OPTIMIZATION, ISOLATION AND PARTIAL CHARACTERIZATION OF PROTEASES FROM UNDERUTILIZED AND COMMON FOOD LEGUMES

Anupama V., Marimuthu M., Uma Sundaram, Gurumoorthi P*

Nutraceutical Lab, Department of Food Process Engineering, School of Bioengineering, SRM University, Kattankulathur, Tamil Nadu, India

*Corresponding Author Email: p_gurumoorthi@yahoo.com

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ABSTRACT

The present study aims at the isolation and partial characterization of proteases from six legume seeds, horse gram, jack bean, lima bean and velvet bean (white and black seeds). The extraction of these seeds was optimized using response surface methodology. The best buffer for extraction was seen to be 10 mM Tris HCl at pH 8 with 25 mg/ml-45 mg/ml protein release. The ideal extraction parameters exhibiting highest protease release (91.9112 U) were pH 4.86, 10-15 °C with 70 hours incubation time. The optimized extracts were subjected to $(\text{NH}_4)_2\text{SO}_4$ precipitation to obtain crude enzyme solution. The proteolytic activity of the precipitated enzyme was again checked. The effect of pH and temperature of protease activity in the crude enzyme solution were also determined. The pH profile of proteases showed proteolytic activity at pH 7.5 to 9.0. The paper concludes that leguminous seeds can be source of proteases for industrial purposes.

Keywords: *Macrotyloma uniflorum*, *Phaseolus lunatus*, *Canavalia ensiformis*, *Mucuna pruriens*, Protease.

INTRODUCTION

Proteases, a group of hydrolytic enzymes are mostly sourced from microorganisms and the fermentation processes they are involved in. Ashton, F.¹ and Ichishima² reported proteases which are widely distributed in plants, especially leguminous seeds and found that such proteases were mainly alkaline or neutral in nature with optimal pH of about 2-3. The activity of proteases in green gram has been studied and reported during germination^{3,4}. Such proteases play a very important role in the physiology of seed proteins, such as the formation of enzymes that eliminate or reduce the anti nutritional and indigestible components of leguminous seeds during germination⁵. Apart from this, such proteases if isolated may benefit many an industrial process. The protease enzymes are currently used to a great extent in the pharmaceutical, leather and various foods processing industries⁶. Dry legumes are an excellent source of protein, energy and other nutrients in developing countries and also contain varied amounts phytochemical compounds including antioxidants⁷. However their use is limited due to their high dietary bulk; presence of anti nutritional factors, mainly phytic acid in most of the legumes; low protein and carbohydrate digestibility⁸⁻¹¹. During germination of seeds, α -amylase and protease, that are developed, degrade starch granules and reserve proteins, respectively; thereby reducing the dietary bulk and improving the digestibility of starch and protein¹²⁻¹⁴. Germination was also shown to increase the monosaccharide and decrease the disaccharide contents of legumes due to α -amylase¹⁵. Hence, enhanced amylase and protease activities in germinated legumes may improve the nutritional quality, which makes them important in improvement of nutritive value of leguminous seeds. The malting capacity and enzymatic changes of different cereals have been studied in great deal with regard to their use, especially the amylases in germinating cereals in several cereal species^{16,17}. But a comprehensive data on key enzymes (phytase, α -amylase and protease) in legume seeds is scarce, so the present was planned with an objective to obtain more information on enzymatic activities in legume seeds. Thus, in this context the

current study aims at the isolation and partial characterization of a novel protease from underutilized tribal legumes, with a view of better utilization and commercialization of these as an alternative source of proteolytic enzymes.

MATERIALS AND METHODS

Material Collection

Natural strands of mature pods of *Macrotyloma uniflorum* (horse gram), *Phaseolus lunatus* (lima bean) and *Canavalia ensiformis* (jack bean) were purchased from local markets of Madurai, Tamil Nadu, India. *Mucuna pruriens* (L.) DC (velvet bean) black and white coat seeds were collected as mature pods from the different agro-climatic regions of Western Ghats, Tamil Nadu, India. After drying in the sun, the pods were thrashed to separate mature seeds. After thorough cleaning and removal of broken seeds and foreign materials, the seeds were stored in plastic containers at room temperature (25°C) until further use.

Isolation and preparation of crude protease enzyme

The initial step in the extraction process was germination of the seeds. The seeds were sterilized with 0.1% mercuric chloride and washed, were soaked in distilled water at 4°C for 12 h. The soaked seeds were allowed to germinate for 24, 48, 72, 96 and 120 h at 30°C. The seeds were moistened with distilled water at regular intervals of 12 h. The sprouts were rinsed with distilled water and freeze-dried¹⁸. The germinated freeze dried seeds were then ground in a Wiley Mill (Scientific Equipment Works, New Delhi, India) to 60-mesh size with acetone to eliminate fat content. The mixture thus obtained was filtered through double layer Whatmann No.1 filter papers. The homogenates were dried in a tray drier overnight at 32 °C. The dry homogenate, was further coarsely ground using mortar and pestle at 1- 2 °C and the powder obtained was suspended in three kinds of buffer solutions and the efficacy of each buffer was estimated by checking the protein release in each after an incubation of 24 h. The three buffers used were the phosphate buffer (10 mM pH 8), Tris HCl (10 mM, pH 8) and deionized water. The extracts

obtained were tested for protein content using method of Bradford¹⁹. From these, the buffer that showed maximum protease release was used in further processing. The mixture of the optimized buffer, homogenate powder was spiked with 2 M NaCl and incubated for 24 h at room temperature. This mixture was further filtered and centrifuged at 10000 rpm for 8 minutes below 4°C in a KUBOTA 6500 high speed refrigerated centrifuge. The supernatant was collected and further processed^{20,21}.

Optimization of Extraction Parameters

A full factorial design including all possible factor combinations in each factor is a powerful tool in understanding complex processes and to describe factor interactions in multifactor systems. Response Surface Methodology is an empirical statistical technique employed for multiple regression analysis by using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously²². The powder homogenate was suspended in 10 mM Tris HCl buffer with variations in pH, time of incubation and temperature of incubation.

The coded values of the process parameters were determined by the following equation.

$$X_i = \frac{X_i - X_0}{\Delta x} \quad (1)$$

Where x_i -coded value of the i^{th} variable, X_i -uncoded value of the i^{th} test variable and X_0 -uncoded value of the i^{th} test variable at center point.

The range and levels of individual variables are given in Table 1. The experimental design is given in Table 2, along with experimental data and predicted responses. The regression analysis was performed to estimate the response function as a second order polynomial.

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^k \beta_{ij} x_i x_j \quad (2)$$

Where Y is the predicted response, β_i , β_j , β_{ij} are coefficients estimated from regression. They represent the linear, quadratic and cross products of X_1 , X_2 , and X_3 on response.

A statistical program package Design Expert 8.0.7.1, was used for regression analysis of the data obtained and to estimate the coefficient of the regression equation. The equations were validated by the statistical tests called the ANOVA analysis. The significance of each term in the Equation is to estimate the goodness of fit in each case. Response surfaces were drawn to determine the individual and interactive effects of the test variable on the protease production.

Ammonium Sulphate precipitation

The optimized extracts were saturated with 50% solid $(\text{NH}_4)_2\text{SO}_4$ for overnight precipitation. After precipitation, they were centrifuged at 10000 rpm for 30 min below 4°C²³. The amount of salt required for effective precipitation was calculated by the Ammonium Sulphate Calculator from EnCor Biotechnology Pvt. Limited, Florida. The collected precipitated were dissolved in 10 mM Tris-HCl buffer (pH 8) and centrifuged at 5000 rpm for 10 min. The supernatant was

used as crude enzyme for the assay of specific activity of enzyme and characterization.

Water Soluble Protein measurement

Protein concentration was determined method described by Lowry²⁴, using bovine serum albumin (BSA) as standard protein. The amount of the soluble protein was calculated from the standard curve as mg of protein per ml of test samples.

Protease Activity Estimation

Protease activity in the optimized extracts was assayed using the method of Kuntz²⁵ using casein as a substrate. In this, a 1% (w/v) solution of casein was prepared in 0.5 M Carbonate Buffer, pH 9.5 to this; 1 ml of suitably diluted extract was added and incubated at 37 °C for 10 min. The reaction post incubation was terminated by adding 5% TCA solution and the mixture was incubated at room temperature for 10 minutes. The precipitate was filtered using Whatmann No. 1 filter paper and the absorbance of the filtrate was read at 280 nm using a CECIL CE-7200 UV-Visible Spectrophotometer. A standard curve of tyrosine activity upon casein was prepared and used to estimate the protease activity of the extracts. The enzyme is assayed in terms of its activity and the activity is expressed in "units".

$$1 \text{ U} = 1 \text{ mg/10min}$$

$$\text{Protease Activity (U)} = \frac{(\text{test-control}) \times 850 \times \text{dilution factor}}{0.488 \times \text{Volume of Enzyme}} \quad (3)$$

Effect of pH and temperature specific activity of proteases: Partial characterization

Optimum temperature and pH of protease activity were determined using various temperatures (10 °C, 37 °C and 70 °C) and pH (2, 7 and 14). This was done to establish the stability and to partially characterize the crude enzyme²⁶.

RESULTS AND DISCUSSION

Optimization and Selection of Buffer

Two buffers and de-ionized water were used to extract the homogenate powders in this study. The extraction was carried out in a ratio of 10:1 of buffer and powder respectively. The Bradford's assay was used to determine the protein released in each solution after incubation of 24 h at room temperature. The results are as seen in Figure 1. The highest protein release was seen in 10 mM Tris HCl at pH 8 (25 mg/ml-45 mg/ml) and thus, this was used in the further studies carried out.

Optimization of Extraction Parameters

To examine the combined effect of the different process parameters (independent variables), on the release of protease, a Box Benken design with 5 center points in Quadratic design mode was used. A total of 17 trials were performed according to the design generated. The extraction of horse gram, lima bean, jack bean and velvet bean (black and white) was carried out with reference to the mathematical model relating the protease production to the parameters using 2 factor interaction manual models. The equations for the same were as seen below.

$$\text{Horse gram Protease Activity} = 85.22052 + 1.48491 * \text{TIME} + 1.66965 * \text{TEMP} - 1.57266 * \text{pH} - 0.068486 * \text{TIME} * \text{TEMP} - 0.055796 * \text{TIME} * \text{pH} + 0.15347 * \text{TEMP} * \text{pH}$$

(5)

$$\text{Lima bean protease activity} = 85.70052 + 1.48491 * \text{TIME} + 1.66965 * \text{TEMP} - 1.57266 * \text{pH} - 0.068486 * \text{TIME} * \text{TEMP} - 0.055796 * \text{TIME} * \text{pH} + 0.15347 * \text{TEMP} * \text{pH}$$

(6)

$$\text{Jack bean protease activity} = 84.94052 + 1.48491 * \text{TIME} + 1.66965 * \text{TEMP} - 1.57266 * \text{pH} - 0.068486 * \text{TIME} * \text{TEMP} - 0.055796 * \text{TIME} * \text{pH} + 0.15347 * \text{TEMP} * \text{pH}$$

(7)

$$\text{Velvet bean (black) protease activity} = 85.81052 + 1.48491 * \text{TIME} + 1.66965 * \text{TEMP} - 1.57266 * \text{pH} - 0.068486 * \text{TIME} * \text{TEMP} - 0.055796 * \text{TIME} * \text{pH} + 0.15347 * \text{TEMP} * \text{pH}$$

(8)

$$\text{Velvet bean (White) protease activity} = 84.93052 + 1.48491 * \text{TIME} + 1.66965 * \text{TEMP} - 1.57266 * \text{pH} - 0.068486 * \text{TIME} * \text{TEMP} - 0.055796 * \text{TIME} * \text{pH} + 0.15347 * \text{TEMP} * \text{pH}$$

(9)

The Analysis of Variance for the above defined model in 2 factor interaction mode was performed and the results were as indicated in Table 3. According to the ANOVA results, the models for all five responses were significant. The Model F-value of 4.27 implied the model to be significant with only a 2.16% chance of such a value occurring due to noise. Model F-value was calculated as a ratio of mean square regression and mean square residual. The p value at 0.0216 lays further emphasis on the significance of the model. The most significant model terms in the current design were seen to be AB wherein A is the time of extraction, B the temperature and C the pH of extraction media. The predicted R^2 of 0.3340 is in practical agreement with the adjusted R^2 of 0.5505. Adequate precision is the ratio that measures the signal to noise ratio. A ratio greater than 4 is generally considered to be most desirable. The adequate precision ratio of the experimental model at 7.414 indicates an adequate signal and that this model can be used to navigate the design space adequately. The fit of the model was also expressed by the coefficient of regression R^2 , which was found to be 0.7191 indicating that almost 72% of the variability in the response could be explained by the model. This implies that the prediction of experimental data is quite satisfactory. To investigate the interactive effect of the factors on the release of protease, three-dimensional plots were drawn. Figure 2 represents the contour plot of the experimental model. Figure 2a shows the contour plot of the influence of time and temperature on the activity of protease in the extracts. The analysis for the generation of this response was performed, considering pH as a slice in the factorial of time vs. temperature space. From this graph, it was concluded that at higher limit of time and middle level of temperature (72 h, 10-20°C) the release of protease was heightened. As indicated from Figure 2b, a response with temperature as the variable factor or a slice in the time vs. pH space, the protease release is highest at higher time, temperature values and pH up to 7.4. Figure 2c shows the interaction of the protease release with respect to pH and temperature with time as the variable factor. From this graph, it is evident that a higher value of time increases the release of protease along with temperatures within 20°C and pH up to 7.4 as seen before also. In order to further emphasize the results seen in the contour plots, the 3D response surface plots were generated. The 3 D response plots are as seen in Figure 3. From Figure 3a, it can be deduced that the information from the contour plots is further emphasized. The plot had pH as variable factor and the best response was obtained at a pH of about 4.86. From Figure 3b, it can be concluded that the temperature that is most optimal for the release of protease is about 10.19°C this is also in

relative agreement with the contour plot. In Figure 3c, the indication is of ideal time for maximum protease release. This is observed at about 70 h which again lays emphasis on the results observed in the corresponding contour plot. Thus, with the agreement between the contour and 3D surface plots, we can identify the ideal points of highest protease release (91.9112 U) at 4.86pH, 10-15°C and 70 h incubation time.

Ammonium Sulphate Precipitation

The amount of solid ammonium sulphate added to obtain a 50% saturated solution was 15.26 g/ 50 ml of extract. The precipitation was carried out at 10°C and was incubated overnight, followed by the washing of the precipitate. The precipitate after washing was dissolved in 10 mM Tris-HCl buffer (pH 8) and then centrifuged again. The supernatant thus obtained was used as the crude enzyme.

The efficiency of the precipitation, was estimated by checking the protease activity in the extract and crude enzyme. The results (Table 4) of this showed the protease activity in the crude enzyme solution to be greater than that of the extract by around 0.5 in each seed.

Water Soluble Protein

The protein content in the crude enzyme solutions were analysed. It was seen that almost all the seeds showed a high concentration of proteins. Among the seeds under study here, horse gram was found to have highest protein content at 224 mg/ml. The results were as indicated in Figure 4.

Protease Activity Estimation

The amount of protease was measured and estimated using tyrosine as the standard. After the calculations as per eq. 3 were done, the results obtained were represented as a bar graph as shown in Figure 5. The highest protease release was observed in Jack bean (92.39 U).

Effect of pH and temperature on protease activity

The sensitivity of the crude enzyme solution, was tested to ascertain the stability of the crude enzyme isolate. The enzyme solution exhibited maximum stability at pH 2 and 7 when compared to pH 14 (Table 5). The enzyme solution was also stable at 37°C but started to show a marked reduction in activity at temperatures above 37°C (Table 6). A very similar result (pH stability at 8.4 and 9.2) was also reported by Evans *et al.*, 2011 for the extract of palm weevil (*Rhynchophorus palmarum*). The results from effect of pH indicate that the alkaline proteases involved in all the seeds were more potent than the acidic proteases. This alkaline protease may be playing an important role in industrial food applications such

as production of soy sauce, digestion of soy bean protein and in leather and detergent industries^{27,28}. Dahot²⁹ investigated on plant protease and found that the optimum temperature for enzyme reaction was 35°C, which was a good consistent with our results but Evans³⁰ reported optimum temperature for the palm weevil was 23°C on 0.03% casein. Kamini³¹ and Aoki³² have shown that the protease activity with optimum

temperature less than 20°C is considered as a cold protease. Therefore, the proteases isolated in the present study may be an excellent source of enzymes for industrial purposes that require low or mild temperatures as a vital factor in the processing steps.

Table 1: Extraction Parameters and limits used in design of optimization experiment

Parameter	Levels
pH	3.0 - 14.0
Time	24 - 72
Temperature	10.0 - 30.0

Table 2: Designed experiment with actual and predicted values

Run	Time (hour)	Temp. (°C)	pH	Protease Activity (U) *									
				Horse Gram		Lima Bean		Jack Bean		Velvet Bean (White)		Velvet Bean (Black)	
				Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	72	30	8.5	41.847	46.7138	42.327	47.193	41.567	46.433	42.437	47.3038	41.457	46.3238
2	72	20	14	50.128	48.8189	50.608	49.298	49.848	48.538	50.718	49.4089	49.738	48.4289
3	48	10	14	46.403	54.6928	46.883	55.172	46.123	54.412	46.993	55.2828	46.013	54.3028
4	24	10	8.5	57.019	60.613	57.499	61.093	56.739	60.333	57.609	61.203	56.629	60.223
5	48	30	3	67.483	66.2256	67.963	66.705	67.203	65.945	68.073	66.8156	67.093	65.8356
6	48	30	14	67.139	70.1108	67.619	70.590	66.859	69.830	67.729	70.7008	66.749	69.7208
7	48	20	8.5	74.764	68.9	75.244	69.38	74.484	68.62	75.354	69.49	74.374	68.51
8	48	20	8.5	76.754	68.9	77.234	69.38	76.474	68.62	77.344	69.49	76.364	68.51
9	24	20	3	75.375	74.251	75.855	74.731	75.095	73.971	75.965	74.841	74.985	73.861
10	48	10	3	80.511	84.5709	80.991	85.050	80.231	84.290	81.101	85.1609	80.125	84.1809
11	72	20	3	73.625	76.5455	74.105	77.025	73.345	76.265	74.215	77.1355	73.235	76.1555
12	24	30	8.5	80.711	89.6227	81.191	90.102	80.431	89.342	81.301	90.2127	80.321	89.2327
13	48	20	8.5	56.415	68.9	56.895	69.38	56.135	68.62	57.005	69.49	56.025	68.51
14	72	10	8.5	79.101	78.6506	79.581	79.130	78.821	78.370	79.691	79.2406	78.711	78.2606
15	24	20	14	81.339	75.9847	81.819	76.464	81.059	75.704	81.929	76.5747	80.949	75.5947
16	48	20	8.5	81.339	68.9	81.819	69.38	81.059	68.62	81.929	69.49	80.949	68.51
17	48	20	8.5	81.339	68.9	81.819	69.38	81.059	68.62	81.929	69.49	80.949	68.51

*1 U = 1mg/10min.

Table 3: ANOVA of experimental design in 2 FI model

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	Comments
Model	2081.98	6	346.996	4.26609	0.0216	significant
A-TIME	309.291	1	309.291	3.80253	0.0797	
B-TEMP.	4.28409	1	4.28409	0.05267	0.8231	
C-pH	337.816	1	337.816	4.15323	0.0689	
AB	928.615	1	928.615	11.4167	0.007	
AC	216.979	1	216.979	2.66762	0.1335	
BC	284.99	1	284.99	3.50377	0.0907	
Residual	813.382	10	81.3382			
Lack of Fit	388.359	6	64.7264	0.60916	0.7205	not significant
Pure Error	425.023	4	106.256			
Cor Total	2895.36	16				

Table 4: Influence of Ammonium Sulphate precipitation on protease activity in extracts

Seed	Extraction/Precipitation	Protease Activity (U) *
Horse Gram	Extract	93.932
	Precipitate	94.192
Lima Bean	Extract	93
	Precipitate	93.861
Jack Bean	Extract	98.5
	Precipitate	98.67
Velvet Bean (Black)	Extract	96
	Precipitate	96.8
Velvet Bean (White)	Extract	96.23
	Precipitate	97

*1 U = 1mg/10min.

Table 5: Effect of pH on protease activity in crude extract

pH	Seed Powder #	Protease Activity (U) *
2	<i>Phaseolus lunatus</i>	81
	<i>Canavalia ensiformis</i>	87
	<i>Macrotyloma uniflora</i>	80
	<i>Mucunna Puriens (White Seed)</i>	74
	<i>Mucunna Puriens (Black Seed)</i>	74
7	<i>Phaseolus lunatus</i>	83
	<i>Canavalia ensiformis</i>	96
	<i>Macrotyloma uniflora</i>	85
	<i>Mucunna Puriens (White Seed)</i>	76
	<i>Mucunna Puriens (Black Seed)</i>	77
14	<i>Phaseolus lunatus</i>	83
	<i>Canavalia ensiformis</i>	92
	<i>Macrotyloma uniflora</i>	83
	<i>Mucunna Puriens (White Seed)</i>	75
	<i>Mucunna Puriens (Black Seed)</i>	76

Macrotyloma uniflorum - horse gram, *Phaseolus lunatus* - lima bean, *Canavalia ensiformis* - jack bean, *Mucuna pruriens* - velvet bean black and white coat seeds, *1 U = 1mg/10min.

Table 6: Effect of temperature on protease activity in crude extract

Temperature	Seed Powder #	Protease Activity (U) *
10	<i>Phaseolus lunatus</i>	79
	<i>Canavalia ensiformis</i>	85
	<i>Macrotyloma uniflora</i>	78
	<i>Mucunna Puriens (White Seed)</i>	72
	<i>Mucunna Puriens (Black Seed)</i>	72
37	<i>Phaseolus lunatus</i>	81
	<i>Canavalia ensiformis</i>	94
	<i>Macrotyloma uniflora</i>	83
	<i>Mucunna Puriens (White Seed)</i>	74
	<i>Mucunna Puriens (Black Seed)</i>	75
70	<i>Phaseolus lunatus</i>	81
	<i>Canavalia ensiformis</i>	90
	<i>Macrotyloma uniflora</i>	81
	<i>Mucunna Puriens (White Seed)</i>	73
	<i>Mucunna Puriens (Black Seed)</i>	74

Macrotyloma uniflorum - horse gram, *Phaseolus lunatus* - lima bean, *Canavalia ensiformis* - jack bean, *Mucuna pruriens* - velvet bean black and white coat seeds, *1 U = 1mg/10min.

Figure 1: Protein release in buffer solutions and water

Figure 2: Contour surface plots of optimized extraction parameters. 2a Contour surface plots of influence of Temperature and Time on Protease Activity. 2b Contour surface plots of influence of pH and Time on Protease Activity. 2c Contour surface plots of influence of Temperature and pH on Protease Activity.

Figure 3: 3 D Surface plots of optimized extraction parameters. 3a Effect of temperature and time on protease activity. 3b Effect of pH and time on protease activity. 3c Effect of temperature and pH on protease activity.

Figure 4: Water soluble protein in crude enzyme solutions of various seeds

Figure 5: Estimation of protease activity in crude enzyme solutions

CONCLUSION

From the present study, we conclude that the leguminous seeds under study here are a good alternative to commercial sources of protease enzymes. The abundance of these seeds also plays a role in drastically cutting the cost of enzyme production. The isolation protocol demonstrated in the present study is a very simple, inexpensive method, further reducing production costs. The characterization of the isolated enzyme and their applications are under study. The same protocol may also be applied to examine other seeds as possible enzyme sources.

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REFERENCES

- Ashton F. Plant Endopeptidases, In plant Proteolytic Enzymes, Michael J Dalling, Ed., CRC Press, Florida 1976; 1(119): 140
- Ichishima E. J Ferm Assoc Japan 1964; 22: 393.
- Yaw Huei Lin and Wen Hsiang Yao. Green Gram *Vigna radiata* L. Wilczek contains some high proteolytic activities already before germination. Bot Bull Acad Sin 1996; 37: 1-7.
- Rahman M, Mahberbar L, Arjunmand Bau, Mashiar Rahman M and Fatema Shahjada U. Changes of the enzymes activity during germination of different green gram varieties. Bangladesh J Sci Ind Res 2007; 42(2): 213-216. <http://dx.doi.org/10.3329/bjsir.v42i2.474>
- Bau HH, Villanme C, Nicholas JP and Mejean L. Effect of germination on chemical composition, biochemical constituents and anti nutritional factors of soy bean *Glycine max* seeds. J Sci Food Agric 1997; 73: 1-9. [http://dx.doi.org/10.1002/\(SICI\)1097-0010\(199701\)73:1<1::AID-JSFA694>3.0.CO;2-B](http://dx.doi.org/10.1002/(SICI)1097-0010(199701)73:1<1::AID-JSFA694>3.0.CO;2-B) [http://dx.doi.org/10.1002/\(SICI\)1097-0010\(199701\)73:1<1::AID-JSFA694>3.3.CO;2-2](http://dx.doi.org/10.1002/(SICI)1097-0010(199701)73:1<1::AID-JSFA694>3.3.CO;2-2)
- Fox PF and Morrissey PA. Industrial and Clinical Enzymology. Editors: LJ Vitale, V Simon, Pergamon Press Oxford 1980; 61: 43.
- Uma Sundaram, Marimuthu M, Anupama V, Gurumoorthi P. Comparative antioxidant quality evaluation of underutilized /less common South Indian legumes. Int J Pharm Bio Sci 2013; 4(2): (B) 117-126.
- Sumathi, NG Malleshi and Rao SV. Elaboration of amylase activity and changes in past viscosity of some common Indian legumes during germination. Plant Foods Hum Nutr 1995; 47: 341-347. <http://dx.doi.org/10.1007/BF01088272> PMID:8577652
- Preet K and Punia D. Proximate composition, phytic acid, polyphenols and digestibility *in vitro* of four brown cowpea varieties. Int J Food Sci Nutr 2000; 51: 181-193.
- Mubarak AE. Nutritional composition and anti nutritional factors of mung bean seeds *Phaseolus aureus* as affected by some home traditional processes. Food Chem 2005; 89(4): 489-495. <http://dx.doi.org/10.1016/j.foodchem.2004.01.007>
- Negi P, Boora and Khetarpaul N. Starch and protein digestibility of newly released moth bean cultivars: Effect of soaking, dehulling, germination and pressure cooking. Nahrung 2001; 45(4): 251-254. [http://dx.doi.org/10.1002/1521-3803\(20010801\)45:4<251::AID-FOOD251>3.0.CO;2-V](http://dx.doi.org/10.1002/1521-3803(20010801)45:4<251::AID-FOOD251>3.0.CO;2-V)
- Lasekan OO. Effect of germination on α -amylase activities and rheological properties of sorghum *Sorghum bicolor* and acha *Digitaria exilis* grains. J Food Sci Tech 1996; 33(4): 329-331.
- Muntz K. Proteases and proteolytic cleavage of storage proteins in developing and germinating dicotyledonous seeds. J Exp Botany 1996; 47: 605-622. <http://dx.doi.org/10.1093/jxb/47.5.605>
- Kataria A, Chauhan BM and Punia D. Digestibility of proteins and starch *in vitro* of amphidiploids, black gram X mung bean as affected by domestic processing and cooking. Plant Foods Hum Nutr 1992; 42(2): 117-125. <http://dx.doi.org/10.1007/BF02196464> PMID:1374184
- Akinlosottu A and Akinyele IO. Effect of soaking, dehulling and fermentation on the oligosaccharides and nutrient content of cowpeas *Vigna unguiculata*. Food Chem 1991; 41: 43-53. [http://dx.doi.org/10.1016/0308-8146\(91\)90130-G](http://dx.doi.org/10.1016/0308-8146(91)90130-G)
- Malleshi NG, Daodu MA and Chandrasekar A. Development of weaning food formulations based on malting and roller drying of sorghum and cowpea. Int J Food Sci Tech 1989; 24: 511-519. <http://dx.doi.org/10.1111/j.1365-2621.1989.tb00674.x>
- Helland MH, Wicklund T and Narvhus JA. Effect of germination time on alpha-amylase production and viscosity of maize porridge. Food Res Int 2002; 35: 315-321. [http://dx.doi.org/10.1016/S0963-9969\(01\)00202-2](http://dx.doi.org/10.1016/S0963-9969(01)00202-2)
- Gurumoorthi P and Uma S. Heat-stable and heat-labile anti nutritional profile in *Mucuna pruriens* var utilis: Effected by germination. Intl Food Res J 2011; 18(4): 1421-1426.
- Marion M Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 76: 248-254.
- Dahot M Umar. Investigation of protease in plant seeds. J Islamic Acad Sci 1992; 5: 4, 241-244.
- Tapash Paul, Rubel Mozumdu NHH, Syed MA and Aktaruzzman M. Proximate composition, mineral contents and determination of protease from green gram (*Vigna radiata* L. Wilczek). Bangladesh Res Pub J 2011; 5: 207-213.
- Srimanta Ray. RSM: A statistical tool for process optimization; 2006. <http://www.indiantextilejournal.com/articles/FAdetails.asp?id=393-> Accessed on 15/01/2013.
- Akhtaruzzaman M, Rubel Mozumder NHM, Ripa Jamal, Atikur Rahman and Tanjina Rahman. Isolation and Characterization Protease Enzyme from Leguminous Seeds. Agri Sci Res J 2012; 2(8): 434-440.
- Ammonium sulphate calculator; EnCore Biotechnology Limited Gainesville, Florida; 2013. <http://www.encorbio.com/protocols/AM-SO4.htm> - Accessed on 15/01/2013.
- Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ. Protein Measurement with the Folin reagent. J Biol Chem 1951; 193: 265-275. PMID:14907713

26. Kunitz M. Crystalline soybean Trypsin Inhibitor, II. General properties. J Gen Physiol 1947; 30(4): 291-310. <http://dx.doi.org/10.1085/jgp.30.4.291> PMid:19873496 PMCID:PMC2142836
27. Alistair Rogers and Yves Gibon. Enzyme Kinetics: Theory and Practice in Schwender J. ed, Plant Metabolic Networks, Springer Science Business Media, LLC; 2009. p. 71-103.
28. Egwim Evans C. Partial characterization of protease activity from *Rhynchophorus palmarum* Palm Weevil. African J Food Sci Tech 2011; 2(6): 140-145.
29. Kamini NR, Hemachander C, Mala JGS, Puvanakrishnan R. Microbial enzyme technology as an alternative to conventional chemicals in leather industry. Financial Time Information Ltd Asia Intelligence Wire; 2004. PMid:14992693
30. Nadafi MF, Deobagkar D. Potential application of protease isolated from *Pseudomonas aeruginosa* PD100. Electron J Biotechnol 2005; 8(2): 122-125.
31. Aoki H, Nazmul HMd, Matsno K, Hagiwara T, Watabe S. Partial purification of protease that are generated by processing of the northern shrimp *Pandalus borealis* and which can tenderize beef. Int J Food Sc. Technol 2004; 39(5): 471-480.

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