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Production and Purification of a Protease Enzyme Produced by a *Streptomyces* Strain Isolated from a Wastewater Treatment Plant

Wael N. Hozzein[1,2], Marzouka S. Abdel Tawab[3], Mohammed B. Ahmed[4], Mohammed A.M. Wadaan[1]

[1]Bioproducts Research Chair (BRC), Zoology Department, College of Science, King Saud University, Riyadh, Saudi Arabia.
[2]Department of Botany, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt.
[3]Beni-Suef Wastewater Treatment Plant, Beni-Suef, Egypt.
[4]Department of Biochemistry, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt.

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A *Streptomyces* strain isolated from Beni-Suef wastewater treatment plant reported previously as a potent producer of proteases was subjected to fermentation for production of the protease, fractional precipitation of the enzyme by ammonium sulphate, purification of the enzyme by sephadex G200 and characterization of the enzyme by SDS-PAGE gel electrophoresis. The results showed that the purified protease enzyme produced by the strain *Streptomyces* C11 gave only a single band on the SDS-PAGE gel with a molecular weight of about 50 kDa reflecting its purity and homogeneity. The results showed that the wastewater actinomycete strain *Streptomyces* C11 have good capabilities for the production of a pure and homogenous protease.

Key words: Protease, production, purification, characterization, *Streptomyces* sp. C11.

Driven by increasing industrial demands for biocatalysts able to cope with industrial process conditions, continuous efforts are being focused on the search for industrial enzymes. Despite the fact that large numbers of different enzymes have been identified and many are being used in various biotechnological applications, the available enzymatic array is still not sufficient to meet the ever increasing demand [1].

Protease constitutes one of the most important groups of industrial enzymes, accounting for more than 60% of the total industrial enzyme market [2]. Proteases are used in many areas of applications, such as the detergent, food, agrochemical, and pharmaceutical industries [3].

* To whom all correspondence should be addressed.
Tel.: 00966569481811; Fax: 00966114675783;
E-mail: hozzein29@yahoo.com

Microorganisms represent the most common candidates as sources of new enzymes because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. While most studies on actinomycetes have focused on antibiotic production, only a few reports are on their enzymes [4]. Among actinomycetes, strains of *Streptomyces* are the preferred source for enzymes production [5].

The possibility of using *Streptomyces* for protease production has been investigated because of capacity to secrete the proteins extracellular in the media which is generally regarded as safe with food and drug administration [6]. *Streptomyces* strains produce variety of extracellular proteases that have been related to aerial mycelium formation and sporulation [7]. Therefore, proteases from *Streptomyces* origin offer an advantage as the mycelium can be easily removed by filtration [8].

During a screening program for producers of commercial enzymes from wastewater actinomycetes, a *Streptomyces* strain was found to be a good producer of proteases. This paper reports the conditions for production of the protease, purification and characterization of the enzyme.

MATERIALS AND METHODS

Microorganism

Isolation of the actinomycete strain from the wastewater samples collected from Beni-Suef Wastewater Treatment Plant (WWTP), characterization of the strain as *Streptomyces* sp. C11 and screening it for protease activity were carried out as described in previous studies [9, 10].

Protease production by strain *Streptomyces* sp. C11

A 500ml conical flask containing 100ml of starch casein broth medium [11] was prepared. The pH of the medium was adjusted to 8, inoculated with a loopful from a culture of *Streptomyces* sp. C11, and incubated at 30°C and 150 rpm for 2 days. Twenty 500ml-conical flasks each one containing 100ml of starch casein broth were inoculated with 10ml of the previously prepared inoculum. The flasks were incubated at 30°C and 150 rpm for 4 days in a shaking incubator. After incubation, the cells were harvested by centrifugation at 15000 rpm for 15 minutes at 10°C to obtain a cell free filtrate. The cell free filtrate was then preserved at – 20°C as a crude protease enzyme. Protease activity in the cell free filtrate, the protein content and the specific activity of the crude enzyme were determined as described below.

Determination of the protein content

Total soluble proteins were determined quantitatively according to Bradford [12]. For this, one ml of the crude enzyme was diluted to 1:1 with distilled water. 0.1ml from the diluted crude enzyme was mixed with 5ml Coomassie Blue reagent (100 mg Coomassie Brilliant Blue G250 in 50ml of 95% ethanol, add 100 ml 85% (w/v) H_3PO_4, complete up to 1 liter by distilled water). After 30 min, the absorbance was measured at 595 nm against water as a blank. The protein concentration was determined by using bovine serum albumin standard curve, and then the free protein content was calculated as mg protein l^{-1} volume.

Purification of the protease enzyme

Fractional precipitation by ammonium sulphate

The chart of Gomori [13] was applied to calculate the solid ammonium sulphate to be used to achieve fractional precipitation of the cell free filtrate under investigation.

Dialysis

The obtained enzyme preparation was concentrated again by dialysis against sucrose to be ready for column chromatography.

Purification of the enzyme by sephadex G200

The dialyzed partially purified enzyme was applied onto column packed with sephadex G200. This was equilibrated with phosphate buffer (0.2 M) adjusted at pH 7.4, then eluted with the same buffer. Preparation of the column and the fractionation procedure could by summarized in the following: A pharmacia column (2.5 X 80 cm) has been used. Sephadex G200 (particle size 20µm) was used as the selected gel for the permeation of the enzyme. Fifteen grams of sephadex G200 were suspended into 500 ml phosphate buffer (0.2 M) at pH 7.4 and allowed to swell overnight at room temperature. Sodium azide (0.02%) was added to prevent any microbial growth. The column was packed carefully by pouring the previously degassed thin slurry of gel into vertical column partially filled with the same buffer.

Testing the purity of the protease enzyme by SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [14]. The protein bands were visualized by coomassie brilliant blue R250 staining.

RESULTS AND DISCUSSION

Within the pool of biochemical reactions that are involved in many industrial applications, proteolysis plays an outstanding role. The microbial proteolytic systems are of great importance for the control of those industrial processes [15]. Therefore, microorganisms account for a two-third share of commercial protease production worldwide [16].

Actinomycetes, the high G+C Gram-positive bacteria, can degrade various macromolecules and compounds. Among actinomycetes, *Streptomyces* species are the most

industrially useful because of their capacity of producing numerous secondary metabolites, particularly antibiotics. Similarly, *Streptomyces* strains offer another industrial interesting use by producing large numbers and amounts of proteolytic enzymes with different applications. *Streptomyces* that produce protease include *S. clavuligerus*, *S. griseus*, *S. rimosus*, *S. thermoviolaceus* and *S. thermovulgaris* [17].

Production of the of the protease enzyme under study was performed by culturing the *Streptomyces* sp. C11 strain on starch casein broth medium prepared based on the optimal conditions (data not shown).

The purification process usually includes essential steps as preparation of the cell free filtrate; precipitation of protein using ammonium sulphate or other precipitants as low molecular weight

Table 1. Ammonium sulphate precipitation of the protease enzyme produced by the wastewater actinomycete isolate *Streptomyces* C11

Ammonium sulphate conc. (% saturation)	Enzyme activity (U/ml)	Protein content (mg protein/ml)	Specific activity (U/mg)
20	400	0.4	1000
40	410	0.48	854.1
60	450	0.5	900
80	890	0.44	2022.7
100	480	0.42	1142.8

Table 2. Fractionation pattern of the protease enzyme produced by the wastewater actinomycete isolate *Streptomyces* C11 on sephadex G200

Fraction number	Protein content (mg)	Enzyme activity (U/ml)	Specific activity (U/mg)
1-3	UD*	UD	UD
4	0.06355	410	6451.6
5	0.1116	540	4838.7
6	0.23405	860	3674.4
7	0.1209	890	7361.4
8	0.1209	450	3722
9	0.0899	400	4449
10	0.04495	260	5784
11-30	UD	UD	UD

* UD= undetected

Table 3. Summary of the purification steps of the protease enzyme produced by the wastewater actinomycete strain *Streptomyces* C11

Purification step	Volume (ml)	Protein content (mg/ml)	Total protein (mg)	Protease activity (U/ml)	Total activity (U)	Specific activity (U/mg protein)	Purification fold
Crude	2000	0.38	760	1200	2400000	3157.8	1
Ammonium sulfate	100	0.44	44	2022	202200	4595	8.4
Dialysis against sucrose	8	9.362	37.448	19080	152640	20380	6.4
Sephadex G-200	5	0.12	0.6	7361	36805	61341.6	1.53

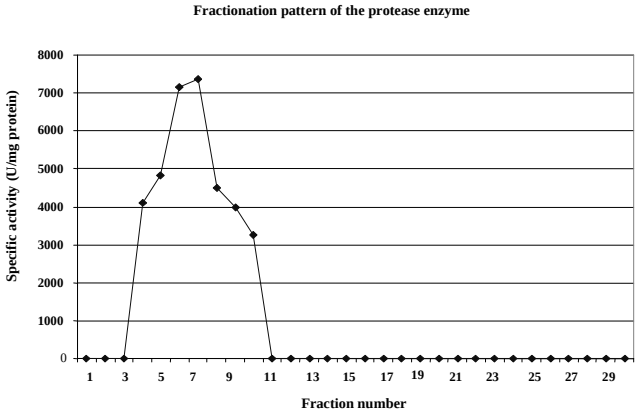

Fig. 1. Fractionation pattern of the protease enzyme produced by the wastewater actinomycete isolate *Streptomyces* C11 on sephadex G200

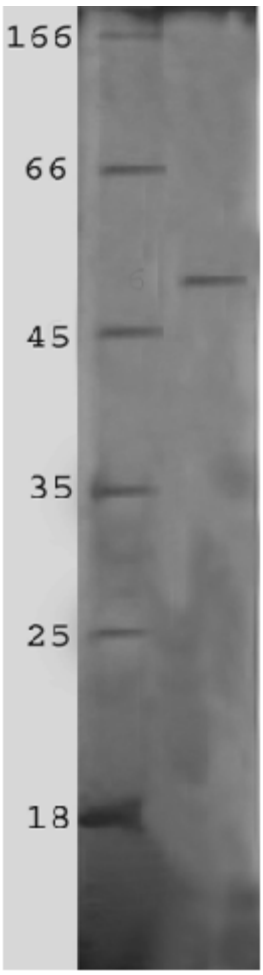

Fig. 2. SDS-PAGE gel photograph showing one band for the purified protease enzyme produced by the wastewater actinomycete strain *Streptomyces* C11 and its molecular weight at about 50 kDa

alcohols; dialysis; and then passing the enzyme preparation through a gel using different column chromatography like sephadex G200 column.

In this study, fractional precipitation of the produced protease enzyme was carried out by ammonium sulphate, since it is highly soluble in water, cheap and has no deleterious effect on the protein structure. For all these reasons, precipitation by ammonium sulphate was selected as a first step in purification of enzymes by many investigators [18-21]. After precipitation and during purification, the protein content was determined against a reference standard curve.

The results revealed that increasing the concentration of ammonium sulphate resulted in an increase in the specific activity of protease up to 80% saturation and then the specific activity decreased at higher concentrations (Table 1). Similarly, Chitte *et al*. [22] found that the crude protease enzyme was concentrated by precipitation with 80% saturation of ammonium sulphate. Also, Hutadilok and others [18] used 80% saturation of ammonium sulphate for protease purification. On the other hand, Uchida *et al*. [23] used 75% ammonium sulphate saturation for protease precipitation from the strain *Bacillus subtilis* CN2.

Isolating pure enzyme is important to use it for medical and industrial purposes [24, 25]. Therefore, the protein preparation, after ammonium sulphate precipitation, was dissolved in the least amount of tris-buffer (pH 9). Then, it was dialyzed against distilled water to get rid of the sulphate ions followed by concentration by dialysis against sucrose crystals to reach a minimum volume

resulting in raising the purification fold for the precipitated enzyme many times.

The dialyzed crude enzyme preparation was applied onto column packed with sephadex G200. Data recorded in table 2 and represented graphically in figure 1 showed that there were one active peak started from fraction 4 to fraction 10 and the maximum specific activity, 7361.4 U/mg protein, was reached at fraction number 7. The purification steps of the protease enzyme under study and changes in its activity, protein content and specific activity are summarized in table 3.

The purified protease enzyme was checked for purity and homogeneity by running on SDS-PAGE. The purity and homogeneity of the enzyme was obvious as it gave only a single band on the gel with a molecular weight of about 50 kDa (Fig. 2). The molecular masses of proteases from actinomycetes are variable. For instance, the alkaline proteases produced by an alkaliphilic *Thermoactinomyces* sp. had the values of 45 and 66 kDa [26]. While, the molecular weights of two proteases from a *Nocardiopsis* strain were 21 and 23 kDa [27].

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