Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(5): 729-732
Received: 10-07-2019
Accepted: 12-08-2019

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Isolation of protease from onion scale leaves and its comperative study with ginger protease

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Abstract
Proteases are group of enzymes which can cleave proteins into smaller fragments. Plant proteases are gaining interest due to their easy availability. Fruits and vegetables are the abundant source of commercially important proteases. We found protease activity in onion scale leaves. We compared the protease activity of onion with ginger protease. Protein isolation was achieved by ammonium sulfate precipitation from the aqueous extract. We compared the enzyme in terms of caseinolytic ability in different temperature, pH and different substrate concentrations. Optimum temperature for both the enzymes is 55 ºC but the optimum pH for protease from ginger is 5 and the optimum pH for protease from onion is 7.5. Specific activity of the protease from onion scale leaves is lesser than ginger but the affinity is higher in case of onion protease.

Keywords: Ginger, onion, proteases, azocasein, skim milk agar and trypsin

1. Introduction
Proteases are the class of enzymes, which occupy key position with respect to their applications in both physiological and commercial fields [1]. They represent one of the three largest groups of industrial enzymes and account for about 60% total worldwide sale of enzymes [1b]. Plant proteases are responsible for protein metabolism, a fundamental network of reactions required during the life cycle. Proteases are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated reactions such as blood cascade, complement system and apoptosis pathways [1a]. Proteases, of all the enzymes, remain the dominant enzyme type because of their extensive application in the detergent, food and dairy industries [1b]. The activities of proteases have been reported in several plant materials. Some reports are on legumes, cereals, vegetables and apricot and grapes [2]. There are also reports on bulbs [2a, 3]. Aspartic proteinase and some aminopeptidase activities are present in ungerminated seeds and some of these enzymes have been purified and cloned. Potato tubers contain about 2% proteins by weight and consist of patatin, storage protein, proteases as well as protease inhibitors. Ginger rhizomes contain protease enzymes, GP-I and GP-II, which were first isolated by Y. Ichikawa in 1973 [4]. Ohtsuki *et al.*, later separated ginger proteases into three fractions by IEF, following which the 3D structure of GP-II was elucidated by X-Ray crystallography by Choi *et al.* in 1999 [5]. According to Choi and Laursen (2000), Ginger proteases are cysteine proteases (EC 3.4.22) and belong to the papain (C1) super family [6]. *Allium cepa* Linn (onion) belongs to the family of Liliaceae (lily) and is a well known medicinally important plant. The Allium plants are used as spices worldwide. The molecular basis for the odour, taste and biological activity of the onion bulb has been studied in detail by Block *et al.* [7] in 1993. Onion enzymes are not well explored and there is one report of protease isolation from onion bulbs [2a]. Here in this paper we have isolated and partially purified protease from onion scale leaves. We have done comparative study of protease from ginger and onion. Caseinolytic property was observed in skim milk agar plate assay and spectrophotometrically by using azocasein as substrate.

2. Materials and Methods
2.1. Protein Extraction and Isolation
Plant sample:
Ginger and Onion were purchased, identified and validated from herbarium unit of Botanical Survey of India, Kolkata situated in Shibpur Botanical Garden, Kolkata. Ginger rhizome and onion scale leaves were used in this study.
Each sample (10 g) was homo-genised in a ratio of 1:1 (w/v) in 50mM phosphate buffer (pH 7.5). The homogenate was filtered and centrifuged at 10,000 rpm for 15 min. The supernatant was used as protein extract.

2.2. Protein Estimation
Total protein content was determined with Bradford reagent spectrophotometrically with the help of standard curve of BSA [8].

2.3. Ammonium Sulphate (AS) Precipitation
The crude extract (after filtration followed by centrifugation) was treated with ammonium sulphate (60%) and so that the protein content can be concentrated by precipitation. This step includes magnetic stirring for 45 mins. to 1 hr. with a magnetic bead and centrifugation at 10,000 rpm for 15 mins.

2.4. Skim Mik Agar Screening (Qualitative Assay)
Skim Milk Agar screening test was used to see whether protease is present in the plant samples. Skim milk agar was prepared by adding skim milk solution (10% skim milk powder in distilled water) and agar agar solution (2% agar agar powder in distilled water). Then the mixture was poured into each petridish. After the agar was solidified, holes were punched and positive control, negative control as well as enzyme were added into their respective wells (previously marked). The plates were allowed for 24 hours incubation in incubator to get results. Presence of protease was detected by the cleavage of casein present in the skim milk.

2.5. Proteolytic assay by using azocasein
The protease assay was done using azocasein as substrate following this reference [9]. The removal of azo dye by the protease was measured spectrophotometrically at 440 nm. We mixed enzyme and substrate in approximately 1:100 ratios at a given temperature and pH that corresponds to the optimum conditions of the enzyme under investigation. The reaction was terminated adding 5% TCA to the enzyme-substrate-buffer mixture. The coagulated protein was removed by centrifugation at 6000 rpm for 5 min at room temperature. The obtained supernatant was then added to a 0.5 N NaOH solution using a 1:1 (v/v) ratio and its absorbance was read at 440 nm.

2.6. Effect of pH & temperature on the protease activity
We have assayed the protease activity in terms of caseinolytic activity at different pH at room temperature (25 ºC) with plant extracts. Azocasein is used as substrate. pH in this case is adjusted by performing the assay at different phosphate buffers ranging pH(5, 7.5 and 10). We have estimated the effect of temperature on the protease activity. The protease assay is performed using the optimum pH at which maximum tyrosine is liberated in different temperature ranging from (4, 25, 37, 55 and 85) °C.

2.7. Enzyme kinetics assay at different azocasein concentration
Enzyme activity is a measure of amount of product formed per unit time. It is always expressed in Unit/mol. the enzyme activity assay for protease is conducted with azocasein as substrate, at pH-8 in 37 °C. Using the optimum temperature and optimum pH the entire protease assay is performed. Here the substrate concentration (azocasein) varied in the range (100 - 900) mg/L keeping the enzyme concentration fixed.
All the experiments were repeated three times and the data is reproducible.

3. Results and Discussion:
3.1. Estimation of protein content by Bradford method:
We have done protein estimation by Bradford method. Here we have taken BSA as a known protein and generated a standard curve. A standard curve was plotted with different known concentrations of BSA versus absorbance at 595 nm. From this linear standard curve, concentration of the unknown samples were determined using the formula y = mx + c. The unknown concentrations for Ginger and Onion were found 0.464 mg/ml and 0.68mg/ml respectively.

3.2. Estimation of protease activity by using skim milk agar assay
Qualitatively protease activity was assayed by using skim milk agar plate assay. The degradation of milk casein was observed after 24 hrs incubation in 37 ^{0}C. Ammonium sulphate precipitated protein solution from Onion crude extract and Ginger crude extract showed clear zones indicating caseinolytic ability of the extracts and thus presence of protease enzyme in the samples (Figure 1).

3.3. Effects of temperature and pH on proteolytic assay by using azocasein as substrate–
Quantitative estimation of protease activity was done by using azocasein as substrate. Degradation of azo dye was measured by absorbance at 440 nm. Enzyme activity was determined from the following equation
Enzyme activity = (Δ Absorbance / min.) / ϵ
[ϵ is the molar extinction coefficient and $\epsilon^{1\%}$ of azocasein is 37 liters g-1 cm-1]

3.3.1. Effect of Temperature
Protease activities in terms of azocasein degradation per unit time at different temperatures 4 ºC, 25 ºC, 37 ºC, 55 ºC and 85 ºC are shown in Figure 2. Both proteases from Ginger rhizome and Onion scale leaves showed optimum protease activity at 55 ^{0}C.

3.3.2. Effect of pH
Protease activities in terms of azocasein degradation per unit time at different pH 5, 7.5 and 10 are shown in Figure 3. Protein from ginger showed optimum protease activity at pH 5.0 and protein from onion showed optimum protease activity at pH 8.0

3.3.3. Substrate concentration
Protease activities at different azocasein concentrations (100, 200, 300, 400, 500, 600, 700, 800 and 900) mg/L were plotted to get the Michaelis Menten kinetics plot of ginger and onion proteases. Michaelis Menten constant K_M and maximum protease activity were calculated from the double reciprocal plot or Line-Weaver Burk plot of 1/s (1/azocasein concentration) versus 1/v (1/ protease activity) showed in Figure 4. Maximum protease activity found from onion scale leaves and ginger rhizome are respectively 961.44 mg/L/min and 224.00 mg/L/min. The K_M value for onion scale leaves proteases and ginger proteases are respectively 0.63 and 0.32 mg/L. The results indicate higher affinity and higher protease activity in onion scale leaves protease.

3.4. Specific Activity Comparison
Specific activity is a measure of enzyme activity for a specific enzyme concentration. The specific activity calculated with the help of protein concentration present in the onion scale leaf extract and ginger extract. Specific activity of protease of ginger (0.47 mg/L/min/µg) is found to be more than onion (0.27 mg/L/min/µg).

4. Conclusion
This study finds that onion scale leaves has alkaline protease

activity at the optimum temperature of 55 ^{0}C. Comparative study with ginger protease showed lesser specific activity for onion protease.

5. Acknowledgement

We would like to acknowledge Techno India University for providing the lab facilities and chemicals. We also want to thank Dr. Sirshendu Chatterjee and Mr. Pranabesh Ghosh for their help and support.

Table 1: K_M and V_{max} values for Ginger and Onion proteases

Protease Samples	$K_{M\ mg/L}$	$V_{max\ mg/L/min}$
Ginger protease	0.3199	224.003
Onion protease	0.6280	961.44

Table 2: Protein Concentration, Enzyme (Protease) Activity and Specific Activity of Ginger and Onion

Protease sample	Enzyme Activity mg/L/min	Protein concentration	Specific Activity mg/L/min/ug
Ginger	**0.2216**	0.464	**0.4776**
Onion	**0.1837**	0.68	**0.2703**

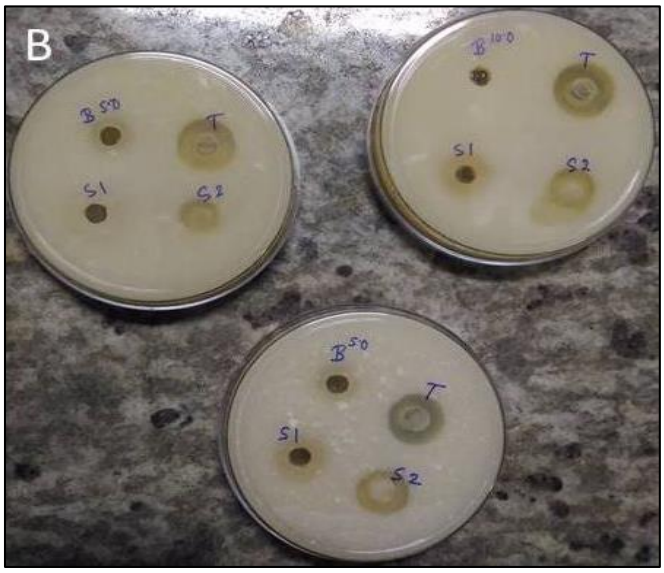

Fig 1: Qualitative protease assay using skim milk agar plates: 2% skim milk was used with 15% agar. The extracts from onion and ginger were paced by making holes in the skim milk agar plates. After 24 hrs incubation at 37 ^{0}C clear zones have been observed for degradation of casein present in skim milk. Trypsin is used as positive control and phosphate buffer as negative control.

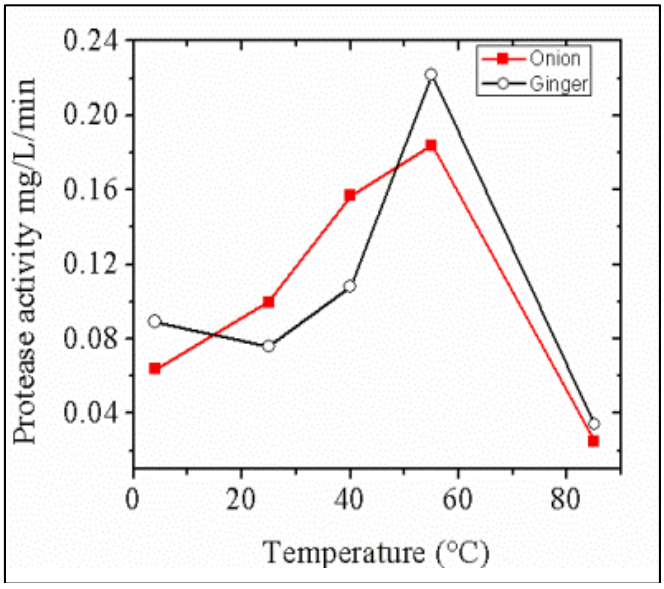

Fig 2: Effect of temperature on quantitative protease activity of Onion and Ginger using azocasein as substrate: Degradation of azocasein was detected with absorbance at 440 nm after the reaction at respective temperatures (4 ºC, 25 ºC, 37 ºC, 55 ºC and 85 ºC). Protease activity of onion (closed square red) and protease activity of ginger (open circle black) was plotted against temperature.

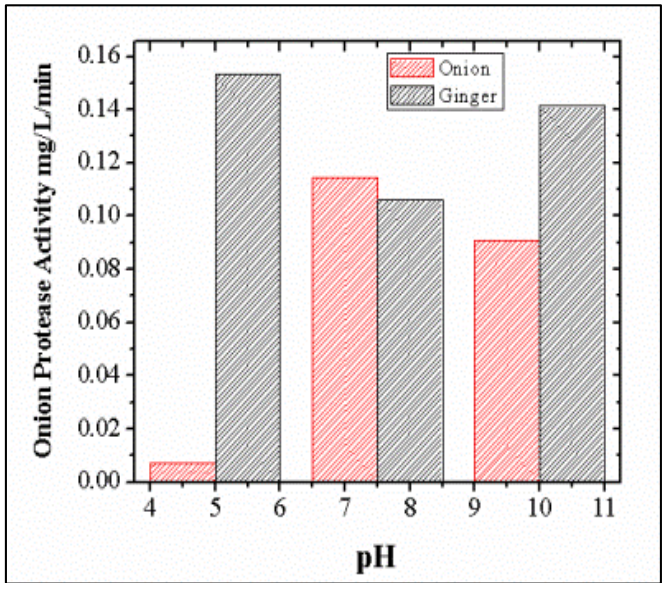

Fig 3: Effect of pH on quantitative protease activity of Onion and Ginger: Degradation of azocasein was detected with absorbance at 440 nm after the reaction at respective pH (pH 5, 7.5 and 10). Protease activity of onion (red column) and protease activity of ginger (black column) was plotted against pH.

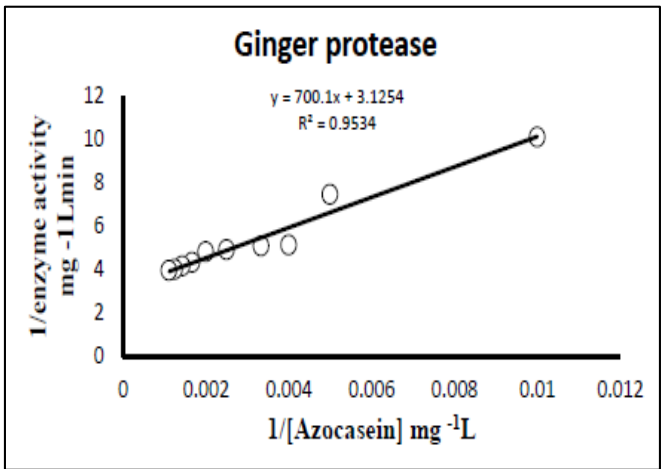

Fig 4: Effect of substrate concentration on quantitative protease activity of Onion and Ginger: Solutions with azocasein concentrations (100 -900) mg/L were used to determine protease activity kinetics with fixed enzyme concentrations at pH 7.5 and 37 ^{o}C. The double reciprocal plot of reciprocal of azocasein concentration versus reciprocal of enzyme activity for ginger protease was shown with open circles and for onion protease was shown with closed squares.

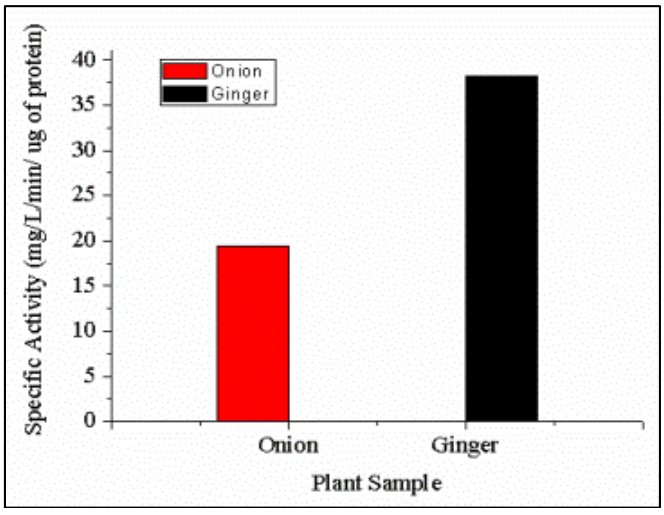

Fig 5: Comparative specific activity of protease activity from Onion and Ginger: Specific activity of onion and ginger proteases are shown as bar diagram.

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