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Optimization and Partial Purification of Protease Enzyme from the Leaves of Citrus Decumana L. (Grapefruit)

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Abstract
A protease from Citrus decumana L. leaves was purified by ammonium sulphate precipitation followed by Sephadex G 20 column chromatography. Molecular weight of the enzyme was estimated to be 55kDa by SDS- PAGE. Optimization of pH for protease enzyme was tested using different buffer solutions and maximum activity was observed at pH 6.8 using Tris buffer (0.01M).

Key words: Protease, ammonium sulphate, column chromatography, X ray film

1. Introduction:

Proteases which are also called "enzymes of digestion" are well known biocatalysts. They are commercially used in various industries such as detergents, food, pharma, diagnostic etc. It is reported that 60% of total enzyme market is covered by the protease and are considered as the most valuable commercial enzyme (1).

The source of proteases are enormous and bacterial proteases are more significant as compared to plant and animal proteases (2) because of their rapid growth and can be easily manipulated genetically. However, plant proteases which has unique substrate specificity are free from undesirable side enzyme activities which is absent in microbial or animal systems (3). This makes the plant based proteolytic enzyme resources as valuable source having profound applications in enzyme industry (4, 5). There are minimal reports about the

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characterization of plant proteases (6, 7, 8,9).

Thus, the arduous search for new potential plant proteases continues in order to make them industrially applicable and cost effective. In the present investigation the plant *Citrus decumana* L. (Rutaceae family) was selected as there is no report available on the protease enzyme and its characterization. The plant selected is well documented with its medicinal uses: antispasmodic, anti-inflammatory, anti-bleeding, bronchodilator, antidiabetic, anthelminthic, disinfectant, etc. Therefore, the plant looked to be a promising candidate for the protease source which can be exploited for its biotechnological applications. Therefore, the aim of the present study is to characterize the protease and partially purify it from the leaves of *Citrus decumana* L. with a view of that these proteolytic enzymes can be commercialized as alternative source.

2. Materials and Methods:

2.1 Material Collection: The 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 10,000rpm for 15 minutes. The supernatant obtained called "crude enzyme", was used for further investigation of Proteolytic activity.

2.3 Protein Estimation: Protein concentration in the enzyme extract was determined using Folin Ciocalteau reagent as per the procedure (10). 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 (11). 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.

2.5 X ray film as qualitative assay: The unused x-ray film degradation assay was performed according to standard method (12). 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. The efficacy of each buffer was estimated by checking protease activity. The two buffers used were: Phosphate Buffer (pH

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of 5,6,7,8) and Tris Buffer (pH of 6.5, 6.8, 7.0, 7.2).

2.7 Partial purification of protease enzyme:

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. (13, 14)

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 (13).

3. Results and Discussion:

Recently, exploration of plant proteases has been widely considered due to their importance in food and pharmaceutical industries (15, 16,17). Considering the importance of the enzyme, protease enzyme has been widely studied by researchers from various plants and food crops (18, 19). Despite its importance in various fields, the extraction of proteases from Rutaceae members remains minimal in current scenario (20, 21). In this study, a well-known medicinal plant *Citrus decumana* . L was considered for extraction of protease and purify it. The enzyme was initially extracted, partially purified and characterized using the existing methods with slight modification.

3.1 Extraction of protease: The samples were fractionated and purified initially by ammonium sulfate precipitation method, a widely used method for purification of protease (22, 23). The optimum ammonium sulphate saturation percentages for highest protease activity were found to be 60% for leaf supernatant. The results were comparable with earlier reported protease isolated with 60% fraction of *Cucurbita maxima* peel (24).

3.2 Purification of protease: In enzyme purification process, dialysis was carried out for the partial purification of leaf sample of *Citrus decumana* L. and the purity was further checked using assay and native PAGE (25). Partial purification of protease enzyme at each step of purification from 30g of leaves is shown in table 1. About 4050 U/ml of the enzyme was obtained and the final yield of the enzyme was about 87.9%. The low specific activity observed in ammonium sulphate fraction indicates a considerable loss of activity during the precipitation of the enzyme by the salt. The SDS- PAGE showed 55kDa band corresponding to the

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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	1.00
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

enzyme (Figure 1) which was confirmed by trypsin assay. Previous literature studies have reported, the presence of proteases in various plant sources like rice, tomato plant, cucumber, squash, potato plant, maize, papaya, etc. The protease enzyme is classified into four types, serine, aspartate, metallo and cysteine protease respectively. Cysteine proteases are reported to be
predominant among all four in plant sources (6).

3.3 Effect of pH on protease activity**:** The enzyme activity is maximum at a particular pH know as optimum pH which is important for any enzyme in terms of its production and activities (26). However, there are reports that few plant proteases are active in wide range of pH and temperature (27). In this study, the pH activity of proteases isolated from leaf samples were calculated at different pH (5, 6, 7 and 8) using Phosphate Buffer and pH (6.5, 6.8, 7.0, 7.2) using Tris Buffer respectively (Figure 2, 3). The pH studies reported an increased enzyme activity at a pH range using Tris Buffer. At pH 6.8, maximum protease activity was observed (95.8 U) thus considered as an optimum pH for protease enzyme production. Similar studies were reported protease purified from *Vicia faba* exhibit the optimum pH at 5.0. (28), Cysteine protease (20kDa) was purified from *Ficus microcarpa* showed maximum activity at pH 8.0 (29).

3.4 X ray film detection of protease enzyme activity: All extract and fractions showed decolourization of gelatin coating of X-ray film (Figure 5). This clearly demonstrates the protease enzyme activity in the selected plant. The method of using photographic film is simple, less tedious and semi quantitative method. The time of incubation of film in buffer solution indicates the activity of the enzyme. The approach can be used in the form of dot blot assay.

4. Table and Figures:

Table 1.: Summary of Partial Purification of Protease enzyme from 30 g Leaves of *Citrus decumana* L.

* Micromoles of tyrosine liberated

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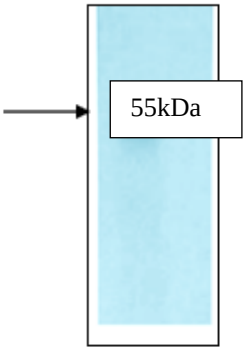

Figure 1. Native PAGE (12%)

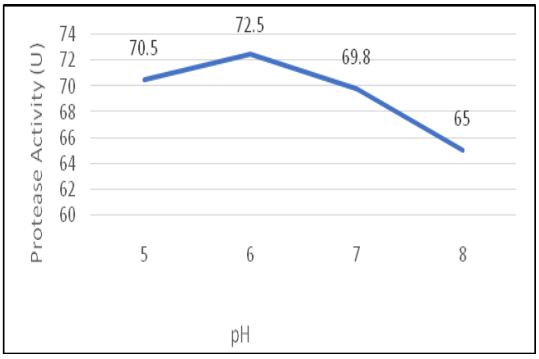

Figure 2. Effect of Buffer solutions of Phosphate Buffer on Protease activity

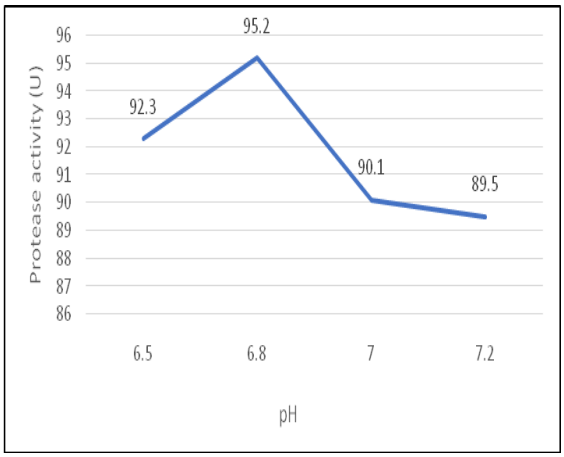

Figure 3. Effect of Buffer solutions of Tris Buffer on Protease activity

Figure 4. Decolorization of X ray film

5. Conclusion

The present investigation first time reports a new, effective method for obtaining protease enzyme from the leaves of *Citrus decumana*. The enzyme showed protease activity with Phosphate buffer and Tris buffer of selected pH used for the study indicating its stability over wide range of buffer solutions. A detailed research is required to purify the protease enzyme from this plant which holds many biotechnological potential.

References

1. Ganesh Kumar C; Hiroshi Takagi 1999. *Biotechnology Advances., 17, 561-594.*
2. Gupta, A., Roy, I., Patel, R.K., Singh, S.P., Khare, S.K., Gupta, M.N. 2005. *One step purification and characterization of an alkaline protease from haloalkaliphilic Bacillus sp. J. Chromatogr., 1075: 103–108.*
3. Gary D. J. and Weiping J. 2005. *Characterization of cathepsin L secreted by Sf21 insect cells, Arch Biochem Biophys, Vol. 444 (1), pp. 7-14.*

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4. Tomar R., Kumar R, Jagannadham M.V. 2008. *Stable serine protease from latex of Wrightia tinctoria J.Agric.Food Chem. 27,4 :1479-87.*
5. Patel, A.K., Singh, V.K and Jagannadham, M.V. 2007. *Carnein: A serine protease from noxious plant weed Ipomoea carnea (Morning glory). Journal of Agricultural Food Chemistry 55; 5809-5818*
6. Domsalla A and Matthias F. Melzig 2008. *Occurrence and Properties of Proteases in Plant Latices. Planta Med 74: 699–711.*
7. Sharmila S, Rebecca L, Das M and Saduzzaman Md 2012. *Isolation and partial purification of Protease from plant leaves. Journal of Chemical and Pharmaceutical Research 4(8):3808-3812, ISSN : 0975-7384.*
8. Vidyalakshmi S and Esaki S 2013. *Protease Activity of Floral Extracts of Jasminum grandiflorum L., a wound healing herb. Journal of Medicinal Plants Studies, Volume: 1, Issue: 4:11-15, ISSN: 2320-3862.*
9. Khurana B et al. (2013). *Various Biochemical Parameters of Protease Isolated from Adhatoda Vasica: A Medicinally Important Plant. International Journal of Genetic Engineering and Biotechnology. ISSN 0974 3073 Volume 5, Number 1 pp. 1-6.*
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. *Protein measurement with the Folin phenol reagent. J biol Chem, Vol. 193(1), pp. 265-275.*
11. Badgujar S. B. and Mahajan R. T. 2011. Proteolytic enzymes of some latex bearing plants belonging to Khandesh region of Maharashtra. Ph D. Thesis, North Maharashtra University, Jalgaon, pp 108-109.
12. Shankar, S., More, S. V., Laxman, R. S. 2010 *Recovery of silver from waste X-ray film by alkaline protease from Conidiobolus coronatus. Kathmandu Univ J Sci Eng Techno, Vol. 6(1), pp. 60-69.*
13. Purwanto, M.G.M. 2016. *The Role and Efficiency of Ammonium Sulphate Precipitation in Purification Process of Papain Crude Extract. Procedia Chemistry 18(1): 127-131.*
14. Cupp-Enyard, C. 2008. *Sigma's non-specific protease activity assay-casein as a substrate. Journal of Visualized Experiments 19 (1): 899-899.*
15. Gupta, R., Beg, Q.K. and Lorenz, P. 2002. *Bacterial alkaline protease: molecular approaches and industrial application. Applied Microbial Biotechnology 59; 15-32.*
16. Kim, M., Si, J.B., Reddy, L.V. and Wee, Y.J. 2016 *Enhanced production of extracellular proteolytic enzyme excreted by a newly isolated Bacillus subtilis FBL-1 through combined utilization of statistical designs and response surface methodology. RSC Advances 6(56): 51270-51278.*
17. Hasan, F., Shah A.A. and Hameed, A. 2006. *Industrial applications of microbial lipases. Enzyme and Microbial technology 39(2): 235-251.*
18. Cheng, Z., Li, J.F., Niu, Y., Zhang, X.C., Woody, O.Z., Xiong, Y., Djonović, S., Millet, Y., Bush, J. and McConkey, B.J. 2015. *Pathogen-secreted proteases activate a novel plant immune pathway. Nature 521(7551): 213-216.*
19. Sequeiros, C., Torres, M.J., Nievas, M.L., Caffini, N.O., Natalucci, C.L., López,

July-August 2020
ISSN:0193-4120 Page No. 6754 – 6760

L.M. and Trejo, S.A. 2016. *The Proteolytic Activity of Philibertia gilliesii Latex. Purification of Philibertain g II. Applied biochemistry and biotechnology 179(2): 322-346.*

20. Kinsella, J.E. 1993. U.S Patent No. 5,192,677. Washington, DC: U.S. Patent and Trademark Office
21. Foss, N.J. and Eriksen, B. 1995. Competitive advantage and industry capabilities. Resource-Based and Evolutionary Theories of the Firm. Towards a synthesis, p. 43-69. United states: Springer Academic Publishers.
22. Drivdahl, R.H. and Thimann, K.V. 1977. *Proteases of senescing oat leaves I. Purification and general properties. Journal of Plant physiology 59(6): 1059-1063.*
23. Antão, C.M. and Malcata F.X. 2005. *Plant serine proteases: biochemical, physiological and molecular features. Plant Physiology and Biochemistry 43(7): 637-650.*
24. Vadivukkarasi, S., Manikandan, M., Pavithra, K. and Vasudevan, M. 2015. *Novel Application of Cucurbita Maxima Peel Protease in Detergents. Journal of Proteomics Enzymolology 4 (22): 1-3.*
25. Krishnan, V.M. and Murugan, K. 2015. *Purification, characterization and kinetics of protease inhibitor from fruits of Solanum aculeatissimum Jacq. Food Science and Human Wellness 4(3): 97-107.*
26. Rao, M.B., Tanksale A.M., Ghatge M.S., Deshpande V.V. 1998. *Molecular and biotechnological aspects of microbial proteases. Microbiology and molecular biology reviews 62(3): 597-635*
27. Uhlig, H. 1998. Industrial enzymes and their applications. New York, USA: John Wiley & Sons
28. Greiner, R., Muzquiz, M., Burbano, C., Cuadrado, C., Pedrosa, M.M. and Goyoaga, C. 2001. *Purification and characterization of a phytate-degrading enzyme from germinated faba beans (Vicia faba var. Alameda). Journal of agricultural and food chemistry 49(5): 2234-2240.*
29. Mnif, I.H., Siala, R., Nasri, R., Mhamdi, S., Nasri, M. and Kamoun, A.S. 2015. *A Cysteine Protease Isolated from the Latex of Ficus microcarpa: Purification and Biochemical Characterization. Applied biochemistry and biotechnology 175(3): 1732-1744.*

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