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Research paper

Characterization of Metalloprotease from *Serratia marcescens*

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ABSTRACT

In this report, we present the properties of the protease that is produced by a newly discovered isolate of Serratia marcescens. The protease activity was tested using casein as the substrate at different temperatures and pH, and with the addition of EDTA. The protease from S. marcescens was partially purified through ammonium sulfate precipitation and size exclusion chromatography. The crude enzyme demonstrated protease activity of 1.572 U/ml, while the partially purified protease showed 0.46 U/ml activity. The S. marcescens protease was identified as a metalloprotease, as EDTA was able to inhibit its activity. The optimal pH and temperature and pH of this protease were determined to be 8.0 and 45 °C, respectively.

KEYWORDS: Proteolytic enzymes, *Serratia marcescens,* Serratia peptidase.

INTRODUCTION

Proteases (and peptidases) are involved in many biological reactions including the degeneration of misfolded proteins, cell cycle, apoptosis and mediate the immune response [1]. The estimated sales of engineered enzymes worldwide are 1 billion US$, among them, 60% of the sales account for proteases internationally. The demand for proteolytic enzymes is increasing every year due to their advantages such as rapid isolation by means of fermentation, quick purification, high catalytic activity, and substrate specificity [2,3,4].

Serratia marcescens (additionally known as *"Chromobacterium Prodigiosum")* is an opportunistic, motile, non-spore-forming, prodigiosin-producing bacterium that grows in both aerobic and anaerobic conditions [6] at room temperature and at 37 °C. In the late 1960s, *Serratia marcescens* was isolated from the intestine of the silkworm (*Bombyx mori)* [7]. It is typically found in the natural environment like soil, water [8], air, plants, and animals [5]. *Serratia marcescens* can also be found in damp environments like washrooms by forming a pink-orange-red coloration at the corners of wash basins and showers [9], and medical equipment like catheters [10] and milk [11].

Serratia peptidase, a well-known proteolytic enzyme is similarly referred to as Serratiapeptase, Serrapeptase, Serratiopeptidase, Serratia E-15 protease, and Serralysin are produced by *Serratia marcescens* (Strain E-15) [7]. It is a zinc-containing metalloprotease with a molecular weight of approximately 45-60 kDa and can be used extensively as a therapeutic agent due to its proteolytic properties. *Serratia marcescens* is described as a good producer of Serralysin extensively [12]. It is made up of 470 amino acids and their sequence is free from sulfur-containing amino acids like methionine and cysteine. Serratia peptidase is used in pharmaceuticals, cleansing [13], bioremediation, tanning, food dispensation, and the cosmetics industry [14].

In this report, we describe the properties of the bacterial peptidase secreted by a novel isolate of *Serratia marcescens*.

MATERIALS AND METHODS

Culturing of *Serratia marcescens*

A clinical isolate of *Serratia marcescens* was obtained from a tertiary care hospital in Karachi, Pakistan. The isolate was maintained on nutrient and tryptic soy agar plates at 4 °C. The subculturing was performed subsequently at 15 days intervals at 30 °C. Preserved the culture of *Serratia marcescens* by using Luria Bertani and tryptic soy broth with 30% glycerol at -20 °C.

Blood agar was used as a differential media to produce hemolysin at 30 °C for 48 hours of incubation. Violet red bile agar was used as a selective media to observe lactose fermentation at 30 °C for 24 hours of incubation [15] with trivial amendments.

Biochemical tests for the identification of *Serratia marcescens* isolate

Simmons citrate agar test, triple sugar iron agar test, gelatin liquefaction test, catalase test, and urease test were performed for the identification of *Serratia marcescens* [16,17] with minor alterations.

Protease assays

Protease assay by carried out by plate assay technique by using skim milk agar [18,21] with slight alterations. Protease assays were also carried out by agar well diffusion method with skim milk agar and gelatin agar plates as described elsewhere [19,20,22]. Spectrophotometric protease assays were carried out using casein as substrate according to [23]. The Bradford assay of precipitated proteins was also performed after the protease assay by taking the protease assay supernatant.

Partial purification *S. marcescens* protease

Ammonium sulfate precipitation

The crude enzyme from skim milk broth was taken and centrifuged at 10,000 x g for 20 minutes at 4 °C. The supernatant was used for ammonium sulfate precipitation with 67% saturation at 10 °C. Ammonium sulfate was added slowly with continuous stirring. The salt was dissolved completely in 1 hour. The precipitated proteins were collected and centrifuged at 13,000 x g for 20 minutes at 4 °C. The pellet was dissolved in 50 mM Tris-HCL buffer, pH 8.0. Vortex and then stored at -20 °C for downstream examination as reported in [22].

Gel filtration Chromatography

Gel filtration chromatography was performed by using the resin (Seplife 6FF, Matrix: 6% crossed linked agarose) and the column was equilibrated with 50 mM Tris-HCl buffer, 100 mM NaCl and 0.03% sodium azide, pH 8.0. The flow rate of the column was 1.4 ml/min. 250 µL ammonium sulfate precipitated protein was inoculated into the column and 0.5 ml of fractions were collected. Measured the absorbance of each fraction at 280 nm against the blank. The fractions having the peaks in the chromatogram were tested for protease activity. The fractions with protease activity were assayed for protease inhibition with EDTA. Protein estimation was done by the Bradford method.

Characterization of *S. marcescens* protease

Protease assay in the presence of EDTA

The activity of protease was determined by using 1 mM EDTA as mentioned in [23,24].

Protease assay at different temperatures

The protease assay conditions were used as above [23,24]. The activity of protease was assessed at different temperatures (35 °C – 60 °C) with the increment of five temperature units for 1 hour.

Protease assay at different pH

The activity of protease was evaluated at different pH ranges from 6.0-9.0 with the increment of 0.5 pH units at the optimum temperature of 45 °C for 1 hour. The buffers include 50 mM phosphate buffer, 100 mM NaCl for pH 6.0-7.0 and 50-mM Tris-HCl buffer, 100 mM NaCl for pH 7.5-9.0. These

buffers were used for the preparation of 1% (w/v) casein solution (substrate).

RESULTS AND DISCUSSION

Subculturing of *Serratia marcescens*

Both nutrient agar and tryptic soy agar resulted in small, circular, shiny, pinpointed, mucoid, orange color pigmented colonies with entire margin at 30 °C after 18-24 hours of incubation (Figure 1) as reported in [25]. Production of pigment by *Serratia marcescens* depends upon the incubation temperature. Longer incubation period of 3-4 days at 37 °C inhibited pigment production [26]. Further, pigment production also depends upon the pH of the agar. In a liquid medium, when the pH is acidic the pigment produced by *Serratia marcescens* appeared as red however in alkaline pH it appears yellow to orange.

Biochemical tests for the identification of *Serratia marcescens*

Serratia marcescens showed a positive Simmons citrate agar slant test by changing its green color into blue color at 30 ˚C for 24 hours of incubation. *Serratia marcescens* utilized citrate as a sole source of carbon [16,17]. Alkaline slant/acidic butt (Red slant (K)/ Yellow butt (A)), gas positive, and no H_2S production was observed by *Serratia marcescens* after 24 hours of incubation at 30 °C on triple sugar iron agar slant. These results represent that the Serratia marcescens ferment only glucose and produced acid in the butt showing yellow color, the red color in the slant indicates that the acid produced is oxidized into CO2 and H2O.

Figure 1: *Serratia marcescens* on Tryptic Soy Agar

Wilkowske et al [28] stated that 94% of the strains isolated from hospitalized patients showed alkaline slant/acidic butt with 73% of gas production. The clinical isolate of *Serratia marcescens* used in the present study showed *an* acidic slant/acidic butt which indicated that the organism ferment lactose, glucose, and sucrose by changing the color of pH indicator phenol red [17,13]. It was concluded that the results of TSI vary from strain to strain.

Serratia marcescens gives the positive gelatin liquefaction test due to the production of gelatinase enzyme which hydrolyzed the gelatin [28]. According to Wilkowske et al [27], 98% of the clinical isolates of *Serratia marcescens* showed positive gelatin liquefaction test. *Serratia marcescens* gives the positive catalase test by producing the catalase enzyme which breaks the hydrogen peroxide into water and oxygen as described in the article [29,17]. The bright pink color shows the positive urease test which indicates that the *Serratia marcescens* produced the urease enzyme which hydrolyze the urea (CH_4N_2O) and converted it into ammonia (NH_3) and carbon dioxide ($CO_{2)}$ in the presence of water. The ammonia combines with the carbon dioxide and water to form ammonium carbonate ($(NH_4)_2 CO_3$) which changes the pH of the medium into alkaline and turns it from orange yellow color to bright pink color. Phenol red was used as an indicator in urea broth.

The zone of hydrolysis was observed around the pigmented single streak of

Serratia marcescens on skim-milk agar plate which indicates that the organism produced extracellular protease enzyme which hydrolyzed the casein protein and showed the large clear zone around the streak (Figure 2A) as reported in [7,18]. The zone of hydrolysis was also observed around the pigmented single streak of *Serratia marcescens* on gelatin agar plate which indicates that the organism produced extracellular protease (gelatinase) enzyme which hydrolyzed the gelatin protein and showed the clear zone around the streak (Figure 2B). By comparing the results of skim milk and gelatin agar it was found that skim milk agar was more efficient for providing clear and visible zones as equivalence to gelatin agar. Conferring to [18] gelatin agar gives clearer zones as compared to skim milk agar for qualitative analysis.

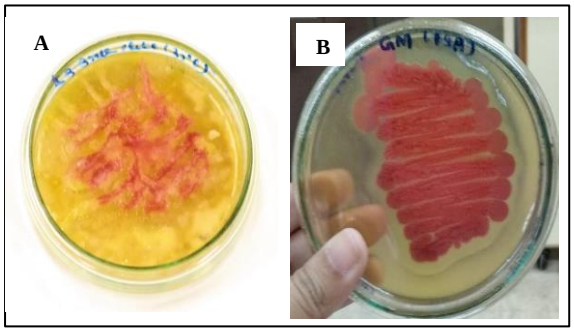

Figure 2: (A) Showing zone of hydrolysis on Skim milk agar. (B) zone of hydrolysis on Gelatin Agar

Protease production

Skim milk broth was used for enzyme production. *Serratia marcescens* isolate was able to produce the protease enzyme by using casein as a substrate as stated in [18,21].

The culture supernatant showed complete hydrolysis all over the skim milk agar plate at 37 °C [21]. It means that the activity of extracellular protease was very high (Figure 3) [19].

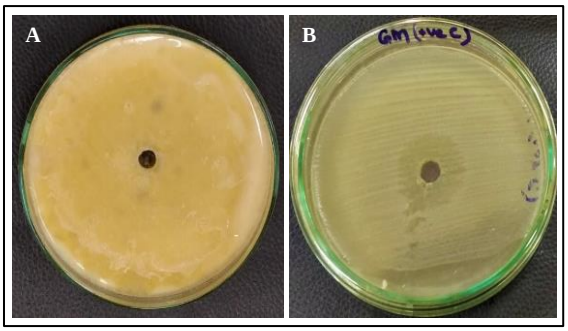

Figure 3: (A) Skim milk agar showing hydrolysis all over the plate. (B) 25 mm zone of hydrolysis on gelatin agar plate.

Partial purification *S. marcescens* protease

Peptidase was partially purified by salt precipitation followed by size exclusion chromatography. Ammonium sulfate precipitation was executed as the first step for the purification of bacterial protease. The crude enzyme was precipitated with 67% saturation of $(NH_4)_2$ SO_4. The collected precipitates were then used for the total protein estimation and protease assay. Rahman et al [30] carried out the ammonium sulfate precipitation from 30-90% and reported that the 60% saturation gives the highest specific activity of 4000 U/mg. Another study testified different levels of saturation from 50-80% and informed that the most appropriate level for the partial purification of protease enzyme is 67% [22].

The precipitated protein fraction was subjected to size exclusion chromatography (SEC) for protein separation. SEC chromatogram was plotted by absorbance of fractions at 280 nm against the fraction number (Figure 6). The peaks in the chromatogram were checked for protease activity (Tables 3 and 4).

Characterization of partially purified S. marcescens protease

Protease inhibition by EDTA

The protease assay was performed in the presence of 1 mM EDTA (Ethylenediaminetetraacetic acid). The results indicated that *S. marcescens* protease is a metalloprotease as enzyme activity was inhibited 83% in the presence of EDTA (Tables 1 and 2). Vélez-Gómez et al. [14] used three inhibitors for the metalloprotease inhibition isolated from *Serratia marcescens* including EGTA (egtazic acid), EDTA (Ethylenediaminetetraacetic acid) and OPHE (1,10-phenanthroline) with 10 mM concentration and informed the percentage inhibition as 48%, 77% and 48%, suggesting that the enzyme was metalloprotease because metals ions are important for the catalytic activity. 2 mM EDTA inhibited 71% of the activity of thermoactive serratiopeptidase while 2 mM PMSP (phenylmethylsulfonyl fluoride; serine protease inhibitor) does not inhibit the enzyme activity [24]. It was also reported EDTA inhibited the activity of extracellular metalloprotease isolated from *Serratia marcescens* NRRL B-23112 while other inhibitors like PMSF, SDS, and Tween 20, etc. have no effect on it [32].

Protease activity at different temperatures

Our results showed the optimum temperature of S. marcescens protease as 45 °C. The enzyme gives the maximum activity of 12.76 units/ml and a specific activity of 149.589 units/mg at 45 °C and loses its activity from 50 °C to 60 °C due to enzyme denaturation as reported in (Figure 4) [31]. Rahman et al. [30] and Tariq et al [13] reported the optimum temperature for the activity of protease produced by *Serratia marcescens* as 40 °C by using a temperature range from 25 °C to 55 °C and 25 °C to 60 °C. Another study described the optimum temperature of extracellular metalloprotease from *Serratia marcescens* as 42 °C [32]. Another study stated the optimum temperature of 45 °C for metalloprotease activity produced by *Serratia marcescens* by using a temperature range from 25°C to 60°C [31].

Protease activity at different pH

The *S. marcescens* protease was characterized as an alkaline protease with optimum pH of 8.0. The enzyme gives a maximum activity of 13.74 units/ml and a specific activity of 161. 078 units/mg at pH 8.0 and loss its activity from 8.5 to 9.0 [13] (Figure 5). Chander et al [24] reported the optimum pH of thermoactive serratiopeptidase from *Serratia marcescens* AD-W2 as pH 9.0. Another published study from [31] reported the optimum pH of 8.5 for metalloprotease activity produced by *Serratia marcescens* by using a pH range from 3.0 to 11.5. Tariq et al. [13] reported the maximum enzyme activity at pH 8.0 of protease produced by *Serratia marcescens* by using a pH range from 2.0 to 11.0.

Conclusion

We characterized a metalloprotease secreted by novel *S. marcescens* isolate. The protease activity was inhibited by EDTA which identified this enzyme as metalloprotease. The optimum temperature of this enzyme was 45 °C and the pH of 8.0 which illustrated the alkaline nature of this protease.

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Table 1: Spectrophotometric Protease Assay

Purification stage	Total protein concentration (mg/ml)	Absorbance (OD) 280 nm (U/µl)	Enzyme Activity (U/ml)	Specific Enzyme Activity (U/mg)
Crude enzyme	0.0853	2.359 U/1500µl	1.572	18.429
Ammonium sulfate precipitated proteins	0.0853	0.023 U/50µl	0.46	5.392

Table 2: Protease Assay in the presence of inhibitor (EDTA)

Purification stage	Total protein concentration (mg/ml)	Absorbance (OD) 280 nm (U/µl)	Enzyme Activity (U/ml)	Specific Enzyme Activity (U/mg)	Inhibition (%)
Ammonium sulfate precipitated proteins	0.0853	0.004 U/50 µl	0.08	0.937	83%

Table 3: Protease Assay of Gel filtration chromatography fractions

Fraction No.	Absorbance at 280 nm (U/100 µl)	Enzyme Activity (U/ml)	Specific Enzyme Activity (U/mg)
3	0.004	0.04	0.468
8	1.080	10.8	126.61
12	1.192	11.92	139.74
15	0.776	7.76	90.973
19	0.411	4.11	48.182
23	0.095	0.95	11.137
27	0.092	0.92	10.785
31	0.086	0.86	10.082
34	0.007	0.07	0.820
38	0.013	0.13	1.524

Table 4: Protease Assay of Gel filtration chromatography fractions with protease activity in the presence of inhibitor (EDTA)

Fraction No.	Absorbance (OD) 280 nm (U/100 µl)	Enzyme Activity (U/ml)	Specific Enzyme Activity (U/mg)	Inhibition (%)
8	0.0096	0.96	11.254	99.12
12	0.225	2.25	26.375	81.13
15	0.206	2.06	24.150	73.56
19	0.193	1.93	22.626	53.1

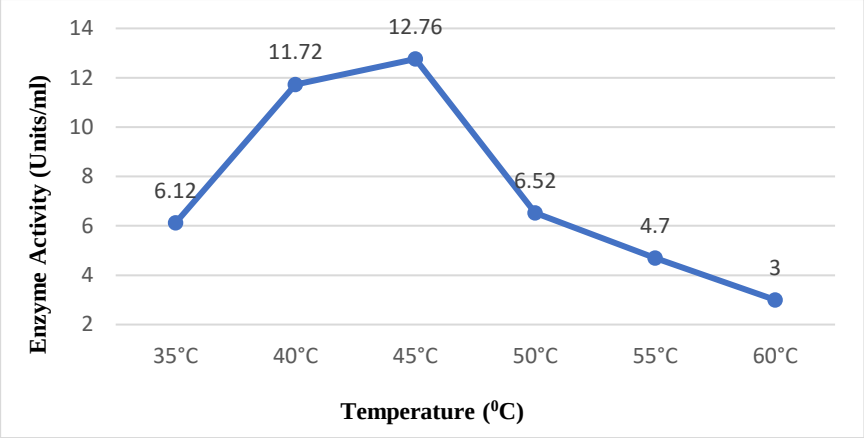

Figure 4: Activity of *S. marcescens* protease at different temperatures

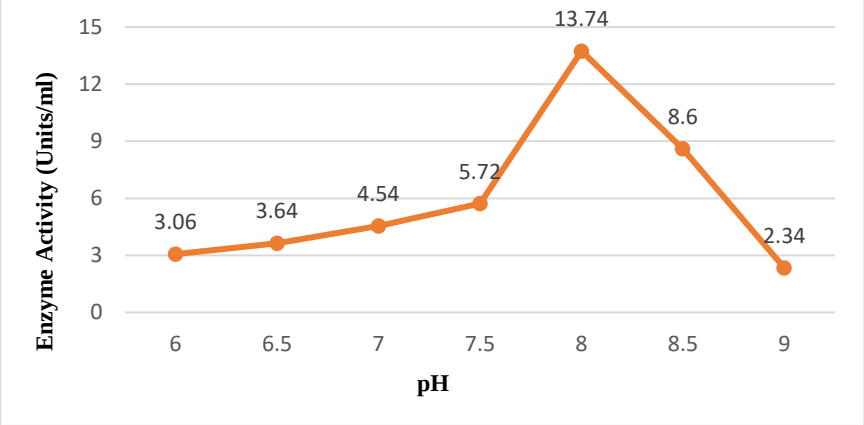

Figure 5: Activity of *S. marcescens* protease at different pH

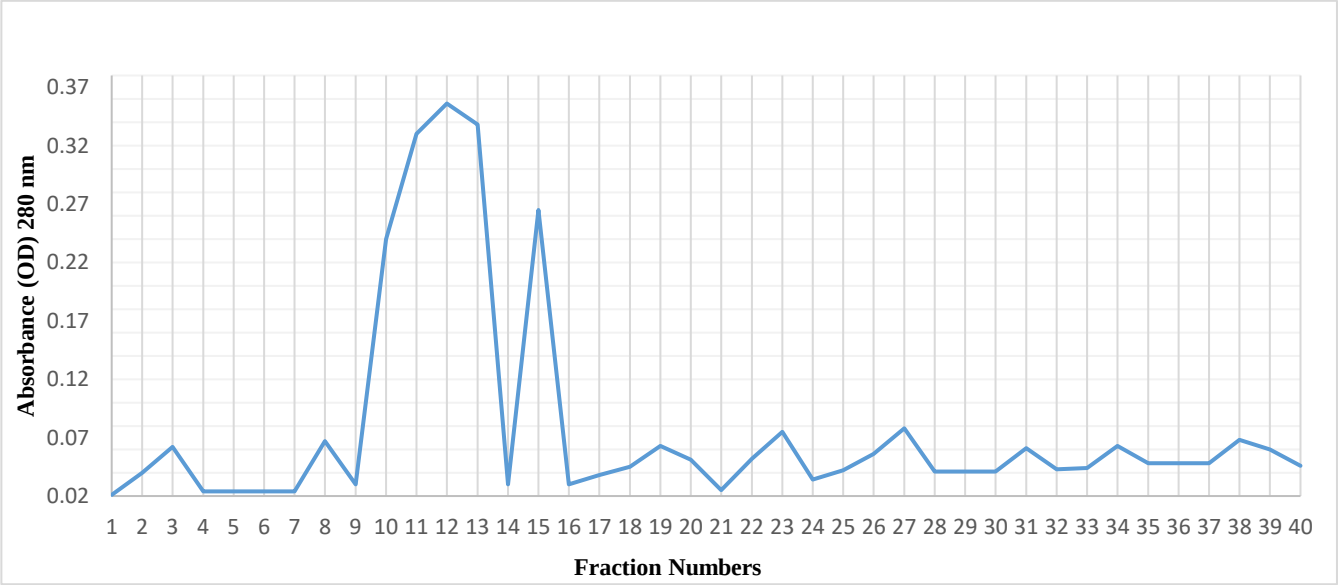

Figure 6: Gel filtration Chromatogram of ammonium sulfate precipitated proteins from culture supernatant of *S. marcescens*.

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