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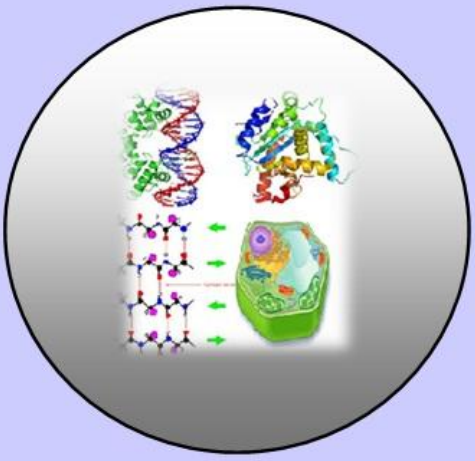

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RESEARCH PAPER

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Isolation of Fungal Protease from *Aspergillus* species and its Application

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ABSTRACT

Some fungi produced fungal protease on screening of soil, the mesophylic species of Aspergillus fumigatus. it was identified that some proteolytic activity is present, thus may assumed that the presence of enzyme protease thereafter the fungal protease isolated and purified from the cultivated A. fumigatus on optimized medium showing a single peak on spectrophotometry with total activity 352.6 U/ ml the molecular weight of isolated fungal protease was estimated of 24 KDA.

The purified fungal protease showed promising efficiency as destaining activity to clean the spot on cloth. The combination of enzyme and detergent resulted the cleaning of cloth pieces and dried blood spot thus can be use as detergent additives.

The purified enzymes can also be use for dehairing of leather.

Keywords: Fungal Protease, Soil, Aspergillus species, Isolation, Proteolytic activity and Biotechological Applications.

INTRODUCTION

Enzymes are the catalysts and catalyzed the biochemical reaction in living cells. Protease represents one of the important most group of enzyme. According to the Nomenclature Committee of International Union of Biochemistry and Molecular Biology, proteases are classified in sub group 4 of group 3 (Hydrolases) (Rao et al., 1998).

Proteases have been isolated from microorganism, animals and plants, out of these, microorganism represent the most common source of enzymes (Zhang and Kim, 2010). About $2/3^{rd}$ of the proteases are from microbial sources. The various microorganisms such as bacteria, fungi and actinomycetes are known to produce these enzymes (Devi et al., 2008). Dane first isolated protease from *Bacillus licheniformis* but Devi et al., 2008 isolated proteases from *genera Aspergillus*, Penicillium and Rhizopus. All the proteases are proteinceous material. The proteases are proven superior and efficient for selective removal of the non collagen part of skin.

The main objective of this work was to isolate, purify and characterized the fungal protease by the application of biotechnology.

MATERIAL AND METHODS

Media and Reagents

Czapek's agar medium (g/l): Sucrose, 30.0, KCl, 0.5, $FeSO_4$, 0.01, $MgSO_4$, 0.5, $K2HPO_4$, 1.0, agar 15.0, Rose Bengal, 1:30.000, Strpomycin 30 µg/ml (Oxoid, 1982).

Production medium (g/l): Malt extract, 20, Fructose, 5, Urea, 0.005, salt solution of (KH_2PO_4, 1.0, KCl, 1.0 $FeSO_4$. $7H_2O$, 0.02, $MgSO_4$. $7H_2O$, 0.5) and pH of the medium adjusted at 8.0 (Saravankumar et al., 2010).

Isolation and Identification of fungi

Isolation of Fungi

Soil samples around local detergent factory and are collected, using clean and sterile containers.

Soil samples (10 g) are transferred into 250 ml conical flasks containing 90 ml sterile distilled water. Serial dilutions, 10^{-1}, 10^{-2} and 10^{-4} were prepared and shaked for 30 min. Aliquotes of 0.5 ml of 10^{-4} dilution were placed in the bottom of two sets of plates each containing three replicates sterile Petri dishes. 15 ml of Czapek's agar medium containing streptomycin and chloramphenicol were poured on the plates and dispersed by gentle rotation. The plates allowed for solidification, and inoculated.

The growing fungal species were purified by streaking repeatedly on slants containing the same medium.

Identification of Fungi

The purified colonies were phenotypically identified up to the species level by microscopic examination. This was made through the help of the several references.

Reference books for Identification

Barnett (1960), Raper and Fenell (1965), Raper and Thom (1968), Barnett and hunter (1972), Raper and fennel (1977), Pitt (1979), Domsch et al., (1980), Kitch and Pitt (1992), Moubasher (1993), Barnett and Hunter (1994), Watanabe (2002).

Assay of Fungal Protease:

The assay was carried out for the five isolated *A. fumigatus* fungi which were cultivated on Czapek's liquid at 45 ^{O}C for five days.

Qualitative Assay

Gelatin liquefaction

This is carried out according to Ali et al., (1977). 2 % gelatin solution was prepared by dissolving the gelatin powder in water on a boling water bath. Aliquots, 2ml each, were poured in test tubes.

The tubes were left in refrigerator till solidification. Standard disc 5 mm diameter from each fungus, previously grown on Czapek's agar medium for 5 days was inoculated onto the surface of the solidified gelatin in the tubes.

These inoculated tubes were incubated at 25-28 ^{O}C for 24-28 hr.

Control tubes were made using water instead of the fungal discs. After the incubation period, the tubes were left again in the refrigerator for four hours to insure that liquefaction is due to gelatin hydrolysis.

Cup Plate Method

The cup plate were prepared according to Dorey et al., (1965) by adding 50 ml of 2.4 % gelatin in 0.2 % thiomersalate to 50 ml of 4 % melted agar in 0.1 M of citrate or phosphate buffer. The pH was adjusted at 8 with 0.1 N NaOH or HCl using pH meter. This mixture was poured into 9 cm diameter Petri dishes to give a 5 mm layer of substrate which was allowed to solidify at room temperature. 5 mm diameter wells were made in the solidified plates using a sterile Cork-borer and the bottom of each well was sealed by gentile heating of the bottom of the dish. Saturated 5 mm fungal disc or 0.15 ml culture filtrate were putted inside wells and the cup plates were incubated at 30 ^{O}C for 24-48h and the clear zones of substrate hydrolysis were measured by treatment with 20 % TCA. Proteolytic activity was detected by development of a clear zone around cells containing the active enzymes.

Quantitative Assay

Determination of Total Proteins

Proteins were determined using the method of (Lowry et al., 1951) using bovine serum albumin as a standard protein

Reagents

Solution A: 2% Na_2CO_3 in 0.1 M NaOH

Solution B: 0.5% $CuSO_4$ in 1% (W/V) sodium or potassium tartarate.

Solution C: Mix 50 ml of reagent A with 1 ml of reagent B, renewed daily

Solution D: Folin, reagent (BDH) diluted with water 1:3.

Protein sample (0.1ml) of the fungal filtrate was added to 5 ml of solution, mixed well and allowed to stand for 10 min at room temperature. Solution D (0.5 ml) was added with immediate mixing then incubated for another 30 min to allow the color developing. Absorbance of sample was measured using spectrometer at 660 nm. Then the concentration of protein was calculated from the standard curve.

Purification of fungal protease

A. fumigates (three fungal discs/50 ml medium) was cultured on the optimized shaking medium at (150 rpm) for 7 days consisting of malt extract 20g/l, urea 7g/l, fructose 5g/l, salt solution 1.25 % and its pH was adjusted to be 9.

The culture filtrate (3 liters) was taken as source for extracellular alkaline protease. The following purification steps represent a typical example for preparation of homogenous *A. fumigates* protease.

Unless otherwise stated 0.1 M Tris-HCl buffer 8.0 was used and all purification steps were performed.

Crude extract

The crude extract was prepared by filtering the both through 4 layers of gauze and then through Prechilled puchner. The solution was then rotated in refrigerated centrifuge at 20000 rpm to remove the muddy matter. The protein content and the protease activity were assayed for this extract preparation.

Ammonium sulfate fractionation

Solid ammonium sulfate was added to the enzyme solution to 40-80 % saturation (Segel 1968). The precipitate was collected by centrifugation, dissolved in 0.1 % Tris-Hcl buffer (pH 8). These steps were repeated at least three times to assure complete elution of the co-precipitate enzyme. Then the precipitate was dissolved in minimal volume of 0.1 m Tris-HCl buffer and dialyzed against same buffer over night at 4^0C, until the enzyme solution inside the dialyzed bag became free of sulphate. This was achieved by changing the buffer outside the bag several times. After complete dialysis, the protein content and the protease activity were assayed. The partially purified enzyme was saved in refrigerator for further purification steps.

Column chromatography

DEAE-cellulose chromatography

The chromatography of protein on a cellulose ion exchanger involves primarily the establishment of multiple electrostatic bonds between charged sites on the surface of the adsorbent and sites bearing the opposite charge on the surface of the protein molecule. The number of such bonds that can be established will determine the concentration of competing ions required for the release of the bound molecule. Thus proteins differing significantly in charge density or in number of charges virtue of size may be expected to differ in their requirements for elution. The charge distribution can also be a factor. However, it is the total effect of these factors that determines affinity of the protein for the adsorbent. So a simple relation between any one of them and the chromatographic behavior of the protein in question will not always be obtained. The situation is further modified by the possibility that in some cases the non-electrostatic forces might play an important role.

Differential elution is accomplished by reducing the number of charges on the protein molecule through the appropriate changes in pH or by decreasing the effectiveness of existing s by increasing the concentration of salt. The precise manner in which this is done is subject of great variation, depending on the specific properties of the proteins involved, the objectives of the experiment, the time and equipment available (Peterson and Sober, 1962).

Selection of adsorbent

Diethyl amino ethyl cellulose (DEAD-cellulose) (micro granular form, fully swollen and supplied wet) was used as an adsorbent (Knight, 1967).

Choice of conditions

Operating temperature: 0-5^{0}C at cold room.

Types of separation: Column separation.

Buffer system: Tris HCl buffer, pH 8.

Flow rate: 150 ml/hr using peristaltic pump.

Equilibration

The ion exchanger was equilibrated with 0.1 M Tris-HCl buffer pH 8.0. The column was poured, the starting buffer was passed through the column until the conductivity and pH of the effluent became exactly the same as the starting buffer (Peterson and Sober 1962).

Column packing

The column was packed as follows

1- A column end unit was fitted into the bottom of a column and tightened into a place. The column was set up vertically and an extension tube via a suitable connector was added, so as to make a smooth joint inside. The extension tube was half as long as the final length of the required bed.

2- The slurry was gently stirred and poured into the column through a wide necked funnel. The effluent was allowed to run to waste.

3- After adding all slurry, a column end unit was inserted into the top of the extension tube, tightened and connected to a peristaltic pump at a flow rate of 150 ml/h/sq. cm. cross sectional area. It is important that the flow rate at the end of the packing operation is greater than the desired elution flow rate to avoid subsequent decrease in bed volume.

4- The column was allowed to pack under these conditions until the bed height attained a stable value. This point is reached with in 60 minutes for 30 cm bed.

5- Once the bed was packed and consolidated, the pump was disconnected and flow from the bottom of the column was shut-off by tap; the top column end unit was released and withdrawn. The buffer was siphoned off in the extension tube and dismantled.

6- The column was filled over flow with buffer and a column end unit was inserted carefully so as to exclude all air.

7- The column end unit was pushed carefully into the column until it firmly touched the top of the bed and tight it. Displaced buffer was allowed to escape freely via the top feed tube, the bottom of the column remaining shut off.

8- The top unit was reconnected to the pump and the bottom tap was opened. One bed volume of buffer was pumped through the column at the desired elution rate and absence of dead space between end unit and column bed was checked before loading with enzyme solution.

Sample loading

The dialyzed enzyme solution was loaded at a controlled flow rate.

Stepwise elution

The enzyme was then eluted in succession with 0.1 M Tris-HCl buffer of pH 8.0 (500 ml), 0.05 M NaCl in 0.1 M Tris-HCl buffer of pH 8 (200 ml), 0.075 M NaCl in 0.1 M Tris-HCl in 0.1 M Tris-HCl buffer of pH 8.0 (200 ml), 0.1 M NaCl in 0.1 M Tris-HCl buffer of pH 8 (200 ml), 0.15 M NaCl in 0.1 M NaCL in 0.1 M Tris-HCl buffer of pH 8 (200 ml), 0.15 M NaCl in 0.1 M Tris-HCl buffer of pH 8 (200 ml), 0.2 M Nacl in 0.1 M Tris-HCl buffer of pH 8 (200 ml).

Ten ml fractions ware collected using fraction collector at a flow rate of 150 ml/h. The protein content and protease activity of each fraction was determined. The fractions which showed protease activity were pooled and lyophilized for further investigation.

Sephadex G-100 column chromatography

The active fractions collected from DEAE-cellulose were then applied to the Sephadex G-100 column previously equilibrated with 0.1 M Tris-HCl buffer at a flow rate of 150 ml/h and the fraction volume of 5.0 ml/tube. The protein content and protease activity of each fraction was determined.

Determination of the molecular weight of the purified enzyme by SDA-PAGE Electrophoresis

Sodium dodecyl-sulphate-polyacrylamide system (Laemmli, 1970) with homogenous gel was used under reducing or non-reducing conditions.

The following solutions were required for the preparation of gels

Acrylamide stock solutions	30 % (W/V) acrylamide 80 % (W/V) methylene bisacryl-amide
Resolving gel buffer (pH 8.8)	1.5 M Tris-HCl
Stackling gel buffer (pH 6.8)	1.0 M Tris-HCl
Sodium dodecyl-suphate solution	(SDS) solution 10 % (w/v) SDS
Ammonium persulphate (APS)	60 mg/ml (always fresh)
N,N,N',N'-tetramethylethylen-Diamine (TMED)	
Sample buffer (5-fold concentrated)	Stacking gel buffer 3.2 ml Glycerol 3.5 ml Mercaptoetha 2.5 ml SDS 1.5 g Bromophenol blue 2.0 g
Electrode buffer (pH 8.8)	50 mM Tris-HCl 6 g/l 384 mM glycine 28.8 g/l 0.1 % (w/v) SDS 1 g/l

Normally, resolving gel with 12.5 % acrylamide was used which had the following constitution

A	Acrylamide stock solution	1.1 ml
B	Stackling gel buffer	1.1 ml
C	SDS-solution (10 %)	80 µl
D	Water (bidist.)	5.82 ml
E	TMED	8 µl
F	APS	40 µl

The stacking gel contained 4 % acrylamide according to the following constitution

A	Acrylamide stock solution	1.1 ml
B	Stackling gel buffer	1.1 ml
C	SDS-solution (10 %)	80 µl
D	Water (bidist.)	5.82 ml
E	TMED	8 µl
F	APS	40 µl

Casting gels

The casting apparatus (thoroughly cleaned glass plates and spacers) was assembled vertically. Resolving gel components (a-d) were mixed and deaerated by applying vacuum, then components (e) and (f) were added and mixed gently. The resolving gel was poured immediately into the space between the glass plate to the desired height, over laid gently with water and was left to polymerize. After polymerization of the resolving gel, the water layer was removed. The stacking gel components (e) and (f) were added and mixed gently. The space over the resolving gel was filled with the stacking gel and an polymerization of the stacking gel, the comb and the spacer were removed. The glass plates were fixed in electrophoresis tank and the upper and the lower reservoirs were filled with electrode buffer. The air bubbles trapped beneath the bottom of the gel were removed by an bent syringe needle.

Sample preparation

A protein sample (12 µl) with protein content of 10-15 µg protein was mixed with 3 µl of sample buffer and heated in boiling water bath for 5 min followed by centrifugation at 14,000 rpm for 5 minutes at 4^0C. The protein samples were loaded into wells in stacking gel under electrode buffer using microliter syringe. Molecular weight standards were purchased for Bioard Chem. Company were prepared by mixing µl of marker protein with 11 µl water and 3 µl sample buffer together and treated similarly as the protein sample.

Electrophoresis run conditions

The electrophoresis was carried out at 4^0C under constant current (16 mA in stacking gel and 20 mA in resolving gel). When the marker dye (bromphenol blue) had reached the bottom of the gel, the electrophoresis was stopped and the glass plates were removed from the tank. The inner and outer glass plates were separated and the gel was detached from them and subjected to staining.

Silver staining of polyacrylamide gels (Morrissey 1981)

This method is more sensitive than coomassie staining. The staining was carried out at room temperature under agitation. The protein in the gel was fixed firstly in a solution containing 50 % methanol and 0.1 % formaldehyde in water for 30 min followed by soaking gel in a reduced solution containing 2.5 mg dithioerythrital (DTE) in 500 ml water for 30 min. the reduced solution consisting of 3 % (w/v) sodium carbonate and 0.45 % (v/v) formaldehyde in water, then was placed in fresh developer solution and was left to develop was stopped by adjusting the pH of the developer solution to neutrality with 2.3 M citric acid solution.

The gels were removed from the staining solution and de stained with 7.0 % acetic acid until a clear background, then photographed wet.

Calculation

The mobility was calculated as follows:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{Length of gel after destaining}} \times \frac{\text{length of gel before destaining}}{\text{distance of dye migration}}$$

The mobility was plotted against known molecular weight standard proteins and calculated the molecular weight.

Biotechnological Applications

Despotting property of purified fungal protease relative activity of fungal protease depending upon types of detergent, the detergent mixed with 10 mg / ml in ariel showed its compatibility and showed relatively activity 70.2 % and when mixed with tide it showed 36.7 % activity thus the relative activity of enzyme was highest in ariel.

The combination of enzyme and detergent resulted in cleaning of blood spot from cotton cloth.

Dehairing activity of enzyme in leather the purified enzyme was tested for dehairing of leather when leather was treated with enzymes solution 20 mg / ml complete removal of hair from leather takes place with 24 hrs.

RESULTS

The *A. fumigatus* was recorded highest in soil samples the fungal colonies therefore isolated from culture. The qualitative and quantitative assay of fungal protease activity showed that *A fumigatus* has high protease production with protease activity 398.0 U/ml. The results showed that lysing zone was larger in *A. fumigatus* with 1.1 cm diameter. The highest proteolytic fungal species was then further identified based on current universal keys, data were analyzed by T-test, the analysis variance (ANOVA) and significant of coefficient determinant by F test and P value.

Table 1. Full purification protocol of A*. fumigatus* protease.

Purification steps	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg)	Purification fold	Recovery %
Crude	151.28	882.0	5.8	-	100
Ammonium sulphate	54.4	790.2	14.5	2.5	89.5
DEAE-Cellulose	3.21	560.6	174.6	30.1	63.5
Sephadex G100	1.989	555.00	200.1	34.5	45.1

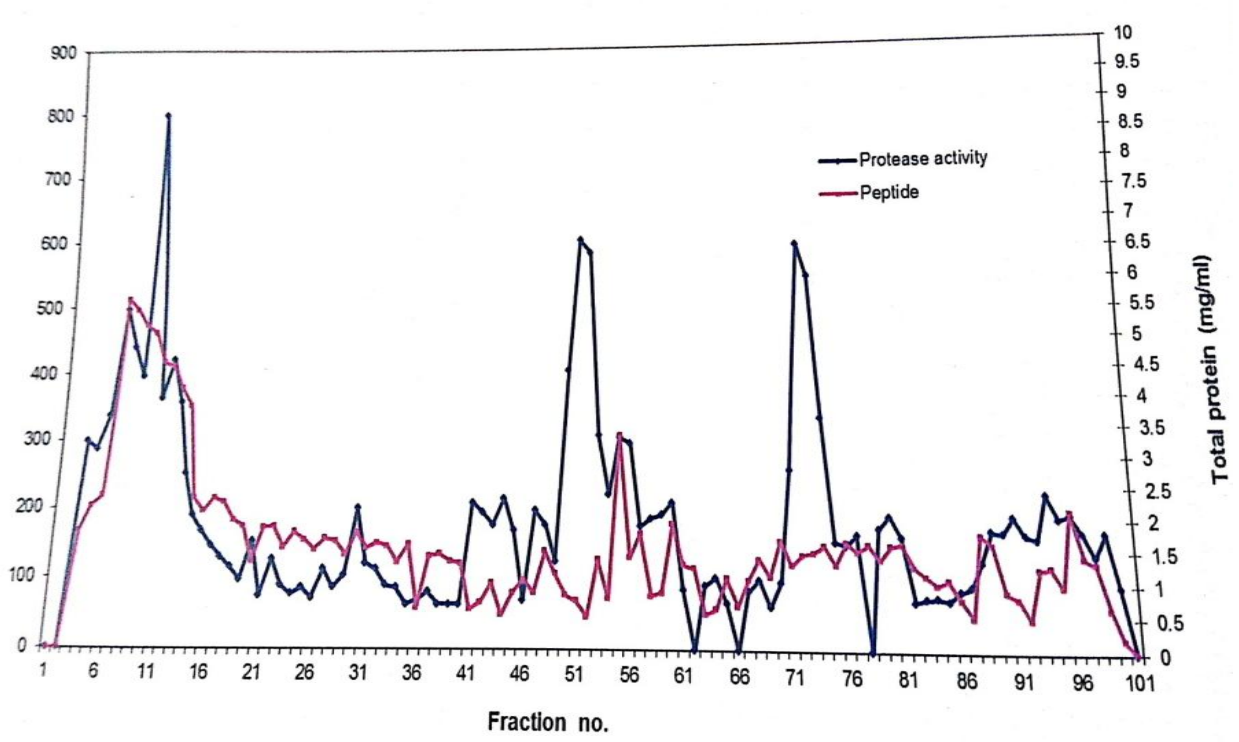

Figure 1. The anion exchange profile obtained on DEAE-Cellulose chromatography for fungal protease from *A. fumigatus.*

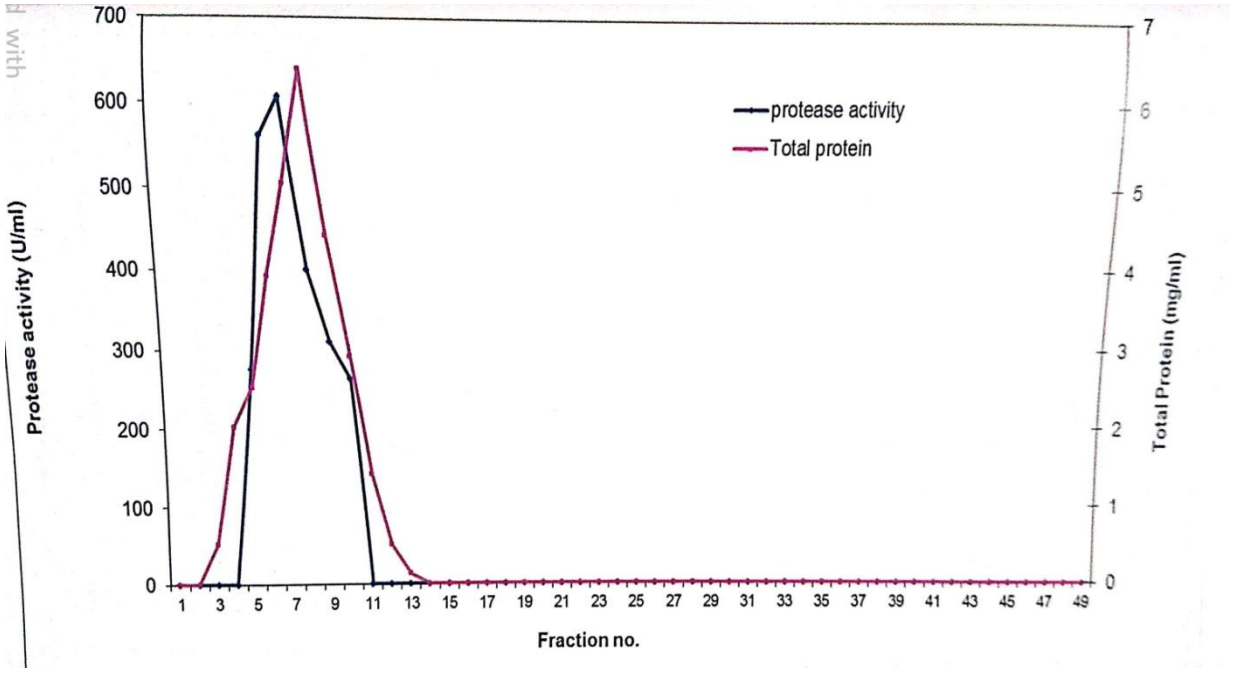

Figure 2. The obtained chromatographic profile from Sephadex G-100 for alkaline protease produced by *A. fumigatus.*

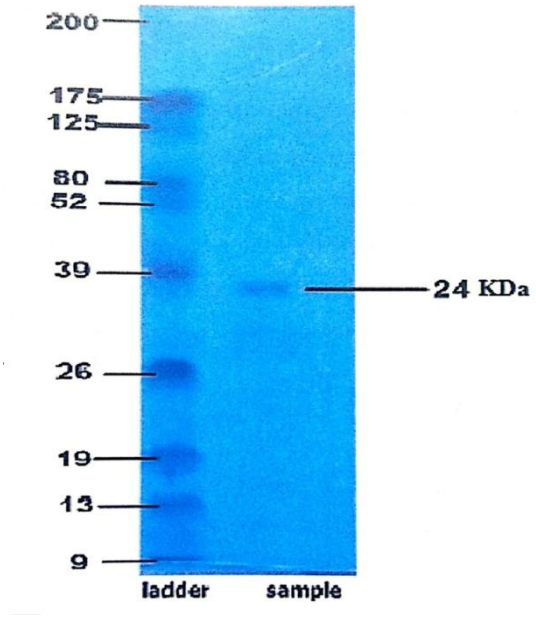

Figure 3. SDS-PAGE showing the molecular weight of fungal protease.

Purification of fungal protease produced by *A. fumigates*

The purification steps and yield of a typical experiment are summarized in table. The final step of purification resulted in single peak of proteolytic activity with 200.1 U/mg specific activity, 34.5 purification fold and 45.1 % recovery from the crude enzyme as given in table 1.

The molecular weight of enzymes was determined using SDS - polyacrylamide gel electrophoresis by the method is Laemmli, 1970 (Fig. 3).

The isolated samples of enzyme were spectrophotometrically examined and their protease activity was determined (Figure 1, Figure 2).

Biotechnological applications of enzyme fungal protease clearly showed highest relative activity of protease was in ariel 70.2 % and complete dehairing of goat leather takes place with in 24 hrs.

DISCUSSION

Fungal proteases are produced by a wide range of microorganism (Singh et al., 2001) fungi elaborate a wider variety of enzyme production than bacteria and such proteases obtained from fungi were more thermostable than bacterial enzyme (Fogarthy, 1996) in present studies fungus is cultured from soil and identified as *Aspergillus fumigatus* the study conducted to determined most promising *A. fumigatus* species for production of fungal proteases, (Monod et al., 1991) found that isolate of *A. fumigatus* is most suitable for this enzyme production, several other fungal species have already been reported to produce fungal protease (Malathi and Chakraborty, 1991, Den Belder et al., 1994, Dixit and Verma, 1993, Coral et al., 2003, Mirza et al., 2010 and Dubey et al., 2014).

Optimization of the Culture Condition

No definite medium has been established to optimized the production of fungal proteases from different microbial sources (Gupta et al., 2005), therefore optimization of the culture medium from maximum production of fungal protease was performed using Taguchi Factorial Design System.

Purification of Fungal Protease

Fungal protease from *A. fumigatus* was isolated and purified by conventional method using DEAE- cellulose method Sephadex G 100 method and characterized by using SDS-PAGE the molecular weight of fungal protease was estimated to 24 KDA by the method Tremacoldi et al., 2007 and Anandan et al., 2007.

The DNA and protein elucidated secondary structure and function relationship was similar to Rao et al., 1998 the isolated fungal proteases was biotechnologically very useful in despotting and dehairing.

Fungal proteases are biochemical catalysts with molecular masses lying in the range of 15 to 30 KDa (Kumar and Takagi, 1999). There are very few reports with fungal proteases of higher molecular masses 31.6 kDa, 33 kDa, 36 kDA and 45 kDa (Kumar abd Takagi, 1999), 32 Kda (Huang et al., 2003), 36.0 KDa (Durham et al., 1987), 45 KDa (Kwon et al., 1994) and 63 KDa (Namrata and Kantishree, 2001). However an enzyme of extremely low molecular weight of 8 KDa has been reported from *Kurthia spiroforme* (Steele et al., 1992).

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