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Characterization of a new protease produced by a thermohaloalkali tolerant Halobacillus strain

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An extracellular protease was produced by a thermohaloalkali tolerant bacterial strain, designated B300, which was isolated from a salt-affected soil sample collected at Beni-Suef city, Egypt. The bacterial strain was assigned to genus Halobacillus based on the phylogenetic analysis of the 16S rRNA gene. It was found that the protease was produced at the end of the exponential growth phase. The enzyme was purified and characterized by SDS-PAGE and its molecular mass was about 56 KDa. The enzyme had a wide salt range and was dependent on salt concentration for activity, with optimum activity at 55°C and pH 10 in the presence of 10% NaCl. It was inhibited by leupeptin, aprotinin, E-64 and PMSF, while chymostatin had an extremely low inhibition effect. Therefore, the protease was characterized as a trypsin-type serine or subtilisin-type, but not as a chymotrypsin-type. It was obvious also that the protease under investigation is not metalloproteinase-type or aspartic-type. The production of the enzyme in the culture medium was influenced by the medium composition, temperature, pH and NaCl concentration; and it was induced by the presence of yeast extract in the medium.

Key words: Protease, Characterization, Thermohaloalkali Tolerant, *Halobacillus sp*.

Enzymes that degrade polymers, such as amylases, cellulases, lipases, proteases and xylanases, have important roles in food, chemical, pharmaceutical, paper, pulp and other industries[1]. In recent years, extremozymes have been a focus of researchers' attention. The great interest of scientists is attracted to the mechanisms of biochemical adaptation of microorganisms to the extreme environmental conditions and the use of extremozymes in biotechnology[2].

Heat-stable enzymes are currently the most investigated of all extremozymes. Such extremozymes not only serve as excellent models for understanding protein stability but also carry significant biotechnological potential[3]. They usually obtained from either thermophiles, which grow optimally at temperatures above 60oC, or from hyperthermophiles with optimal growth temperatures above 90oC[4]. Also, halophilic enzymes that are stable in high salt concentrations serve as models for biocatalysis in low-water media. The industrial potential for halophilic enzymes is increasing as the approaches to study the genetic processes of halophiles and the understanding of haloadaptation become more sophisticated[5]. On the other hand, alkaliphiles thrive in alkaline environments have made a great impact in industrial applications, especially alkaline proteases in biological detergents[6].

Microbial proteases play an important role in many industrial processes and several workers

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have done a great deal of work on microbial proteases[7,8,9]. Many proteases from extremophiles have been studied because of their utility in the laundry detergent industry[10]. The potential use of thermostable proteases in a range of biotechnological applications is widely acknowledged. Their inherent stability at elevated temperatures and in the presence of organic solvents and denaturing agents enables their use in processes that restrict the use of conventional enzymes[11].

Moreover, alkaline proteases are commercially important as a major enzyme constituent in laundry detergents. Also, the most significant and useful work published to date with halophilic enzymes concerns the application of an extracellular protease from Halobacterium halobium[12].

Proteases have been classified according to the nature of their catalytic site into the following groups: serine, cysteine, aspartic proteases or metalloproteases. It has been found that most proteases from extremophiles belong to the serine type and are stable at high temperatures even in the presence of high concentrations of detergents and denaturing agents[13].

The exploration of proteases that can catalyze reactions under extreme conditions of temperatures, salinity and pH will be valuable for industrial applications. Therefore, the present study focused on characterization of a new protease isolated from a thermohaloalkali tolerant Halobacillus strain.

MATERIALS AND METHODS

Bacterial strain isolation

A thermohaloalkali tolerant strain, designated B300, was isolated from a soil sample collected from salt-affected area in Beni-Suef Governorate, Egypt on Sato agar medium14 adjusted at pH 10 and amended with 10% NaCl after incubation at 55°C for 3 days. The isolate was maintained in 20% glycerol at -20ºC.

Identification of the microorganism

The strain was identified based on typical cultural, morphological, biochemical and molecular characteristics. The phenotypic and phylogenetic analysis based on the 16S rRNA gene sequence were done as described previously[15].

Production media and growth conditions

The culture was grown in 500 ml flasks containing 100 ml of Czapek dox broth 16 as the production medium. The medium was amended with 10% NaCl and the pH was adjusted to 10 with sodium carbonate after autoclaving. The bacterium was grown for 24 h at 55°C on a shaker at 150 rpm. The culture was centrifuged at 10,000 × g for 10 min and the supernatant was used as the crude enzyme for further work.

Protease activity and protein content

Protease activity was measured by a modification of the method described by[17]. Reaction mixture (2ml) containing 1ml of casein 1% (w/v) (dissolved in 50mM glycine NaOH buffer, pH 10.0) and 0.95 ml of 50mM glycine NaOH buffer, containing 20% NaCl (thus, the final concentration of NaCl is 10%), was preincubated at 55ºC. The reaction was initiated by the addition of 0.05 ml enzyme solution and kept at 55ºC for 20 minutes. Two ml of trichloroacetic acid 10% (w/v) was added to terminate the reaction and the mixture was allowed to stand at room for one hour. The reaction mixture was centrifuged at 12000 g for 10 minutes and the absorbance of the supernatant was determined at 280nm by using Shimadzu UV-1601 spectrophotometer. One unit of protease activity is defined as the amount of enzyme required to liberate 1?g of tyrosine residue per minute under experimental conditions. The absorbance of the supernatant at 280nm was compared against a standard tyrosine solution dissolved in water. The protein content of the enzyme preparations was determined by the method of Bradford 18 with Quick Start Bradford Protein Assay reagent and bovine serum albumin as the protein standard.

Fractionation by salting-out with ammonium sulphate

After 36 h of submerged fermentation in Czapek dox broth under the optimum conditions (10% NaCl, pH 10 and 55ºC), the crude enzyme was collected by centrifugation at 8500 g for 20 min at 4ºC. The cell free supernatant containing the protease was partially purified by salting-out with different concentrations of ammonium sulphate, 20- 80%, in a sequential manner until the desired saturation of ammonium sulphate was reached. The ice cold enzyme solution was left for 2 h and then centrifuged for 15 minutes at 10,000 rpm in a cooling centrifuge. The enzyme preparation was

dialyzed overnight using the same buffer (50 mM glycine NaOH buffer of pH 10.0). The enzyme obtained was centrifuged at 10000 g for 20 min at 6ºC to remove any insoluble residues. The dialyzed enzyme was used for further characterization studies. The protein content and the proteolytic activity of the enzyme solution were determined after this step.

Purification by gel Column filtration chromatography

Sephadex G-150 was used for gel filtration with column (45 X 1.8 cm). Elution of the enzyme was performed at a flow rate of 36 ml/h by using a Bio-Rad Econo pump. Buffer used for elution was the same as that of the sample. The eluent was collected in 5ml fractions using a Bio-Rad fraction collector (Modle 2128). The protein content and the enzyme activity of each fraction was determined.

Polyacrylamide gel electrophoresis (PAGE)

The relative molecular weight and purity of the enzyme was determined and monitored by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by 19 on a vertical gel electrophoresis unit (Bio-Rad Laboratories). The gels were stained with 0.1% Coomassie blue R-250 (in water-methanol-acetic acid, 50: 40: 10, v/v) followed by destaining with water-methanol-acetic acid (50: 40: 10, v/v).

Two dimensional PAGE

The protease sample was allotted equally into strip holder for the 2D-PAGE analyses and then the isoelectric focusing was carried out as described before 20. The second dimensional electrophoresis was performed at 200 voltages for 55 minutes. The protein spots were visualized by Coomassie brilliant blue.

Effects of inhibitors and surfactants

The effects of different inhibitors and surfactants were determined 17, where the enzyme solution was pre-incubated with different concentrations of the used inhibitors and surfactants and then the remaining activity was measured. The used inhibitors were the serine proteinase inhibitors: PMSF (0.1, 0.5 and 1 mM), leupeptin (10, 50 and 100 ?M), aprotinin (0.03, 0.15 and 0.3 ?M) and chymostatin (10, 50 and 100 ?M); the cysteine proteinase inhibitor E-64 (1, 5 and 10 ?M); the metalloproteinase inhibitor EDTA (1, 5 and 10 mM); and the aspartic proteinase inhibitor pepstatin A (0.1, 0.5 and 1 ?M). The tested surfactants were triton X-100 (1, 2.5 and 5 %) and SDS (0.1, 0.25 and 0.5 %). Also, the effect of H2O2 (0.5, 2.5 and 5 %) was tested. All the previously used materials were dissolved in 50 mM glycine NaOH buffer (pH 10.0) at 40ºC for 30 min. After 30 min pre-incubation, the reaction was initiated by the addition of 1 ml casein 1% (w/v) and kept at 55ºC for 20 min. After 20 min reaction, protease activity was assayed as described earlier.

RESULTS AND DISCUSSION

The bacterial strain B300 was isolated from salt-affected soil collected at Beni-Suef Governorate, Egypt. It is a strictly aerobic Gram-positive, rod-shaped, motile and spore-forming bacterium with a polar spore. Biochemical tests showed positive reactions for oxidase, catalase, urease, and negative for nitrate reduction and H2S production. It has a pH range of 8- 12 with optimum pH at 10, with a wide salt range from 0- 25 % NaCl and required 10% NaCl for optimal growth. Also B300 could grow from 25- 65oC with optimum temperature at 55oC. Therefore, it can be considered as a polyextremophilic bacterium. The results of the phenotypic characters are given in Table 1.

The partial 16s rRNA gene sequence analysis revealed that strain B300 is most closely related to members of the genus Halobacillus with 100% sequence similarity to the strain Halobacillus sp. BH178 (data not shown). These results revealed that it is a member of genus Halobacillus.

In a study on proteases from seed borne fungi, More et al.[21] reported that the enzyme production was affected by the pH and temperature. The purified protease enzyme under study showed maximum enzyme activity at pH 10. These findings are in accordance with several earlier reports showing pH optima of 10-10.5 for protease from Bacillus sp., Thermus aquaticus, Xanthomonas maltophila and Vibrio metschnikovii[6, 22].

The optimum temperature for both good growth and enzyme activity was found to be 55ºC. This value is higher than those of mesophilic alkaline proteases produced from alkaliphilic Bacillus sp. reported earlier[23].

Similarly, the optimal pH and temperature was found to be 10 and 55oC for a purified

Table 1. Phenotypic properties of the poly-extremophilic bacterial isolate B300

Character	B300
Cell morphology	Rod
Spore shape	Spherical
Spore position	Polar
Temperature range (°C)	25- 65
pH Range	8- 12
NaCl Range (%)	0- 25
Oxidase reaction	+
Catalase production	+
Nitrate reduction	-
H2S production	-
Hydrolysis of:	
Aesculin	-
Casein	+
Gelatin	+
Starch	+
Tween 80	-
Urea	+
Acid production from:	
D-xylose	+
D-fructose	-
D-galactose	-
D-glucose	-
D-mannitol	-
Trehalose	+
Maltose	-
Sucrose	-

+ = Positive result; - = Negative result.

Table 3. Gel filtration fractions from 25% Ammonium Sulphate fraction

Tube number	Protein concentration (mg/ml)	Protease activity (Unit/mg)
5	0.030	200.0
6	0.070	3071
7	0.103	3359
8	0.100	9070
9	0.079	11480
10	0.055	16490
11	0.029	31275
12	0.017	53353
13	0.007	129571
14	0.006	151166
15	0.005	43000
16	0.005	26600
17	<0.005	10200
18	<0.005	6200
19	<0.005	6200
20	<0.005	1400
21	-	-

Table 2. Protein content and proteolytic activity at different ammonium sulphate fractions

Amm. Sulphate (%)	Protein content (mg/ml)	Proteolytic activities (Unit/mg)
20	0.335	343
25	0.472	1612
30	0.284	652
35	0.221	520
40	0.16	656
60	0.098	1071
80	0.10	1050

Table 4. Effects of different protease inhibitors and surfactants on protease activity

Inhibitor	Concentration	Inhibition (%)
EDTA	10mM	18
	5.0mM	10
	1.0mM	4.0
Leupeptin	100µM	75
	50.0µM	70
	10.0µM	52
Aprotinin	0.30µM	69
	0.15µM	67
	0.03µM	55
E-64	10.0µM	70
	5.00µM	59
	1.00µM	58
PMSF	1.00mM	63
	0.5mM	48
	0.1mM	47
Chymostatin	100µM	5.0
	50.0µM	5.0
	10.0µM	5.0
Pepstatin A	1.00 µM	7.0
	0.50 µM	6.0
	0.10 µM	3.0
SDS	0.50%	83
	0.25%	77
	0.05%	66
Triton X100	5.00%	44
	2.50%	30
	1.00%	20
H_2O_2	0.50%	73
	0.25%	68
	0.05%	65

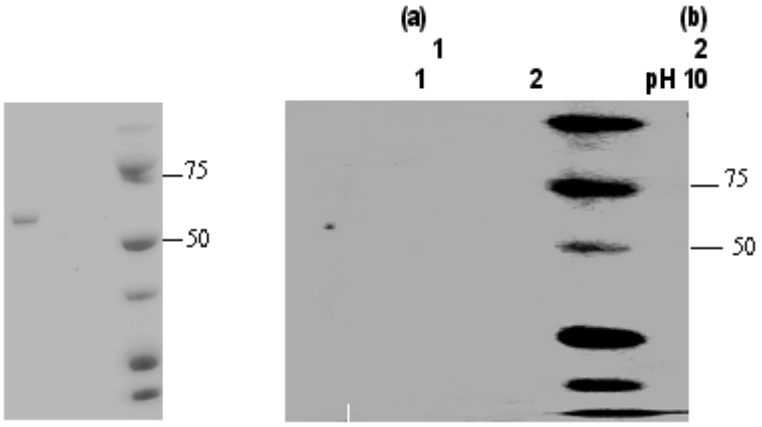

Fig. 1. (a) SDS-PAGE of the purified protease. Lane 1, purified protease after gel filtration chromatography showing one band; Lane 2, molecular weight protein marker. (b) 2D-PAGE, Lane 1, purified protease after IPG stripe showing one spot around pH 9; Lane 2, molecular weight protein marker

thermostable extracellular alkaline protease produced by from Bacillus subtilis K-30[24].

On the other hand, the highest proteolytic activity was obtained by the isolate B300 at 10% NaCl. The requirement of high salt concentration for the activity and stability of the enzymes of halotolerant and halophilic microorganisms was recorded by many researchers before 7,25. This indicates that the enzyme under investigation produced by the selected isolate is not only halophilic enzyme but also alkalophilic and thermophilic enzyme.

As shown in Table 2 the highest values of both protein content and protease activity were obtained at 25% of ammonium sulphate. The lowest protein content value was recorded at 60% of ammonium sulphate and the lowest value of protease activity was at 20% of ammonium sulphate.

The gel filtration chromatography revealed that the protease activity was detected from tube number 5 until tube number 20. Table 3 showed that among the tubes which have protease activity, the highest value of protein content was obtained in tube number 7, while, the lowest value was obtained at tubes 17- 20. The highest value of the protease activity was obtained in tube number[14], while the lowest value was in tube number [5].

After purification of the protease under investigation, SDS-polyacrylamide gel electrophoresis was used for molecular weight determination. The SDS-PAGE revealed a single band of about 56 KDa as shown in Fig 1a. The protein profile of the purified enzyme revealing that the molecular weight is around 56 KDa and the isoelectric focusing point lies in the alkaline range around pH 9 as shown in Fig 1b.

Similarly, it was reported that an alkaline protease isolated and purified from B. licheniformis, has a wide pH range of 4-12 with optimum pH at 10, temperature range of 30-90oC with optimum temperature at 50oC and a molecular weight of 55kDa[26].

As shown in Table 4, various protease inhibitors were used to determine the type of protease enzyme under investigation. Among these inhibitors, leupeptin, aprotinin, E-64, and PMSF were found to inhibit the protease activity to a high extent ranging from 47% to 75%. Leupeptin showed the maximum inhibitory effect on the activity, while pepstatin A, EDTA and chymostatin exhibited less inhibitory effect. Therefore, the protease from the bacterial isolate B300 is identified as trypsin-type serine or subtilisin-type, but not chymotrypsin-type. The protease enzyme under investigation is not metalloproteinase-type or aspartic-type.

These results are similar to the thermostable alkaline protease isolated from the thermophilic and alkaliphilic Bacillus sp. JB-99 which was a trypsin-type serine protease[17].

In the presence of 1 and 2.5% Triton X-100, the enzyme retained 80 and 70% of activity, respectively, while 0.5% H2O2 inhibited the enzyme

activity by 73%. Also, the enzyme activity was greatly affected by SDS. These results indicated that the alkaline protease under study to some extent is not bleach stable.

In the present study, characterization of a new protease isolated from a thermohaloalkali tolerant Halobacillus strain was achieved. This new protease which can work under extreme conditions of high temperature, moderate salinity and pH could be valuable for future industrial applications.

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