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Research Article

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# Purification and Characterization of Alkaline Protease Isolated from Cotton (*Gossypium hirsutum*) Seeds

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## Abstract

Proteases are widely utilized both in physiological and commercial fields such as medicine, food, detergent, and leather. Plant-originated proteases play a significant role in several biomedical fields due to their easy accessibility and activity. Pakistan is an agro-based country and can be an ideal place for the isolation of industrially important proteases from plant sources such as cotton, which is the main crop and frequently available and low cost. Purification of protease was carried out by fractionation with two-fold acetone, ethanol, methanol and various concentrations (40-80%) of ammonium sulphate. The precipitates formed were collected after centrifugation and dialyzed for 24 hours against universal buffer pH 7.0 and was centrifuged in a cooled refrigerated. The dialyzed sample was loaded on Sephadex G–100 gel column. The fractions of the samples were collected and their absorbance of protein was monitored at 280 nm. The homogeneity of the purified enzyme was checked by SDS gel electrophoresis The purified protease enzyme has optimum activity at 30°C and pH 8.0 when casein was used as substrate. The Km and Vmax values of purified cotton seed's alkaline protease activity was recorded as 0.03M and 17 µmol/minute respectively. Protease activity was increased by the addition of cysteine but inhibited by Iodoacetic acid and β-Mercaptoethanol and decreased with some metal ions. These characteristics of the purified enzyme allowed classifying it as a cysteine protease. In conclusion, this study suggests that the alkaline protease enzyme is the best choice for commercial use.

Keywords: Cotton seed, Protease, Purification, Alkaline, Cysteine

## Introduction

Protease enzymes are widely distributed nearly in all plants. Plants are consistent and low-cost production sources of stable and significant protease activity (Banerjee & Bhattacharyya, 1992; Matagne, Bolle, El Mahyaoui, Baeyens-Volant, & Azarkan, 2017). Plant proteases are applied in various industries, such as medicine, food, detergent, and leather (Diana R Baidamshina *et al.*, 2021; Sarkar & Suthindhiran, 2020). On the basis of their significant characteristics like a broad range of temperatures, pH, high stability in extreme conditions, substrate specificity and good solubility have been applied in various biotechnological fields and research (Kim, Si, Reddy, & Wee, 2016; Kumar, Anjana, & Sharma, 2019). Nowadays, the isolation of protease enzymes from plants has been augmented considerably by its subsequently. Alkaline proteases which are active at pH 7.0 or above are found everywhere in nature and present in animals, plants and microorganisms (Devi & HemaLatha, 2014). Alkaline proteases have broad applications at different industrial scales as compared to neutral and acidic proteases (Sharma *et al.*, 2019). Worldwide, the production of proteases on an industrial scale is about 60% of the enzyme marketing commercially (Ashouri, Abad, Zihnioglu, & Kocadag, 2017). However, the accessibility of protease is not satisfying the intact requirement of industries yet. These situations escort some researchers to continue to explore novel sources for the production of protease, particularly from low-cost raw materials. Nowadays, plants are used in novel protease enzyme investigations due to their advantages as the cheapest source and simple extraction than animals and microorganisms (Akcan & Uyar, 2011). Furthermore, the vast variety of proteases, based on their characteristics like the specificity of their action, thermal stability, etc. attracted worldwide attention to exploring the cheapest 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 proteases from plant sources, which are frequently available and low cost. Several reports are available on the isolation and characterization of protease from different plant sources like Solanum dubium seeds (Abbas & Abdelrahman, 2021), *Gliricidiasepium* seeds (da Silva *et al.*, 2020), *Viciafaba* L. seeds (Sathya Prabhu, Apoorva, Nandita, Chamy, & Devi Rajeswari, 2018), sprouted seeds (Rawski, Sanecki, Dżugan, & Kijowska, 2018), and *Nigella Sativa* seeds (Yang, Song, Gu, & Li, 2011). However, available literature

shows a lack of stress related to the isolation of protease from cotton seeds. In the present study, alkaline cysteine protease was isolated, purified and characterized from cotton seeds.

**Material and Methods**

Cotton seeds (BS-15) were purchased from the local market. Casein acid hydrolysate, ammonium sulphate and other chemicals were used in high analytical grade and purchased from Sigma, Merck, Fluka etc.

**Preparation of Enzyme Powder and Soluble Enzyme**: Cotton seeds were de-coated and pulverized through pastel and motor to obtain seed grains and prepared a homogeneous solution in 0.2 M Tris-HCl buffer. However, 10% enzyme solution from extracted cotton seed enzyme and 2% casein acid hydrolysate substrate were prepared in chilled 0.2 M Tris-HCl buffer as reported earlier (A. Ali & Dahot, 2009).

**Determination of Protein:** The protein content of the enzyme solution was determined by the reported method (Lowry, Rosebrough, Farr, & Randall, 1951; Reagent, 1951), using bovine serum albumin as a standard. During purification, protein absorbance was monitored at 280nm

**Assay of Protease Activity:** Protease activity was determined as described in earlier report (A. Ali & Dahot, 2009). One unit of protease activity was defined as the amount of enzyme that liberated 1ug of tyrosine under the standard assay conditions.

**Statistical analysis:** All experiments were carried out in triplicate and average along with standard deviation are presented.

**Isolation, dialysis and purification of protease from cotton seed:** Purification of protease was carried out by fractionation with two-fold acetone, ethanol, methanol and various concentrations (40-80%) of ammonium sulphate. All these materials were kept at 4°C for 4-5 hours. The precipitates formed were collected after centrifugation using refrigerated centrifuge (Model 7000, KUBOTA Japan) at 7000rpm at 4°C for 20minutes. The collected precipitates were dissolved separately in 10ml of Universal buffer (Tris-HCl, Bis-Tris and sodium acetate) pH 7.0 and protease activity was determined by the standard reported method. The enzyme sample was dialyzed in a cellulose membrane, having molecular weight-cutoff 10K, for 24 hours against universal buffer pH 7.0 and was centrifuged in a cooled refrigerated centrifuge at 7000 rpm for 20 minutes as described earlier(A. J. P. J. o. A. Ali & Chemistry, 2022)

**Sephadex G-100 column chromatography:** 10.0 grams of Sephadex G-100 (Sigma Chemical, Pore size 40-120µm diameter, with bed volume 15-20 ml/g dry weight) was suspended in an excess of distilled water to swell for 3 days at room temperature. A column of (60 x 2 cm) was carefully packed with a deaerated slurry of swelled Sephadex G-100. The packed column was equilibrated with Universal buffer pH 7.0 for overnight. 5.0 ml of dialyzed sample was loaded on this Sephadex G–100 gel column. The sample was eluted with the same buffer pH of 7.0 at the flow rate of 40.0ml /hour. The fraction of 5.0ml was collected using fraction collector EYELA UV–9900, UV-visible detector. The absorbance of protein was monitored at 280 nm.

**SDS-Gel electrophoresis:** The homogeneity of the purified enzyme was checked by SDS gel electrophoresis using a buffer system. The two layers of gels at various pH and pore size were required in discontinuous buffer system. The resolving gel of pH 8.8 was first prepared and allowed for polymerization. Similarly the stacking gel pH 6.8 was allowed to polymerize on the top of first gel using 7.5 % acryl amide and bis-acryl amide concentration as described in the literature (Alhashem *et al.,* 2022; Davis, 1964; Kavoosi & Ardestani, 2012).

**Ion exchange chromatography:** Out of five, one pooled fraction (fraction no.5) was not homogenized and this was separated by CM-Sephadex C-50 column (26 x 2cm), which was previously equilibrated with sodium phosphate buffer pH 7.0. The enzyme was eluted with NaCl (0.0 – 0.9 N) gradient using same buffer. The flow rate was adjusted at 20.0ml/hour and 5.0 ml fraction was collected in each tube. The protein absorbance was monitored at 280nm by EYELA UV–9900, UV - Vis. Detector.

**Kinetic Study and Characteristic Properties of Protease Enzyme:** The kinetic properties such as substrate specificity, thermostability, optimum pH and temperature, molecular weight determination and activators/inhibitors, of the purified and homogenize enzyme sample was characterized by early reported method(A. J. P. J. o. A. Ali & Chemistry, 2022).

**Effect of substrate concentration and substrate specificity on protease activity:** The effects of substrate concentration were observed on the rate of enzymatic reaction of protease by using various concentrations of casein acid hydrolysate and peptone as a substrate ranging from 0.5-3%. The reaction mixture consists of a 1.0ml of purified fraction sample 1.0 ml substrate of different concentrations and the reaction mixture was incubated at 30°C for one hour. The substrate specificity towards different substrates was measured by using different substrates. The desired amount of enzyme and substrate was incubated for one hour at 30°C and the protease activity of the purified fraction sample was checked as previously described standard protease assay method.

**Effect of pH on protease activity:** The effect of pH on the activity of protease was checked by measuring the enzyme activity of purified Fraction at various pH ranges from 4 to 10 using universal buffer and casein acid hydrolysate as a substrate. The activities of the purified protease in the different pH values of the casein solution are measured respectively, and expressed as percentages of the highest activity, which was taken as 100%. The purified protease

activities were estimated by the standard protease assay method.

**Effect of temperature and temperature stability on protease activity:** The protease activity of the purified enzyme sample was determined at various temperatures in the range of 15°C to 55°C. Whilst, the assessment of the thermal stability of purified fraction by measuring the residue activity after incubation of the enzyme at various temperatures ranging between 20°C to 90°C for 10 minutes with and without the addition of an activator at 30°C. The purified enzyme was heated at various times ranging between 5–20 minutes with and without the addition of an activator at 40°C. The remaining activities were determined by the standard assay method as reported earlier.

**Effect of various metal ions / reagents on protease activity:** Various metal ions and chemicals in 5mM concentration were reacted with purified protease for 10 minutes at optimum temperature prior to the addition of substrate and the remaining activities were determined by the previously described standard assay method.

## Results and Discussion

**Purification of protease by chromatography:** In this study enzymatic protein from cotton seeds extract (20%) of pH 7.0 was precipitated with solvents such as acetone, ethanol, methanol and different concentrations of ammonium sulphate (40, 60 and 80%). More protease activity was recovered with 60% ammonium sulphate precipitation in compression to solvent precipitation as illustrated in table 1. Therefore, the desired concentration of ammonium sulphate (60% saturated) was selected for precipitating enzymatic protein for further work.

**Table- 1:** Enzyme Protein and activity of precipitates of solvents

| **Precipitation With** | **Total protein mg** | **Total protease activity units** | **Specific Activity units/mg protein** |
|---|---|---|---|
| Control | 253 | 8500 | 33.6 |
| Ethanol | 134 | 5000 | 37.3 |
| Methanol | 199 | 5750 | 28.9 |
| Acetone | 167 | 5500 | 33 |
| **Ammonium sulphate** | | | |
| 40% | 51.9 | 1200 | 23.12 |
| **60%** | **115** | **6000** | **52.17** |
| 80% | 83 | 1300 | 15.66 |

The typical elution pattern of cotton seeds protease purification by Sephadex G-100 column chromatography is shown in figure 1. The precipitates obtained from 60 % ammonium sulphate were dissolved in 20ml Universal buffer pH 7.0 and then dialyzed overnight at 4°C. The analyzed sample was applied on the Sephadex G-100 column (60×2cm), which was packed and equilibrated with the same buffer at a flow rate of 40ml/hour with 10ml of fraction volume. The elution of enzymatic protein was monitored at 280nm. The protease activity was checked in each tube and the active fractions were pooled for further characterization. These pooled fractions were named Fraction-I, Fraction-II, Fraction-III, Fraction-IV and Fraction-V. The Fraction-I, Fraction-II, Fraction-III and Fraction-IV were found homogeneous showing a single protein band by SDS gel electrophoresis while Fraction-V was not homogeneous. For homogenization, the Fraction-V was loaded on CM-Sephadex C-50 column (26 x 2cm), which was previously equilibrated with sodium phosphate buffer pH 7.0. The enzyme was eluted with NaCl (0.0 – 0.9 N) gradient using same buffer. The flow rate was adjusted at 20.0ml/hour and 5.0 ml fraction was collected in each tube. The protein absorbance was monitored at 280nm by EYELA UV–9900, UV - Vis. Detector. From this procedure two fractions were obtained named VI and VII as exhibited in figure 2.

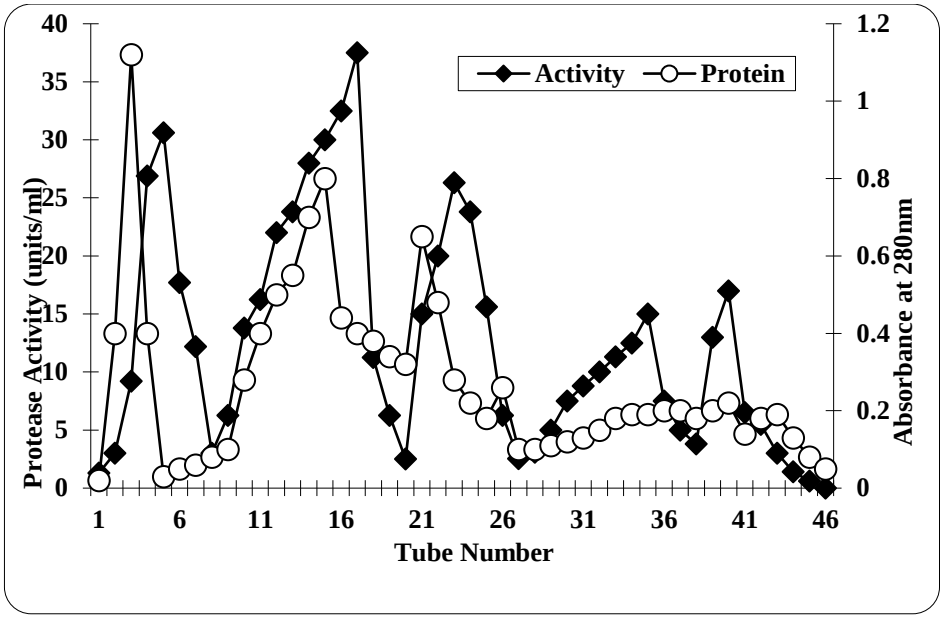


**Figure-1.** Purification profile of protease of cotton Seeds by Sephadex G-100

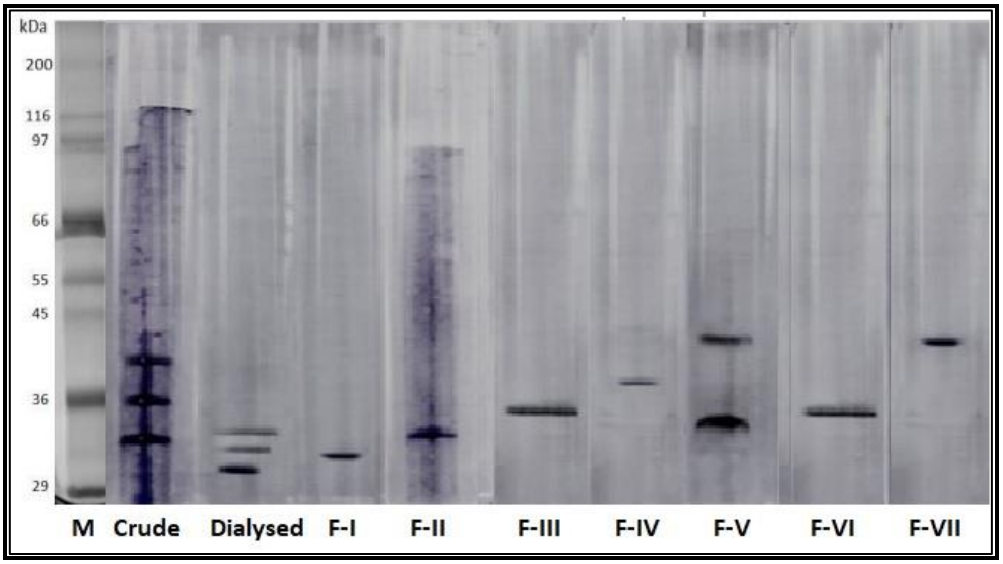


**Figure 2.**SDS Polyacrylamide Gel Electrophoresis of Cotton Seeds Protease M=Molecular mass marker

All fractions were individually characterized and found alkaline, acidic and neutral. However, on the basis of terms of optimum temperature, pH, substrate concentration, thermostability, substrate specificity and effect of various reagents Fraction-I protease, which was alkaline in nature further characterized and reported in the present study. The protease was purified to 1.43-fold with a percent yield of 12.2. While specific activity was found to be 48 Units /mg of protein. The molecular weight of alkaline fraction was estimated to be 32 KDa. The overall purification profile of alkaline protease fraction is presented in Table 2. The maximum protease activity precipitates were recovered with 60% ammonium sulphate as compared to solvent precipitation as reported in table 1. 60% saturated ammonium sulphate precipitation gives the highest protease activity from the leaf pellet of *Viciafaba* L (Sathya Prabhu *et al.*, 2018). While several researchers have used higher concentrations of ammonium sulphate for maximum protease activity i.e. 50–70%, and 60% (Abd-ElKhalek, Seoudi, Ibrahim, Abd-Rabou, & Abd ElAzeem, 2020). On the other hand, low protease activities recovered in acetone, ethanol and methanol may be due to the change in pH or temperature, which may cause denature of the proteins during the process (Doi & Nojima, 1975; D. Holme & Peck; D. J. Holme & Peck, 1983)

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**Table-2** Purification Profile of Protease from Cotton Seeds

| Purification Steps | Total Protein mg | Total Protease Activity µg | Specific Activity µg /mg | Purification Fold | % Yield |
|---|---|---|---|---|---|
| **Enzyme Crude** | 253 | 8500 | 33.6 | 1 | 100 |
| **After dialysis** | 190 | 6750 | 35.5 | 1.06 | 79.4 |
| **Sephadex G-100** | 123.8 | 5800 | 46.8 | 1.39 | 68.2 |
| **Fraction-I** | 21.6 | 1039 | 48 | 1.43 | 12.2 |

**Effect of substrate concentration on protease activity:** In addition to pH and temperature, substrate concentrations are considered as one of the main factors for determining the activity of the enzymes. In addition, it can be used for determining the enzyme kinetics ($K_m$ and $V_{max}$) to illustrate the enzyme affinity and specificity. For this purpose, various concentrations of casein acid hydrolysate ranging from 0.5 to 3% were used with respect to their optimum time. Blank determinations were carried out simultaneously at the specific substrate concentrations without the enzyme. In each case a Lineweaver–Burk plot was plotted and value of Michaelis–Menten constant (Km) were calculated. The hydrolytic activity of protease was investigated and the results are represented in figure 3. Furthermore, it was noted that the substrate concentration increased up to 2% when both casein hydrolysates which was used throughout the study and peptone of soymeal and then declined. The present study revealed that the rate of reaction declined after 2% substrate concentration as exhibited in figure 3. The decline in the rate of reaction after optimum concentration could be due to the alteration of the enzyme-substrate concentration ratio. The activity of protease increases with the increase of substrate concentration also reported from *Carica papaya* latex using azocasien as a substrate (Diana R Baidamshina *et al.*, 2021). It is also reported by other workers and correspondence with reported values from *Mentha piperita* L and *Thymus capitatus* L using egg albumin as a substrate and from *Moringa oleifera* leaves using β-casein as a substrate (Atrooz & Alomari, 2020; Banik, Biswas, & Karmakar, 2018).

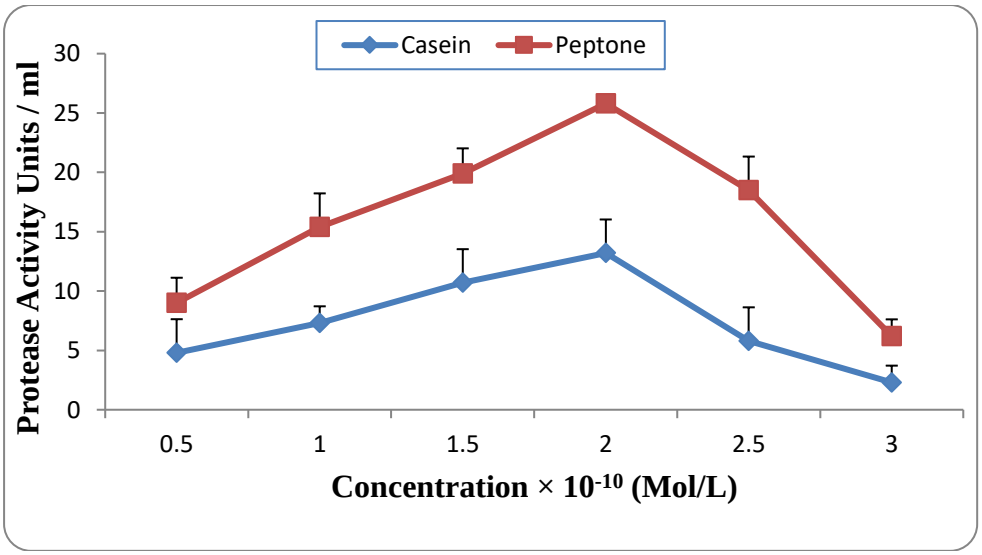


**Figure -3.** Effect of substrates concentration on purified Protease activity of cotton seeds

**Determination of $K_m$ and $V_{max}$:** The value of Michaelis constant ($K_m$) for the activity of purified cotton seeds alkaline protease was estimated. A Line weaver Burk plot was plotted and the value of the Michaelis Menten constant (Km) was calculated (Segel, 1976) which is 0.03 M as depicted in table 3. It is clear, from the result obtained in the present study revealed that the purified alkaline protease enzyme has a greater affinity for the substrate. On the other hand, maximum velocity ($V_{max}$) shows the catalytic efficiency, the higher the $V_{max}$ the higher the efficiency. The value of $V_{max}$ of cotton seed purified alkaline protease is17µmol/minute. However, the activation energy of purified protease was obtained at 4.61 KJ/mol as summarized in table 3. The kinetic study in terms of $K_m$ and $V_{max}$ were calculated on the basis of the specificity of an enzyme for a particular substrate. In the special condition /case, $K_m$ becomes a measure of the strength of the ES (Enzyme-Substrate) complex. A Large $K_m$ indicates weak binding while a small Km indicates strong binding of substrate. $K_m$ depends on pH, temperature and substrate, etc. therefore, its value serves to characterize a particular enzyme-substrate system under certain specific conditions. A Lineweaver-Burk plot for casein was linear suggesting that the rate of proteolysis was

directly proportional to the substrate concentration and permitted the calculation of the kinetic constant (figure 4).

**Table- 3.** $K_m$, $V_{max}$ values, Arrhenius plot of the activation energy of purified cotton seeds protease. Ea=slope,where R=8.314KJ/mol

| **$K_m$ values** mol/L | **$V_{max}$ values** µmol/min | **Activation energy** KJ/mol |
|---|---|---|
| 0.03 | 17 | 4.61 |

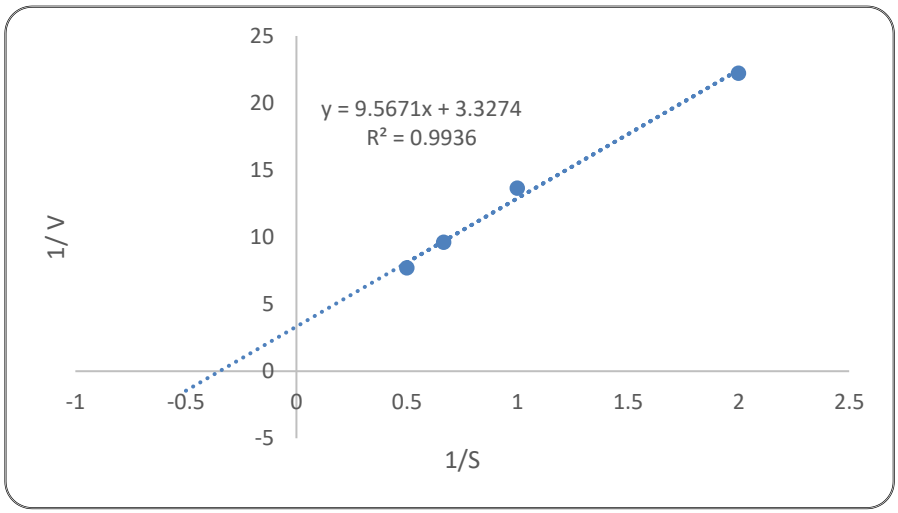


**Figure- 4.** Double reciprocal plot for casein hydrolysis by purified protease. The enzyme activity in the reaction mixtures containing variable amounts of casein (0.5 – 2.5 $\times 10^{-3}$M/L) at pH 8.0 was determined.

The $K_m$ value of the present report is also lower than reported by other workers obtained from protease enzymes of some other plants like field bean seeds enzyme 0.0105M (Paul & Gowda, 2000), 0.107M from *Moringa oleifera* leaves (Banik *et al.*, 2018), 0.02M from the seeds of *Cucumismelovaragrestic* (Devi & HemaLatha, 2014) and 0.19M from Yemeni bean seeds (Maqtari, Naji, & Ali). However, the value of $V_{max}$ is higher in the present study as reported in Table 3 than those of calculated from Sorghum malt protease which was 8.2 µmol/minute (Ogbonna & Okolo, 2005), *Mentha piperita* L protease, 2.94 µmol/minute and *Thymus capitatus* L protease 1.3 µmol/minute (Atrooz & Alomari, 2020).

**Determination of the optimum pH:** The pH influences the enzymatic activity and the determination of the optimum pH are useful to support the protease classification. The effect of pH was determined from purified samples of cotton seeds protease. Maximum pH was determined by mixing enzyme with Universal buffer. The pH ranges used were 4-10. Universal buffer was first made with pH 7. Low pH 4-6 were made by adding 0.1 M HCL, and high pH 8-10 were made by adding 0.1 M NaOH to universal buffer. The enzyme was mixed with a buffer of varying pH then stored at 30°C for 30 minutes, the activity of the enzyme was measured as reported method. The results presented in figure 5 shows that the enzyme is active over a wide range of pH and maximally active in alkaline pH (8.0). Most plant proteases are known to be active over a wide range of pH and temperature values. These results are in good agreement with the results obtained from *Gliricidia sepium seeds* (da Silva *et al.*, 2020), green chickpea seeds (Banik *et al.*, 2018) from *Canavalia ensiformis* seeds (Gonçalves, Gozzini Barbosa, & Silva-López, 2016), *Cucumis melovaragrestis* seeds (Devi & HemaLatha, 2014) and Black gram seeds, Lentil seeds and Green gram seeds (Anupama, Marimuthu, Sundaram, & Gurumoorthi, 2013). The excellent activity and stability over wide pH range shows that the alkaline nature of the protease, which makes it suitable for use in alkaline environments and with detergents (Zanphorlin *et al.*, 2010).

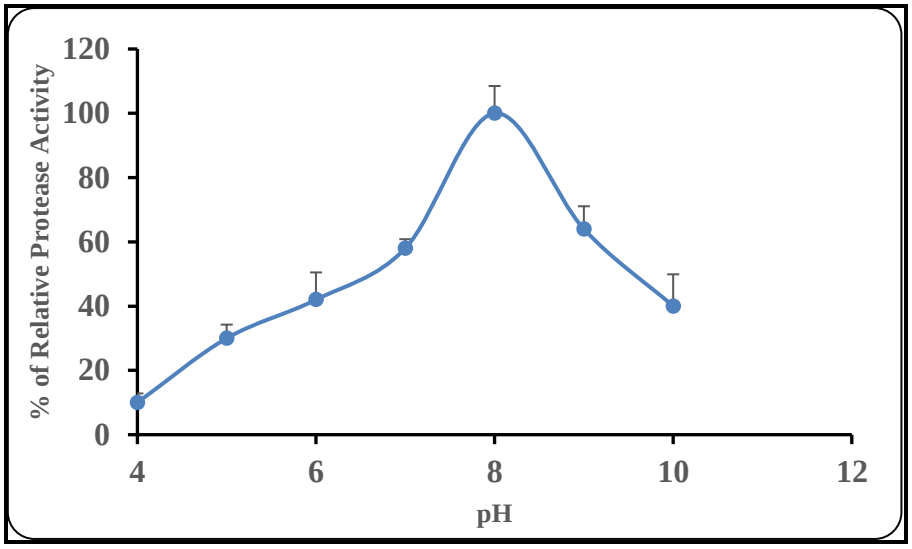


**Figure 5.** Effect of pH on purified protease activity of cotton seeds

**Determination of the optimum temperature:** Temperature is the main factor for the maximum activity of enzymes as well as for numerous industrial applications. The effect of temperature on the activity of purified protease was examined when the enzyme was assayed at different temperatures ranging from 10°C to 50°C as shown in figure 6. It was observed from the experiments that the optimum temperature for the protease is 30°C. It was observed from the experiment of the present study that the optimum temperature for the cotton seeds protease was noted 30°C , which is in the range (20–50°C) of reported most other plants proteases (Antao & Malcata, 2005). It is already known that protein conformation changes at higher temperatures, which causes a decrease in protease activity (Iqbal *et al.*, 2018; Matkawala, Nighojkar, Kumar, & Nighojkar, 2019).

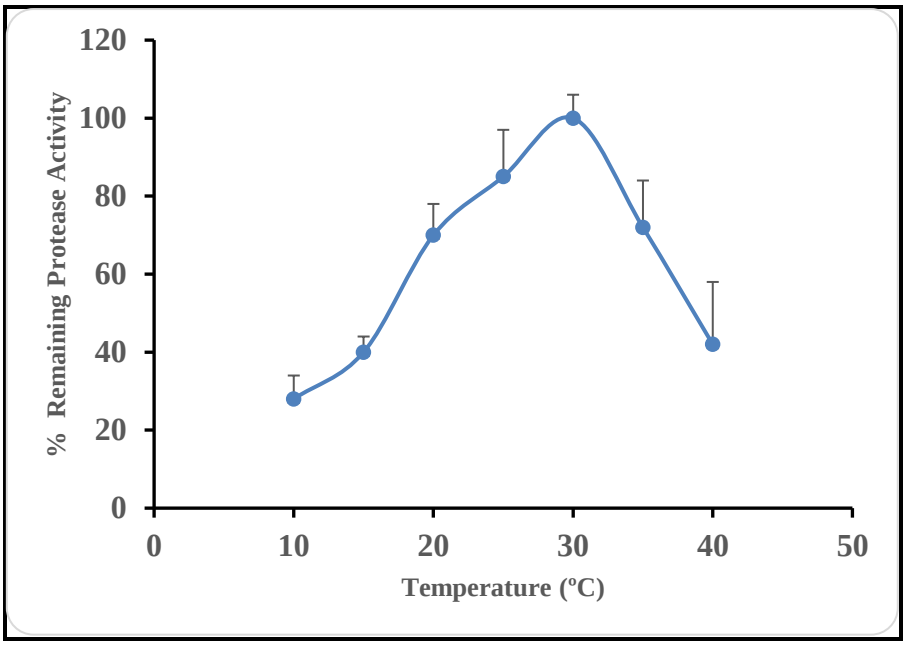


**Figure 6.** Effect of temperature on purified protease activity of cotton seeds

**Effect of some metal ions and chemical agents:** The effect of various metal ions and chemicals of 5mM concentration on purified cotton seeds protease activity was investigated. The thiol nature of purified protease is clearly indicated by the 54% activation with the addition of cysteine, which acts as reducing agent. On the other hand, both Iodoacetic acid and β-mercaptoethanol inhibited the enzyme activity. The other results presented in table 4 do not show any obvious metal ion requirement of the purified enzyme due to strong inhibition by metal ions. The detergents sodium dodecylsulphate (SDS) and sodium deoxycholate (SDC) completely inhibits the protease activity while triton X-100 and tween- 80 did not have any appreciable effect. It was also mentioned previously as exhibited in table 4 the thiol nature of

cotton seed purified protease activity enhanced by cysteine as compared to other metal ions/reagents. The enhancement of protease activity by cysteine is due to the regeneration of an SH-group from an S-S bond (Moo-Young, 2011). On the other hand, protease activity was almost completely inhibited by Iodoacetic acid and β-Mercaptoethanol, both are considered inhibitors of cysteine proteases. The molecular weight of the purified alkaline protease (32KDa) also confirm the cysteine nature of the enzyme because majority of plant cysteine proteases are small proteins with molecular weight in the range of 20 to 35 kDa (Turk, Turk, & Turk, 1997). These findings correlated to literature, in which partial purified fibrinolytic and fibrinogenolytic protease from *Gliricidia sepium* seeds were explored and the hemostatic and thrombolytic potential of heynein, a latex cysteine protease from *Ervatamia heyneana* and purified cysteine protease from the latex of *Euphorbia nivulia* (Badgujar, 2014; da Silva *et al.*, 2020; Uday *et al.*, 2017). Several researchers have also isolated cysteine protease from different plant sources such as from *Gliricidia sepium* seeds, Jackfruit seeds, *Caricapapaya*latexas, baby kiwi, *Nigella Sativa* L seeds, Latex of *Ficus microcarpa* and Latex of *Plumeriarubra* Linn (Diana R. Baidamshina *et al.*, 2021; Chanda, Basu, Dutta, & Das, 2011; Gangaraju *et al.*, 2015; Jinka, Ramakrishna, Rao, & Rao, 2009; Miyazaki-Katamura *et al.*, 2019; Silva *et al.*, 2020). It was also mentioned previously as exhibited in Table 4 the thiol nature cotton seed purified protease activity enhanced by cysteine as compared to other metal ions/reagents. The enhance of protease activity by cysteine is due to the regeneration of an SH-group from an S-S bond. On the other hand, protease activity was almost completely inhibited by Iodoacetic acid and β-Mercaptoethanol, both are considered inhibitors of cysteine proteases. These findings correlate with reported in which, they partially purified fibrinolytic and fibrinogenolytic protease from *Gliricidia sepium* seeds and explored the hemostatic and thrombolytic potential of heynein, a latex cysteine protease from *Ervatamia heyneana* and those of tested a purified cysteine protease from the latex of *Euphorbia nivulia*. Several researchers have also isolated cysteine protease from different plant sources such as from *Gliricidia sepium* seeds, Jackfruit seeds, *Caricapapaya*latexas, baby kiwi, *Nigella Sativa* L seeds, Latex of *Ficus microcarpa* and Latex of *Plumeriarubra* Linn. (Badgujar, 2014; Chanda *et al.*, 2011; da Silva *et al.*, 2020; Mnif *et al.*, 2014; Uday *et al.*, 2017)

**Table- 4.** Effect of various reagents on purified protease activity of cotton seeds

| **Reagents 5mM conc.** | **Activity units/ml** | **% of relative protease activity** | **% of activation/ [inhibition]** |
|---|---|---|---|
| Control (without activator / Inhibitor) | 13 | 100 | 100 |
| CoCl2 | 6.8 | 52 | [48] |
| CaCl2 | 2.95 | 22.6 | [77.4] |
| MnCl2 | 2.95 | 22.6 | [77.4] |
| ZnCl2 | 8.3 | 63.8 | [36.2] |
| **Iodoacetic acid** | **1.4** | **10.8** | **[89.2]** |
| **β-mercaptoethanol** | **2.95** | **22.6** | **[77.4]** |
| **Cysteine** | **20** | **154** | **54** |
| SDS | 0 | 0 | [100] |
| SDC | 0 | 0 | [100] |
| Tritron X-100 | 14.2 | 109 | 9 |
| Tween 80 | 14.2 | 109 | 9 |

**Determination of the thermal stability for the enzyme:** Studies of thermal stability of the purified cotton seeds protease activity was performed by heating the enzyme samples at various temperatures ranging from 10°C to 100°C for10 minutes with and without the addition of activators. On the other hand, thermostability was checked at various times from every interval of 5 minutes (5 to 20 minutes) at a fixed incubated temperature with and without the addition of activators. The protease activity of cotton seeds was retained at 15% at 50°C. It was noted that 47% at 50°C protease activity was increased by the addition of cysteine in the enzyme sample heating for 10 minutes and results are shown in figure 7. On the other hand, the data summarized in figure 8 indicates the effect of heat treatment on protease activity at a variable time (5-20minutes) and fixed temperature with and without the addition of activator. However, protease activity was retained at 30% and 51% for 20 minutes at 40°C with and without the addition of an activator respectively. The enzyme was stable at a temperature 30°C. However, it lost its activity completely at 70°C when incubated for a period of 10 minutes possibly due to thermal denaturation as revealed in figure 6. The optimal temperature of the present study result (30°C ) of the purified protease is also in harmony with that of the protease isolated from durum wheat from *Cajanus cajan* seeds and from *Pseudoalteromonas* sp. Strain A28. Omar and Fatmah have also isolated a protease from leaves of *Menthapiperita* L have an optimum temperature of 35°C. Kaminiet and Aoki have shown that the

protease activity with an optimum temperature of less than 20°C is considered as a cold protease. Akhtaruzzaman suggested that plant protease may be the best source for commercial demands where need optimum mild or low temperature as a chief factor in the processing of food at an industrial scale.

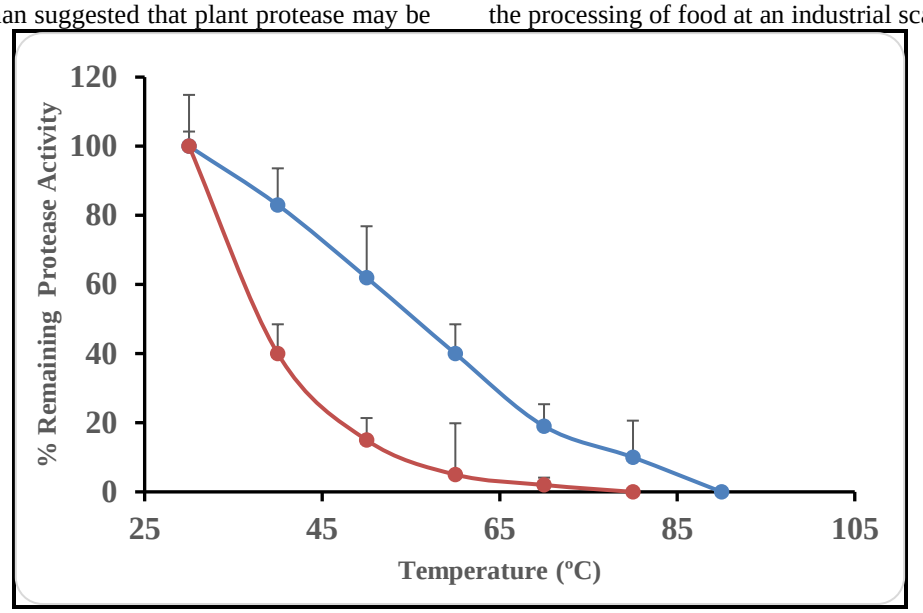


**Figure- 7.** Thermostability of Purified protease activity of cotton seeds with and without cysteine for 10 minutes

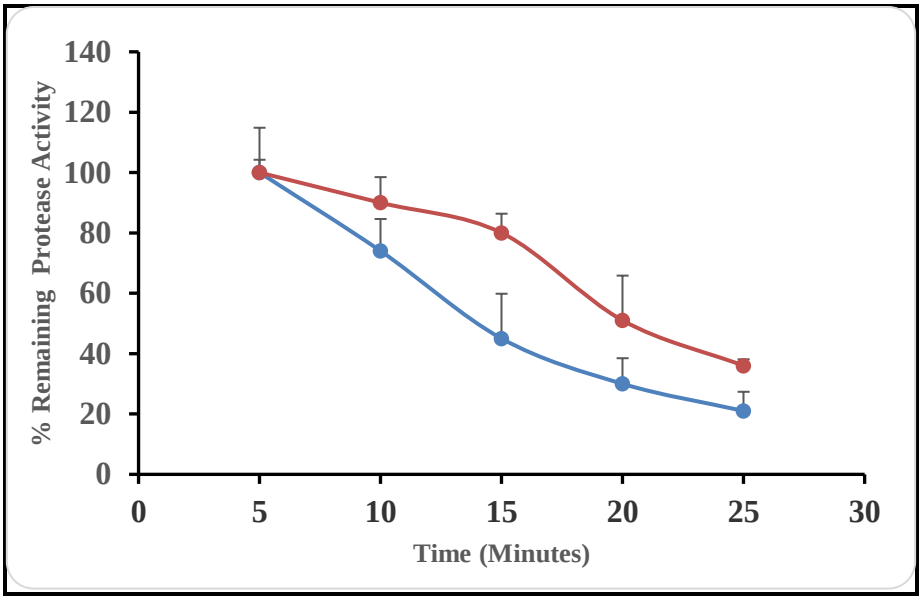


**Figure 8.** Effect of heat treatment at (40ºC) with & without cysteine at various time period on purified protease activity

**Effect of substrate specificity:** The substrate specificity profile of the purified protease indicates that the enzyme has a wide range of hydrolytic activity on various protein substrates. It was reported that the ability to hydrolyze different substrates together with other properties is important for various industrial applications such as pharmaceutical, food, detergent and biotechnology industries. The specificity of cotton seeds protease was tested with different substrates prepared in universal buffer pH 7.0 and results are presented in figure 9. The greatest protease activity was obtained with peptones while low substrate specificity was observed with other proteins used as a substrate. It was noted from the above-reported results as depicted in figure 9 that cotton seed protease prefers hydrolyzed peptones rather than other proteins. However, our finding is in variance with the reports of other researchers who investigated the substrate specificity of protease isolated from different plants such as cysteine protease from the fruit of Babi Kiwi exhibited high activity toward the synthetic substrate ZLeu-Arg-MCA and moderate activity toward Z-Val-Leu-LysMCA, Z-Val-Val-Arg-MCA, Z-Phe-Arg-MCA and Pro-PheArg-MCA, protease from miswak roots (Abdulaal, 2018)showed that the enzyme acted on casein, hemoglobin, egg albumin, gelatin and bovine serum albumin, three proteases

from *citrus sinensis* fruit peel prefer to hydrolyzed gelatin than casein and bovine serum albumin and protease from seeds of *Citrulluscolocynthis* identified significantly high rates of hydrolysis for natural proteins such as hemoglobin, casein, gelatin and bovine serum albumin compared to modified proteins such as azoalbumin and azocasein (Khan, Khan, Shah, & Khan, 2016)

.

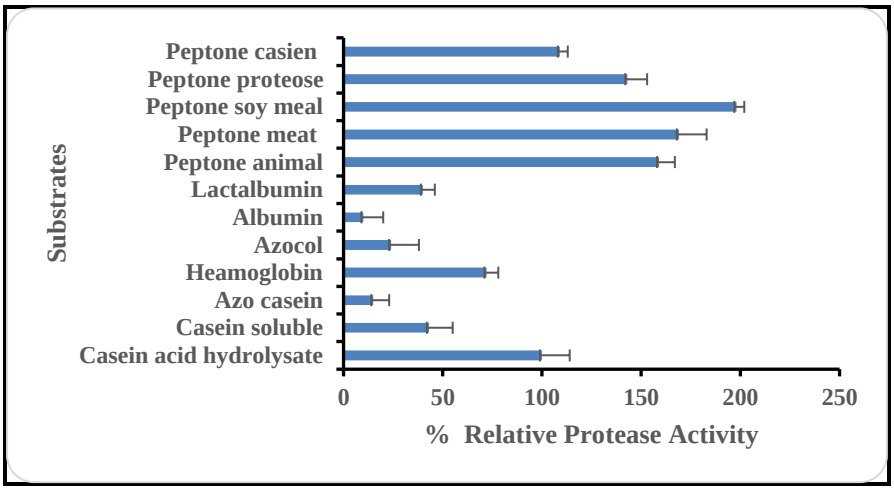


**Figure- 9.** Substrate Specificity of purified protease activity of cotton seeds

## Conclusion

It is concluded from reported finding research work that during the purification of cotton seeds proteases by Sephadex G-100 column chromatography, five fractions of proteases (Fraction-I, II, III, IV, and V) were eluted. On the basis of optimum pH, the Fraction-I was found alkaline in nature (pH 8) which was characterized on the basis of different kinetic parameters. The activity of the protease was stimulated in the presence of cysteine. The activity of purified protease was found to heat labial and increase 47% at 50°C in the presence of an activator (cysteine). The protease prefers to hydrolyze peptone as compared to other substrates. On the basis of broad pH range and substrate specificity, it is suggested that the purified alkaline protease could be used in food, detergents, medicine and leather industry where this enzyme plays vital role. However, the effectiveness of alkaline purified protease need to be tested by incorporating different industrial products.

In future study there is a need to explore amino acid composition and their sequence in purified alkaline protease for elucidation of structure.

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## Conflict of Interest

The authors declare that there is no conflict of interest.

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