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Purification of protease enzyme from the leaf, seed and pod samples of *Vicia faba* L.

Sathya Prabhu, D., Apoorva, S., Nandita, J., Palani Chamy and *Devi Rajeswari, V.

Department of Biomedical Sciences, School of Biosciences and Technology, VIT University, Vellore – 14, Tamilnadu, India

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Abstract

Protease is a well-known plant enzyme, used widely in the field of medicine and industry in various potential and economical applications. Available literatures show a lack of study related to the isolation of protease enzyme from *Vicia faba* L (Faba bean), hence, this study focuses on the isolation of protease from faba bean. Initially, the extraction process was carried out using overnight ammonium sulphate precipitation method, followed by protease assay to determine the enzyme activity in seed, leaf and pod samples of faba bean. Apparently, dialysis was carried out for partial purification, later High-Performance Liquid Chromatography (HPLC) for further purification. Finally, the molecular weight of the protease enzyme was determined from each sample extracted by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) respectively. The outcome of the study demonstrates that all the samples of faba bean contain protease enzyme with 60-70% saturation. The enzyme extracted from different parts of the sample show an optimum activity at pH 6 along with a molecular weight ranging between 60 to 100 kDa. In conclusion, the study suggests faba bean can serve as best source of protease enzyme.

Introduction

Recently, the extraction of protease enzyme from plants has increased significantly by considering its consequentiality (González-Rábade *et al*., 2011; Sun *et al*., 2016). Globally, the industrial production of proteases accounts for 60% of the enzyme sales economy (Kim *et al.*, 2016). Protease enzyme has been utilized widely in industries to engender a wide range of products such as, detergent, leather, waste management, brewing, meat softening, milk-clotting, food, pharmaceutical, cancer treatment, diagnostics, digestion, viral disorders and silver recovery (Gupta *et al*., 2002; Naidu and Devi, 2005; Roy and Kumar, 2014; Kuddus, 2015). Proteases belong to a class of enzymes that can be classified based on their physical and biological property (Rooprai and McCormick, 1996; Arpigny and Jaeger, 1999; Ellaiah *et al.*, 2002; Gupta *et al.*, 2012). Apart from this, the plant proteases have many biological roles, such as anti-cancer activity, avert edema, avails in the digestive process, procoagulant activity and many more. (Van der Hoorn, 2008; González-Rábade *et al.*, 2011). However, the molecular mechanism defining the biological activity of the enzyme remains obscure (Shivaprasad *et al.*, 2012). Considering the wide range of application of proteases, finding incipient sources for the production of protease draws a greater attention for various scientific communities.

Vicia faba L. commonly named as broad bean, faba bean, horse bean and fava bean belongs to the family Fabaceae (Boukhanouf *et al*., 2016). The fruit of faba bean is highly nutritious, that serves as a rich source of carbohydrates, proteins, and lipids (Fabbri and Crosby, 2016; Topal and Bozoğlu, 2016). It also has many biological properties like anti-diabetic (El-Maksoud *et al.,* 2013), anti-cancer activity (Jordinson *et al*., 1999) and plays an adjuvant role in curing Parkinson's disease (Apaydin *et al*., 2000; Randhir and Shetty, 2004). Faba bean has served as a best choice for cultivation among farmers, as it is a beneficial crop that gives more production with less investment (Duc, 1997; Crépon *et al.,* 2010). A contemporary literature review shows isolation of protease enzyme among different plant sources and also in the legume plants. However, no literature reports on the isolation of protease enzyme from Faba bean.

The present study was designed to extract protease from faba bean utilizing the well known existing method with slight modification (Morikawa *et al.,* 1994; Henry, 2013). The extraction and purification of protease enzyme was carried out by utilizing different assays such as ammonium sulphate precipitation, protease assay, dialysis, HPLC and SDS page. The ammonium sulphate precipitation

*Corresponding author.
Email: *sdevirajeswari@gmail.com, vdevirajeswari@vit.ac.in*

was performed in different concentrations ranging from 30% to 80%, to find the ideal concentration for the extraction of protease in high concentration. The presence of the protease is confirmed using casein as a substrate (Wingfield, 2001; Gerze *et al*., 2005; Orhan *et al.,* 2005). Once the presence is substantiated, partial purification was done using overnight dialysis using phosphate buffer. After dialysis, the enzyme was characterized using HPLC. Finally, the molecular weight of the enzyme from the samples was determined using SDS-PAGE. Further, the effect of pH of the enzyme was studied in order to find the optimum activity of the enzyme.

Material and Methods

Materials

The chemicals of analytical grade required for this study were purchased from Hi-Media Laboratories, Mumbai, India and S.D Fine Chemicals Pvt. Ltd., Mumbai, India.

Sample preparation

The fresh faba bean samples of pod, seeds, and leaf were bought from the vegetable market in Vellore, Tamil Nadu, India. Taxonomist identified the samples at Department of Biological Sciences, VIT University. A voucher specimen was deposited at VIT plant repository for further reference. The seed and pod samples were separated and washed with distilled water extensively, wiped with sterile cotton. The seeds were kept overnight for germination. Fresh seed samples (60 g) were taken and ground using 100ml acetone to remove fat content, using mortar and pestle. Pod samples (60 g) were mixed using a mixer along with acetone. Both samples were mixed with chilled 10 mM Tris-Hydrochloride (Tris-HCl) buffer at pH 8.0 and 2M NaCl for three hours on an orbital shaker. The samples were centrifuged at 10,000 rpm for 10 minutes at 4°C and filtered through Whatmann filter paper; the filtrate was collected. The leaves of faba bean (60 g) were washed properly in sterile water and ground with the help of a mechanical mixer. Phosphate buffer was added and left in the orbital shaker for three hours. The seed, pod and the leaf samples were centrifuged at 10,000 rpm for 10 minutes at 4°C. The samples were filtered using Whatmann filter paper (Sindhuja and Mohanraj).

Ammonium sulphate precipitation

Ammonium sulphate precipitation was performed for the seed, pod and leaf samples, in a stirrer maintained at 4°C. Overnight ammonium sulphate precipitation was performed at 6 saturation percentages consecutively 30%, 40%, 50%, 60%, 70% and 80% to determine the saturation percentage. After precipitation, centrifugation at 10,000 rpm for 30 minutes at 4°C was done. Both, the supernatant and pellet are collected after each saturation percentage, and the pellet was resuspended in phosphate buffer (pH 7.0) (Purwanto, 2016).

Protease assay

In protease assay, a standard curve was plotted, and protease activity using casein as a substrate was performed. A 5 ml of casein is added to four test tubes labeled – Test 1, Test 2, Test 3 and Blank. It was incubated in a water bath at 37°C for 5 minutes. 1ml of enzyme solution (supernatant and pellet samples obtained after ammonium sulphate precipitation is diluted with sodium acetate buffer) was added to the three test samples and then mixed and incubated at 37°C for 10 minutes. A 5ml of trichloroacetic acid is added to all test tubes, and 1ml of enzyme solution was added to the test tube labeled blank. After mixing and incubating at 37°C for 30 minutes, the samples were centrifuged at 10,000 rpm for 10 minutes at 4°C. To 2 ml of the supernatant, 5ml of sodium carbonate and 1 ml of Folin-Ciocalteu reagent was added. Spectrophotometric readings were determined at 660 nm. Enzyme activity was calculated for each saturation percentage, to measure the amount of active enzyme present. It was determined at what saturation percentage highest protease activity is detected for seed supernatant, seed pellet, pod supernatant, pod pellet, leaf supernatant and leaf pellet. After protease assay was performed for samples, enzyme activity values for each sample was calculated using the standard formula (Cupp-Enyard, 2008).

$$\mu\text{mole tyrosine equivalents released} = \frac{(\text{Test OD} - \text{Blank OD})}{\text{Slope}} = \frac{(\text{Test OD} - \text{Blank OD})}{1.691}$$

Dialysis

Partial purification by dialysis was carried out for the six samples obtained after the extraction step. A 2 ml of each of the six samples was pipetted into pretreated dialysis membranes and kept in beakers with 200 ml phosphate buffer in a magnetic stirrer. The buffer was changed once every 1.5 hours for 3 hours and then kept overnight. The initial and final volumes were then compared (El-Khonezy *et al*., 2015).

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 six samples

were loaded in 1:1 dilution along with loading sample buffer in each well. The samples were run for electrophoresis 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 (Heussen and Dowdle, 1980; Purwanto, 2016). Further, reverse phase HPLC was performed for each of the 6 samples after dialysis to check purity. Isocratic elution was followed with two solvents – Solvent A (0.052% Formic Acid in Water) and Solvent B (0.05% Formic acid in 80% Acetonitrile). Both the mobile phases were degassed by ultrasonication followed by filtration with Whatman filter paper. The flow rate was 0.500 µl/min, and pressure was maintained at 200 kg. The samples were filter sterilized, and about 20 µl of each sample was injected with 1mg/ml Bovine Serum Albumin (BSA) as standard (Krishnan and Murugan, 2015; Banerjee *et al.*, 2016).

Effect of pH on protease activity

Effect of pH on protease activity was measured using a Tris-HCl buffer. The pH was adjusted to 5, 6, 7 and 8 respectively. Each of the six samples was mixed with Tris-HCl buffer in 1:1 ratio. After an incubation period of 24 hours at room temperature, protease assay was performed with each of the six samples at the pH mentioned above, and enzyme activity values were calculated (Das and Prasad, 2010).

Results and Discussion

Recently, exploration of plant proteases has been widely considered due to their importance in food and pharmaceutical industries (Gupta *et al.*, 2002; Hasan *et al.*, 2006; Kim *et al*., 2016). Considering the importance of the enzyme, protease enzyme have been widely studied by researchers from various plants and food crops (Ryan, 1990; Kim and Hwang, 2015; Chen *et al.,* 2015; Cheng *et al*., 2016; Mandujano-González *et al.*, 2016; Niemer *et al.*, 2016; Sequeiros *et al.*, 2016). Despite its importance in various fields, the extraction of proteases from legumes still remains minimal in current scenario (Kinsella *et al*., 1993; Foss and Eriksen, 1995; Rao, *et al.*, 1998). In this study, a well-known legume plant *Vicia faba.* L was considered for identification and extraction of protease. The enzyme was initially extracted, partially purified and characterized using the existing methods with slight modification.

Extraction of protease

The samples were fractionated and purified initially by ammonium sulfate precipitation method, a widely used method for purification of protease (Drivdahl and Thimann, 1977; Antão and Malcata, 2005; Esposito *et al.*, 2016; Nam *et al.*, 2016). The optimum ammonium sulphate saturation percentages for highest protease activity were found to be 70% for seed pellet and supernatant, 80% for pod pellet and supernatant, 80% for leaf supernatant and 60% for leaf pellet, as shown in Figure 1. It was observed that seed pellet had a high protease activity of 0.049 µ/ml at 70% saturation. Protease assay was performed for the samples from 30% to 80% saturation and the enzyme activity values for each sample were calculated. The saturation percentage of the six samples ranged from 30% to 80% respectively. Similar results were earlier reported in protease isolated from *Bacillus subtilis* (Salem *et al*., 2016), *Vipera venom* (40-50%) (Moroz *et al*., 2016), traditional Chinese fish sauce (70%) (Xie *et al*., 2016), and *Cucurbita maxima* peel (60 %) (Vadivukkarasi *et al*., 2015) comparatively. Among the samples, seed pellet sample had the highest enzyme activity followed by leaf supernatant, leaf pellet, pod supernatant, seed supernatant and lastly pod pellet. Particularly, the seeds of *Vicia faba* L. contain high quantity of protease than other parts of the plant.

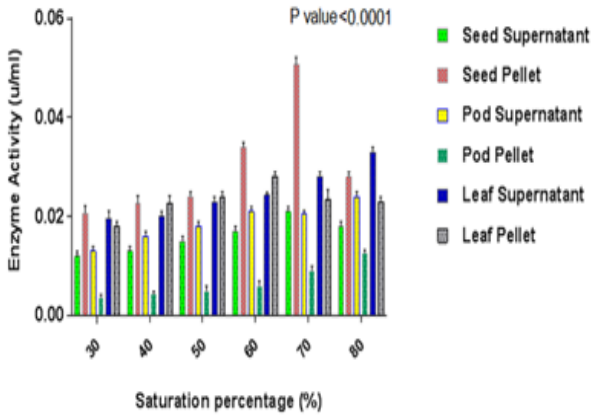

Figure 1. Effects of enzyme activity were carried out for faba bean samples at various saturation values. Two way ANOVA analysis shows the values are statistically significant (P value <0.0001). The bars represent means ± standard deviation for triplicates. The bars represent means ± standard deviation for triplicates.

Purification of protease

In enzyme purification process, dialysis was carried out for the partial purification of six different samples of *Vicia faba*. L and the purity were further checked using reverse phase HPLC at 280 nm and 350nm, as shown in Figure 2 and 3, corresponding with the standard BSA (Krishnan *et al.*, 2015). Finally, SDS-PAGE was carried out to determine the

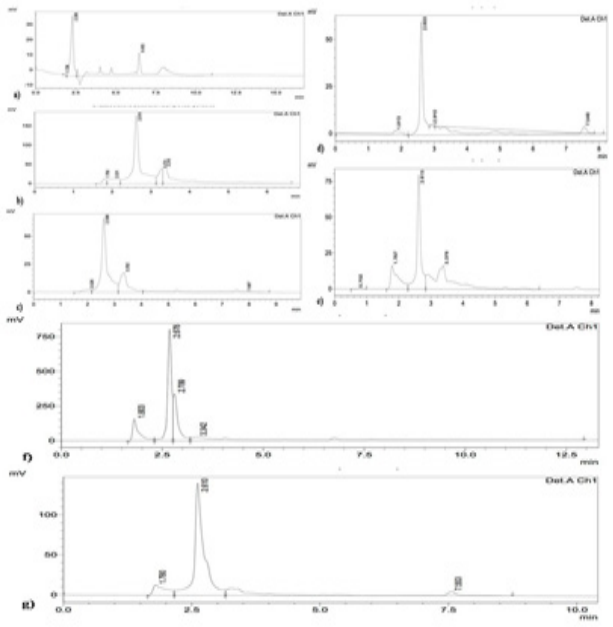

Figure 2. HPLC analysis chromatogram of faba bean samples analyzed at optical density 280 nm. a) Standard Peak b) Leaf pellet c) Leaf supernatant d) Pod pellet e) Pod supernatant f) Seed pellet and g) Seed supernatant.

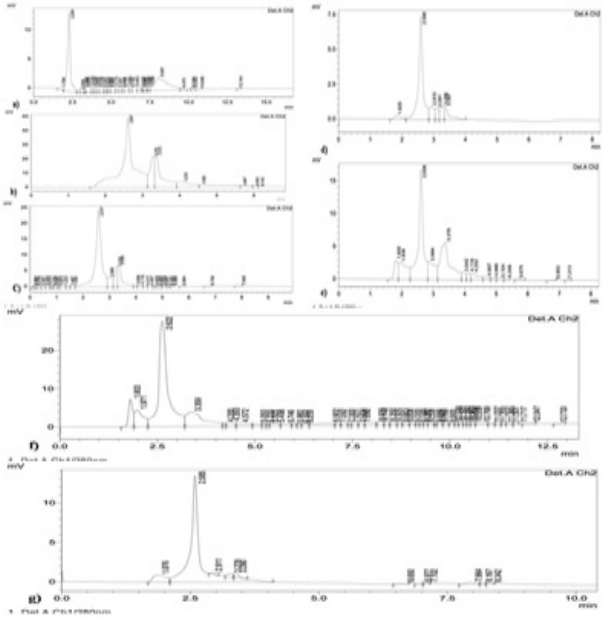

Figure 3. HPLC analysis chromatogram of faba bean samples analyzed at optical density 350 nm. a) Standard Peak b) Leaf pellet c) Leaf supernatant d) Pod pellet e) Pod supernatant f) Seed pellet and g) Seed supernatant.

molecular weight of the protease extracted from the samples. It was found that the proteases were found in the range from 60 to 100 kDa with an average of 80 kDa, as shown in Figure 4, with the tabulations representing the approximate molecular weight of protease extracted from each sample. 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 can be classified into four types, serine, aspartate, metallo and cysteine protease respectively. Among these groups, cysteine proteases are predominantly found in plant sources (Domsalla and Melzig, 2008; Rawlings *et al*., 2010; González-Rábade *et al.*, 2011). Accordingly, glutamate decarboxylase (58kDa) involved in Gamma-aminobutyric acid synthesis was purified from faba bean (Yang *et al.*, 2013); Trypsin inhibitor (15kDa) was isolated from faba bean (Fei Fang *et al*., 2011); Phytate-degrading enzyme (65 kDa) is purified from *Vicia faba* Var. Alameda (Greiner *et al.*, 2001); Serine protease was isolated from the *Cucumis trigonas* (Asif-Ullah *et al.*, 2006), *Helicoverpa armigera* (Johnston *et al*., 1991); Cysteine protease from *Horse gram* (Jinka *et al.,* 2009); alkaline protease from *Bacillus subtilis* (Sathishkumar *et al*., 2015) and many more.

Effect of pH on protease activity

Proteases are pH dependent, pH is a consequential factor for the enzyme activity. The production of the enzyme will be maximum at a particular pH know as optimum pH. The optimum pH is important for any enzyme in terms of its production and activities (Rao *et al*., 1998), variation in pH leads to enzyme inactivation (van der Hoorn and Jones, 2004; Bah *et al*., 2016). However, few plant proteases are exceptional, they are active in wide range of pH and temperature (Uhlig, 1998). In this study, the pH activity of proteases isolated from *Vicia faba*. L samples were calculated at different pH (5, 6, 7 and 8) respectively. The pH studies reported an increased enzyme activity at a pH range of 5-7, which shows similar range (pH 6-7) of activity in experiments carried out earlier for protease comparatively (Clemente, 2000). Proteases which are highly active at acidic pH are widely used as a milk coagulating factor in industrial firm (Silva and Malcata, 2005). At pH 6, a maximum protease activity was observed in all the samples, hence pH 6 is considered as an optimum pH for enzyme production, which is illustrated in Figure 5. The remarkable ranges of pH activity of enzymes favor the acidic and neutral environments. Similar studies were reported in different studies, where protease enzyme is purified from different sources. Such as, the phytate-degrading enzyme (65kDa) purified from *Vicia faba* Var. Alameda exhibit the optimum pH at 5.0. (Greiner *et al*., 2001), Cysteine protease (20kDa) was purified from *Ficus microcarpa* showed maximum activity at pH 8.0 (Mnif *et al*., 2015), protease was extracted from Jatropha seed cake showed maximum pH activity at

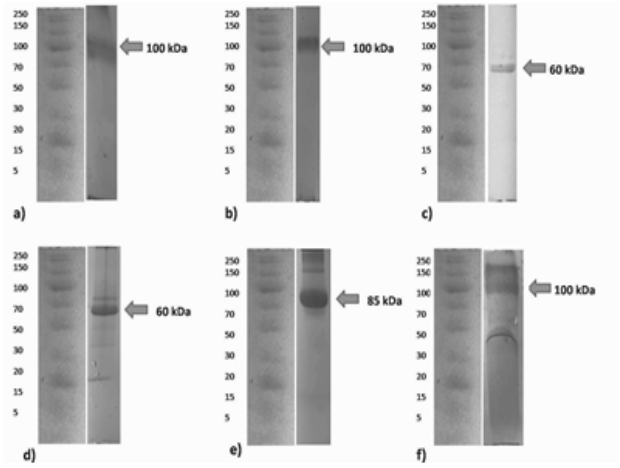

Figure 4. Electrophoretic analysis of different parts of faba bean of partially purified protease a) leaf supernatant, b) leaf pellet, c) Seed supernatant, d) Seed pellet, e) Pod supernatant, and f) Pod pellet along with mid range marker (15-300 bp) as standard.

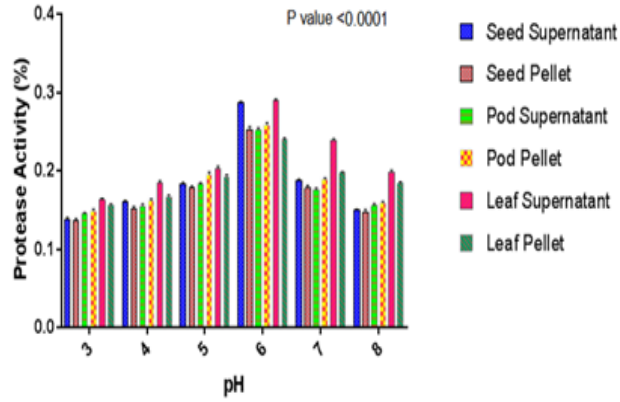

Figure 5. Effects of pH on faba bean samples were shown in graphical representation. Two way ANOVA analysis shows the values are statistically significant (P value <0.0001). The bars represent means ± standard deviation for triplicates.

7.0 (Veerabhadrappa *et al*., 2014).

Statistical analysis

A two-way ANOVA statistical analysis was performed using GraphPad prism (version 7.00 for windows, GraphPad software, San Diego California USA, www.graphpad.com) for saturation percentage and effect of pH on faba bean samples. The obtained data showed a statistical significant P-value less than 0.0001.

Conclusion

It is well-known that proteases are used for various purposes in industrial, biological and medical applications. Protease is a well known commercial enzyme with wide range of applications; hence, this study was intended to check the presence of protease content in faba bean. The protease enzyme was successfully extracted and partially purified from the leaves, pods, and seeds of faba bean. The percentage of protease was high at 70% saturation for all the samples. The saturation percentage denotes that faba bean contains protease enzyme in all the parts of the plant. Whereas, the optimum pH was found to be slightly acidic (pH 6), suggesting the maintenance of pH at this point to gain a maximum enzyme production. The determination of pH plays a vital role in enzyme production for large scale of industrial interest. The successful identification of protease from faba bean will be beneficial to mankind. As humans consume faba bean as stable food, the effect of protease in human metabolism may play a key role. Studying metabolic role of this enzyme will help in better understanding of proteases in human health and diseases. Apart from this, the protease from faba bean should be studied in detail for various other industrial applications.

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