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CHARACTERIZATION OF CRUDE ALKALINE PROTEASE OF SOYBEAN (GLYCINE MAX) SEEDS

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Abstract

The present work has been carried out to isolate protease from the crude extract of *Glycine max* seeds. The activity of *Glycine max* seeds protease was found optimum at pH 9.0 and at temperature 30°C. The activity of crude *Glycine max* seed protease was strongly enhanced in the presence of $CaCl_2$ and $MnCl_2$ while inhibited by cysteine and mercaptoethanol. The enzyme was found thermostable.

Keywords: Protease activity, *Glycine max* seeds, effects of $CaCl_2$ and $MnCl_2$.

1. Introduction

Proteases are the leading class of enzymes, which have importance both in commercial and physiological fields. Proteases are degradative enzymes, which catalyze the total hydrolysis of proteins. Proteases are the oldest enzymes which have been used in the food and detergent industries. Proteases have been used for centuries in the manufacturing process for soy sauce, tamari sauce and miso (Tatsumi, 1988). Now a days proteases are extensively used in numerous fields including protein engineering (Durrschmidt, 2001), food processing industry, leather industry, silk industry, detergent industry (Joo, 2005) and waste processing industry (Pastor, 2001). Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes (Mala, 1998). The protease activities of seeds have been investigated as a storage protein of the seeds. Many proteases have been isolated, purified and characterized from different plant seeds such as Indian bean seeds (Vadde, 2005), Kidney beans (Vitalyi, 1997), Vetch seeds (Becker *et. al*., 1995), Rice seeds (Asakura and Abe, 1995) and Cocoa seeds (Biehl *et. al.*, 1993). The vast diversity of proteases, in contrast to the specificity of their action, hence attracted worldwide attention to explore low cost sources. As a matter of fact, a country like Pakistan marked by an agro-based economy can be an ideal place for the isolation of industrially important enzymes from plant sources, which are frequently available and low cost. In this regards in present study, some characteristic properties of crude soybean seeds protease were investigated.

2. Material and Methods

Material:

The soybean seeds were purchased from local market in dry state. All reagents were of analytical grade and were used without further purification.

Methods:

Enzyme powder preparation: After removing seed coats, the seeds were crushed in Pestle and mortar and defatted with Diethyl ether. The defatted sample was homogenized in chilled acetone. The suspension was filtered through Whatman No.1 filter paper through suction pump and residue was separated on filter paper and allowed to dry at 20°C.

Preparation of soluble enzyme: The crude enzyme solution was prepared by accurately weighing 10g of acetone dried residue (crude enzyme) and crushed to fine powder using pestle and mortar and dissolved in 30 ml ice cold 0.2M Tris-HCl buffer pH 8.25. The solution was

centrifuged at 7000 rmp in Kubota refrigerated centrifuged at 4°C for 15 minutes and the supernatant was transferred to a 100 ml volumetric flask. This procedure was repeated twice and total volume was made up to 100 ml. Protease activity was determined from this solution.

Substrate preparation: 2% solution of Casein acid hydrolyzed (Sigma chemicals) was prepared in 0.2 M Tris-HCl buffer, pH 8.25.

Determination of protein: Protein content from the crude extract was determined by the method of (Lowry *et. al.,* 1951) using bovine serum albumin as standard.

Determination of Protease activity: Protease activity was determined by the method of Anson (1938) with slight modification. One ml of enzyme solution was incubated with 1.0ml 2% casein acid hydrolyzed (Sigma Chemicals) in 0.2 M Tris-HCl buffer (pH 8.50) at 35°C for 30 minutes. The enzyme reaction was stopped by the addition of 2.0 ml 15% trichloroacetic acid, which precipitated the residual protein. The contents of the tubes were centrifuged after 30 minutes at 6000 rpm for 10 minutes. In 1.0 ml of clear supernatant, 4.0 ml of 0.5 N sodium hydroxide and 1.0 ml Folin phenol reagent (diluted 1:1 v/v in water) were added. The total volume was then made up to 10.0 ml by adding 4.0 ml double distilled water. A blue colour developed was determined after 5minutes at 625 nm.

The quantity of enzyme that released 1ug of tyrosine under the standard assay conditions called one unit of protease activity.

3. Results and Discussion

The different parts of plants contain different protease while seeds serve as a preferred source of these enzymes. To ensure whether these enzymes are suitable for commercial level, it is important to determine some parameters such as pH, temperature, substrate concentration, activators and inhibitors, which effect on enzyme catalyzed reaction.

It was observed that the activity of the enzyme increased with the increase of time of incubation upto 60 minutes and then decreased **(Fig.1)**. This declination in activity after 60 minutes reaction time may be suggested to the presence of other proteolytic enzymes in crude sample of soybean seeds (Dahot, 1993). The decline in activity after 60minutes could not be ruled out due to self-digestion or product inhibition (Weil, 1966).

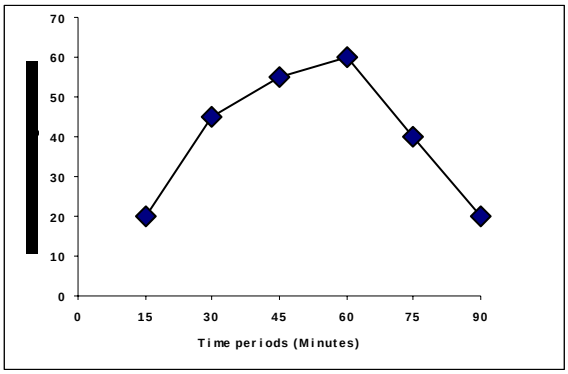


Fig. 1: Effect of time period on protease activity of Soybean seeds

Generally the rate of enzymatic reaction increased linearly with the increase in the substrate concentration until an optimum point is appeared where the concentration of the substrate saturated with enzyme present in reaction mixture. The hydrolytic activity of protease was investigated and results are represented in **(Fig.2)**.

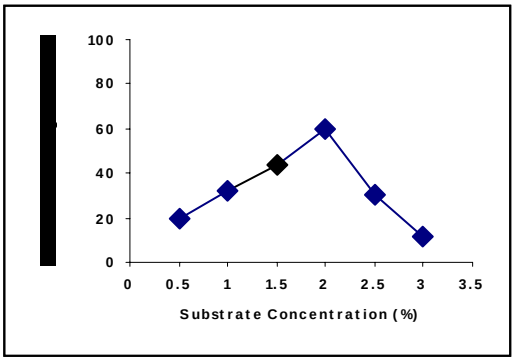


Fig. 2: Effect of substrate concentration on soybean seed protease

when various concentrations of substrate (casein acid hydrolyzed) ranging from 0.5 to 3 % with respect to its optimum time. It was observed that the rate of reaction declined after 2% substrate concentration. The declination in the rate of reaction after optimum concentration could be due to the alteration of enzyme substrate concentration ratio. The activity of protease increases with the increase of substrate concentration also reported by (Hiroshi *et. al.,* 2000) using 22-kDa protein as

a substrate obtained from Osmunda Japonica, (Yong and Linda, 2000) using Leu-Gly as substrate and (Isabelle *et. al.,* 1996) using Glutenins and Gliadins as substrates .

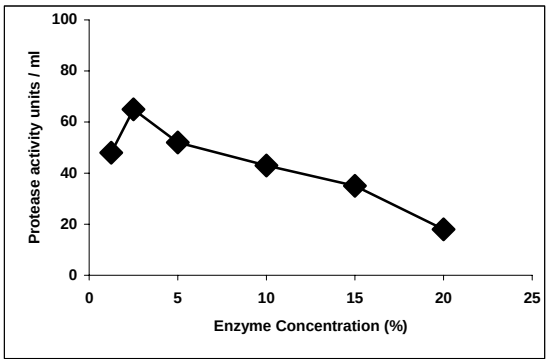


Fig.3. Effect of enzyme concentration on Soybean seed protease

(Fig.3) shows the effect of various enzyme concentrations (2.5-20 %) on protease activity of soybean seeds and observed that the activity increased with the increase of enzyme concentration up to 10 % and then decreased. The maximum reaction velocity at 10 % enzyme concentration is due to the utilization of total amount of substrate in enzyme-substrate complex (Sandra and Pant, 2005). In subsequent experiments 10% enzyme concentration (10 % crude enzyme solution of soybean seeds) and 2 % casein acid hydrolyzed was used as substrate.

The hydrolysis of casein was measured in a series of universal (Britton and Robinson type pH 2.69-12.0.) buffer in an attempt to cover the range of pH 3.0 to 11.0. Protease activity was found to be most active at the lower side of the alkaline pH. The enzyme has an optimum pH 9.0

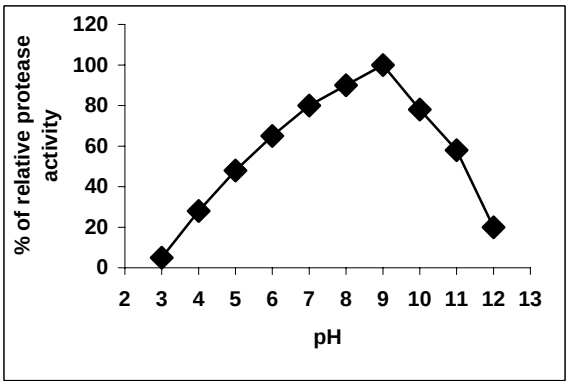


Fig. 4. Effect of pH on soybean seed protease activity

as shown in **(Fig. 4)**. (Gupta *et. al.,* 2002) have reported that alkaline proteases are the leading detergent enzymes. A large number of researchers have isolated alkaline proteases from different parts of plants such as tomato (Jian, 2005), alga *chara corallin* (Yuji and Ladislav, 2004) wheat leaves (Irma, 2003), roots of sweet potato (Tzeng *et. al*., 2004), fruits of *Bromelia hieronymi* Mez (Mariela *et. al*., 2006) and pea plant (Iwona *et .al*. 1996).

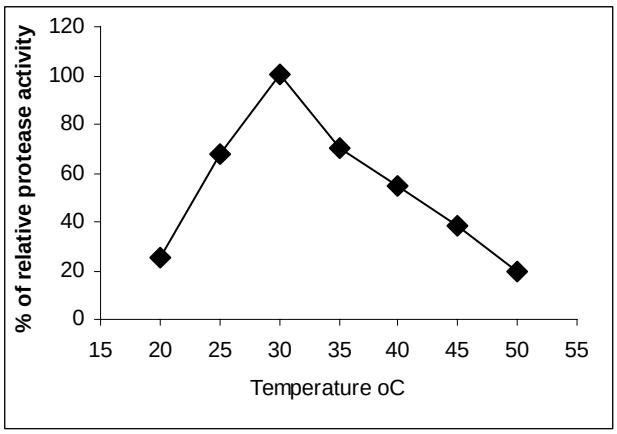


Fig. 5. Effect of temperature on soybean seed protease activity

The effect of temperature on protease enzyme activity was observed and the results are shown in **(Fig.5)** the temperature profile indicates that the optimum temperature of action was found to be 30°C. An initial increase in temperature up to 30^{o}C increased the rate of the enzyme-catalyzed reaction due to the increase in number of collision between the reacting molecules, but the enzyme probably got denatured on exposure to higher temperature than its optimum temperature. This declination of activity after optimum temperature may also be due to the alteration of the structure of enzyme (Acevedo *et. al*., 2002). Optimal temperature result (30^{o}C) of soybean protease is in agreement with that of the protease isolated from *Cajanus cajan* seeds (Dahot, 1992) and from Durum wheat (Isabelle *et. al.,* 1996). It has been reported that most plant proteases have optimum temperatures above 30^{o}C (Ogbonna and Okolo 2005; Senarath *et. al*., 2004; John *et. al*., 2003) but soybean seeds protease optimally active at temperature 30^{o}C. The effects of some reagents and metals (5m) on soybean seeds protease activity are summarized in (**Table-1)**.

Table–1. Effect of different reagents on Soybean seed protease activity

Reagents 5mM Conc.	Activity units/ ml	% of relative activity	% of activation/ [inhibition]
Tween 80	25	41.66	[56.34]
Triton X-100	14	23.33	[76.67]
SDC	28	46.66	[53.34]
SDS	26	43.33	[56.67]
$AgNO_3$	46	76.66	[23.34]
$HgNO_3$	53	88.33	[11.67]
Mercapto-ethanol	8	13.33	[86.67]
Cysteine	10	16.66	[83.34]
O-phena-nthroline	35	58.33	[41.67]
EDTA	38	63.33	[36.67]
$CaCl_2$	82	136.66	36.66
$MnCl_2$	98	163.33	63.33
$CoCl_2$	58	96.66	[3.34]
$ZnCl_2$	55	91.66	[8.34]
Control	**60**	**100**	**-**

It was noted that the activity of soybean seeds protease was enhanced 58% with $CaCl_2$ and 70% with $MnCl_2$. On the other hand $AgNO_3$ and $HgNO_3$ slightly accelerate the enzyme activity. Where as detergents SDS, SDC, Tritron-X-100, Tween-80, EDTA, O-phenonthroleine, cysteine and mercaptoethanol retarded the enzyme activity. The inhibition effect of EDTA and O-phenonthroleine and the stimulation effect of divalent cation on the protease activity were reported by (Becker *et al.,*1995). The increase of activity by Mn^{2+} was also observed by (Wellingta and Meire, 2004) and (Rahman *et. al.,* 1994) and Ca^{+2} by (Rahman *et. al.,*1994 and Gianna *et. al.,* 2001). (Schaller, 2004) has reported that the metallo proteases depend on divalent cations, most commonly zinc and manganese. Several researchers have isolated and characterized the metallo protease from different plant sources such as Arabidopsis, (Junping *et .al.,* 2006), Tomato (Hauser *et. al.,* 2001), Sorghum malt (Ogbonna and Okolo, 2005), Potato tuber (Stahl *et .al.,* 2002), Arabidopsis thaliana (Yu *et. al.,* 2004) and Pea seeds (Distefano *et. al.,*1997).

On the other hand protease activity significantly inactivated in the presence of cysteine, mercaptoethanol, EDTA and O-phenonthroline. EDTA removes the metal from the vicinity of the active site of the enzyme by chelation. The inhibitory effect of EDTA and O-phenonthroline supports the assumption that alkaline protease of soybean seeds belongs to a metallo type enzyme (Hampson *et. al.,* 1963; Morihara and Tsuzuki, 1970 and Manabe *et. al.,* 1985). Studies of thermal stability of purified soybean seeds protease activity was performed at pH 9.0 by heating ambient volume of enzyme solution at different temperatures ranging from 30 to 100°C for 10 minutes with and without addition of activators. The remaining activities were examined under standard assay conditions as shown in **(Fig.-6)**.

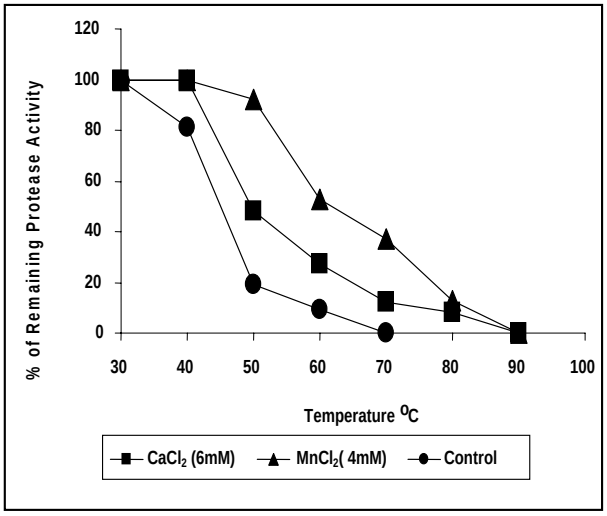


Fig. 6. Thermostability of soybean seed protease activity with and without activator

The protease activity of soybean seeds was retained about 20% at 50°C. It has noted that at 50°C, 48% and 92 % protease activity was present by the addition of $CaCl_2$ (6mM) and $MnCl_2$ (4mM) respectively. These results suggested that the soybean seeds protease is a thermostable protease. (Tzeng *et. al.,* 2004) have isolated a protease from roots of sweet potato, which was stable upto 40°C while (Kenneth *et. al.,* 2003) have isolated a thermostable protease from *Arabidopsis thaliana*, which was stable above 60°C and (Yonezawa *et. al.,* 1998) have characterized a thermostable *Asparagus* protease, which was fairly stable up to 50°C.

In order to obtain information about the primary specificity of alkaline protease, soybean seed was tested with different substrates prepared in universal buffer pH 7.0 and results are presented in **(Fig.7).**

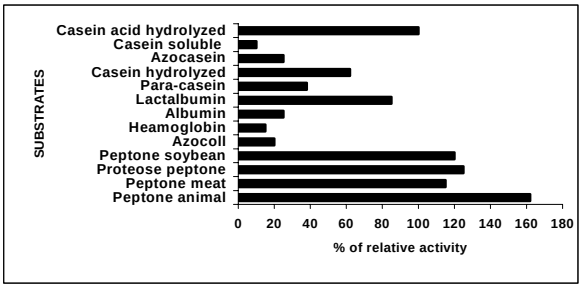


Fig. 7. Effect of substrate specificity of soybean seeds protease activity

The greatest protease activity was obtained with peptones obtain from animal, soybean and meat while low substrate specificity was observed with other proteins such as Hemoglobin, azocoll, albumin and azocasien used as a substrate. It is concluded from the above results that soybean seeds protease prefer to hydrolyzed peptones rather than other proteins.

A large number of researchers investigated the substrate specificity of protease isolated from different plants such as the *Osmunda Japonica* protease hydrolysis only 22-KDa protein but not the other proteins (Hiroshi *et.al.,* 2000), the favorable substrates for Cucumisin, a Prince melons protease are Glt-Ala-Ala-Pro-Leu- *p*NA and Suc-Ala-Ala-Pro-Phe-*p*NA (Tetsuya *et.al.,*1995), Durum wheat protease specific for reduced Gliadin as compared to other substrates such as Casein, Hemoglobin and Gelatin (Isabelle *et.al.,* 1996), a Maize protease prefer to hydrolyze Boc-Val-Leu-Lys-MCA and Suc-Leu-Leu-Val-Tyr-MCA than other oligopeptide substrates (Takafumi *et.al.,* 2000), Tomato proteases, His_6-LAP-A prefer to hydrolyze Leu-β-NAP and His_6-LAP-N significantly hydrolyzed Met-β-NAP than other acyl-β-naphthyl-amide (β-NAP) substrates (Chao *et.al.,* 2003) and LAP-A hydrolysis Xaa-Leu substrate than other dipeptides and Leu-Gly-Gly substrate than other tripeptides(Yong and Linda, 2000) and Winged-bean seeds protease prefer to hydrolyze Gelatin as compared to other substrates (Rajamma and Manoranjan, 1996) .

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