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OPTIMIZATION OF THE PARTIAL PURIFICATION PROCESS OF LEAF PROTEASE FROM CITRUS DECUMANA L. (GRAPEFRUIT)

Biological Science

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ABSTRACT

A protease enzyme was partially purified from the leaves of Citrus decumana L. using ammonium sulphate precipitation followed by gel filtration chromatography on a Sephadex G 20 column. The molecular weight of the purified enzyme was estimated to be approximately 55kDa by SDS-PAGE analysis. The enzyme's pH activity profile was assessed using various buffer systems, revealing optimal activity at pH 6.8 in 0.01M Tris buffer. These findings suggest that Citrus decumana leaves are a potential source of protease with moderate molecular weight and optimal activity under near neutral pH conditions.

KEYWORDS

Protease, ammonium sulphate, column chromatography, X ray film

INTRODUCTION

Proteases, also known as "enzymes of digestion," are highly versatile biocatalysts with broad commercial applications in industries such as detergents, food processing, pharmaceuticals, and diagnostics. It is estimated that proteases account for approximately 60% of the global enzyme market, making them the most commercially valuable class of enzymes (Acrofan 2021; Naveen et al 2021).

Although bacterial proteases are generally preferred due to their rapid growth rates and ease of genetic manipulation (Akram et al 2023); plant-derived proteases offer distinct advantages. Notably, exhibit unique substrate specificities and free from undesirable side activities commonly found in microbial or animal enzymes (Akram 2013). These properties make plant proteases attractive candidates for industrial applications (Naveen et al 2021; Feijoo-Siota and Villa 2011, Troncoso et al 2021). However, studies characterizing plant proteases remain limited (Balakireva et al 2019).

In this context, the search for novel plant proteases with commercial potential remains a priority. Citrus decumana L. is documented for its therapeutic uses including antispasmodic, anti-inflammatory, bronchodilator, antidiabetic, anthelminthic, and disinfectant properties (Sood et al 2009). Its potential as a source of industrially viable protease enzyme remains unexplored and thus prompted this investigation. The objective of this study was to partially purify and characterize a protease enzyme from the leaves of Citrus decumana L.

2. MATERIALS AND METHODS:

2.1 Plant Material Collection: Fresh mature leaves of Citrus decumana L. were collected from the plant growing in the college campus. All the chemicals used in the study were of analytical grade from SRL Company Mumbai, India.

2.2 Crude Enzyme Preparation: All experiments were carried out at low temperature. The leaves of the plant part were homogenized using Tris Buffer (0.01M) pH 6.8 in a homogenizer under chilled condition and filtered and centrifuged at 10,000 rpm for 15 minutes. The supernatant obtained as crude enzyme was used for further investigation for proteolytic activity.

2.3 Protein Estimation: Protein concentration in the enzyme extract was determined using Folin Ciocalteau reagent as per the procedure (Sharmila et al 2012). Crystalline Bovine Serum Albumin was used as standard protein for preparation of standard curve.

2.4 Protease Assay: The protease activity was assayed as described by Sharmila et al 2012 with slight modifications. One ml of crude enzyme was incubated with 2 ml of substrate (1 % casein) in presence of 0.01 M Tris buffer (pH 6.8) for 60 minutes at 37°C. The residual protein precipitated by adding 5% ice chilled trichloroacetic acid and filtered. One ml aliquot of the diluted filtrate was mixed with 2.5 ml 15% sodium carbonate. Then 0.5 ml of diluted Folin Ciocalteau reagent was added and the contents were mixed. The blue coloured complex formed was read by a spectrophotometrically at 650 nm exactly after 15 minutes. The protease activity was expressed as µg of tyrosine equivalents liberated/minutes/mg protein under standard conditions (Cupp-Enyard 2008).

2.5 X-ray Film as Qualitative Assay: The unused x-ray film degradation assay was performed according to standard method (Shankar et al 2011). The x-ray films were cut into 2 × 2 cm and were incubated with 10 ml of diluted protease of plant in 0.01M Tris buffer pH 6.8 (such that the film is completely submerged in the enzyme) at 30°C. The removal of coating is observed comparing with controls.

2.6 Effect of pH: The crude extract was subjected to different buffer solutions (Phosphate and tris buffer). The efficacy of each buffer was estimated by checking protease activity.

2.7 Partial Purification of Protease Enzyme (Purwanto 2016):

2.7.1 Salt Precipitation: The crude enzyme solution was precipitated by ammonium sulphate (0-30%, 30-60%, 60-90% w/v). The solution was kept overnight in cold condition (4 °C) and then centrifuged (8000 rpm, 30 min., 4 ºC). The precipitate was dialyzed in 0.01M Tris buffer (pH 6.8). Following dialysis, the fraction obtained was used for protein estimation and enzyme assay as mentioned above.

2.7.2 Column Chromatography Using Sephadex G 20: Dialyzed samples were passed through Sephadex G 20 column, pre equilibrated with 0.01M Tris buffer (pH 6.8) and eluted with the same buffer. Each fraction was assayed for enzyme activity and fractions showing high protease activity were pooled and subjected to molecular weight determination.

2.7.3 Determination of Molecular Weight: The molecular weight of purified protease was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Tris-glycine buffer (pH 8.2). The samples were run in 12% polyacrylamide gel and stained with Coomassie Brilliant Blue stain and molecular weight was estimated by comparing it with the mid - range protein marker.

RESULTS AND DISCUSSION

Plant-derived proteases are increasingly gaining attention for their potential applications in food, pharmaceutical, and industrial processes (Troncoso et al 2022). Numerous plants have been studied for protease production, yet limited research exists on proteases from members of the Rutaceae family (Ibraheem at al 2017). This study explores the potential of plant Citrus decumana L., with significant ethnomedicinal value for proteolytic activity.

3.1 Protease Extraction

Protease was successfully extracted and partially purified using ammonium sulfate precipitation, a common method for enzyme concentration (Vidyalakshmi and Esaki 2013). Maximum activity was recorded in the 60% saturation fraction, similar to reports for protease from Cucurbita maxima peel (Vadivukkarasi et al 2015).

3.2. Enzyme Purification

Dialysis of the ammonium sulfate fraction enhanced enzyme purity. Enzyme activity and protein concentration were evaluated at each purification step (Table 1). From 30 g of leaf tissue, approximately 4050 U/ml of protease was obtained, with a final yield of 87.9%. SDS-PAGE revealed a single band corresponding to 55 kDa (Figure 1), confirmed through a trypsin activity assay. Previous studies have documented plant proteases of similar molecular weights in latex

yielding plants (Badgujar and Mahajan 2011). Among the four major types—serine, aspartate, metallo, and cysteine—cysteine proteases are often predominant in plants (Mnif et al 2015).

Table 1.: Summary of Partial Purification of Protease Enzyme from 30g Leaves of Citrus Decumana L.

Purification step	Total volume (mL)	Total Protein (mg/mL)	Enzyme activity (U/mL)*	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	200	1050	4050	3.85	100	100
Ammonium sulphate precipitation (30-60%)	30	430	1125	2.62	27.7	0.68
Column chromatography	15	110	989	8.99	87.9	2.33

* Micromoles of tyrosine liberated

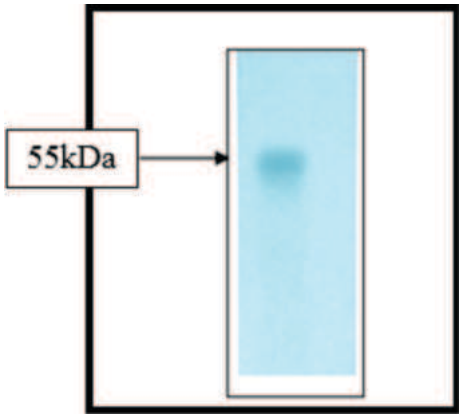

Figure 1. SDS PAGE (12%) Showing the Purified Band After Ammonium Sulfate Purification

3.3. pH Optimization of Protease Activity

Determining the optimal pH is essential for understanding enzyme function and suitability for industrial use (Antão and Malcata 2005). The enzyme showed significant activity across a range of pH values in both phosphate and Tris buffers. Maximum activity (95.8 U) was observed at pH 6.8 in Tris buffer (Figure 2, 3), indicating this as the optimum pH. Comparable results have been reported for protease from Vicia faba (optimum pH 5.0) (Greiner et al 2001) and a 20 kDa cysteine protease from Ficus microcarpa (optimum pH 8.0) (Mnif et al 2015).

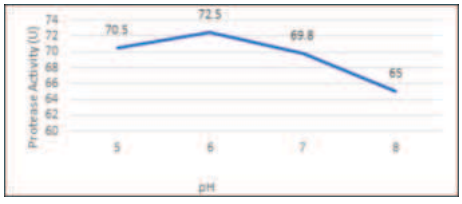

Figure 2. Effect of Buffer Solutions of Phosphate Buffer on Protease Activity

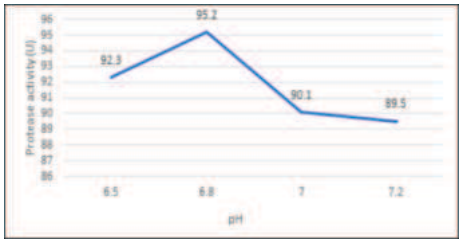

Figure 3. Effect of Buffer Solutions of Tris Buffer on Protease Activity

3.4. Qualitative Protease Activity by X-ray Film Assay

Proteolytic activity was visually confirmed by the degradation of gelatin on X-ray film in all tested extracts and fractions (Figure 5). This qualitative assay offers a simple, cost-effective, and semi-quantitative method to detect protease activity. The intensity and timing of gelatin removal serve as indicators of enzyme strength and potential application in rapid screening formats like dot blot assays.

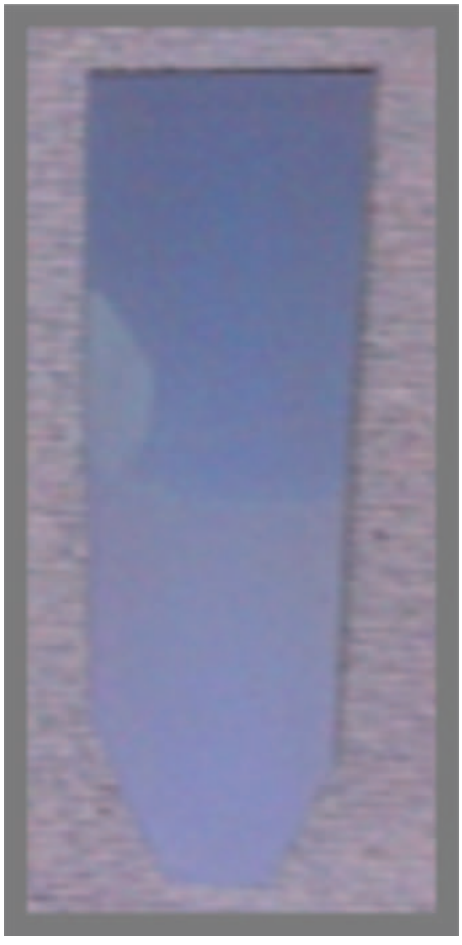

Figure 4. Decolorization of X ray Film

4. CONCLUSION

This study is the first to report the extraction and partial purification of a protease enzyme from the leaves of Citrus decumana L. The enzyme exhibited significant proteolytic activity and demonstrated stability across a range of pH values using both phosphate and Tris buffers, indicating its functional versatility. These findings suggest that Citrus decumana is a promising novel source of plant-based protease with potential biotechnological applications. Further in-depth studies focusing on complete purification, characterization, and application-specific assessments are warranted to fully explore its industrial relevance.

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