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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 5(3): 317-320, 2002

Partial Purification and Characterization of Protease from Germinating Wheat Seeds (*Triticum aestivum* L.)

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Abstract: Enzyme with proteolytic activity was found in the germinating wheat seeds (*Triticum aestivum* L.,). The enzyme activity appeared after 40 h of dark germination and reached its maximum value after 72 h and then declined sharply during further germination. After ion exchange chromatography, three protein peaks (pro-I, pro-II & pro-III) showed proteolytic activity and the degree of purification attained nearly 29-42 fold. Properties like optimum pH and optimum temperature of pro-I, pro-II and pro-III were 7.1, 6.4, 6.8 and 45°C, 43°C and 42°C, respectively. Mg^{2+} and Mn^{2+} had no effect on enzyme activities while EDTA, urea, Ca^{2+} had an activating effect. On the other hand Hg^{2+} and Fe^{2+} had strong inhibitory action on the extracted enzyme. The enzymes were found to be homogeneous as judged by Polyacrylamide disc gel electrophoresis. Molecular weights of pro-I, pro-II and pro-III were 1.08, 1.04 and 99kD and Km values were found to be 0.027, 0.032 and 0.037 mM respectively.

Key words: Germination, wheat seeds, protease, Km value

Introduction

Seeds are the main source of protein in plant. Wheat is the most widely used of all cereals, owing to high nutritive value combined with the dough forming properties of gluten. The protein stored in seeds are used chiefly in the formation of protoplasm in new cells when seeds germinate (Harry, 1989). Protein content rather seeds size may be the main factor influencing seedling development (Lowe and Ries, 1972). The reserve proteins are stored in two separate sites of the cereal grain : the aleurone grains (bodies) of the aleurone layer, and the protein bodies (sometimes disrupted) of the endosperm. Protein of the aleurone layer are much in the basic amino acids. Proteolysis within the cells of the aleurone grain proteins appear to be important for the provision of amino acids from which hydrolytic enzymes are synthesized. High and increased activity during seed germination may also indicate a high probability of the participation of the protease in the degradation process. Some researchers investigate about the degrading enzymes which act on storage substance during germination (Shinamo and Fukushima 1968 & William, 1960). The modification of the 11S and 7S proteins in germinating seeds is caused mainly by limited proteolysis (Hashinaga *et al.*, 1983). Bangladesh is an agriculture dependent country. Agricultural commodities are used in food industries for the production of various kinds of food products. But presently, many good quality proteins are lost in food industries through wastage due to their less solubility and other poor functional properties.

Till now there is no detailed study about the degrading enzymes from germinating wheat seeds. The objective of the present work was to study the proteolytic enzyme from germinating Akbar and Khanchan varieties of wheat seeds. Further more, the proteolytic enzyme from Akbar variety only was purified and characterized.

Materials and Methods

Collection of seeds: Two varieties of wheat seeds (Akbar & Khanchan) were collected from Bangladesh Agricultural Research Institute (BARI) , Irshardi, Pabna. The seeds were cleaned, dried in the sunlight, sealed in a polythene bag and stored in a desiccator for experimental purpose.

Germination of wheat seeds: Good and mature seeds of wheat were soaked in distilled water within a glass beaker for six hours which were scattered on a wet filter paper and placed on a plastic tray containing little amount of distilled water. The tray was then covered by a glass lid and placed in the dark room at 25°C for 120 hours including soaking time. The germinating seeds at different hours (24, 48, 72, 96 and 120 h) were collected and stored separately in the deep freeze (-10°C) for further experimental purposes.

Measurement of proteolytic activity: The proteolytic activity was measured following the method of Kunitz (1947). The crude extracted enzyme from Akbar variety shows higher proteolytic activity than Kanchan variety. Only seeds of Akbar variety were used for further purification and characterization of the enzyme.

Preparation of crude enzyme extract: Firstly the germinated seeds were taken in a mortar and ground uniformly into fine powder. The powder was mixed uniformly with pre-cooled distilled water (4g/50ml) and homogenized uniformly. The extract was then transferred into a beaker and kept overnight at 4°C with occasional gentle stirring. The clear filtrate was collected by centrifuging at 7000xg for 15 min at 4°C. The clear supernatant was adjusted to 100% saturation by adding solid ammonium sulfate. The precipitate was dissolved in minimum volume of pre-cooled deionized water and dialyzed against 5 mM phosphate buffer (pH 8.0) for 24 h. After centrifugation, the clear supernatant was used as crude enzyme extract.

Gel filtration: The crude extract after dialysis with 5 mM phosphate buffer (pH 8.0) was loaded on Sephadex G-75 gel column which was equilibrated with the same buffer. The column was washed with 5 mM phosphate buffer, pH 8.0, containing 1M sodium chloride. The flow rate was 30 ml/h.

DEAE cellulose chromatography: The enzymatically active protein fractions after gel filtration were collected and dialyzed against 10 mM Tris-HCl buffer (pH 8.4) for overnight and then concentrated to its $1/4^{th}$ volume and loaded into DEAE- cellulose column. The column was washed with 10 mM Tris-HCl buffer (pH 8.0) and then elution was done with sodium gradient (0-0.5 M) containing the same buffer.

Characterization of the protease's:

Determination of optimum pH: The activity of the protease enzyme was measured at different pH values (4.0-10.0) using phosphate buffer at 45°C.

Determination of optimum temperature: The activity of the protease enzyme was measured by the method of Kunitz (1947) at different temperatures using phosphate buffer, pH 8.0.

Determination of protein concentration: Protein concentration of each fraction was determined by UV-visible spectrophotometer at 280 nm. The amount of protein was determined by the method Lowry *et al.* (1951) .

Electrophoresis: Protein containing fractions were analyzed by

sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Orstein (1964).

Molecular weight determination: The molecular weight of the protease were determined by gel filtration on a Sephadex G-75 column (90x0.9 cm^2). Egg albumin (MW 45,000), bovine serum albumin (MW 66,000), galactosidase (MW 116,000) and β–amylase (MW 200,000) were used as marker proteins (Weber and Osborn, 1966).

Determination of Km value: The initial velocity is equal to the amount of product formed per unit time. The initial velocity (Vi) was determined by measuring quantitatively the amount of one of the products at various times (Robyt and White, 1990).

Chemicals: Glucose, magnesium chloride, casein and SDS were purchased from Sigma Chemicals Ltd., USA. DEAE-cellulose was purchased from Pharmacia Chemicals Ltd., Sweden. All other chemicals used for this research purpose were of analytical grade.

Results

Time course study of protease from germinating seeds: The protease from two varieties of germinating wheat seeds showed their maximum activity after 72 h of germination and then declined rapidly (Fig. 1). The activity of Akbar variety of protease was higher than Kanchan variety. So, in further studies, we used the extracts of Akbar variety of wheat seeds germinated at 72 hours.

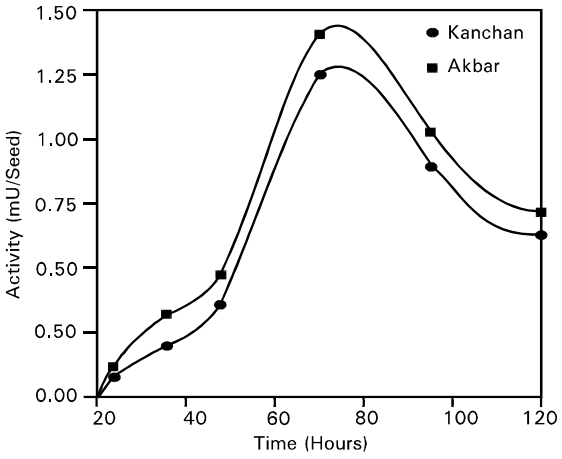

Fig. 1: Time course of two varieties of wheat protease activity during germination (Crude extract)

Gel filtration: The enzyme was eluted as two main peaks namely F-1 (11-40) and F-2 (41-60) (Fig. 2). It was found that only F-1 fraction contained the proteolytic activity while the F-2 peak showed no activity. The F-1 fraction containing the proteolytic activity was pooled, concentrated and further purified by DEAE-cellulose column chromatography.

DEAE-cellulose column chromatography: The components of F-1 fraction were separated into four peaks : pro-I, fraction no. (5-32), pro-II, fraction no. (33-54), and pro-III, fraction no. (55-66) containing proteolytic activity while the peak pro-IV, fraction no. (67-81) possessed no proteolytic activity. All fractions having proteolytic activity were collected separately, dialyzed against 10 mM Tris-HCl buffer (pH 8.0) for overnight and then concentrated. Table 1 summarizes the purification of proteolytic enzymes from germinating wheat seeds (Akbar variety). The specific activities of the extracted enzyme increased at each purification step and the purification fold was nearly 30-40 (Fig. 3).

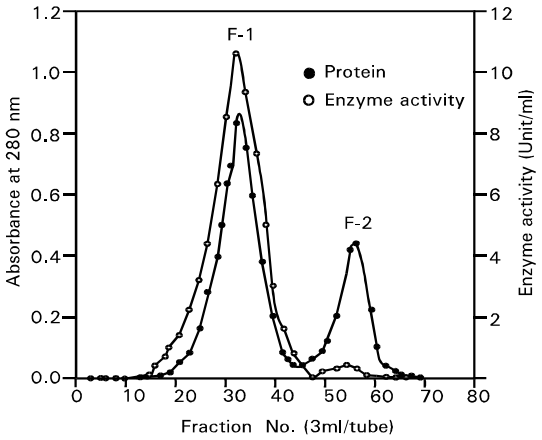

Fig. 2: Gel filtration pattern of ammonium sulfate saturated crude extract of Akbar variety on Sephadex G-75 column.

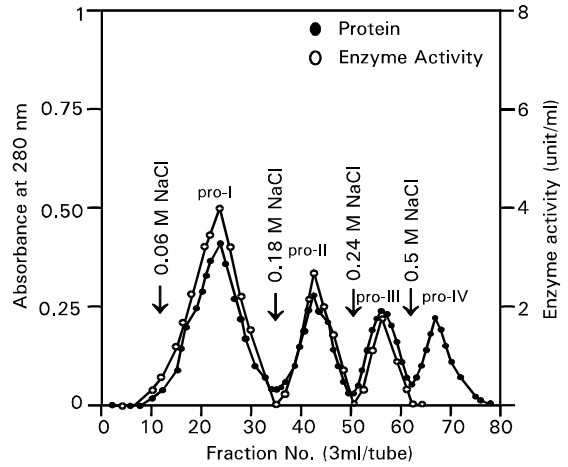

Fig. 3: DEAE-cellulose chromatography of wheat protease (Akbar variety) from F-1 fraction.

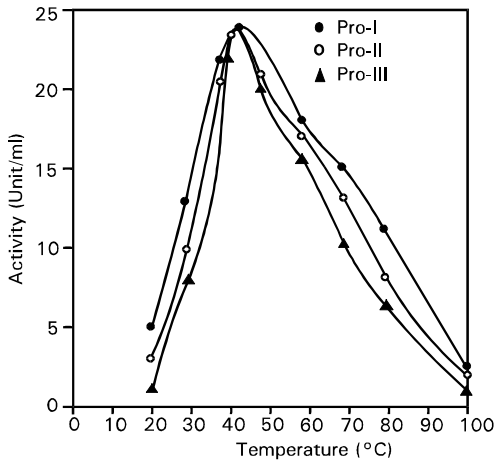

Fig. 4: Effect of temperature on the protease activities from germinating wheat seeds.

Determination of optimum temperature: The effect of temperature on the proteolytic were examined in the range of 10-

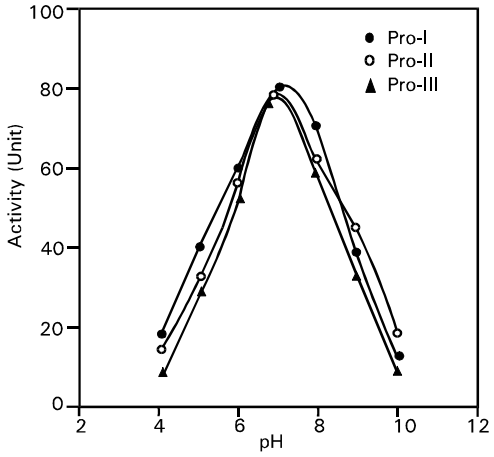

Fig. 5: Effect of pH on the protease activities from germinating wheat seeds.

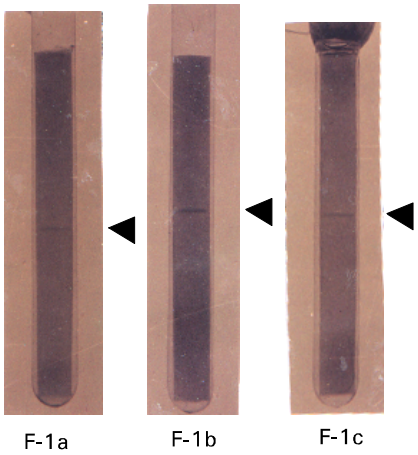

Fig. 6: Polyacrylamide disc gel electrophoresis of germinating wheat seeds protease from Akbar variety

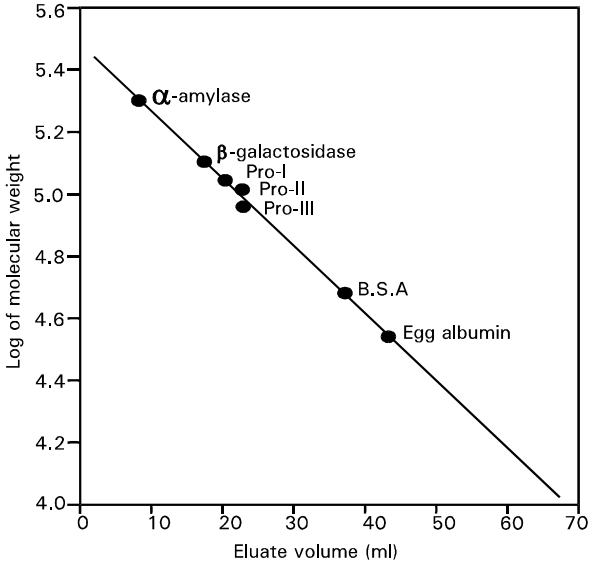

Fig. 7: Standard curve for the determination of molecular weight of protease enzyme by gel filtration methods.

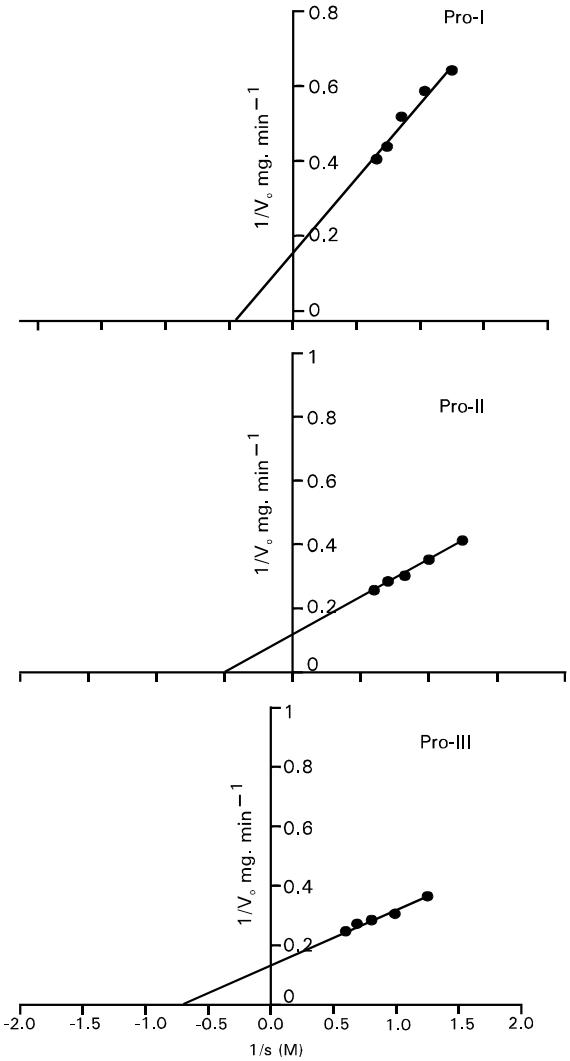

Fig. 8: Lineweaver-Burk double reciprocal plots for the determination of Km value of protease enzyme.

100°C. The activities of the purified enzyme increased gradually up to 30°C and then increased remarkably with the maximum activities at 45, 43 and 42°C for pro-I, pro-II and pro-III, respectively (Fig. 4). The activities began to decrease gradually with the rise in temperature at 60°C. Very little activities were found at or above 90°C and at or below 10°C.

Determination of optimum pH: The activities of the protease's were greatly influenced by pH changes. The enzymes pro-I, pro-II, and pro-III gave maximum activities in the ranges of pH 7.1, 6.9, and 6.8, respectively (Fig. 5). From the results it might be concluded that enzymes pro-I and pro-II isolated from germinating wheat seeds belong to the category of slightly acid protease's while pro-III was slightly alkaline proteolytic enzyme.

Homogeneity test: The homogeneity of the enzymes were judged by Polyacrylamide disc gel electrophoresis. All the fractions gave single band on the gel indicating that they contained pure enzyme (Fig. 6).

Molecular weight determination: The molecular weight of the enzyme were determined by comparing their elution volume on Sephadex G-75, with those of the marker proteins under same

Table 1: Purification summary of germinating wheat seeds protease (*Akbar*) variety

Steps of purification		Total protein (mg)	Total activity (Units)	Specific activity (Unit/mg)	Yield (%)	Purification folds
Crude extract		460	1015	2.21	100	1
Ammonium sulphate		370	890	2.41	87.68	1.09
After gel filtration		4.12	122.57	29.07	12.08	13.46
DEAE	pro-I	0.87	76.34	87.75	7.52	39.71
Cellulose	pro-II	0.66	52.71	79.86	5.19	36.14
fraction	pro-III	0.45	30	66.66	2.95	30.16

Table 2: Effect of EDTA on the activities of proteolytic enzymes

Concentration of EDTA (molar)	Relative activity (%) pro-I	Relative activity (%) pro-II	Relative activity (%) pro-III
0.000	100	100	100
0.001	99	98	96
0.002	104	102	99
0.005	109	108	106
0.010	112	109	108
0.020	116	113	110

Table 3: Effect of calcium on the proteolytic activities

Concentration of EDTA (molar)	Relative activity (%) pro-I	Relative activity (%) pro-II	Relative activity (%) pro-III
0.000	100	100	100
0.001	101	100	100
0.002	104	102	102
0.005	107	104	103
0.010	109	108	107
0.020	113	111	108
0.050	116	114	110
0.100	120	117	115
0.500	127	122	119

experimental conditions. The molecular weights were calculated from the standard curve of the reference proteins, constructed by plotting log of molecular weight against elution volume on gel filtration. The molecular weights were found to be 108000, 104000 and 99000 for pro-I, pro-II and pro-III respectively (Fig. 7).

Effect of EDTA on the enzyme activities: EDTA, a metal chelating agent had an activating effect on the extracted enzyme from germinating wheat seeds (Table 2). Hence the enzymes present in the germinating wheat seeds homogenate shared a characteristic indicative of cysteine type proteolytic enzyme, specifically the enhancement of activity by EDTA.

Effect of calcium on the proteolytic activities: The effect of calcium as metallic salt on the activities of enzymes, purified from germinating wheat seeds are presented in Table 3. The activity of pro-I, pro-II and pro-III were gradually increased with the increase in concentration of calcium.

Determination of Km values of enzyme: The activities of purified enzyme were analyzed using different concentrations of casein as substrate. The Km values for pro-I, pro-II and pro-III were estimated to be 0.027, 0.032 and 0.032mM respectively (Fig. 8).

Discussion

Proteolytic enzymes pro-I, pro-II and pro-III were purified from germinating wheat seeds. They have molecular weights of about 108000, 104000 and 99000 respectively. The enzymes pro-I, pro-II and pro-III showed maximum activities in the pH ranges of 7.1, 6.9 and 6.8 and at temperatures 45, 43 and 42 °C respectively. The optimum pH of protease around 6.3-7.5 were also reported by Keay *et al.* (1970), which is nearly similar to pH value obtained from our investigated enzymes. Metallic ions like Mg^{2+} and Mn^{2+} had no effects on proteolytic activities while EDTA, urea and Ca^{2+} had an activator effect. Hg^{2+} and Fe^{2+} had strong inhibitory action on proteolytic activities. Ahmed *et al.* (1990), also reported that protease from the larval gut of *Spodoptera litura,* which were not inhibited by Mg^{2+} and Mn^{2+} while the presence of Hg^{2+} and Fe^{2+} completely inhibited the activities of three enzymes. The Km values of the enzymes pro-I, pro-II and pro-III were found to be 0.027, 0.032 and 0.037mM for casein as substrate respectively. The Km value of protease from tomato juice have been reported to be 0.055 mM reported by Islam (1994). Protease's, extract from microorganism are now used in the food industries to improve the food quality which are very expensive. Our laboratory reported the existence of proteolytic enzyme in the germinating wheat seeds. So, purified proteolytic enzyme from germinating wheat seeds might open new possibilities by changing the functional properties of food proteins. Now further research work is going on in our laboratory regarding this field.

Acknowledgment

This work was done in the Department of Biochemistry and Molecular Biology, Rajshahi University, Bangladesh in the session 1999-2000 and was supported by the Grant-in-Aid (Grant-1-99-335) for Science and Technology, Bangladesh.

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