found to increase linearly up to 62.28µg of protein in CFE.

   ![Fig2: Effect of enzyme concentration on proteolytic activity](image)

   50µg BSA (1mg/ml) and varying amount of protein (CFE) was added. The final volume was made 200µl by adding phosphate buffer. It was then incubated for 30 min at room temperature and Direct method was performed.

2. **Optimum incubation time:**
   The proteolytic activity was maximum at 30 min incubation period (Fig. 3). After 30 min, activity decreased gradually.

3. **Effect of temperature:**
   The proteolytic activity was optimum at 30°C (room temperature). The activity gradually decreased with increasing temperature but even at 100°C nearly 50% activity compared to that of the room temperature was observed (Fig. 4). Enzyme incubated at 0°C also showed the activity close to that shown at the room temperature. It suggests that it can be stored in freeze without loss in its proteolytic activity.

   Even after subjecting to autoclave conditions (120°C and 15 pound pressure for 15 min) the CFE retained almost 34% of the original activity (data not shown).
Fig 3: Effect of incubation time on proteolytic activity

![Graph showing proteolytic activity over time](image)

200µl reaction system containing 50µg BSA (1mg/ml), 20µl CFE (5.49µg protein) and 130µl PB was incubated at room temperature for different time intervals and Direct method was performed.

Fig 4: Effect of temperature on proteolytic activity

![Graph showing proteolytic activity over temperature](image)

The enzyme was incubated at different temperatures. 200µl reaction system was mixed with 20µl CFE (5.49µg protein), 50µg of BSA (1mg/ml), and 130µl phosphate buffer pH7 (0.1M). Direct method was performed after 30 min incubation at room temperature.

4. Effect of pH on the proteolytic activity

Proteolytic activity was optimum at pH 8. However, comparable activity was seen even at extreme acidic and basic pH (pH 1 and pH 9, 10). Different buffers used were

- Glycine/HCl buffer (pH1)
- Acetate buffer (pH 3,4,5)
- Phosphate buffer (pH 6,7)
- Tris buffer (pH 8,9)
- Glycine/NaOH buffer (pH 10)
Reaction mixture contained 50µg BSA (1mg/ml), 20µl CFE (5.49µg protein) and 340µl buffer (0.2 M) of different pH. After 30 min incubation at room temperature indirect method was performed.

5. Dependence of proteolytic activity on substrate concentration:
Lineweaver-Burk plot was used to determine the Km and Vmax values of lapsi fruit protease. Km and Vmax values were determined to be 13.09µM and 15.87 pmoles/min respectively.

The reaction mixture contained 20µl CFE (5.49µg protein), different amount of substrate (BSA) and phosphate buffer (pH7, 0.1 M) was added to make the final volume 200µl. The reaction mixture was incubated at room temperature for 30 min and direct method was performed.

6. Effect of SDS on proteolytic activity:
Increased proteolytic activity was observed on increasing the concentration of Sodium Dodecyl Sulphate (SDS) in the reaction mixture (Table 2 & Fig. 7). The experiment above SDS concentration of 0.5% could not be done due to difficulty in removing SDS.
Table 2: Effect of SDS on proteolytic activity.

<table>
<thead>
<tr>
<th>SDS %w/v</th>
<th>Abs 595nm 0 min</th>
<th>Abs 595nm 30min</th>
<th>BSA chopped (µg)</th>
<th>Activity (pmoles/min)</th>
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<tr>
<td>0</td>
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<td>16.10</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Fig 7: Effect of SDS on proteolytic activity

To the reaction mixture containing 50µl BSA (1mg/ml) and 20µl CFE (5.49µg protein) required volumes of SDS (2%w/v in 0.1 M phosphate buffer, pH 7) and 0.1 M phosphate buffer pH 7 were added to make the final volume 200 µl having desired concentrations of SDS in the reaction mixture. After 30 min incubation at room temperature, acidified acetone/methanol was performed.

Discussion and Conclusion

A protease enriched extract has been isolated from the fruit of Choreospondias axillaris. It is relatively easy to process unlike the lapsi leaf protease. The fruit skin extract showed no significant proteolytic activity so our research was carried out with fleshy pulp extract. The enzyme activity increased linearly even up to 62.28 µg protein. Further experiment needs to be carried out with higher protein concentration. Like leaf protease fruit protease also showed its tolerance to extreme conditions of pH and temperature. The high thermostability of the fruit protease may be due to the presence of considerable amount of disulphide bond in the protease.

The optimum pH for the fruit protease was pH 8 whereas that of the lapsi leaf protease was pH 7. However, the activity shown at pH 1, pH 9 and pH10 was close to the activity shown at its optimum pH. It might be due to the presence of both acidic and basic amino acyl groups as functional units in the active site. The proteolytic activity may be attributed to the protonation of the free amino group of basic residue at acidic pH or the deprotonation of the free carboxyl group of acidic residue at basic pH. The proteolytic activity may also be metal dependent. Further works with EDTA and EGTA need to be done for its confirmation.

The Km value of 13.09 µM and Vmax 15.87 pmoles/min was observed which is significantly lower than that of lapsi leaf protease (Km 29.1µM and Vmax 52.63pmoles/min). The increased proteolytic activity in the presence of SDS could be due to denaturation of substrate (BSA) or due to the inhibition of protease inhibitors by SDS.
This protease if purified opens door for its commercialization. It can be used for digestion of proteins, removal of blood stains, contact lens cleaning and removal of body hairs. Further works need to be done for determining its mechanism of action and specificity which may widen its application. Commercialization of larsi fruit protease in large scale will help uplift the economic standard of the local farmers.

References


