Pak. J. Biotechnol. Vol. 2 (1-2) 49-55 (2005) ISSN. 1812-1837

NEUTRAL PROTEASE ACTIVITY IN THE CRUDE EXTRACT OF COTTON (GOSSYPIUM HIRSUTUM) SEEDS

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ABSTRACT:

Ten plant seeds were analyzed for protease activity. During the course of screening, cotton seeds (Gossypium hirsutum) was found more potent for protease activity. The enzyme was most active at pH 7.0 and the optimum temperature for the enzyme activity was found to be 30°C. Cotton seeds protease was found heat stable and retains more than 15% activity at 80°C within 10 minutes. Protease activity was increased in the presence of cysteine and mercaptoethanol but activity decreased by the addition of $CaCl_2$, $MnCl_2$, $ZnCl_2$ and $CoCl_2$ in the reaction mixture. Protease isolated from Cotton seeds strongly hydrolyzes peptones than other substrates.

INTRODUCTION:

Proteases are multienzyme complex in nature, which hydrolyze poly-peptide and proteins stepwise to smaller peptides and finally amino acids [1]. Proteases are classified into three groups on the basis of pH such as acidic, neutral and alkaline [2]. Proteases are widely distributed in different plant cellular components. These proteases play some important physiological roles and the level of these plant proteases are affected by many factors like seed maturation and the breakdown of storage protein during seed germination [3], protein remobilization upon the onset of leaf senescence [4], concomitant with the relocation of nitrogen source to reproduction organs, and combined with environmental conditions such as carbon starvation, stress responses, infection and wounding [5]. Proteo-lysis involves not only in an important recycling system of amino acids but also the final step of a complex cascade of regulatory function [3].

The applications of proteases increases day by day and have great importance in a number of biological processes and numerous practical applications in biotechnology and industry [6], including protein engineering [7], detergent industry, food industry, leather industry, silk industry [8] and waste processing industry [9]. The major source of these proteases is microorganism [2] but plants are also good sources of protease [10]. Work is under way in a large number of research laboratories to explore and develop a low cost process for the isolation of protease, amylase, lipase etc by the use of indigenous source. Pakistan is a country with calculated resources and variegated economical constrains, the import of industrially important enzymes from abroad costs millions of rupees each year. As a matter of fact a country like ours 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. Looking to the increasing application and demand of protease, work has been undertaken to screen different plant seeds for protease activity. In present study, some characteristic properties of Cotton seeds protease have been reported.

MATERIAL AND METHODS:

MATERIAL:

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

METHODS:

Enzyme powder preparation: After removing seeds coats, the seeds were crushed using pestle mortar. The crushed seeds were defatted with diethyl ether. The defatted residue was further crushed in ice-cold acetone. Acetone was removed by filtration through Whatman No.1 filter paper and the residue was dried at low temperature (20°C).

Preparation of soluble enzyme: The crude enzyme solution was prepared by taking 10 g of acetone dried enzyme powder and crushed to fine powder using pestle mortar and 30 ml ice cold 0.2 M Tris-HCl buffer pH 8.50 was added. The solution was centrifuged at 7000 rpm in Kubota refrigerated centrifuged at 4°C for 15 minutes. The supernatant was transferred to a 100 ml volumetric flask. This procedure was repeated twice and total volume was made up to 100ml.

Substrate preparation: 1% solution of Casein acid hydrolyzed (Sigma chemicals) was prepared in 0.2 M Tris-HCl buffer, pH 8.50.

Determination of protein: Protein content from the crude extract was determined by the method of Lowry et al., [11] using bovine serum albumin as standard.

Determination of protease activity: Protease activity was determined by the method of Anson [12] with slight modification. One ml of enzyme solution was incubated with 1.0 ml 1% 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% trichloro acetic acid, which precipitated the residual protein. The content of the tubes were centrifuged after 30 minutes at 6000 rpm for 10minutes.

In 1.0 ml supernatant, 4.0 ml of 0.5N sodium hydroxide and 1.0ml of 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. The absorbance of blue colour developed was determined after 5 minutes at 625nm.

One unit of protease activity was defined as the amount of enzyme that liberated 1µg of tyrosine under the standard assay condition.

RESULTS AND DISCUSSION:

First of all the presence of protease activity in various plant seeds extract was determined and it was found that cotton seeds were more potent for protease activity as shown in Table-1. The nature of Cotton seeds protease was checked when different casein

substrates were disso-lved in buffers of different pH values. It was observed that casein acid hydrolysate showed optimum protease activity at pH 7.0 as shown in Table-2.The effect of time period on the rate of enzymatic reaction of cotton seeds protease is shown in Figure-1. The rate of reaction of cotton seed protease increased with the increase of incubation time up to one hour and then declined. This declination in activity after one hour reaction time may be suggested due to presence of other enzymes in crude enzyme sample of cotton seed [13] or presence of natural protease inhibitors in the sample could not be ruled out.

Table-1. Protein content protease activity of different plant seeds extract in universal buffer pH7.0

Botanical Name of Plants	Common Name	Protein Mg/ml	Protease Activity Units/ml
Gossypium hirsutum	Cotton	2.50	83
Glycine max	Soybean	2.60	50
Brassica campesteris	Mustard	0.97	12
Malia indica	Neem	2.30	06
Mangifera indica	Sindhri mango	1.40	10
Mangifera indica	Chosa Mango	1.25	08
Mangifera indica	Desi Mango	0.85	05
Dolichos lablab	Sem	1.20	12
Pongamia pinnata	Such chen	1.50	15

Table-2: Effect of extractable media and Substrates on cotton seeds protease activity when incubated at 35°C for 30 minutes.

Substrate (1%)	Universal buffer			
	H_2O	pH 5	pH 7	pH 8.25
	Activity units/ml			
Casein acid hydrolysate (Oxide)	28	30	30	64
Casein acid hydrolysate (sigma)	75	27	83	58
Soluble casein	0	3	10	15
Azo casein	5	4.5	16	13.5

The effect of substrate concentration (0.5-3%) on protease activity of cotton seeds was investigated and result is shown in Figure-2. It is observed that the rate of reaction increased proportionally to substrate concentration up to 2.0% and then declined.

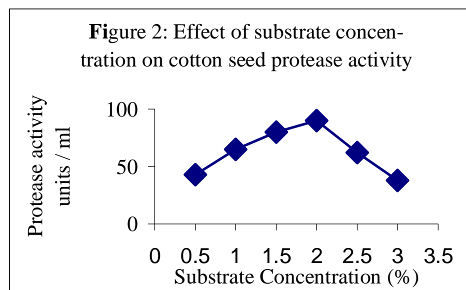


Figure 2: Effect of substrate concentration on cotton seed protease activity

Figure-3 shows the effect of various enzyme concentrations (2.5-20%) on protease activity of cotton seeds and observed that the activity increases with the increase of enzyme concentration up to 5% and then decrease. The maximum reaction velocity at 5% enzyme concentration is due to the utilization of total amount of substrate in enzyme–substrate complex [14]. In subsequent experiments 5% enzyme concentration (5% crude enzyme

solution of cotton seeds) and 2% casein acid hydrolyzed was used as substrate.

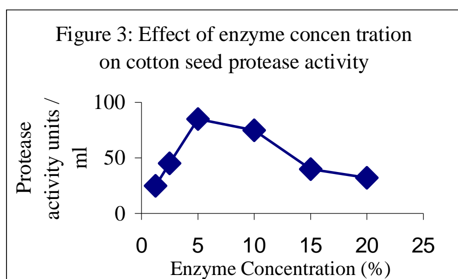


Figure 3: Effect of enzyme concen tration on cotton seed protease activity

The effect of pH on protease activity of Cotton seeds is presented in Figure-4. Protease activity was found to be optimum at pH 7.0. Dahot [13] reported neutral proteases from different plant seeds. A number of researchers [10, 15, 16] have isolated a neutral protease from different sources. The effect of temperature plays an important role on an enzyme-catalyzed reaction.

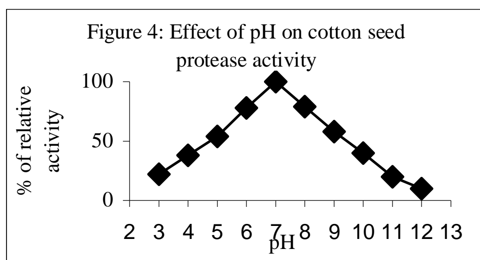


Figure 4: Effect of pH on cotton seed protease activity

The temperature profile (Figure-5) indicates that the optimum temperature for enzyme activity was 30°C. An initial increase in temperature up to 30°C increased the rate of the enzyme-catalyzed reaction due to the increase in number of collisions between the reacting molecules, but the enzyme probably got denatured on exposure to temperature higher than its optimum temperature [10]. This declination of activity after optimum temperature may also be due to the alteration of the structure of enzyme [17]. This result is in agreement with the finding of Rollan [18], Lee et al. [19] and Dahot [13]. In this study, the activation energy of the protease was calculated 23.75 kJ/mol.

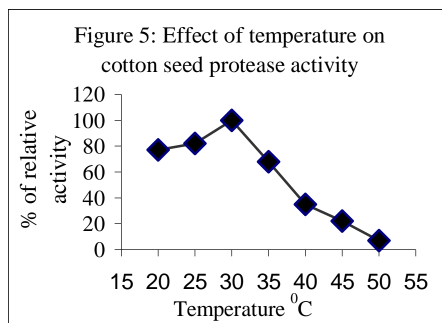


Figure 5: Effect of temperature on cotton seed protease activity

It is known that metal ions coordinate to the active site residue and activate enzyme while, in some cases coordination of metal ion results in inhibition. In this study, the effect of various metal ions, reagents and detergents [5 mM] on cotton seeds protease activity was checked and results are summarized in Table-3. The activity of crude cottonseeds protease is highly stimulated with cysteine and mercaptoethanol. This indicates that crude cotton seeds neutral protease possesses -SH group at active site or near to active site. The inhibitory effect of Ca^{+2}, Hg^{+2}, EDTA and O-phenanthroline suppor-ted this assumption. Several authors have reported cysteine protease from various sources [13, 20, 21]. Effect of different concentrations of cysteine and mercaptoethanol on protease activity of Cotton seeds was checked and results are shown in Figure – 6. The results showed that cotton seed crude protease requires 4 mM

cysteine and 6 mM mercaptoethanol for maximum activity.

Table – 3: Effect of different reagents on Cotton seed Protease activity.

Reagents 5 mM Concentration	% of relative activity	% of activation/ [inhibition]
Control	100	-
Tween 80	85	[15]
Triton X- 100	87	[13}
SDC	40	[60]
SDS	45	[65]
AgNO3	10	[90]
HgNo3	20	[80]
Mercaptoethanol	160	60
Cysteine	158	58
O-phenanthroline	68	[32]
EDTA	70	[30]
$CaCl_2$	65	[35]
$CoCl_2$	78	[22]
$MnCl_2$	70	[30]
$ZnCl_2$	78	[22]

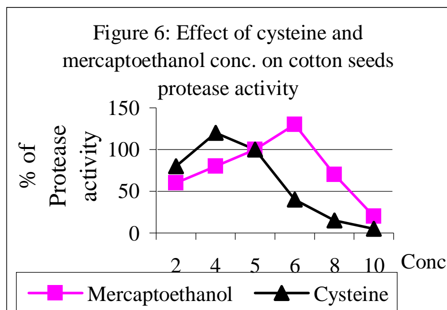
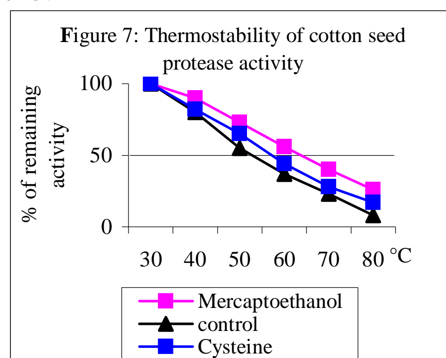
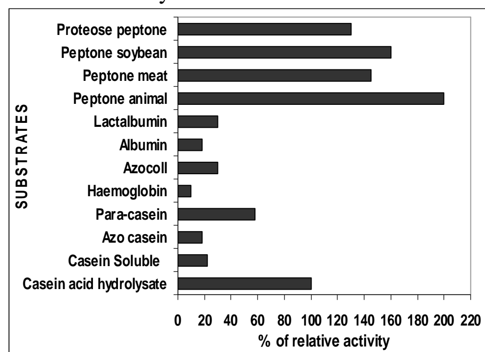


Figure-7 represents the results of thermostability of Cotton seeds protease activity in the presence and absence of cysteine (4mM) and mercaptoethanol (6mM). Thermo-stability was studied after pre-incubation of enzyme solution with and without cysteine (4mM) and mercaptoethanol (6mM) at various temperatures ranging from 30°C to 80°C for 10 minutes and the remaining activity after heat treatment was determined according to standard assay methods. It was noted that the enzyme is heat stable and retains more than 10 and 15% activity respectively with and without cysteine and mercaptoethanol at 80°C within 10 minutes. Yonezawa et al. [22] have reported a neutral thermostable cysteine protease from young stems of *Asparagus*. In order to detect the primary specificity of neutral protease of Cotton seeds, the activity was checked against various substrates at pH 7.0 at temperature 30°C.



It was observed from Figure-9 that the enzyme is highly specific towards peptones in comparison to other substrates.

Figure –8: Effect Of Substrate Specificity On Protease Activity Of Cotton Seeds



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