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STUDIES ON PROTEASE ENZYME FROM SOIL BACTERIA AND THEIR APPLICATIONS IN DRUG DISCOVERY AND DEVELOPMENT

Sara Fawzi Sahm*

Middle Technical University, Continuous Education Center, Baghdad – Iraq.

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***Corresponding Author: Sara Fawzi Sahm**

Middle Technical University, Continuous Education Center, Baghdad - Iraq.

ABSTRACT

One of the three main dietary groups required for a healthy diet is protein. The class of enzymes called proteolytic enzymes, also called proteinases, is responsible for proteins' catalytic hydrolysis. *Bacillus licheniformis* Strain Sara produces protease enzyme and partially purifies it was the study's objective. Protease is the most valuable microbial enzymes after amylase. Protease have been used extensively and in a variety of applications due to their ability to break down complex proteins into simple proteins or short peptides. Current research focuses on Purification, characterization, and application of proteases isolated from *Bacillus licheniformis* Strain Sara species. Fractional Ammonium sulfate precipitation was administered in different quantities to induce the precipitation of enzymes. In purification system, anionic exchange simple technique was utilized by using column to separate protease from other proteins. The necessary partially purified protease molecular weight was determined using SDS-PAGE with 8-fold purity and 6.9 % yield. Molecular weight of the protease estimated to be around 36 kDa. Along with this partially purified protease used in variety of application like production of small peptides from soy protein and antimicrobial activity of that small soy protein petides.

INTRODUCTION

A broad category of enzymes called proteases, which hydrolyzes or break down proteins or peptides. The peptide bonds that hold adjacent amino acid residues together in a protein molecule are targeted by proteases, who cleave the bonds to produce shorter peptides and amino acids.[1]

Almost 60% of all enzyme sales are made up of proteases, one of the most significant classes of industrial enzymes. These are a special class of enzymes and biocatalysts that hydrolyze the peptide bonds in proteins. They play a crucial role in the detergent, pharmaceutical, leather, food, poultry feed, meat processing, and agricultural industries.[2,3]

Naturally all bacteria, animals, and plants consist proteases but here are very little enzymes synthesized by plant and animal sources.[4] Due to their wide range of enzyme production, microorganisms have been identified as being crucial for the development of technologies enabling the industrial production of extracellular enzymes.[5]

Because they can improve quality control and are more effective and stable in producing peptides with particular molecular weights and peptide profiles, companies choose higher amounts of in vitro enzymatic hydrolysis[6] Under the right pH and temperature circumstances, in vitro enzymatic hydrolysis can also use a combination of specific and nonspecific proteases, including papain, chymotrypsin, trypsin, pepsin, and peptidase, to extract peptides from soy protein digestion.[7] By means of physiological and biochemical investigations as well as 16S rDNA sequence analysis, the strain was defined. Using ammonium sulfate precipitation, dialysis and enrichment, DEAE-cellulose. Partially purified protease is used in breakdown of bulk proteins from soy protein into small peptides then antimicrobial activity of that obtained peptide were done. Thus, *Bacillus licheniformis* Strain Sara has potential to produce an extracellular protease, active at neutral and alkaline pH. This protease may be useful in various industrial processes.

MATERIAL AND METHODS

Collection of soil sample and Screening of potent protease producer

Samples of soil have been collected from milk processing industry effluent treatment plant area in Bagdad. The soil sample was taken at a depth of 5 to 6 cm from the plan surface of land in sterile sample collection container and kept at 4 °C for further study.

After that Serial dilution was prepared by slight modification of jalkute et.al.,[8] up to 10^{-5} and soil sample from each dilution was spread on nutrient agar medium containing Skimmed milk 20 ml and simple nutrients like peptone powder 0.5 gm, extract of yeast 0.2 gm, agar powder 2.4 gm and incubated room temperature for 18- 24 hrs in which gelatin is replaced by skimmed milk. Clearance zone around the isolated colony as a result of proteolytic activity was observed after 24 hrs incubation. Isolates seen with a clearance zone (due to casein hydrolysis) of more than 7-10 mm were selected and proceeded for further study of protease producer.[9]

Morphological characterization of bacterial isolates

Bacterial growth on skimmed milk agar plates shows colony characters of potent isolate| Microscopy-based morphological research included gram stain, motility test, and endospore stain.[10]

Colony characters, Biochemical characterization of isolates

By using the pour plate technique, the selected isolates were inoculated on nutrient agar plates. They were incubated for 24 hours at 30 °C and examined for colony characteristics like color, shape, surface, and opacity. Biochemical tests like Catalase test, Starch hydrolysis, Gelatin hydrolysis, Urea hydrolysis test etc standard biochemical tests done and results are noted down.[11]

Protease production and biochemical testing of isolate

The 18 h. fresh culture of isolated *Bacillus licheniformis* Strain Sara was inoculated into broth containing simple nitrogen source as peptone – 0.6 gm, yeast extract – 0.3 gm, pure casein powder – 2.0 gm. Flasks were incubated at room temperature for 36 to 48 hrs at 120 rpm in rotary shaker. Enzyme production was checked at 8 hrs time interval.[9]

Protease enzyme assay

Protease activity was measured using modified Tran and Nagano method [quantitative method][12,13] Where is the reaction combination incubated (0.5 ml of (0.5%) casein solution, 1.5 ml of 0.2M phosphate buffer [pH 7.2]) with 0.2 ml of crude enzyme were all included in the reaction mixture, which was incubated for 30 minutes then reaction was stopped by using 0.5 ml of 0.5 N all reaction was carried at room-temperature. After that the released free amino acids by protease action were measured by using a modified ninhydrin colorimetric analysis method for free amino acids estimation at 530 nm wavelength was used to determine.[14] Enzyme activity of an enzyme was calculated using following way the quantity of enzyme needed to release 1 µg of leucine per minute under typical experimental circumstances was considered one unit of enzyme activity of an enzyme.[15]

Ammonium sulphate precipitation

Crude protease enzyme was obtained by fractional precipitation method by using ammonium sulphate salt[16] in this the inoculum of protease enzyme producer bacteria *Bacillus licheniformis* Strain Sara were made by using 18 hrs old bacterial isolate, using this seed culture crude protease enzyme produced in liquid nutrient media which containing casein as a substrate source for protease enzyme. After proper incubation (48 hrs) hrs of incubation the cell free broth was obtained by centrifugation process at 10,000 rpm for 15 min at 4 °C.[17]

After collection of cell free broth it is used for fractional precipitation of protein by using of ammonium sulphate salt. This involved gradually adding ammonium sulphate to the supernatant from 30% to 80% in order to properly saturate the salt and aid in the precipitation of the desired enzyme. Enough to gradually reach saturation. The aforementioned mixture was stored in a laboratory freezer at 4°C to 10°C for the entire night. The protein precipitate was collected by centrifuging the mixture at 8000 rpm for 20 minutes at 4°C. The precipitate was then dissolved in sterile 0.2 M sodium-phosphate buffer (pH 7.2) and excess salt was eliminated using a straightforward dialysis procedure using a dialysis membrane [Hi-Media]. The dialysis process was carried out at 4°C for the entire night.[18]

Purification of protease enzyme by anionic exchange chromatography

For Purification of crude protease enzyme from ammonium sulphate precipitation simple anionic exchange chromatography was employed. About 25 gm of activated DEAE-cellulose matrix beads are used for packing of column. After packing of column activated beads are saturated with 0.2 M sodium-phosphate buffer having pH-7.2. The height of packed column was about 20 cm with diameter of 2.0 cm. The protein was eluted from crude protein by using sterile NaCl gradients in this 0.1 to 0.5 Molar NaCl gradient (50 ml each) used, totally fifty fractions of eluted gradient were collected having five ml volume with constant flow rate of one ml per minute. All the purification steps were carried out at in closed system having 4 °C constant temp.[19] Protein content from eluted fractions were checked with a UV visible double beam spectrophotometer.[LAB-India] at 280 nm wavelength, after that all observations are plotted and peaks are obtained. Obtained peaks fractions were studied for protease activity.[20] Fraction which showing protease activity further subjected to molecular weight detection by SDS-PAGE.[21]

Characterization of partially purified Protease

Fraction which showing protease activity further subjected to molecular weight detection by using Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE).[22] This gives an idea about purity of the eluted fractions which are showing protease activity. Purity is expressed in terms of sharpness of protein bands and the bands were visualized naked eyes by using simple staining method of polyacrylamide gel i.e. The procedure of Coomassie Brilliant Blue R-250 staining.

Protease's molecular weight was determined through contrasting it to established protein indicators [Hi-Media]. Molecular weight was determined using conventional molecular weight markers and a 12% resolving gel concentration.[9]

Application of partially purified protease

1] Soy protein small peptide formation: Partially purified protease is subjected to the soy protein small peptide in which 10 ml of partially purified protease is mixed with 50 ml of soy protein then it's kept at 37 °C for 3 hrs then all mixture is centrifuged at 10,000 rpm for 15 min at 4 °C and supernatant is collected and used for antimicrobial activity against different pathogens along with this protein concentration of protease treated soy protein was estimated by using Lowery method.[23]

2] Antimicrobial activity of protease treated soy protein

Antimicrobial activity of protease treated soy protein supernatant was checked by using agar well diffusion method[24] in which one Gram positive and one Gram negative test pathogen is selected as indicator microorganisms. A Gram-positive bacterium used for experimentation was *Bacillus subtilis* NCIM 2635 and Gram-negative bacterium used is *E.coli* NCIM 2832. In this only partially purified protease and only soy protein without treatment of partially purified protease kept as negative control and streptomycin [100 µg/ml] as positive control and all results are noted in table 3.

RESULTS

Screening and Morphological characterization of bacterial isolates

Milk processing industry effluent treatment plant area soil samples was examined for extracellular protease enzyme producing bacteria using medium containing skimmed milk as raw source of casein protein as a substrate. In these 3 potent isolates are isolated and purified which are capable of producing protease enzyme which are extracellular in nature. From this, potent microorganism showing maximum zone of breakdown [zone of breakdown] on milk agar plates. This isolate is used for further biochemical tests and the 16s gene sequencing, this analysis and biochemical tests gives single that the organism are arrived from the *Bacillus* group.[Table 1]

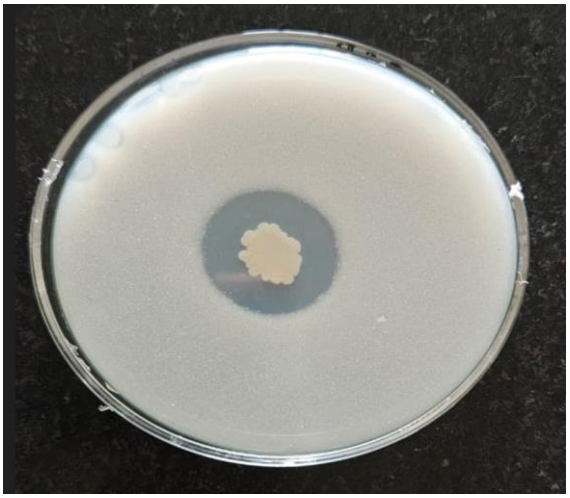

Fig. 1: Potent protease producer i.e. *Bacillus licheniformis* Strain Sara showing zone of clearance (28 mm) around its growth due to casein degradation.

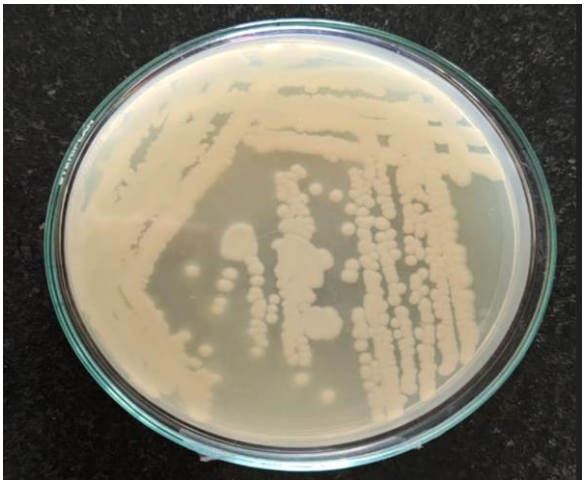

Fig. 2: Pure colony culture of protease producer *Bacillus licheniformis* Strain Sara.

Table 1: Biochemical test of isolated protease producing bacteria.

Name of the test	Observation
Gram's nature	Positive
Shape	Long Rod
Spore formation	Non spore former
Motility	Non motile
Catalase	Positive
Glucose	Positive
Arabinose	Positive
Galactose	Positive
Lactose	Positive
Indole	Negative
Sorbitol	Positive
Maltose	Positive
Gas formation	Positive
Ribose	Positive
Mannitol	Positive
Urea hydrolysis	Negative
Starch	Positive
Casien	Positive

Purification by ion-exchange chromatography

During production one thing is noticed is that as time period of incubation during fermentation increases enzyme production increased,18-24 hrs incubation gives proper amount of protease enzyme. As the *Bacillus licheniformis* Strain Sara produces extracellular protease enzyme, so the cell free broth was subjected for fractional ammonium sulphate precipitation from 30–80% gradual increase. It was concluded that 70% ammonium sulphate concentration able to precipitate protease enzyme in broth. Precipitated and dialyzed crude protease was loaded on DEAE-cellulose column which yields over all 6 peaks (Fig. 3), from them, only one pick shows major protease activity. During protein purification, gradual increase in specific activity of protease was achieved that confirms purity of fractions (Table 1).

At the end of purification, around seven-fold purity and 8.3% yield of protease was achieved [Table 1]. Purified fraction appeared as single band on SDS-PAGE and molecular weight was estimated to be around 36 kilo Dalton.

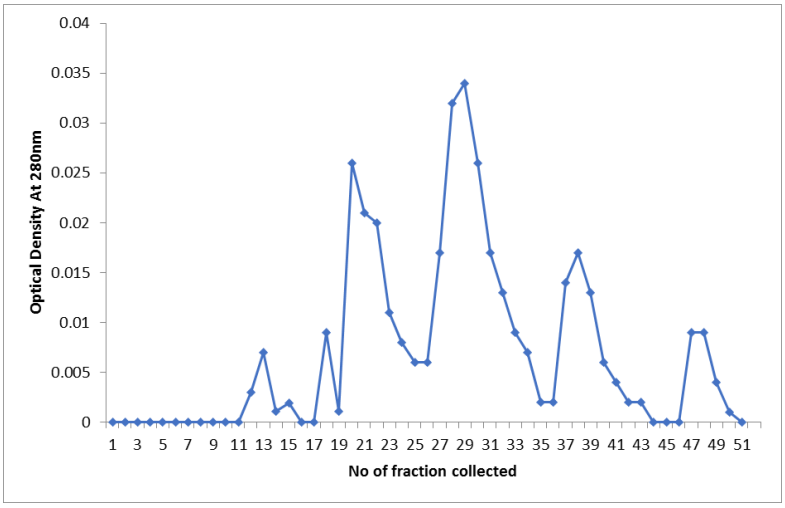

Fig. 3: Anion exchange column chromatography fraction profile of partially purified protease enzyme from *Bacillus licheniformis* Strain Sara.

Table 2: Purification summary of the protease from *Bacillus licheniformis* Strain Sara.

Purification Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Cell free extract	4960	8.3	566.3	99	0.9
Ammonium sulphate pre	1250	1.3	1127.6	25.3	1.6
Anion exchange column	420	0.11	4025	8.3	6.9

Characterization of partially purified Protease

SDS-PAGE analysis

The partially purified protease was analysed by SDS-PAGE, it was found that the band was observed of approximately of 36 kDa. But the purified fraction shows two bands at close proximity, so this needs to purify further for highest purity.

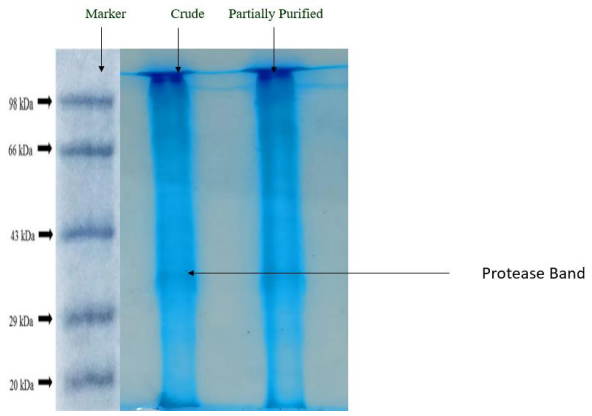

Fig. 4: SDS-PAGE of the purified protease. From left to fright Lane represents molecular weight standard protein markers, crude protease and partially purified enzyme.

Application of partially purified protease

After treatment of partially purified protease concentration of soy protein is increased that indicates large peptide is converted to small peptides.

Antimicrobial activity of protease treated soy protein

In this only partially purified protease and only soy protein without treatment of partially purified protease don't show any zone of inhibition but protease treated soy protein show antimicrobial activity against test pathogens. [Table 3] that confirms small peptides from protease treated soy protein show antimicrobial activity.

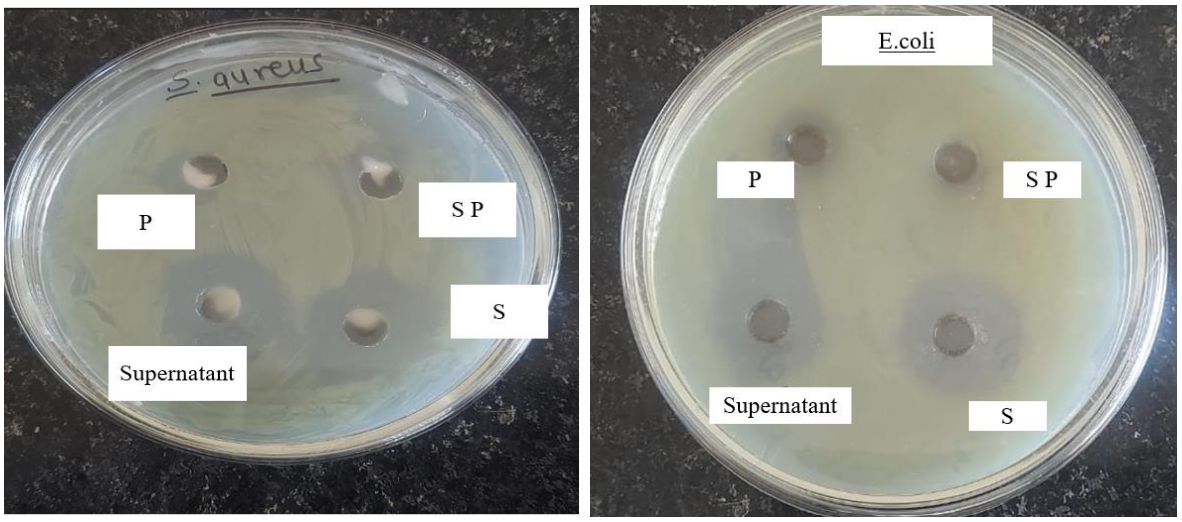

Fig. 5: Antimicrobial activity of protease treated soy protein. Here P is only partially purified protease, SP is only soy protein, supernatant is protease treated soy protein, S is streptomycin.

Table 3: Zone of Inhibition in mm against test pathogens.

Test organism	Zone of Inhibition in mm			
	P	S P	supernatant	streptomycin
S. aureus	00	00	19	26
E.coli	00	00	21	24

CONCLUSION

The current finding states that *Bacillus licheniformis* Strain Sara isolated from milk processing industry effluent treatment plant soil consist a potent microorganism which are capable of producing protease enzyme in extracellular manner. Soy protein treatment and antimicrobial activity of protease treated soy protein confirms that the purified enzyme has ability to produce small peptides after protease treatment to crude soy protein, which show antimicrobial activity against test pathogens. that confirms small peptides from protease treated soy protein show antimicrobial activity. Thus, the extracted protease from *Bacillus licheniformis* Strain Sara might be a potential contender for various industrial level applications specially in breakdown of large protein into small peptides which are especially antimicrobial in nature.

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