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Evaluation of Cotton Seeds as Environmentally Liable Source for Neutral Protease

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Abstract

Proteases are considered one of the most imperative groups of enzymes and are used in bioremediation processes, leather, detergents, pharmaceutical and the food industry. The foremost aim of this study was to extract, purify, and characterize the protease enzyme from cotton seeds. Purification of the neutral protease was achieved with ammonium sulphate, which gave the best results at 60% concentration with a specific activity of 51 units/mg protein and 1.52 purification fold with a percentage yield of 10.5%. The protease was active and stable at a wide range of pH from 4.0–10.0 with an optimum pH of 7.0. The highest activity of the purified enzyme was found at 20 °C. The enzyme was thermally stable and retained 25% of its activity at 50° C. The activity of cottonseeds protease Fraction-IV was enhanced by 20% with $ZnCl_2$ and 15% with $CoCl_2$ as enzyme samples were heated for 10 minutes. The Casein and peptone assays were also performed to check its catalytic activity. Furthermore, the maximum hydrolysis rate (V_{max}) and apparent Michaelis–Menten constant (K_m) values of the purified protease were 19 µmol/min and 0.08 mol/L, respectively, while activation energy was found to be 12.47 KJ/mol.

Keywords: Neutral, Thermostable, Cotton seeds, Metallo Protease

Introduction

Enzymes, due to their low production cost, easy methods of production and environmentally friendly behavior are broadly used in several commercial fields [1]. in proteins, these enzymes accelerate the degradation of peptide bonds present between amino acids [2]. Researchers are focusing to isolate new enzymes which are suitable for commercial applications [3]. In 2018, the world market for industrial enzymes extended by around $5.6 billion in 2018, which will increase by an estimated $7.0 billion in 2023 [4]. Among these enzymes, proteases cover 65% of the total industrial enzymes market [5]. Proteases may be classified as endopeptidases, which degrade protein substrate from the inner site and exopeptidases, which degrade protein substrate from the end but can still be classified according to the substrate specificity, source of isolation (vegetable, animal or microbial), molecular size, catalytic action, charge and active site [6].

Proteases, according to their optimum pH reactions, are classified as neutral protease, acidic protease and alkaline protease [7]. Neutral proteases are broadly applied in the food [8,], feed [9], pharmaceutical [10] and leather [11] industries due to their divergent benefits, including low pollution level, a mild catalysis process and high yield. Neutral protease is usually used for brewing beer [12] and de-bittering soy sauce in the

food industry [13]. For commercial applications, several researchers have focused on isolating new enzymes having satisfactory properties [14]. Due to good solubility, high stability in extreme conditions, substrate specificity and activity over broad-ranging temperatures and pH scales, plant proteases had been used in numerous industries [15]. In several food industry procedures, plant sources proteases are mostly used, therefore, the proteases are cost-effective for use in industries [16]. Furthermore, researchers are searching for novel proteases from plant sources which have cost-effective in industries and have important physiological roles [17].

At present, the important focus is to find a neutral protease from economical sources which have a high affinity to protein, acid–alkali resistance and good thermal stability. Because Pakistan is an agricultural country and cotton is a major crop and cotton seeds are frequently available and cheap sources, therefore, in this study, a neutral protease from cotton seeds was purified and characterized.

Material and Methods

Cotton seeds (BS-15) were purchased from a local market. Ammonium sulphate, Casein acid hydrolysate, peptones and other reagents/chemicals were used in high analytical grade and purchased from a local vendor of Sigma (USA), Merck (USA), and Fluka Company (Switzerland).

Preparation of Soluble Enzyme and determination of Protein and Protease activity

Cotton seeds were de-coated, crushed, defatted and homogenized in chilled acetone. On the other hand, 10% enzyme solution and 2% casein acid hydrolysate substrate were prepared in chilled 0.2 M Tris-HCl buffer as reported earlier [18,19]. Protein content was calculated by the method of Lowry [20], with bovine serum albumin as a standard. Enzymatic solution protein absorbance was monitored at 280 nm. The activity of Protease was checked by the method of Anson [21] with slight modification. In this method, 2% casein acid hydrolysate was used as a substrate. At the end of the reaction, protease activity was checked by spectrophotometer at 625 nm, as reported earlier [18].

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

Isolation, Dialysis and Purification of cotton seeds Protease by using Sephadex G-100 Column Chromatography

Two-fold ethanol, acetone, methanol and 40%, 60% and 80% saturated ammonium sulphate was used to purify protease. All these chemicals were retained at 4 ^{o}C for 4-5 hours. The collected precipitates from different chemicals were dissolved separately in 10 ml of Universal buffer of 7.0 pH and their protease activity was determined by an earlier reported method. On the basis of the highest protease activity, precipitates were obtained from 60% saturated ammonium sulphate dialyzed for 24 h in a universal buffer of 7.0 pH. The collected precipitates and dialyzed samples were centrifuged in a cooled refrigerated centrifuge at 7000 rpm for 20 minutes and protease activity was determined by the reported method.

Sephadex G–100 gel column (60 × 2cm), previously packed and equilibrated with Universal buffer pH 7.0. On this Sephadex G–100 gel column, 5.0 ml of dialyzed sample was loaded. The dialyzed sample was eluted with the same buffer pH 7.0 at the flow rate of 40.0 mL/h. The fraction of 5.0 mL was collected using fraction collector EYELA

UV–9900, UV-Visible detector. The protein absorbance was monitored at 280 nm.

SDS-Gel Electrophoresis

SDS gel electrophoresis using buffer system, as described by Peterson [22] and Hames [23] was used to determine homogeneity of the purified enzyme.

Kinetic Study and Characteristic Properties of Protease Enzyme Effect of Substrate Concentration and substrate specificity on Protease Activity

Casein acid hydrolysate and peptone animal were used as substrates of different concentrations from 0.5-3% and checked their effects on the rate of enzymatic reaction of protease. 1.0 mL of purified fraction sample and 1.0 mL substrate of different concentrations were incubated at 20°C for one hour.

The substrate specificity with various substrates was also calculated by using different substrates. The mixture of a fixed amount of enzyme and substrate was incubated for one hour at 20°C. After incubation protease activity was checked as previously described by the standard protease assay method.

Effect of pH, Temperature, Thermal stability and metal ions / reagents on Protease Activity

To check the effect of pH on the protease activity of purified Fraction-IV, various pH ranges from 4 to 10 by using a universal buffer pH 7.0 were used. The enzyme was incubated with casein acid hydrolysate as a substrate. The optimum temperature of the purified protease enzyme was determined by incubating the enzyme-substrate mixture at various temperatures in the range of 10°C to 50°C. On the other hand, the thermal stability of protease was achieved from purified fractions by measuring the residue activity after incubation of the enzyme at various temperatures ranging between 20°C to 80°C for 10 min with and without the addition of activators at 20^{o}C. The effect of time period on enzyme thermal stability was also noted by heating enzyme samples at 5–20 min with and without the addition of an activator at 40^{o}C. The different concentrations (mM) of several chemicals and metal ions were incubated with purified Fraction-IV for 10 minutes at optimum temperature before adding substrate. The remaining protease activities of each parameter were determined by the previously described method.

Results and Discussions

Purification of Protease by Chromatography

The solvent extraction of cotton seeds enzymatic protein extract (20%) at pH 7.0 was done. For this purpose, cotton seeds, and enzymatic protein extract (20%) precipitated with different solvents such as methanol, acetone, ethanol and various concentrations of ammonium sulphate (40, 60 and 80%). It was observed that cotton seeds enzymatic protein extract with 60% ammonium sulphate precipitation produced the highest protease activity in compression to solvents precipitation, as illustrated in Table 1. Therefore, ammonium sulphate (60% saturated) concentration was selected for precipitating enzymatic protein for further investigation.

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

Purification of cotton seeds protease was done by Sephadex G-100 column chromatography and the elution pattern is shown in Fig.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 dialyzed sample was applied to Sephadex G-100 column (60 × 2 cm), which was packed and equilibrated with the same buffer at a flow rate of 40 mL/h with 10 mL of fraction volume. The elution of enzymatic protein was monitored at 280 nm. 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 as exhibited in Fig. 2.

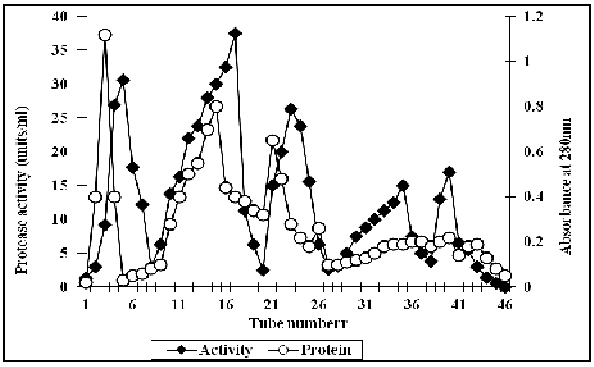

Figure 1. **Purification profile of protease of cotton Seeds by Sephadex G-100**

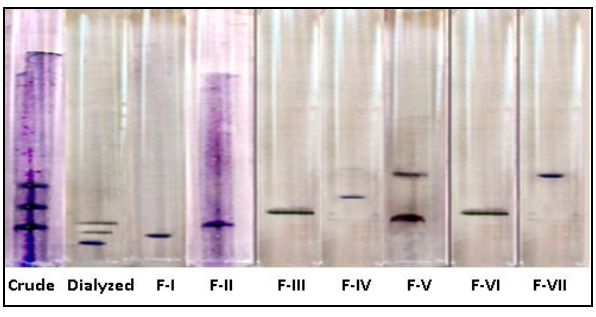

Figure 2. **SDS Polyacrylamide Gel Electrophoresis of Cotton Seeds Protease (Fraction-IV)**

All fractions were individually characterized and found alkaline, acidic and neutral. However, in terms of optimum temperature, pH, substrate concentration, thermos ability, substrate specificity and effect of various reagents, Fraction-IV was neutral in nature, further characterized and reported in present study. The protease of Fraction-IV was purified to 1.52 fold with the percent yield of 10.5. While specific activity was found to be 51 units /mg protein. The overall purification profile of alkaline protease fraction is presented in Table 2.

Table 2. **Purification profile of protease from cotton seeds (Fraction- IV).**

Purification Steps	Total Protein mg	Total Protease Activity µg	Specific Activity µg /mg	Purific-ation 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-IV	17.5	895	51	1.52	10.5

Effect of Temperature on Protease Activity

From the industrial point of view, the optimum temperature of an enzyme, especially proteases is considered a significant factor. Temperature affects the speed of enzymatic reactions by disturbing the structure of the enzyme and boosting the meeting of the substrate with the active site [24]. Hence, as the temperature is elevated, the speed of enzymatic reaction also increases as long as the natural structure of the protein is spoiled. Higher temperatures denaturation the enzymes mainly by breaking hydrogen bonds. To determine the optimum temperature of Fraction-IV protease, the enzyme-substrate mixture was incubated at a different temperatures ranging from 10 to 50°C. Fig. 3, shows the maximum relative activity (100%) for Fraction-IV protease at a temperature of

20°C. It is mentioned that the protease activity with an optimum temperature 20°C or less than 20°C is considered a cold protease [25]. After that optimum temperature, enzyme activity starts to decrease as temperature increases and is completely lost at a temperature reached 40°C. After 20°C decline in activity was observed and this is due to the thermal denaturation of Fraction-IV protease. Huston et al. have isolated a protease from *PsychrophileColwellia psychrerythraea* Strain 34H, which has a low optimum temperature (19°C) [26]. The activation energy for the protease of Fraction-IV is calculated at 12.47 KJ/mol.

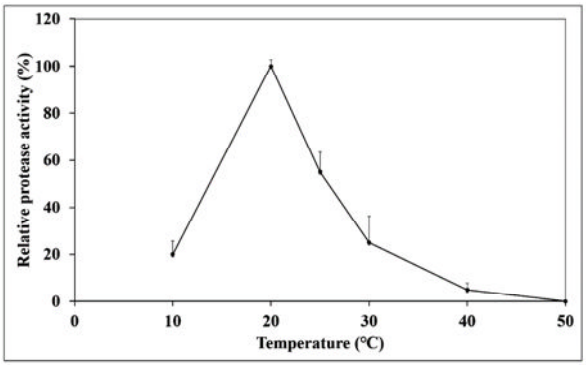

Figure 3. **Effect of temperature on purified protease activity of cotton seeds (Fraction- IV)**

Effect of pH on protease activity

pH is an important factor for all enzyme activity. The pH at which the enzyme showed maximum activity, is called optimum pH. The optimum pH is considered a key factor for all enzymes in terms of their activities and production, variation in pH leads to enzyme inactivation [27]. Nevertheless, some proteases extracted from plants are exceptional, they are active in a wide range of pH and temperature. In this study, the pH activity of proteases isolated from cotton seeds protease samples was calculated at different pHs (5, 6, 7, 8, 9 and 10). The enzyme activity increases as pH increases to 7 and then declines steadily up to pH 10. This trend showed that the enzyme has optimum activity at pH 7 as presented in Fig. 4. It is documented that Metallo-proteases are in the range of neutral to alkaline [28], and pH studies reported an increased enzyme activity at a pH range of 5-7. The functions of an enzyme rely on its structural integrity, as the structure of the enzyme is changed, the enzyme loses its functions. The remarkable ranges of pH activity of enzymes favor acidic and neutral environments. Bijina isolated protease inhibitor from Moringa oleifera with potential application as therapeutic drug and as seafood preservative at pH 7. [29], Siritapetawee isolated and purified protease from *Artocarpus heterophyllus* (jackfruit) latex and found maximal activity between 55 and 60 ℃ at pH 8. [30].

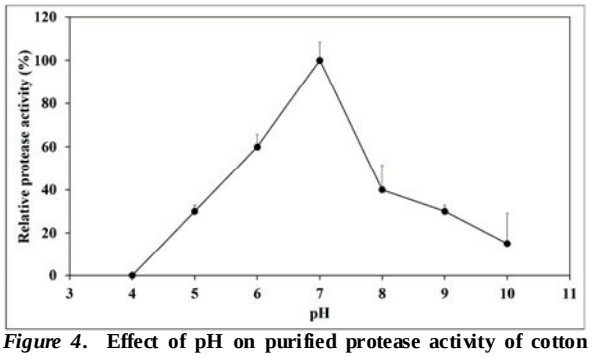

Figure 4. **Effect of pH on purified protease activity of cotton seeds (Fraction-IV)**

Effect of Metal ions / chemical reagents on Protease activity

The effects of different metal ions on protease activity are presented in Table 2. The activity of the purified protease was enhanced up to 15 and 20% by Ca^{2+} and Zn^{2+} respectively, while metal ions Ag+, Hg^{2+}, Co^{2+} and Mn^{2+} decrease enzyme activity with the proportions of decrease 30, 35, 70 and 65%, respectively. On the other hand, tested chemicals such as Tween 80, Triton X-100, Sodium dodecylsulphate (SDC), Sodium deoxycholate (SDS), Mercaptoethanol and Cysteine also have strong inhibition effects 35%, 20%, 85%, 75%, 70% and 50%

respectively on the enzyme activity in variable degrees in comparison with the control as exhibited in Fig.5. It was documented that Plant metalloproteases are generally zinc and calcium-dependent enzymes and calcium are well-known to interfere with the three-dimensional structure and catalytic properties of enzymes [31]. These findings showed that Fraction-IV protease belongs to Metallo protease and Zn^{2+} and Ca^{2+}ions stabilize the enzyme structure and protect the protease against thermal denaturation [32]. Ca^{2+} and other metal ions dependent protease from leaves of *Moringa oleifera* was also reported. Ramakrishna has isolated Metallo-protease from Dry Grass Pea Seeds [33].

Table 3. **Effect of various metals / reagents on purified protease activity of cotton seeds (Fraction-IV).**

Reagents	Activity	% of relative	% of
5mM Conc.	*units/ mL*	*protease activity*	*activation/ [inhibition]*
Control	8	100	
Tween 80	5.2	65	35
Triton X- 100	6.4	80	20
SDC	1.2	15	85
SDS	2	25	75
$AgNO_3$	5.6	70	30
$HgNo_3$	5.2	65	35
Mercaptoethanol	2.4	30	70
Cysteine	4	50	50
O-Phenanthroline	7.4	93	07
EDTA	7.6	95	5
$CoCl_2$	2.4	30	70
$MnCl_2$	2.8	35	65
$CaCl_2$	9.2	115	15
$ZnCl_2$	9.6	120	20

Effect of Thermostability on Protease Activity

The stability of an enzyme depends on the sum of numerous weak, non-covalent interactions such as Van der Waal interactions, hydrogen bonds and hydrophobic effects. All of these non-covalent interactions are upset by environmental conditions, such as temperatures. The enzyme degradation mechanisms are significantly speeded at high temperature, and thus impart an important role in the thermo-inactivation of enzymes. Thermo-stability is considered a crucial characteristic of protease enzymes required in various industries. Those enzymes which are thermostable are considered beneficial from the industrial point of view. It was mentioned in the literature that the thermal stability of an enzyme is a key factor for its biotechnological applications.

Thermal stability of purified cotton seeds Fraction-IV protease was accomplished by heating the enzyme samples at various temperatures ranging from 20°C to 70°C for 10 minutes with and without the addition of activators ($ZnCl_2$ and $CaCl_2$). After heating, the enzyme samples were cooled, and the remaining activities were determined by standard method. Moreover, thermostability was also determined at various times from every interval of 5 min (5 to 20 min) at fixed incubated temperature with and without the addition of activators. The protease activity of cotton seeds Fraction-IV was retained at 25% at 50°C. Fraction-IV protease activity was increased by 25% at 50°C by the addition of 5mM $ZnCl_2$ in the enzyme samples heating for 10 min, and results are shown in Fig. 5. Furthermore, the data depicted in Fig. 6 showed the effect of heat treatment on protease activity at a variable time (5-20 min) and fixed temperature (40°C) with and without the addition of activators. However, Fraction-IV protease activity was retained at 60% without and 72% with $ZnCl_2$ for 20 minutes of incubation. These results are comparable with other sources of proteases reported in the literature. Protease from *Salvadorapersica* was Stable up to 50°C [34], protease from Baby Kiwi was stable up to 45°C [35] and protease from *Citrullus colocynthis* was stable

up to 40°C. Some other researchers, such as Gonçalves et al. have isolated thermostable proteases from the leaves, seeds, roots, and stem of *Canavalia. Ensiformis* [24].

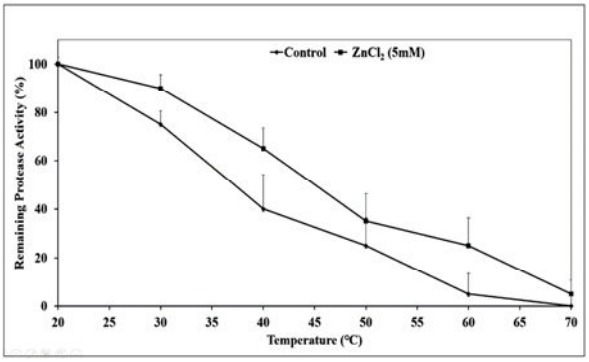

Figure 5. **Thermostability of Purified protease activity of cotton seeds (Fraction-IV) with and without $ZnCl_2$ for 10 min**

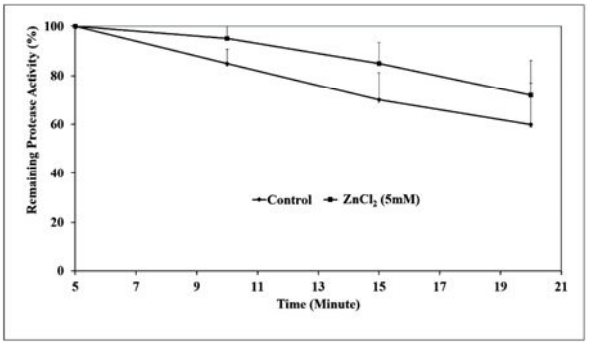

Figure 6 **. Effect of heat treatment at (40°C) with and without $ZnCl_2$ at various time period on purified protease of cotton seeds (Fraction-IV)**

Effect of Substrate Concentration on Protease activity

It is well-known consideration that substrate concentration is a key factor for determining the enzyme's activity beside pH and temperature. Furthermore, it is also used for the calculation of enzyme kinetics (K_m and V_{max}) to elucidate the enzyme specificity and affinity. In this connection, different concentrations of Casein acid hydrolysate from 0.5 to 3% concentration at 20°C were used with respect to their optimum time period. The activity of Fraction–IV protease was examined, and results are signified in Fig. 7. Moreover, 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 dropped. Praiwala et al. [36] have used several amounts (0.2, 0.4, 0.6, 0.8 and 1.0ml) of Hemoglobin as a substrate incubated with protease obtained from Green gram seeds and Soybean seeds. N-α-tosylL-arginine methyl ester (L-TAME) was used as substrate with proteases isolated from leaves, seeds, roots and stem of *Canavalia ensiformis.*

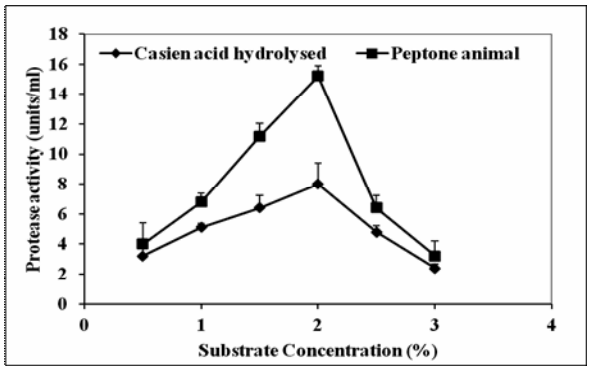

Figure 7. **Effect of substrates on purified Protease activity of cotton seeds (Fraction-IV)**

Estimation of K_m and V_{max} of protease

The K_m value is a tool to measure the enzyme affinity to the substrate. It is fact that smaller k_m values indicate greater Enzyme-Substrate affinity [37]. The value of K_m shows the highest rate achieved when the enzyme sites are saturated by the substrate of the reacted enzyme. The K_m value of Fraction-IV protease was calculated at 0.08M (Table 4) by double reciprocal plots according to Lineweaver and Burk [38]. The K_m value of (0.08) of the present work is lower than reported by other researchers who isolated protease enzymes from different plants like Yemeni bean seeds [28]. K_m is 0.19M, field bean seeds enzyme 0.0105M [39], *Moringa Oleifera* leaves is 0.107M [40] and from the seeds of *Cucumis melovaragresticis* 0.02M [41] and seeds of ash groud *Benincasa hispida* (thumb) Cogn is 0.01M [42]. It is clear from

the results obtained in this work, that the K_m value of Fraction-IV protease is lower than other proteases hence greater affinity towards substrate.

On the other hand, the value of V_{max} of Fraction-IV protease was also calculated which comes out 19 µmol/min as presented in Table 4. This value of V_{max} is higher than proteases isolated from seeds of Green gram(V_{max} 2 µmol/min) and Soybean(V_{max} 1.01 µmol/min) but lower than those obtained from proteases isolated from seeds of *Citrullus colocynthis* (557 µmol/min) [43]. Ibraheem and Malom have reported two neutral proteases, Pll and Plll from *Citrus sinensis* fruit peel, having a value of V_{max} are 192.31 and 111.11 µmol/min, respectively [44].

***Table 4*. K_m, V_{max} values, Arrhenius plot of the activation energy of purified cotton seeds protease (Ea=slopxR, where R=8.314KJ/mol).**

	Km values mol/L	Vmax values µmol/min	Activation energy KJ/mol
Fraction-IV	0.08	19	12.47

Effect of substrate specificity of protease

The substrate specificity has great importance in biotechnological fields. The substrate specificity of purified protease Fraction-IV was determined by incubating the enzyme with different protein substrates prepared in universal buffer pH 7 and results are depicted in Fig.8. The comparative activity of Faction-IV protease for the degradation of casein was taken as 100%. Experimental results showed that Fraction-IV protease strongly hydrolyzed different peptones proteins such as Peptone soy meal 198%, Peptone meat 169%, Peptone animal 159%, Peptones protease 143%, Peptone casein 109% but less hydrolyzed Hemoglobin 72%, Caseinn soluble 43%, Lactalbumin 40%, Azocol 24%, Azocasien 15% and Albumin 10%. These findings reflect that peptones are favorable substrates for Fraction –iv protease. It is the obvious conclusion from the above results that cotton seed Fraction-IV protease strongly favors hydrolyzed peptones as compared to other substrates. Some other investigators have also demonstrated substrate specificity. Raghunath has isolated protease from the latex of the *Euphoriba Prunifolia* Jacq, which shows proteolytic activity of 72.14% with keratin, 58.61% with egg albumin, 53.18% with gelatin, 38.61% with hemoglobin and 27.51% with bovine serum albumin used as substrates [45]. It was reported in the literature that *Salvadora persica*protease hydrolyzed proteins as casein 100%, hemoglobin 95%, egg albumin 72%, gelatin 68% and bovine serum albumin 53%. Ademola Ibraheem and Malom have purified two neutral proteases Pll and Plll from *Citrus Sinensis* fruit peel. Both enzymes Pll and Plll exhibited relative activities are 75% and 91%, but for gelatin 125% and 109%, respectively [44].

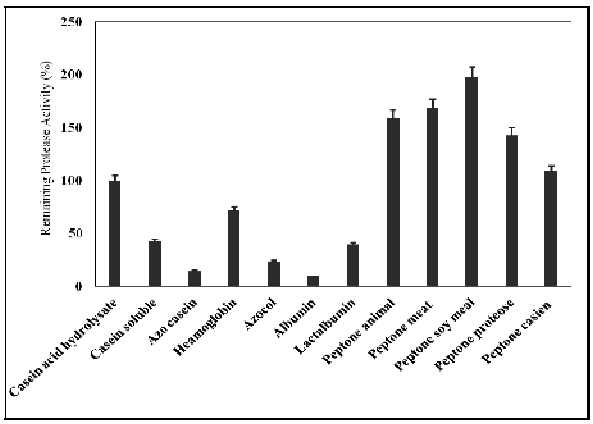

***Figure 8*. Substrate Specificity of purified protease activity of cotton seeds (Fraction-IV)**

Conclusion

It is concluded that the obtained protease Fraction-IV from cotton seeds by Sephadex G-100 column chromatography was found neutral, having pH 7. On the basis of kinetic parameters, protease activity of

Fraction-IV was found heat stable and increased by 20% at 50°C in the presence of an activator ($ZnCl_2$). Both $ZnCl_2$ and $CaCl_2$ enhance activity of Fraction-IV protease. The Fraction-IV was found Metallo protease. This enzyme favors degraded peptones as compared to other substrates. On the basis of investigational results, it is concluded that the neutral protease of Fraction-IV is successfully used in food, pharmaceutical and leather industries because of their diverse benefits, including low pollution levels.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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