Partial Purification of Gelatinase Enzyme from Local Isolate of *Brevibacillus laterosporus*

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Abstract

A solid state fermentation system consisting of 1:2 w/w wheat bran/fish scales was used for the production of gelatinase enzyme activity. The activity has been screened from a local isolate of ***Brevibacillus laterosporus*** at 37 ºC and using Tris-HCl buffer pH 7.5.

Maximum activity obtained was 3.5 U/ml. Gelatinase protein content was precipitated using 60% ammonium sulfate. The enzyme has been partially purified on column chromatography technique using Sephadex G-150 column (1.5X30 cm).

The elution was carried out using buffer phosphate pH 7.4. Three isoform peaks were recorded from degraded gelatin. The obtained purification fold and recovery were (10.58) and (43.42) % respectively.

Results indicated the possibility of using purified gelatinase in industry fields.

Key words: ***B. laterosporus***, Gelatinase, Purification, Characterization.

الخلاصة

انتج انزيم الجيلاتينيز من البكتريياالمحلية ***B. laterosporus*** باستخدام نظـام تخمـرات الحالـة الصلبة عند استخدام 2:1 وزن/وزن نخالة الحنطة/قشور السمك. كانت فعالية الانزيم بحدود 3.5 وحدة/مـل بدرجة 37م˚ عند استخدام المحلول المنظم الترس HCl عند قيمة الاس الهيدروجيني 7.5 . رسب الانـزيم باستخدام 60% كبريتات الامونيوم ثم نقي جزئيا باستخدام كروماتوغرافيا الترشيح الهلامي بعمود السيفادكس

G-150 واسترد الانزيم من العمود باستخدام بفر الفوسفيت 0.1 مولار و الاس الهيدروجيني 7.4 وتـم الحصول على ثلاث قمم ذات فعالية تحللية للجيلاتين. وكان عدد مرات التنقية 10.58 وبحـصيلة انزيميـة مقدارها 43.42% .

تشير هذه الدراسة الى امكانية استخدام انزيم الجيلاتينيز في مجالات الصناعة المختلفة.

Introduction

Proteases are a unique class of enzymes, since they are of immense physiological as well as commercial importance. They possess both degradative and synthetic properties, and the degradative enzymes catalyze the total hydrolysis of proteins. The current estimated value of the worldwide sales of industrial enzymes is 1$ billion [1]. The industrial enzymes are 75% hydrolytic. Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes, and microbial proteases account for approximately 40% of the total worldwide enzyme sales [2]. Proteases from microbial sources are preferred to the enzymes from plant and animal sources, since they possess almost all the characteristics desired for their biotechnological applications [1].

Collagenases and gelatinases are important metalloproteases and their action are very specific, i.e., they acts only on collagen and gelatin substrates. Gelatinases are hydrolyze both native and denatured collagens (gelatin), and the enzymes are widely used not only in chemical and medical industries but also in food and basic biological science [3]. To classify an enzyme in the broad class of metalloproteases, a requirement for metal ions, such as Ca^{2+}, Mg^{2+}, or Zn^{2+}, for activity must be demonstrated [4]. Alderton *et al* (2004), confirmed that gelatinolytic activity of the enzyme when incubated with excess $CaCl_2$, $MgCl_2$, and $ZnCl_2$, and with the addition of sufficient EDTA to decrease cation concentrations, the majority of enzymatic activity was inhibited and only faint enzyme activity was observed [5]. Several ***Bacillus*** spp. secrete two major types of protease, a subtilisin or alkaline protease and a metalloprotease or neutral protease, which are industrial importance. It was reported that endspore-forming bacteria like ***Bacillus coagulans***, ***B. fumarioli***, ***B. pumilus***, ***B. gelatini***, ***B. thermoamylovrans***, and ***Anoxybacillus***, ***Brevibacillus***, and ***Geobacillus*** are the major concern in gelatin contamination and they have gelatinase enzyme activity [6]. In general ***Bacillus*** spp. and related genera could be potentially useful in industry because they produce high levels of enzymes such as amylase, protease, lipase and pectinase; also they are good source of antibiotics and other secondary metabolites [7 and 8].

Traditionally the purification of microbial enzymes from fermentation media has been done in several steps which include centrifugation of the culture broth (a step of extraction may be required for solid media), selective precipitation of the enzyme by ammonium sulfate or organic solvents such as ethanol in the cold. Then the crude enzyme is subjected to chromatography (usually affinity or ion exchange and gel filtration) [9].

The present study describes the partial purification of gelatinase enzyme produced from local isolate of ***B. laterosporus*** as a first step towards understanding its properties.

Experimental

1/ Organism and culture conditions:

Brevibacillus laterosporus strain was isolated and identified by Hamza [10]. It was cultured in a fermentation medium contains

wheat bran and fish scales (1:2) w/w moisted with water (30 ml/flask). The medium was adjusted at pH 7.0 with (1 N NaOH) before sterilization. After inoculation with $2X10^8$ cell/ml broth culture of the isolate, the flask was incubated at 37ºC for 72 hrs. The gelatinase enzyme was extracted from the fermentation medium by adding 30 ml distilled water to the fermentation flasks with stirring using magnetic stirrer for about 20 min. The mixture was centrifuged at 5000 rpm for 25 min to remove the cells and the residues medium. The supernatant (crude enzyme) was clarified by filtration through glass wool and the filtrate was stored at -15 ºC for further works [10].

2/ Enzyme assay:

Gelatinase activity was determined by two methods:

- Semi-quantitative method using a plate with nutrient agar supplemented with 0.4 % gelatin. Bacterial cells were streaked as a single line across the center of the plate, after incubation for 2-4 days at the optimal temperature; the medium was flooded with a 10 % HCl-15% $HgCl_2$ solution. A clear zone around the growth of the bacteria was indicated to gelatinase activity [11].

- Quantitative method according to Tran and Nagano [12]. The reaction mixture contained 0.3 ml of (0.2%) gelatin in water, 0.2 ml of (150 mM) Tris-HCl, pH 7.5, containing 12 mM $CaCl_2$, and 0.1 ml sample (crude enzyme). The reaction mixture was incubated at 30˚C for 30 min and stopped by the addition of 0.6 ml of (0.1 N) HCl. The released free amino group's amount was measured by the Ninhydrin method [13]. Gelatinase activity is expressed as µmol of leucine equivalent per min/ml of the culture filtrate. The same mixture except gelatin was used as a control.

3/ Total Protein Assay:

Protein concentration was determined by the Biuret method [14], using bovine serum albumin as a standard protein.

4/ Enzyme purification:

The gelatinase enzyme was extracted from the fermentation medium as above. Partial purification of gelatinase from the crude culture filtrate was done by adding 60 % of ammonium sulfate at 4˚C. The precipitated enzyme was dissolved in the minimum amount of phosphate buffer (0.005 M, pH 7.0). The protein fraction so precipitated, possessing gelatinase activity, was loaded on Sephadex G-150 column (1.5X30 cm), previously equilibrated with (0.1 M, pH 7.4) phosphate buffer and eluted with the same buffer at flow rate of 40 ml/hr. Fractions of (2ml) were collected and monitored for protein (A_{280} nm) and gelatinase activity using the spectrophotometer type (TU-1800S UV-VIS).

Results and Discussion

Metalloproteases can be generally classified on the basis of their substrate specificity, gelatinase that practically degrade gelatin [5]. Various bacteria and fungi produce gelatinase and the isolates belonged to member of ***Bacillus*** or related endospore-forming genera (like ***Brevibacillus***) have gelatinase activity [6and15]. Studies of this extra-cellular protease are significant not only from the point of view of overproduction but also for understanding their mechanism of secretion.

In the present work, an attempt was made to partially purified the gelatinase enzyme present in the culture filtrate obtained from fermentative action by ***B. laterosporus*** using solid state fermentation technique(wheat bran/fish scales). The activity of gelatinase enzyme recovered at 60 % ammonium sulfate saturation fraction and it was 3.6 U/ml. Ammonium sulfate was used to concentrate the enzyme and to

obtained a degree of purification [9]. The purification protocol developed for gelatinase enzyme of ***B. laterosporus*** is summarized in table 1. The ammonium sulfate removed amount of the contaminating proteins and it gave a 53.48 % yield with 1.25 purification fold.

The commonly used compound for precipitation of proteins in solution is ammonium sulfate because its high solubility in water and it dose not denature proteins [16]. Four protein peaks were found after gel filtration of gelatinase enzyme in Sephadex G-150 column, but only three enzyme activity peaks were found (fig 1). Gelatinase activity purification techniques, the data obtained reflects peaks purity which were confirmed by the close related protein content obtained during the partial purification of gelatinase enzyme. Nakagawa *et al* (1990) reported that a peak of gelatinase activity and it showed a much higher molecular mass (greater than 200 KDa) on Sephadex G-150 gel filtration [17]. Hamza (2005) showed three peaks of gelatinase activity after culture filtrate was loaded on the same column [10].

Purification of enzymes can be carried out to various degrees. The degree of purity of the product is usually related to its intended use, very high purification of enzyme is usually recommended only when the enzyme has special uses like therapeutic and medicinal applications in most other applications, partial purification is sufficient[18].

Conclusions

The results of this study suggest the possibilities of purification of bacterial gelatinases by using ammonium sulfate fractionation (60%) and gel filtration chromatography on Sephadex G-150 column. The present work is the first, to our knowledge, to report the production and purification of gelatinase enzyme from ***B. laterosporus*** in Iraq.

This study may provide a valuable source of experimental materials for further investigations, such as study of the enzyme kinetics, screening of natural substrates and enzyme immobilization and also applications. Further studies on the purified enzyme are currently on progress because the system may provide a good substitution for practical use in the batch and continuous fermenters using raw materials as a supporting and production media like (wheat bran and fish scales) in biotechnological applications.

Table 1: Summery of purification of gelatinase from local isolate of *B. laterosporus.*

Step	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification fold
Culture supernatant	25	3.5	11.7	0.299	87.5	100	1
Redissolved ppt.60% $(NH_4)_2SO_4$	13	3.6	9.6	0.375	46.8	53.48	1.25
Gel filtration elute	10	3.8	1.2	3.16	38	43.42	10.58

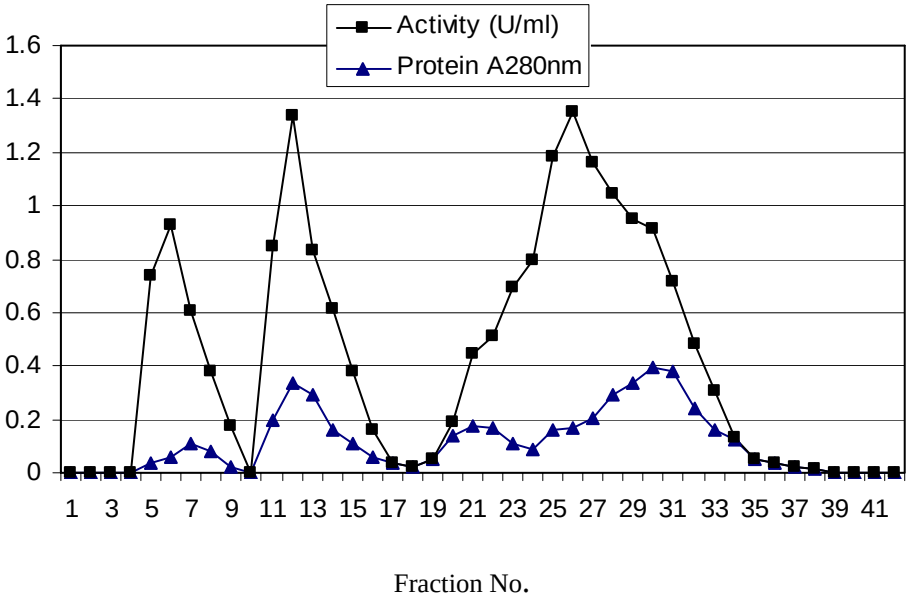

Fig 1: Elution profile of gelatinase enzyme using 1.5 x 30 cm Sephadex G-150 column.

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