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Production, isolation, purification and partial characterization of an extracellular acid protease from *Aspergillus niger*

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Abstract

Now a days, commercially available enzymes are not economically comparable to the chemical process. Hence, any substantial reduction in the cost of production of enzymes will be a positive stimulus for the commercialization of enzymatic depilation. Proteases are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme sale. Many thermophilic fungi produced thermostable proteases, such as in genus *Aspergillus*. The aims of this study are isolation, purification and characterization of the protease from a thermoplilic fungus isolated from fertilized hay. Fermentation, filtrate was estimated for acid protease activity, bovine serum albumin used as a substrate found that the broth showed proteolytic activity. As like observed that acetone precipitate also assayed for acid protease activity showed protease activity. From the Sephadex-200 gel filtration process the enzyme acid protease was separated the 13th fraction showed highest acid protease activity. The molecular weight of produced acid protease was revealed by SDS-PAGE, when compared with standard molecular weight markers the relative molecular weight of *A.niger* extracellular acid protease is between 40kd and 50kd molecular weight markers.

Keywords: Enzyme, Acid Protease, *Aspergillus niger*, fermentation, thermophilic.

Introduction

Enzymes commercially available now are not economically comparable to the chemical process. Therefore, any substantial reduction in the cost of production of enzymes will be a positive stimulus for the commercialization of enzymatic depilation. Proteases are one of the most important groups of industrial enzymes and count for nearly 60% of the total enzyme sale (1). The major uses of free proteases occur in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds(2). Solid-substrate fermentation (SSF) was chosen for the present research because it has been reported that previously as much greater productivity than does submerged fermentation (3). Economically, SSF offers many advantages, including superior volumetric productivity, use of simpler machinery, use of inexpensive substrates, simpler downstream processing and lower energy requirements with submerged fermentation.

Protease is an enzyme that hydrolyzes peptide bonds (4) and its usage in various industries is very significant. In dairy industry, protease is being used to coagulate the milk protein forming curds and ready to be used for cheese preparation. In food industry, proteases were used for improving the functional, nutritional and flavour properties in proteins especially in baking where it is used to degrade proteins in flour for biscuits, crackers and cookies. In pharmaceutical industry also, protease give a wide application such as in treatment of clotting disorder. It has been used for treatment of clotting disorder in pharmaceutical

industry while in detergent industry, used for protein stain removal. For leather industry, its usage is for unhairing and bating. Protease can easily isolate from various sources as well as plants, animals and microbial via fermentation process (4).

Enzyme production is a good value added to agro-industrial residues since they can be used in the production of enzymes by bioprocesses (5). This issue was related to "From waste to wealth" concept which is an idea that had been practiced nowadays by almost all people in the world. This statement gives an idea of using unwanted materials that can be recycled or becoming alternatives resources for other processes. The unwanted materials will basically refer to the wastes. An example of wastes come from agricultural and food industry because there are most abundant of agro-industrial residues on the Earth that actually possess such good potential as renewable resources (6). The utilization of wastes as substrate in the industrial enzyme production has made the fermentation process of industrial enzymes economically feasible.

Proteases are enzymes that hydrolyze peptide bonds of protein into peptides and amino acids, That can be classified into four major groups according to the character of their catalytic active sites and action: serine protease, cysteine (thiol) protease, aspartic protease and metalloprotease. Proteases constitute one of the most important groups of industrial enzymes accounting for about 60% of the total worldwide enzyme market because of their various applications in many industries such as the detergent, food, leather, silk, dairy and pharmaceutical industry (7).The value of worldwide sales of industrial enzymes was estimated to be US$ 1.7-2.0 billion (8). Many industrial processes take place at high temperature and therefore, the thermostable proteases are suitable for such industries. For example, the detergent industry is performed at 20-60C at a pH ranging from 7.5 to 10.5. The alkaline proteases are particularly important for this application, because they are both stable and active under high temperature, alkaline condition and in the presence of surfactants (9). The proteases in detergent industries account for 30% of the total worldwide enzyme production and represent one of the largest and most successful applications of modern industrial biotechnology. An alkaline protease with elastolytic and keratinolytic activities was in leather processing, this enzyme plays an important role as catalysts in technical process at 50-70°C (10).

Microorganisms including bacteria, fungi and yeasts are the important sources for thermostable proteases. Many thermophilic fungi produced thermostable proteases, such as in genus *Aspergillus* (11 &12). A large proportion of commercially available thermostable proteases were derived from Bacillus (13), an alkaline protease from *Bacillus licheniformis* API was suitable for tannery industry (14). Now, the thermostable protease from fungi having their major applications enzyme because fungal proteases offer a distinct advantage over the bacterial protease in terms of easing the downstream processing, the fungal cells can be easily removed from the final product by simple filtration, ability of fungus to grow on cheap substrate, easy immobilization of mycelium for repeated use, and they can grow in a broad range of pH, 4-11 (15).

Aspergillus niger is a member of the genus *Aspergillus* which includes a set of fungi that are generally considered asexual, although perfect forms (forms that reproduce sexually) have been found. Aspergilli are ubiquitous in nature. They are geographically widely distributed, and have been observed in a broad range of habitats because they can colonize a wide variety of substrates. *A. niger* is commonly found as a saprophyte growing on dead leaves, stored grain, compost piles, and other decaying vegetation. The spores are widespread, and are often associated with organic materials and soil (11, 12 & 16).The primary uses of *A. niger* are for the production of enzymes and organic acids by fermentation. While the foods, for which some of the enzymes may be used in preparation, are not subjected to TSCA, these enzymes may have multiple uses, many of which are not regulated except under TSCA. Fermentations to produce these enzymes may be carried out in vessels as large as 100,000 liters (17). *A. niger* is also used to produce organic acids such as citric acid and gluconic acid.

The history of safe use for *A. niger* comes primarily from its use in the food industry for the production of many enzymes such as amylase, amyloglucosidase, cellulases, lactase, invertase, pectinases, and acid proteases (11, 12& 16). In addition, the annual production of citric acid by fermentation is now approximately 350,000 tons, using either *A. niger* or *Candida* yeast as the producing organisms. Citric acid fermentation using *A. niger* is carried out commercially in both surface culture and in submerged processes. *A. niger* has some uses as the organism itself, in addition to its products of fermentation. For example, due to its ease of visualization and resistance to several antifungal agents, *A. niger* is used to test the efficacy of preservative treatments. In addition, *A. niger* has been

shown to be exquisitely sensitive to micronutrient deficiencies prompting the use of *A. niger* strains for soil testing. There is also interest in using this fungus to perform certain enzymatic reactions that are very difficult to accomplish by strictly chemical means, such as specific additions to steroids and other complex rings.

The aims of this study are isolation, purification and characterization the protease from a thermophilic fungus isolated from fertilized hay. Proteases, also known as proteolytic enzymes or proteinases, belong to a group of enzymes whose catalytically function is to hydrolyze or breakdown the peptide bonds of proteins and they can either be limited proteolysis which break specific peptide bonds or unlimited proteolysis which break down a complete polypeptide chain to amino chain residues (17).These enzymes can be found from various sources such as plants, animals and microorganisms (18). Protease can be classified according to three major criteria which are the type of reaction catalyst, chemical nature of the catalytic site and evolutionary relationship, with reference to structure. Here, in this research, I have studied about microbial proteases. There are two major fungi that are responsible in the production of protease: filamentous fungi and yeast. They can be further classified to acidic protease, alkaline protease, serine protease and metalloprotease (filamentous fungi)/other protease (yeast).

Mostly they were produced by fungi, bacteria and viruses (microbial proteases) (19) because of the inability of plants and animal's proteases to satisfy the world demands (20). Also it has been reported that two third of industrial proteases are microbial proteases. This is due to their broad biochemical diversity and susceptibility to genetic manipulation (20) and also because of the characteristics of microbial proteases that can satisfy the need in biotechnological application (21).

Materials and Methods

Preparation of Active culture of *A. niger*

Potato dextrose agar media has been prepared, its pH was adjusted to 5.6, autoclaved media and poured in a sterilized Petri dishes. After solidification, the media was seeded with spores of *A. niger* and incubated for seven days at room temperature. The spore suspension was prepared in sterile distilled water containing about 0.01% (v/v) Tween 80. This solution was used as a source of inoculum.

Primary Screening for Acid protease production

The isolated Fungal strains were screened for acid protease production using a qualitative plate medium at pH 5 in Petriplates (90mm) diameter containing (in%): glycerol 0.5, casein 1, yeast extract 0.3, NaCl 0.5 and agar 2. The medium was inoculated with a loopfull of fungal spores and incubated at room temperature for 48-72 hours. The plates were then stained with Coomassie Brilliant Blue R-250 for 2 hours, and then followed by destaining overnight to observe the hydrolysis zone by acid protease. The fungal strain giving maximum clearance zone was identified as *Aspergillus* sp. on the basis of colony, morphology and microscopy and was selected for further studies.

Production of Extracellular acid protease by submerged fermentation process

5g of wheat floor was taken and poured in a 250ml Erlenmeyer flask, 100ml of salt solution (composition (%,w/v)): sodium nitrate 0.2, potassium dihydrogen phosphate 0.1, magnesium sulfate 0.05, potassium chloride 0.05, ferrous sulfate trace, zinc sulfate trace, pH 7.0, sterilized at 121^0C at 15 psi for 15 mins, cooled, inoculated with 1 ml of fungal spore suspension (10^8 spores/ml) and incubated at 30^0C for 72 hours. At the end of incubation period, the mycelia mats were aseptically skimmed off and further separation of spores and mycelia fragments was achieved by filtration through Whatman No.1 filter paper. The resulting clear culture filtrate were assayed for protease activity.

Assay for acid protease

The acid protease activity in crude enzyme extract was assayed according to the modified method of Anson (1938) using BSA as substrate. Reaction mixture containing 0.5 ml of enzyme solution and 0.5 ml of 1% (w/v) BSA in 0.2M phosphate buffer was incubated at 30^0C for 10 min. Except where specified, enzyme reactions were carried out at pH 5.0. The enzyme reaction was stopped by adding 1 ml of 10% trichloroacetic acid containing 0.22 M acetic and 0.33M sodium acetate. The reaction mixture was allowed to stand for 30 min at 30^0C and then was filtered. To 2ml of the filtrate, 5ml of 0.55 M sodium carbonate was added, followed by the addition of 1ml x 3 times diluted phenol reagent. The blue colour was measured at 660 nm by using a spectrophotometer.

Acetone precipitation of enzyme from fermented broth:

The solubility of protein among other things depends on the dielectric constant of the solution. In general, solvent molecules with large dielectric constants, e.g. water and dimethyl sulphoxide, can stabilize the interaction between themselves and protein molecules and be associated with the dissolution of protein. On the other hand, organic solvents with small dielectric constants, e.g. acetone and methanol, discourage the dispersion of protein molecules in the media. Thus, the solubility of proteins can be lowered and precipitation can be induced by lowering the effective dielectric constant of the media. This is commonly achieved by adding a water-soluble solvent such as acetone to an aqueous solution of protein. Acetone has the advantage that it is relatively inexpensive and is available in a pure form with few contaminants that may inhibit or poison the enzyme. It is also frequently used in sterol extraction.10 ml fermented broth sample was placed in acetone-compatible tube, such aspolypropylene tube and able to hold six times the sample. Added six times the sample volume of cold (-20°C) acetone to the tube. We vortexed tube and incubated overnight at -20°C. We centrifuged for 15 minutes at 10,000 rpm at 4°C. We carefully collected precipitate by decant the acetone and washed the pellet with cold 90% acetone. Centrifuge 5 minutes at 10,000 rpm at 4°C. The pellet was dried by air for 30 minutes and resuspended in a 10 ml 0.2M phosphate buffer.

Separation of enzyme by gel filtration Chromatography

Gel filtration can be applied to separate a wide range of molecules according to size including proteins and enzymes, polysaccharides, and nucleic acids. There are two major categories of gel filtration: group separation and fractionation. In group separation, samples are separated into two major groups, for example in desalting to remove salts and other low molecular weight contaminants. A gel filtration medium was chosen that excludes larger molecules from the pores of the bead, while smaller molecules were retained in the bead and then eluted. Fractionation is used for the separation of macromolecules of different sizes. The fractionation range of the medium defines the range of molecular weights that should be separable. Typical applications of fractionation using gel filtration include purification and molecular weight determination of proteins, peptides, and nucleic acids. The column was packed with sephadex G-200, washed with 30ml of Tris-Hcl buffer, and loaded Tl ml of sample in a column. Then, we collected 3ml fractions in test tubes (total 20 fractions) and read the each fraction absorbance at 280nm by using UV-Vis Spectrophotometer. Fractions showed absorbance at 280nm were subjected to protease assay. Fraction showed protease activity, estimated concentration of protein in the fraction by Lowry's method and determined its molecular weight by SDS-PAGE.

Estimation of protein concentration by Lowry's method

0.1ml of crude solution was taken, 0.1ml of acetone precipitate solution and l ml of gel filtration fraction showed enzyme activity in a test tubes. The volume was made up to 1ml with distilled water. We added l ml of alkaline copper sulphate, 0.5 ml of FC reagent, 5ml of 7.5% sodium carbonate solution and left it for 20 minutes at room temperature.

Table 1. Estimation of protein concentration in the crude filtrates, acetone precipitate solutions and gel filtration fraction showed highest protease activity by Lowry's method

S.No.	Volume of standard in ml (BSA)	Volume of Distilled water in ml	Volume of Alkaline Copper sulphate	Volume of FC reagent in ml	Volume of Sodium Carbonate in ml	Concentration of standard /Unknown (µgms)	OD
1.	0.2	0.8	1.0	0.5	5.0	20	0.10
2.	0.4	0.6	1.0	0.5	5.0	40	0.24
3.	0.6	0.4	1.0	0.5	5.0	60	0.36
4.	0.8	0.2	1.0	0.5	5.0	80	0.48
5.	1.0	--	1.0	0.5	5.0	100	0.60
6.	Blank	1.0	1.0	0.5	5.0	--	--
7.	Test 1 (0.1ml)	0.9	1.0	0.5	5.0	50	0.30
8.	Test 2 (0.1ml)	0.9	1.0	0.5	5.0	10	0.05
9.	Test 3(1.0ml)	0	1.0	0.5	5.0	4	0.02

Simultaneously, aliquots of working standard solution (100ug/ml) of bovine serum albumin were taken as shown in the table 1, made up with distilled water to lml, added l ml of alkaline copper sulphate to all tubes followed by 0.5ml of FC reagent and 5ml of 7.5%sodium carbonate. Mixed well and read the optical density at 660nm after 20minutes for all tubes (Table 2). We constructed standard graph for BSA and calculated the concentration of protein in the each crude solution and gel filtration fraction with protease activity.

Table 2. indicates spectrophotometric readings of gel filtration fraction at 280nm

Fraction Number	Optical density at 280nm	Fraction Number	Optical Density at 280nm
01	0.000	11	0.020
02	0.000	12	0.015
03	0.000	13	0.015
04	0.010	14	0.013
05	0.010	15	0.010
06	0.015	16	0.010
07	0.010	17	0.021
08	0.010	18	0.012
09	0.000	19	0.000
10	0.019	20	0.000

Determination of molecular weight of enzyme produced by *Aspergillus niger* by SDS-PAGE

Sodium dodecyl sulfate (SDS) is an amphipathic detergent. It has an anionic headgroup and a lipophilic tail. It binds non-covalently to proteins, with a stoichiometry of around one SDS molecule per two amino acids. SDS causes proteins to denature and disassociate from each other (excluding covalent cross-linking). It also confers negative charge. In the presence of SDS, the intrinsic Process of polymerization and formation of gel by Acryl amide and Bisacryl amide presence of ammonium per sulfate and TEMED(N,N, N1, N'-Tetramethyl ethylene diamine) charge of a protein is masked. During SDS PAGE, all proteins migrate toward the anode (the positively charged electrode). SDS-treated proteins have very similar charge-to-mass ratios, and similar shapes. During PAGE, the rate of migration of SDS-treated proteins is effectively determined by molecular weight.

Thoroughly we cleaned and dried the glass plates and assembled them in gel casting assembly. Two glass plates were sealed with the help of silicon grease, clamped and placed the whole assembly in an upright position. We mixed various components of resolving gel as indicated in the table 3. We poured the gel solution into the mould in between the clamped glass plates. Care must be taken to avoid entrapment of any air bubbles. We overlaid distilled water on the top gently and left for 30 min for setting of the gel. When the gel was polymerized, we removed the water layer and rinsed the gel surface with stacking gel buffer. We mixed the stacking gel components in the same way as described for the resolving gel. We poured the stacking gel and immediately insert the comb in the stacking gel, taken care that no air bubble entrapped. Allowed the gel to polymerize for about 20 min. After the stacking gel was polymerized, we removed the comb, washed un polymerized materials from the wells by flushing with electrode buffer using a syringe. We removed the bottom spacer carefully and installed the gel plate assembly to electrophoretic apparatus. We poured reservoir buffer in the lower chamber. Then, equal volume of sample and loading buffer was loaded in each well (table4). Electrophoresis of sample was done by Loaded Soul of gel filtration fraction with protease activity, 20ul diluted crude sample, 20ul diluted Buffered acetone precipitate sample and loaded molecular weight marker proteins in the wells. We switched 'ON' the current maintaining it at 10-15 mA for 30 min until the samples have traveled through the stacking gel. Then we increased the current to 30 mA until the bromophenol reaches near the bottom of the gel slab. After the electrophoresis is completed, we turned off power supply and carefully remove the gel slab from the electrophoresis unit and carefully removed the gel from between the glass plates. Placed the gel in a staining solution for 3hours, destained the gel with destaining solution.

Table 3. Preparation of stacking and resolving get mix

S.No.	Stock solution	Stacking gel (2.5%)	Resolving gel (12.5%)
1.	Acrylamide and Bisacrylamide mix	2.0	12.5
2.	Stacking gel buffer	5.0	-
3.	Resolving gel buffer	-	3.75
4.	10% SDS	0.20	0.30
5.	1.5% APS	1.0	1.50
6.	Water	1130	11.95
7.	TEMED	0.015	0.015

Table 4. Sample buffer composition

S.No.	Component	Quantity
1.	1 M Tris – HCI, pH6.8	12.5ml
2.	SDS	4.0g
3.	β-Mercaptoethanol	10.0ml
4.	Glycerol	20.0ml
5.	1% Bromophenol blue	4.0ml

Results and Discussion

After completion of fermentation, filtrate was estimated for acid protease activity, bovine serum albumin used as a substrate found that the broth showed proteolytic activity. Consequently after our observation, acetone precipitate also was assayed for acid protease activity which shows protease activity.

From the Sephadex-200 gel filtration process, the enzyme acid protease was separated the 13th fraction showed highest acid protease activity. The molecular weight of produced acid protease was revealed by SDS-PAGE. When compared with standard molecular weight markers, the relative molecular weight of *A.niger* extracellular acid protease is between 40kd and 50kd molecular weight markers. Based on the appeared band the relative molecular weight of extracellular enzyme is approximately 40kd (21).

Conclusion

By this research, the fungal species *Aspergillus niger* producing extracellular acid protease was studied which is available in wheat flour as growth material. One extracellular acid protease enzyme from filtrate was precipitated with acetone and purified by Sephadex G -200 gel filtration. The relative molecular weight of enzyme determined by SDS-PAGE revealed that the approximate molecular weight of extracellular lipase produced by *Aspergillus niger* is 40 kilo Daltons.

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