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RESEARCH ARTICLE

ISOLATION, OPTIMIZATION AND CHARACTERIZATION OF PROTEASES FROM LEGUMINOUS SEEDS

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ABSTRACT

The present study was conducted to isolate, optimize and characterize the proteases from two leguminous seeds, Green gram (*Vigna radiata*) and Soybean (*Glycine max*). Isolation of proteases was performed by using Ammonium sulphate precipitation and then it was dialysed. The protein concentration of enzyme was 52µg & 69µg. The Optimum P^H was found to be 9 and optimum temp. was 50^0C. The enzyme activity of soybean proteases and Green gram proteases was found to be o.16µg/ml/min and 0.23 µg/ml/min respectively. In Machaleis menten graph Km was found 0.485 & Vmax was 1.01. In Line weaver Burk Plot Km was found 5.55 & Vmax was 2. The Casein & Heamoglobin assays were also performed to check its catalytic activity. Molecular weight of proteases according to SDS-PAGE found to be 26.0KD Soybean & 19.0KD Green gram. FT-IR characterization was also done. The paper concludes that leguminous seeds can be source of proteases for industrial purposes.

Key words: Proteases, optimization, characterization, leguminous seeds.

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INTRODUCTION

The research work is regarding extracting natural protease from leguminous seed by using ammonium sulfate. The leguminous seeds plant have legume or small space in their roots where a special kind of bacteria lives, these bacteria are called nitrogen fixing bacteria. Plant need nitrogen to make amino acids which make protein, i.e. responsible to make cell which is important for growth, but nitrogen in the atmosphere cannot be used directly, so they absorb nitrate ions (NO_3^-) from the soil as source of nitrogen. These bacteria converts nitrogen to nitrate ions. The legume is belongs to family leguminosae. The fruits or seeds of such a plant, legumes are grown agriculturally. Primarily their grain seeds are called pulses, for livestock foliage and silage and soil enhancing green manure. Well known legumes include Soybean (Glycine max), Green gram (Vigna radiate), Ground nut (Arachishypogaea), Pea bean(Phaseolus vulgaris). The present research work is regarding extract of natural extract protease from leguminous seeds. Legume is a plant or fruits or seed in the family facbaceae (leguminosae). Legumes are grown agriculturally, primarily their grain seed are called pulse, these are salt-soluble globulins of which the leguminas are formed by two chains, linked by disulfide bond the seeds of plants are rich stores of protein, carbohydrates, lipids and they are used as a valuable food sources. Leguminous seed can be use as a source of proteases for industrial purposes. Proteases extract from leguminous seeds; Soybean (*Glycine max*)and Green gram (*Vigna radiata*) in having high content of proteases. Proteolytic enzyme are also called as protease enzyme. Any group of enzymes that break the long chain molecule like proteins into shorter fragements (peptidase) and enventully into their compound are aminoacid. The present research work is regarding extracting natural proteases from leguminous seed by using Ammonium sulphate solution. The Ammonium sulphate help in increasing the stability of proteases. These reagents will breakdown disulfide bonds, hydrogen bond and salt linkage of the protease in order to dissovle it into protein solution. Currently there is an increasing interest in development of materials that are naturally obtain from renewable resources. The main natural materials are obtained from polysaccharides, lipid and protein. Proteases are polymers formed by various amino acids capable of promoting intra and inter-molecular bonds, allowing the resulting material to have a large variation in their functional properties. Proteases are one of the largest groups of industrial enzymes that catalyze of hydrolytic reaction by cleaving peptide bonds in protein. Proteases may be classified as two major group, Exopeptidases and Endopeptidases based on their ability to degrade N or C terminal peptide bond. Endopeptidases, which have more potent industrial application that exopeptidases,

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(The proteases, which can be cleave the hydrophobic bond into amino acids are valuable in debittering of protein hydrolysates in soya-based products. Protease are mainly obtained from microbial sources for industrial purpose. To study the in-vitro degradation of protein, several studies have been worked on purification and characterization of proteases and peptidases. Protease constitute one of the most important group of industrial enzymes, accounting for about 60% of the total enzyme market. Protease is an enzyme that conducts proteolysis, that is begins catabolism by hydrolysis of the peptide bonds, the linking of amino acid together in the polypepetide chain forming the protein. For several physiological processes the action of the proteolytic enzyme is essential, Eg. digestion of food protein, protein turnover, cell division, blood clotting cascade, signaling transduction is important.Protease is commercially valuable and having various industrial applications. They are widely used as detergents in food**,** pharmaceutical and leather tanning industries. The vast variety of protease, with their specificity of their action and application has attracted worldwide attention to explore it. They also have physiological as well as biotechnological applications. It has been considered as eco-friendly because the appropriate producers of the enzymes for commercial exploitation are non toxic and non pathogenic that are desingnated as safe.

The major application of protease is in the dairy industry in the manufacturing the cheese. The enzyme exhibited two major drawback.1) The presence of high level of nonspecific and heat stable proteases which lead to bitterness in cheese after storage and 2) Poor yielding, and primary fuction of protease is to hydrolyze the specific peptide bond to generate macro peptidase. Chymosin is perfrerred due to it high specificity for casein, which is responsible for its performance in cheese making. To study the application SDS-PAGE (sodium dodecyl sulphate- polyacrylamide gel electrophoresis) i.e. most widely used method for analysis of protease mixture qualitatively. To study the degradation of Casein and Gelatin protein by protease enzymes. FTIR spectroscopy has traditionally been used for material analysis within the chemical industry. However, more recently, it has been applied to biological and biomedical analysis. The use of immobilized enzymes in industry is based on the potential advantages they confer over their soluble counterparts, including increased stability to temperature, PH and organic solvents. Recovery or reduction of autolysis or denaturation. Furthermore, immobilized enzyme render continuous production processes possible.

Germination ability of the seeds

Evenari (1981) studied the germination process and% of germination in different cultivated crop seeds in order to understand the physiology and mechanical behind it. similary workers considered different crop including seeds belonging to leguminosae family. for example Atak *et al.* (2008) studied the germination processes in peas and concluded that dark green colourd seeds increases the seed vigor and germination ability in dry green pea (pisumsativum L.) and carpici *et al.* (2009) investigated the effects of salt stress ongermination ability of some maize (*zea mays* L.) cultivars of Africa. Mehrafarin *et al*(2010*)* studied the cultivayion and biotechnology of fenugreek *(trigonellafoenum – graecum* L.*)* as valuable medicinal plant. Similar study on the interaction between seed size and NaCl on germination ability andearly seedling growth of some Turkish cultivars of chickpea (*cicerarientinum* L) and wheat *(Tricumaestivum* L*)* was done by *kaya et al*.(2008 and 2009) resespectively. In India similar works were done by Dharmalingan and Basu (1990) as worked on the maintenance of viability and vigour of sun flower *(helianthus annus* L) seeds by observing the processes of germination step by step. different conditions applied for better germination % and longer viable storage of seeds was also investigated. study on soyabean (*Glycine mar* L) seeds during storage and germination was done by sigh and Dadlani (2003)and by Sharma *et al.* (2007) and explained the role of various changes on the germination ability of the seeds.

Kakani *et al.* (2008) considered fenufreek for their study as it is the one of the most economically, nutritionally and medicinally important legumes and invented advance production technologies for *Trigonellafoenum- graecum* L. and various processes to increase the germination % of the seeds to encourage their cultivation. Urbano *et al.* (2005b) made an assessment of the nutritional value of raw seeds of peas (*Pisumsativum* L) and the seeds after germination by in vivo and in vitro techniques. Tokiko and koji (2006) considered various economically important leguminous seeds and their sprouts and studied the fatty acid and free amino acid composition in them. such an investigation was done by seeds of cultivation legumes. Haseolus *vulgaris and cajanuscajan* by Sangronis and Machado (2007) and on two varieties of Soybean *(Glycine max* L).seeds by Wang *et al.,* (2007) and Megat-Rusydi *et al*. (2011) estimated the proximate content and fatty acid composition of germinated and non germinated legumes like kidney bean, soyabean, peanut and mung and five varities of rice (red**,** black**,** barrio, brown, and milled). Wang *et al*.(2011) isolated starch from the seeds of seven varieties of field peas (*Pisumsativum L).* and characterized their structural and properties. tree species belonging to leguminosae was studied by Agboola (1998). chemical analysis of seeds of *prosopis Africana* (Gulli and Perr) was done for determination of citation composition**,** total nitrogen and crude protein in the cotyledons in this study.

In India also many workers contributed in same area. Gunesh and Venkataraman (1978) gave a detail account of the amount and types of storage proteins found in chick pea(*cicerarienum)* seeds and by Deshpande and Nielsen (1987) in *phaseolus vulgaris* and studied the major storage protein called phaseoline by the process of *in vitro* hydrolysis. similarly cysteine protease from germinating cotyledons of legume seeds was puirified and thoroughly studied by Sadhasivam and Macikam (2008) kausik *et al*. (2010*)* studied soybean seeds and the variation in their nutritional qualities whendomestically processed by different common methods.Besides commercial legume varieties**,** some of the wild species of the same family were also studied in india. Katiyar *et al.*(1970) took a wild variety of legume that was *clitoriaternatea,* a perennial wild leguminous plant which has a great nutritional value for milk producing animal like shhep. Joshi *et al.*(1981)worked on the seedsof another wild leguminous plant species. *Clitoriaternatea*

Biochemical changes in seeds contents during germination

Germination of seeds, starting from imbibition, initiates numerous physiological activites. important biochemical changes during this time period leads to extensive breakdown of stored reserve nutrients like carbohydrates, lipid and proteins in the storage of seeds to provide energy to the

growing embryo. Different human societies have developed proteolytic processes to produce food stuffer which are often intimately associated with cultures in different countries (Adhler-Nissen, 1986). In Europe, France In particular**,** milk isa more common substrate for fermentation with the range cheeses as its main product. inasia**,** in milk, soya bean, in Africa**,** locust bean is used as in Nigerian fermented product called 'dawadawa' (Hesseltine, 1979;Winrno**,** 1979; Reddy *et al.,*1982). The presence of protease in all living plants signifies their role in the essential metabolic and regulatory functions in various biological processes (lopez- otin and bond, 2008,turk, 2006). Proteolytic enzymes have a long history of application in various biotechnological industries (kumar and tukagi,1999,Rao *et al.,* 1998; sabotic and Kos, 2012). but uncontrolled protease can be hazardous to the system and must be regulated both in time and place. Protease in biological systems are regulated by diverse mechanisms. Inactivation of proteases can be achieved by degradation or by binding with inhibitor molecules. intraction with protease inhibitor constitutes a very important mechanism of protease regulation (Lopez- otin and Bond, 2008; Rawlings *et al.,* 2010). Protease inhibitors are generally small proteins or peptides that occure in storage tissue**,** such as seeds and also arial parts of protease inhibitors inhibitors isolated and studied from plants(Bijina et al.,2011a, green and ryan, 1972;Joshi et al.,1998**,** Lorto el al, 1994, rayan, 1990) and most studied and have isolated from various leguminninosae seeds (Macedo *et al.,* 2002 Macedo and Xavier- Filho, 1992; Mello *et al.,* 2002; olive *et al.,* 2000; Souza et al.,1995). legume seeds contain various PIs classified as kunitz- type, Bowman- Birk- type, potato I, potato II, squash, cereal superfamily.

MATERIALS AND METHODS

Method

Extraction

- **Pretreatment:** The seeds (soyabean and green gram) approximately (50g) in amount, were wash with water and they soaked in distilled water at room temperature for overnight germination.
- **Filtration:** Soyabean and green gram were crushed. Soyabean and green gram were Homogenized by using motor and pestal. Then the homogenized solution was filter by using Whattmann filter paper No.1 and the filtrate was collected. Then the filtrate was used for further purification.
- **Centrifugation:** The filtrate was centrifuged at 10000rpm for10min. at 4^0C. For the centrifugation of soyabean acetone was used as it have low fat content. Centrifugation was repeated 2-3 times. Then the supernatant was collected and pellet was discarded.
- **Precipitation of protease enzymes by ammonium sulfate:** 33g of ammonium sulfate is dissolved in soyabean(50ml) and green gram(50ml) respectively. The solution is stirred in cold condition until all the ammonium sulfate particles are dissolved. Then the solution is filter to make it particle free. After this dialysis is carried out.
- **Parial purification of Protease enzymes (Dialysis):** In Dialysis, (separating of particles is done by diffusion. Dialysis can be used to remove the salts) load the sample into dialysis bag and keep it overnight in cold condition. After this place the dialysis bag on gentle stirring for 10-15mins.After this dialysis bag is kept in sucrose for 2-3hours. In dialysis, with the Exchange of the sucrose, the contaminants within the sample can be decreased to acceptable or negligible levels. Then pure protease enzyme is collected. After this optimization of extracted enzyme is carried out.
- **Protein Measurement:** Protein concentration was determined by the method of lowry *et al.,* 1951 using Bovine serum albumin (BSA) as standard protein. The amount of the soluble protein was calculated from the standard graph as mg & µg of protein/ml of sample.
- **Assay of protease enzyme (Determination of catalytic activity of proteases):** The protease catalytic activity was determined by using casein as a substrate for proteases enzyme. For this assay we have prepared 1% casein, Alkaline P^H buffer 2%, 0.1% enzyme in buffer, 10% Tri-chloro-acetic acid, Lowry's reagent. 0.2 -1ml substrate were taken into that 1ml buffer & 0.5ml protease enzyme were added & then the reaction mixture were incubated for 30min. at room temp. Then reaction was inhibited by adding TCA. Then 0.5ml reaction mixture was taken in each test tube & then into that 3ml Lowry's reagent were added & a mixture kept, all the test tubes for 30min incubation at room temp. After incubation blue color complex was formed which is measured at 540nm on spectrophotometer.

Optimization of Protease

- **Effect of pH on protease enzyme:** The *p*H activity profile of the partial purified enzyme (*p*H 6 to 10.6) was determined. Phosphate buffer (0.2 M, pH range 6.0-8.0) and Glycine-NaOH buffer (0.2 M, pH range 9.0 – 10.6) were used for the studies. The assay was carried out using 0.1 ml of approximately diluted protease enzymes, the optimum pH was measured using the Folin-Lowry's assay. The pH stability of the enzyme at optimum pH was studied by varying the time of incubation from 30 min. to 180 min.

- **Effect of reaction tempreature on protease enzyme:** Similarly, the activity of the enzyme was measured at different temperatures ranging from 15^0C to 70^0C. The cold temp.15^0C and 30^0C, normal temp. 37^0C and 40^0C, high temp..50^0C, 60^0C and 70^0C were taken. The protease activity was measured by Folin-Lowry's assay. The thermal stability at optimum temperature was determined upto 180 min.

- **Effect of substrate conc. On Protease enzyme:** Protease substrate consumption was determined by taking 0.2, 0.4, 0.6, 0.8, 1.0ml Blood (Hb), then 0.8, 0.6, 0.4, 0.2, 0.0ml alkaline P^H buffer were added & then in each test tube 0.5ml protease enzyme were added. Then O.D. was taken at 540nm. It were observed that at ……conc. The Enzyme became fully saturated.

Characterization of Soybean and Green gram protease enzyme

SDS – PAGE: Procedure

- Assemble of electrophoresis unit such that the glass plates slamped to the unit along with the spacer placed in between them at two vertical edges.

- Prepare 1% agarose. Boil to dissolve the agarose and pour thin horizontal layer at the lower edge of the plates to seal the assembly let it solidify by allowing it to cool down for 5-10 min.

Preparation of 12% separating gel: To prepare separating gel add the components as follows:-

- 30% acrylamide-bisacrylamide solution-6ml.
- Distilled water-12ml.
- Ph 8.8- tris SDS buffer-6ml.
- 10% APS solution -125ml.
- TEMED-75µl.
- Pour the gel in between the plate and allow it to solidify for an hour. Immideately after the gel poured. Add D/W to check the leakage at the corner.

Preparation of 5% Stacking gel : To prepare stacking gel, add the compounds as follows:-

- 30% Acrylamide-Bisacrylamide -13ml.
- 5X Tris SDS buffer-1.6ml.
- Distilled water-5.1ml.
- 10% APS solution-75µl.
- TEMED-15µl.

- After addition of TEMED gently mix all the compounds. The Stacking gel poured top on the separating and immediately place the comb avoiding air bubbles allow to solidify for 30 mins.

- Pour 1X Tris glycine, SDS gel runnig buffer in the unit such that the buffer connects the two electrodes hence completes the flow current remove the comb from the stacking gel carefully.

Sample Preparations

Take 3 tubes for protease samples and one tube for protein marker. Label them respectively take 20µl of each sample in the respective tube and add 5µl of 5X sample loading buffer to it. Take 3µl of protein marker in the respective tube and for 4µl of 5X sample loading buffer and 13µl of phosphate buffer saline. Boil the tubes containing protease sample at 100 ^{0}C in a boiling water bath. Do not boil the tube containing protein marker till the considerable amount of stain reaches from the gel keep changing the distilled water for 3-4 times.

Staining and Destaining

- After removing water added 50 ml of staining solution in the tray containing gel till the bands are visible sometimes the gel may have to be kept overnight in the staining solution for visualization of the bands.
- Remove the gel from stainig solution. The staining solution can be reused for 2-3 times.
- Wash the gel by D/W till the considerable amount of stain reaches from the gel kept changing the D/W for 3-4 times.
- Add 50 ml of destaining solution to the gel destainig should be Carried out with constant moderate shaking.
- Continue destaining be carried with constant moderate shaking.
- Remove the gel from destaining.
- The destaining solution can be reused 2-3times.
- Then the bands on gel was observed in gel documentation.

Applications of Protease Enzymes

- **Casein protein degradation by protease enzyme:** In order to observe and compare the zone of clearance of casein by greengram and soyabean protease enzyme respectively, the casein protein was degraded by protease enzyme. In the nutrient agar casein was added and the media was prepared. Then media was poured in petri plates and allow to solidfy. After that with the help of plunger wells were made in media plates and protease enzyme was added in wells. After that plates were kept in incubator at 370C for 24 hrs. Next day plates were observed.

- **Gelatin protein degradation by protease enzyme:** In order to observe and compare the zone of clearance of gelatin by Greengram and Soyabean protease enzyme respectively, the casein protein was degraded by protease enzyme. In the nutrient agar casein was added and the media was prepared. Then media was poured in petri plates and allow to solidfy. After that with the help of plunger wells were made in media plates and protease enzyme was added in wells. After that plates were kept in incubator at 37^{0}C for 24hrs. Next day plates were observed.

- **Immobilization of protease enzyme:** Immobilization is defined as entrapment of enzymes. Immobilization is a widely used technique. We have done immobilization of protease enzyme.

Procedure

- 0.5gm of Sodium alginate is dissolve in 25ml hot water, then allow it to cool.
- Then take 10ml Sodium alginate solution into two beaker.
- Add Greengram and Soyabean protease enzyme added in the beaker respectively with stirring to make slurry.
- 0.2% $CaCl_2$ solution is prepared by dissolving 1gm $CaCl_2$ in 50ml D/W.
- Then $CaCl_2$ solution is taken in two petri plates.
- Then with the help of droper add Sodium alginate solution drop by drop in the petri plates respectively and keep it in the refrigerator for overnight.
- Next day Std. Folin-Lowry's assay method was performed with the beads for confirmation.

FTIR of plant extract and partially purified enzyme

For the characterization of soybean and Green gram protease enzyme, the plant extract and partially purified enzymes are send for FT-IR analysis to Rashtriya Uchahatar Abhiyan (RUSA), Dr. Babasaheb Ambedkar marathwada University, Aurangabad.

RESULTS AND DISCUSSION

Extraction

Fig. 1. Extraction of crude enzyme Extract of Soybean and Green gram

Fig. 2. Partially purified Soybean and Green gram protease enzyme

Protein conc. of proteases:

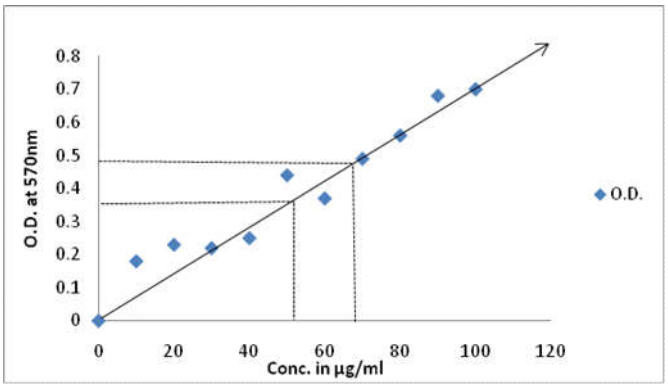


Fig. 3. Protein conc. of Protease enzyme

The amount of protein calculated from standard graph got 51µg of green gram and 69µg of soybean.

- Enzyme activity of Soybean protease was found to be:-
  Enzyme activity = O.D. (Test sample with enzyme) – O.D. (Without enzyme)
  = 0.51 - 0.11
  = 0.41µg/ 0.5ml/ 5min
  = 0.8µg/ 1ml/ 5min
  = 0.16µg/ ml/ min

- Enzyme activity of Soybean protease was found to be:-
  Enzyme activity = O.D. (Test sample with enzyme) – O.D. (Without enzyme)
  = 0.69 - 0.11
  = 0.58µg/ 0.5ml/ 5min
  = 1.16µg/ 1ml/ 5min
  = 0.23µg/ ml/ min

OPTIMIZATION OF PROTEASE:

A) Effect of pH on protease enzyme:

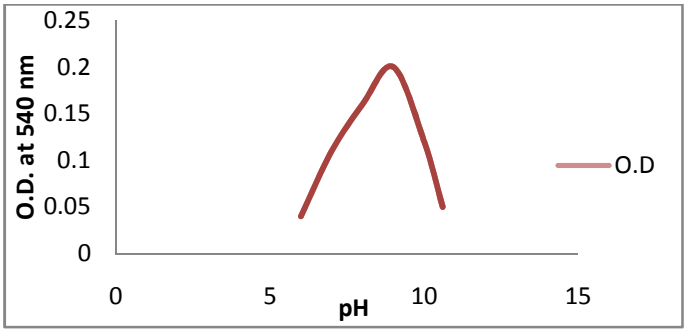


Fig. 4. Effect of pH on Soybean protease enzyme

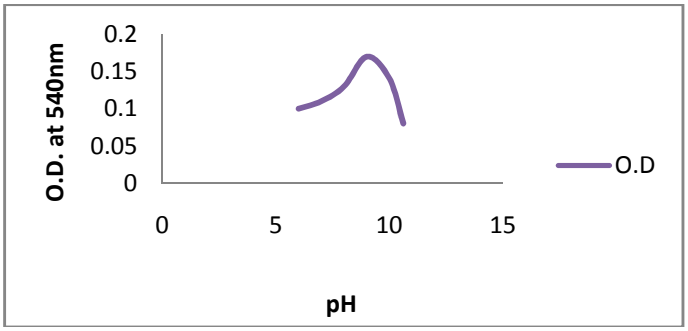


Fig. 5. Effect of pH on green gram protease enzyme

B) Effect of substrate conc. on protease enzyme:

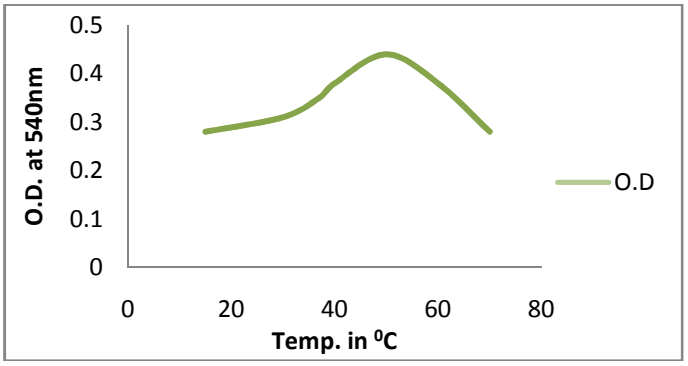


Fig. 6. Effect of temp. on Soybean protease enzyme

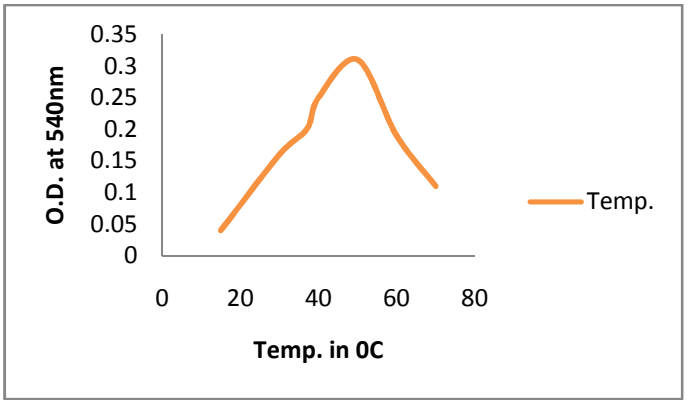


Fig. 7. Effect of temp. on Green gram protease enzyme.

Optimum P^H of Green gram & Soybean protease were found to be P^H 9

C)Effect of substrate conc. on protease enzyme:

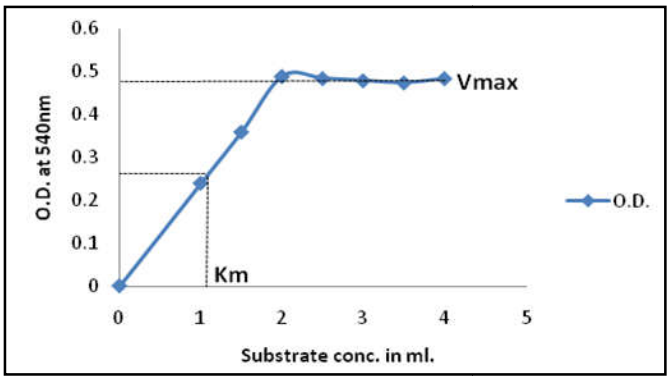


Fig. 8. Machaleis Menten graph of Soybean

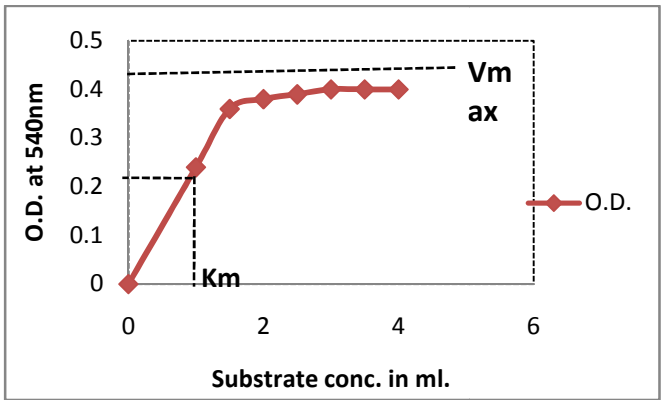


Fig. 9. Machaleis Menten graph for Green gram

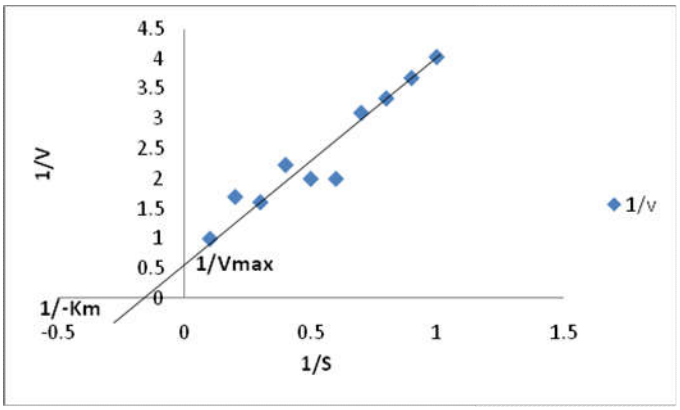


Fig. 10. Line Weaver Burk plot of Soybean

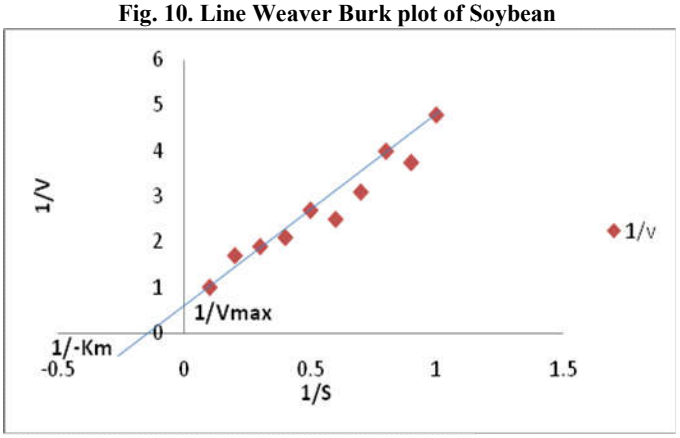


Fig. 11. Line Weaver Burk plot of Green gram

It was found that at 0.8ml conc. enzyme was fully saturated. For Soybean, from Michaleis Menten graph Vmax found to be 0.485 & Km was found to be 1.01µM. From Line Weaves Burk Plot Vmax is 2 & Km is 5.55µM. For Green gram, from Michaleis Menten graph Vmax found to be 0.39 & Km was found to be 0.8µM. From Line Weaves Burk Plot Vmax is 1.72 & Km is 5.26µM.

Characterization of Soyabean and Green gram protease enzyme:-

SDS-PAGE:

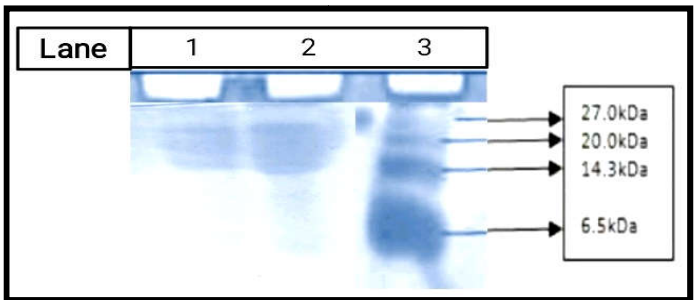


Fig. 12. Gel slab showing the bands of Soybean and Green gram Protease enzyme by SDS-PAGE method

The partially purified Protease enzyme was subjected to determination of molecular weight by using 12% SDS-PAGE and compare with standard protein marker. It shows that Molecular weight of Soybean proteases was found to be 26.0kD & Green gram proteases was found to be 19.0kD as shown in the above figure.

Applications of protease enzyme:

A)Casein protein degradation by protease enzyme:

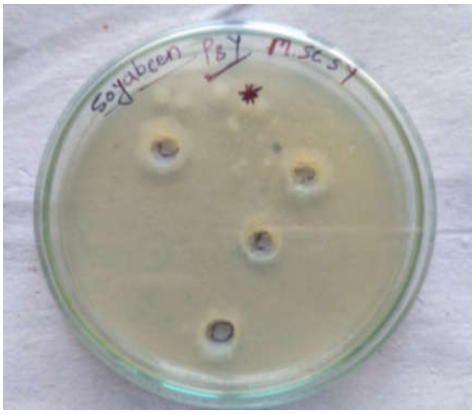


Fig.13. Casein protein degradation by Soybean protease enzyme

Fig. 14. Casein protein degradation by Green gram protease enzyme

Fig.15. Comparison between soybean and Green gram protease enzyme showing the enzyme activity for casein degradation

B) Gelatin protein degradation by protease enzyme:

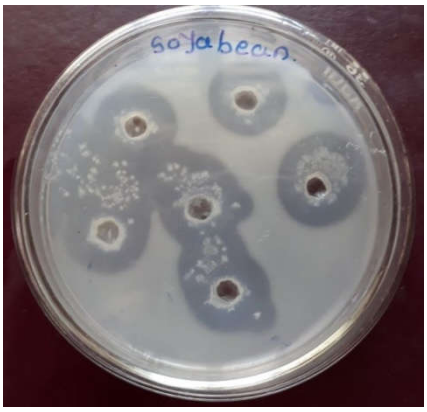


Fig. 16. Gelatin protein degradation by Soybean protease enzyme

Fig 17. Gelatin protein degradation by Green gram protease enzyme

Fig. 18. Comparison between soybean and Green gram protease enzyme showing the enzyme activity for gelatin degradation

C)Immobilization of protease enzyme:

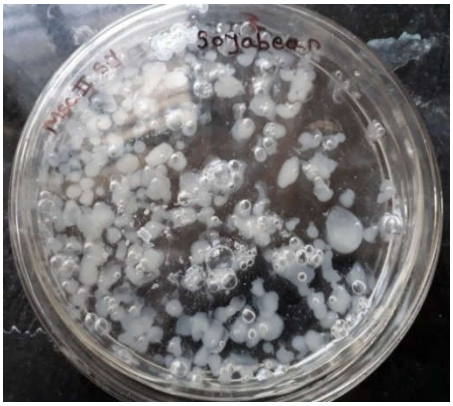


Fig.19. Immobilization of Soybean and Green gram protease enzyme by beads formation in $Cacl_2$ solution

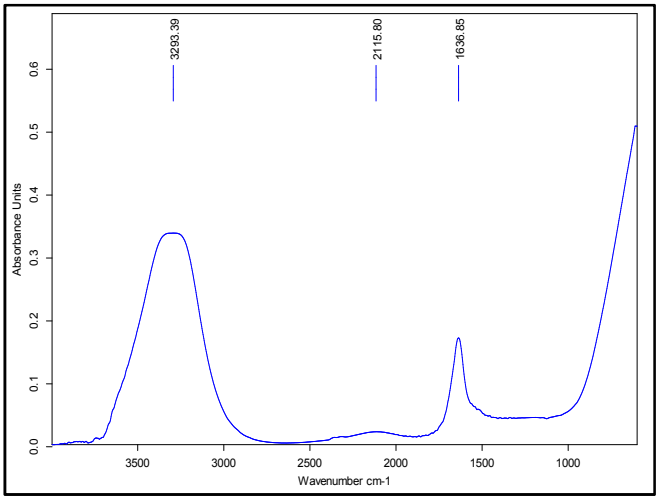
Dialysed proteases were immobilized in Sodium alginate gel by immobilization method which show good enzyme activity. Confirmation of immobilization of soybean and green gram protease enzymes by Folin-Lowry's assay method.

D)FT-IR Analysis:

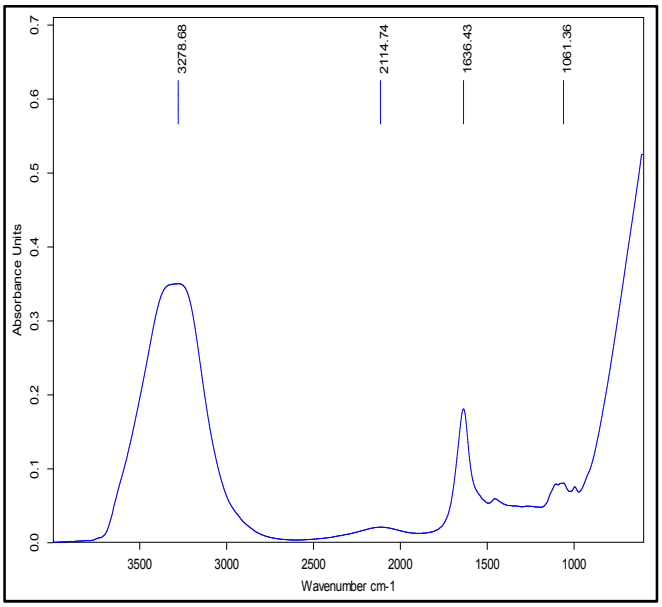
•Characterization of plant extracts and purified enzyme by FT-IR Analysis.

A)Soybean:

Sr. No.	Wave no/cm^2	Functional group
1.	3300	Alkyne
2.	2100	Alkyne
3.	1700	Carboxylic acid



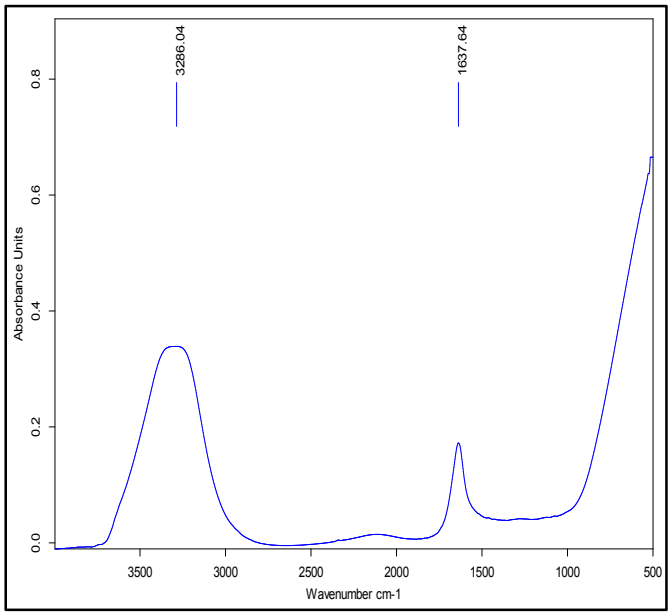
Sr. no.	Wave no/cm^2	Functional group
1.	3300	Alkyne
2.	2200	Alkyne
3.	1700	Carboxylic acid
4.	1450	Methyl group
5.	1300-1000	Alcohols, ethers, esters, Carboxylic acid, Anhydrides, Amines, Fluorides.

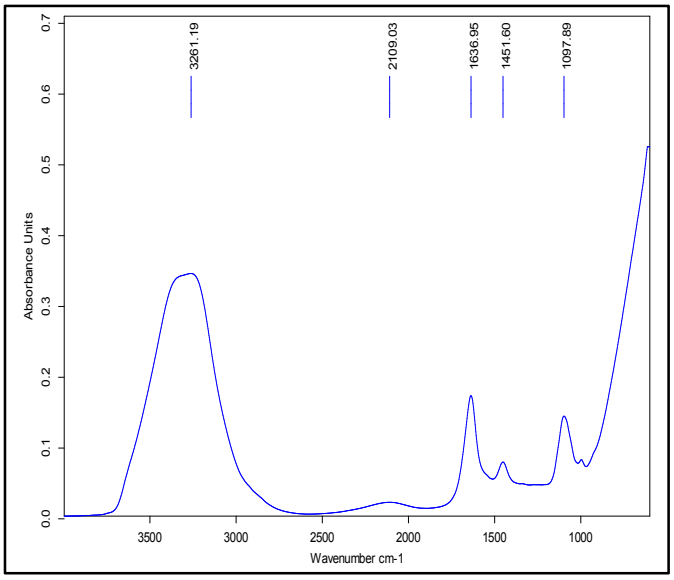


B) Green gram:

Sr. No.	Wave no/cm^2	Functional group
1.	3300	Alkyne
2.	2100	Alkyne
3.	1700	Carboxylic acid

Sr. No.	Wave no/cm^2	Functional group
1.	3300	Alkyne
2.	3200	H-bonded
3.	2100	Alkyne
4.	1700	Fluorides
5.	1330,1400	Amines
6.	1300	Alcohols, ethers, esters, -COOH, Anhydrides
7.	900	Aromatics (out of plane band)





The partially purified Protease enzyme was subjected to determination of molecular weight by using 12% SDS-PAGE and compare with standard protein marker. It shows that Molecular weight of Soybean proteases was found to be 26.0kD & Green gram proteases was found to be 19.0kD as shown in the above Figure. Dialysed proteases were immobilized in Sodium alginate gel by immobilization method which show good enzyme activity. Confirmation of immobilization of soybean and green gram protease enzymes by Folin-Lowry's assay method.

DISCUSSION

The present study investigated the proteases catalytic activity obtained from leguminous seeds by using easy assay system using two substrates Casein and Haemoglobin. The protein conc. ranged between 52µg/ml and 73µg/ml in which Soybean shows highest protein concentration. Dahot M.U. (1992 add another Ref.) investigated on proteases present in some plant seeds & found the ranged of protein conc. between 1.10-276 mg/ml with highest value in Soybean seed which is in a good agreement with our value reported for Soybean seeds. Proteases showed maximum catalytic activity at P^H 9 with substrate casein and haemoglobin which was found to be same reported by M. Akhtar-Uzzaman *et al.,* (2012) and Rahman *et al.,* (2007). This alkaline proteases may be play an important role in industrial food applications such as production of soy sauce, digestion of soybean protein, degradation of casein, gelatin & in leather industries & detergent industries (Kamini *et al.,* 2004) Nadafi & Deobagkal, 2005). The optimum temperature was found in our study was 50 ^{0}C but Evans et.al. 2011 reported optimum temp. 23^{0}C for other leguminous seeds. Kamini *et al.* 2004, and Akoi *et al.,* 2004 have shown that the protease activity with optimum temp. less than 20^{0}C is considered as a cold proteases. So the proteases from present study can be excellent source for industrial purposes.

Conclusion

In present study, we isolated the proteases from leguminous seeds and tried to make an easy assay for protease catalytic activity using casein and heamoglobin as a substrate. This study clearly expressed that all the proteases were edible because extracted from plant sources and showed higher

activities at 9 P^H and 50 ^0C temp. They have the potentiality in food industries.

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