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EXTRACTION AND PARTIAL PURIFICATION OF PROTEASE FROM LOCAL *SERRATIA MARCESCENS* ISOLATE

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ABSTRACT

Serratia marcescens **isolated from a patient with urinary tract infection taken from the Central Health Laboratory. The sensitivity test showed the resistant to the Tetracyclin (30μg), Amoxicillin (25 μg), Gentamycin(30 μg), ampicillin (10μg), and Kanamycin (30μg), but it was sensitive to Ciprofloxacin (5 μg) only. The extracellular protease was extracted in LB broth and purified in two step included precipitation with (30-55%) saturation of ammonium sulfate, dialysis and ion exchange chromatography by DEAE–Cellulose. Only one peak contained the enzymatic activity, and purification fold was 22.6 with 37.92% recovery. Based on the inhibitory effects of EDTA, the protease was characterized as a metalloproteinase. The enzyme activity was increased in the presence of Mg2+ and decreased in the presence of Cu^2+ and Zn2+. The protease could also be activated by the non-ionic surfactants Tween 80 (1.0%) and Triton X-100 (1.0%).**

الإستخلاص والتنقية الجزئية لأنزيم البروتيز من العزلة المحلية
SERRATIA MARCESCENS

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الخلاصة

تمَ عزل بكتريا *Serratia marcescens* من عينة إدرار لمريض مصاب بالتهاب المجاري البولية وشخصت من قبل مختبر الصحة المركزي. أظهر فحص الحساسية للمضادات الحيوية بأن العزلة مقاومة للتتراسايكلين (30 مايكروغرام) وأموكسيسيلين (25 مايكروغرام) وجنتامايسين (30 مايكروغرام) وأمبيسيلين (10مايكروغرام) وكانامايسين (30 مايكروغرام) ولكن حساسة فقط للمضاد سيبروفلوكساسين (5 مايكروغرام). تم أستخلاص إنزيم البروتييز في وسط LB broth وتمت تنقيته بخطوتين بسيطتين تضمنت الترسيب بأستعمال سلفات الأمونيوم وبنسبة أشباع(30- 55%) وديلزة والترشيح بأستعمال المبادل الآيوني DEAE – Cellulose. أظهرت بأن قمة واحدة كانت تحوي على فعالية أنزيمية وبعدد مرات تنقية 22.6 ونتائج التأثيرات التثبيطية بإستعمال EDTA أظهرت بأن الأنزيم من نوع metalloproteinase وأن الفعالية الأنزيمية قد إزدادت بوجود آيونات المغنيسيوم وإنخفضت بوجود آيونات الزنك والنحاس. وقد تم التوصل الى أن إنزيم البروتييز ممكن تنشيطه بإستعمال مادة Tween 80 بتركيز(1.0%) و Triton X-100 بتركيز(1.0%).

INTRODUCTION

Serratia marcescens is a species of Gram-negative, rod-shaped bacterium in the family Enterobacteriaceae. A human pathogen, *S. marcescens* is involved in nosocomial infections, particularly catheter-associated bacteremia, urinary tract infections and wound infections (1,2). *S. marcescens* is a motile organism and can grow in temperatures ranging from 5–40°C and in pH levels ranging from 5 to 9. It is differentiated from other Gram-negative bacteria by its ability to perform casein hydrolysis, which allows it to produce extracellular metalloproteinases which are believed to function in cell-to-extracellular matrix interactions(3). Most *S. marcescens* strains are resistant to several antibiotics because of the presence of R-factors, which are a type of plasmid that carry one or more genes that encode resistance (2). The ability of some *S. marcescens* strains to colonize a wide range of ecological niches has been linked to the production of a spectrum of extracellular products including chitinases, proteases, lipases, nucleases, bacteriocins, surfactants and wetting agents (4,5,6).

MATERIALS AND METHODS

Bacterial strains:

S. marcescens is a non pigmented clinical isolated from a patient with urinary tract infection from the Central Health Laboratory. This strain was identified by classical biochemical test (7,8) and API- system.

Sensitivity test:

It was done according to the method of Kirby-Bauer that mentioned in (9). Pure colonies were inoculated in phosphate buffer, then turbidity were compared with tube number 5 of Mac Farland standard tubes. Then cultured on Mueller – Hinton agar with the following antibiotics Tetracyclin (30µg), Amoxicillin (25µg), Gentamycin (30µg), ampicillin (10µg), Kanamycin (30µg) and Ciprofloxacin(5µg). Incubated in 37°C, then the diameter of inhibition zone was determined (mm) and compared with standard ranges that mentioned in NCCl,2002(10).

Protease detection:

The production media (Casein agar) was inoculated with 1 ml of *S. marcescens* grown in nutrient agar. Incubated the tubes at 37° C for 24 hours. Wash the cell with PBS then centrifuged at 3000 xg for 15 minutes. The supernatant was taken to determine the enzyme activity (protease) and protein concentration.

Protease activity was assayed in LB broth containing non fat dried milk to a final concentration of 2% and distributed into sterilized tubes. It was modified by addition peptone instead of trypton; pH was adjusted to 7.5 and sterilized by autoclave(11).

Determination of protease activity:

It was done by using the standard curve of trypsin (0-100 µmol) and the enzyme reaction was determined according to Murachi, 1970(12). The optical density was 275 nm and the units of enzyme activity were determined according to tyrosin standard curve. Protease activity was determined by casein digestion using a modified method described by Shimogaki *et al*.(13).

The reaction mixture consisted of 200μl of diluted enzyme solution (enzyme:buffer, 1:50) and 200 μl of 0.05 M Tris-HCl buffer (pH 8.0) containing 2% (w/v) casein, which had been preincubated at 40° C for 10 min. The reaction mixture was then incubated at 40°C for 10 min, and the reaction was terminated by adding 500 μl of a trichloroacetic acid (TCA) mixture (containing 0.11 M TCA, 0.22 M sodium acetate, and 0.33 M acetic acid). The mixture was further incubated at 40°C for 10 min, followed by centrifugation at 12,000 ×g for 20 min. The supernatant was harvested, and the absorbance was measured at 280 nm. The "blank" was prepared by the same procedure with TCA being added at zero time. One unit (U) of protease activity was defined as the amount of enzyme required to liberate 1μg of tyrosine per minute under the conditions described above (14).

Determination of protein concentration according to absolute method:
(Whitaker and Granum, 1980)(15).

The protein concentration was determined according to the equation:

$$\text{Protein concentration (mg/ml)} = \frac{\text{O.D. 235 nm} - \text{O.D. 280 nm}}{2.51} \quad \ldots\ldots\ldots\ldots (1)$$

Purification of Protease:

1- Extraction of protease

The enzyme was extracted from the local isolate after growing the isolate in casein medium in optimum conditions (0.5 % peptone, 10^6 cell/ ml of media, pH= 7.5) for 48 hours. Then the cells was harvested by centrifugation at 3000 xg for 15 minutes. The supernatant was taken enzyme purification(16).

2-Ammonium Sulphate precipitation

Ammonium Sulphate was added slowly to the pooled culture supernatant fluids, with gentle stirring, to a final concentration of 30% saturation in ice bath for 30 minutes. Then the solution was centrifuged for 20 minutes at 6000 xg in 4° C. The supernatant was taken and another amount of ammonium sulphate was added to a final concentration of 55% under the same conditions. Then the pellet was dissolved in 0.02 M phosphate buffer (pH=7). The enzyme activity and the protein concentration were determined in this solution. The preparation was dialyzed overnight against distilled water for 24 hours (17).

3-Ion exchange chromatography

A preparation after dialysis was applied to a column (2.6 by 96 cm) of DEAE – Cellulose equilibrated with phosphate buffer (0.02 M, pH= 7), and was eluted, in the downward flow mode, at a flow rate of 2 ml/ minutes. Fractions (5 ml) were assayed for absorbance at 280 nm and for protease activity. Protease peak fractions were pooled and stored at 0° C.

4-Calculation of purification folds:
   The folds of purification were estimated as:

$$\text{Fold of purification} = \frac{\text{Specific activity (unit/ mg protein)}}{\text{Specific activity (unit/ mg protein) of crud extract}} \quad \ldots\ldots\ldots(2)$$

Effects of Metal Ions and Inhibitors on Protease Activity
   The effects of different metal ions, including Ca2+, Co2+, Cu2+, Mg2+, Ba2+, Mn2+, Hg^{2+}, and Zn2+ (in the form of chlorides), was tested by preincubating the purified protease (0.5 ml) with (0.5 ml) 5 mM and 10 mM solutions of these ions at 4° C for 30 min. The residual activity was measured under standard assay conditions. The effects of inhibitors on protease activity was examined by preincubating the protease for 1h at 4°C with phenylmethylsulfonyl fluoride (PMSF), and ethylenediaminetetraacetic acid (EDTA). The final concentration of the inhibitors in the preincubation mixture was 5 mM. The residual activity was calculated with reference to the protease activity in the absence of supplements. A similar assay was conducted to determine the effects of different reagents (β-mercaptoethanol, and urea) and surfactants [sodium dodecyl sulfate (SDS), Tween-80, and Triton X-100 all at 1% (v/v)] on protease activity (14).

RESULTS AND DISCUSSION
Sensitivity test:
   The results showed that the isolate of *Serratia marcescens* isolated from urine infection was resistant to the following antibiotics: Tetracyclin (30µg), Amoxicillin (25µg), Gentamycin(30µg), ampicillin (10µg), and Kanamycin (30µg), but it was sensitive to Ciprofloxacin (5 µg) only according to the NCCL 2002(10). These findings correspond to previous reports concerning the efficacy-of these antibiotics(18,19). Antibiotics are the drugs of choice for the treatment of bacterial infections. Bacterial resistance to antibiotic therapy, however, is becoming a major threat to modern medicine.
   The biological mechanisms which lead to an antibiotic resistant are diverse, but they can be classified into three general categories(20). First, bacterial enzymes can actively degrade the drug. Resistance to the beta-lactam antibiotic ampicillin (Ap), for instance, can be mediated by the enzyme beta-lactamase. Second, bacteria can develop mutations that render the target molecule unable to interact with the antibiotic. Streptomycin (Sm) is an aminoglycoside antibiotic example. Finally, changes in bacterial cell permeability to an antibiotic can confer antibiotic resistance. Ampicillin resistance (Apr) in Gram-negative bacteria can arise from mutations in outer membrane proteins(19). *S marcescens* is naturally resistant to ampicillin, macrolides, and first-generation cephalosporins. In Taiwan, 92% of the strains are resistant to cefotaxime, but 99% are still susceptible to ceftazidime. Extended spectrum beta-lactamases are produced by most *S marcescens* strains (21).

Serratia infections should be treated with an aminoglycoside plus an antipseudomonal beta-lactam, as the single use of a beta-lactam can select for resistant strains. Most strains are susceptible to amikacin, but reports indicate increasing resistance to gentamicin and tobramycin. Quinolones also are highly active against most strains. Definitive therapy should be based on the results of susceptibility testing because multiresistant strains are common (22).

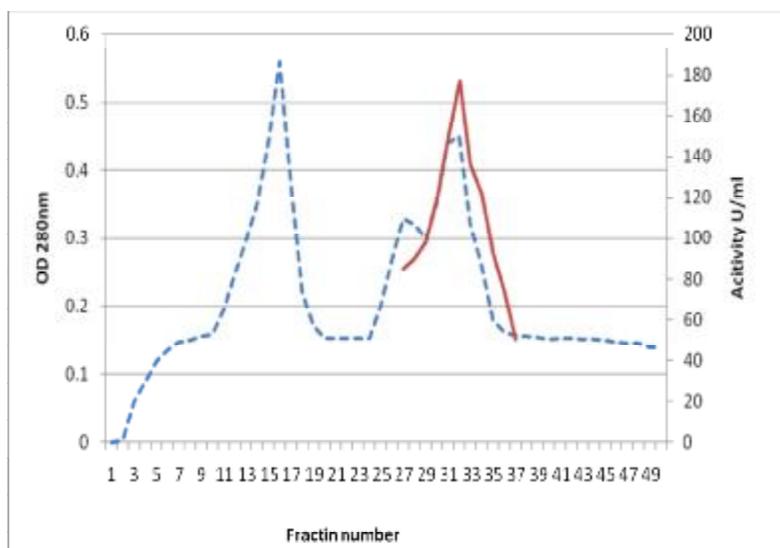**Production of proteases by *S. marcescens***

S. marcescens was able to hydrolyze casein when grown on skim milk agar; a clear zone around the colonies (0.5 mm) was observed indicating protease production. In this study the bacterium was cultured in four different liquid media containing different nitrogen sources to determine which medium is better for protease production. The results showed that L.B broth medium (containing10% yeast extract and pepton as nitrogen source) was the best medium for protease production by *S. marcescens* since it gave maximum protease specific activity (145unit/mg protein) while tryptic soy broth gave minimum specific activity (57unit/mg protein) (data not shown).

It can be concluded from these results that pepton and yeast extract may support the growth of bacteria and encourage protease activity.

Several studies reported that Luria-Bertani broth (L.B) is suitable for protease production from different *S. marcescens* strains, Mao-Hua and his woorkers used L.B broth for production and molecular analysis of a solvent tolerant proteases from *S. marcescens* (14). Castelli *et al*., (11*)*also used L.B broth to produce protease from *S. marcescens*. While Ustariz found that the whey was increased the protease production when used in production media(23). Molla and his coworkers found that the production of protease from *S. marcescens* was in tryptosoy broth (24).

Purification of protease

Serratia marcescens protease was purified by simple steps included precipitation with (30-55%) saturation of ammonium sulfate, dialysis and ion exchange chromatography by DEAE–Cellulose, the eluted fractions of the last step contained three protein peaks Figure(1), only the third peak (fractionation tubes 27-37) contained protease activity about 177 unit/ml and specific activity 1180 unit/mg and the purification fold was 22.6 times with 37.92 % recovery table(1).

Figure(1):Ion exchange for purification of proteases produced by *Serratia marcescens* by DEAE–Cellulose column (2.6 by 96 cm), the resin was equilibrated with phosphate buffer(0.02M, pH=7),the flow rate was 2ml/minutes, the fractions volume was 5 ml.

It was observed in table(1) that the activity and specific activity of proteases was decreased in dialysis step, this may be attributed to auto digestion of protease since this kind of enzymes can breakdown by itself.

The extracellular protease produced by *S. marcescens* was purified by gradient ammonium sulfate precipitation and ion-exchange chromatography and the results are summarized in table(1). The supernatant was fractionated by adding ammonium sulfate to 55%-30% saturation with 82.85% yield. Other studies showed that the yield in this step was 69.1(14).

Table(1): Purification of *Serratia marcescens* protease.

The step	Volume ml	Enzymatic activity unit/ml	Total activity	Protein conc. mg/ml	Specific activity Unit/mg protein	Purification fold	Recovery %
Crude extraction	500	28	14000	0.53	52	1	100
Precipitation with (55-30) saturation of $(NH_4)_2SO_4$	20	580	11600	2.5	232	4.46	82.85
Dialysis	25	320	8000	1.88	170.2	3.27	57.14
Ion exchange with DEAE – Cellulose column	30	177	5310	0.150	1180	22.6	37.92

The ion exchange showed three peaks of protein fig.(1), only one peak contained the enzymatic activity, and purification fold was 22.6 with 37.92% recovery as compared with the result of Mao-Hua,2010 (14) showed 5.98 and 19.87 respectively, whereas Loomes and his co-workers used30%saturation of ammonium sulfate to precipitated protease of *P.mirabilis* and observed one peak for protease in fractions of gel filtration by Sephacryl S-200 (25), also one peak was appeared in Phenyl-Sepharose gel filtration contain protease activity belong to metalloprotease from other strain of *P.mirabilis* BB2000(26). Other local studies revealed that the purification fold of *S. marcescens* protease extracted by DEAE–Cellulose was 12.67 and the specific activity was 2850 U/mg, and the recovery was 23.75% (16). The study of Al-Rubai, 2009 on *Proteus mirabilis* metalloprotease showed 23 purification fold, 22.5% recovery and 1205 U/mg of enzyme activity(27).

   In other studies such as Zinc-containing metalloprotease that produced by *Enterobacter sakazakii* was treated with 70% saturation of ammonium sulfate and purified to homogeneity by gel filtration chromatography with Sephadex G-100 and followed by Phenyl-Sepharose CL-4B(28). also the alkaline protease of *Pseudomonas aeruginosa* PD100 was precipitated by 70% ammonium sulfate saturation and purified with Sephadex G-50 column and CM-Sephadax(29), While *Burkholderia pseudomallei* protease was collected by centrifugation of L.B broth, the supernatant was treated with 65% saturation of solid ammoniumsulfate and dialyzed against ammonium bicarbonate buffer pH=8 and subjected to gel filtration on Sephadex G-100(30), whereas the zinc metalloprotease of *Vibrio angullarum* was precipitated by treated with 80 % saturation of ammonium sulfate and SephadexG-200 was used for gel filtration chromatography (31).

Effects of Metal Ions and some Inhibitors on Protease Activity

The effects of various metal ions and inhibitors on protease activity are summarized in table(2). The results showed that the enzyme activity was weakened in the existence of the ions of mercury in two concentrations 5 and 10 mM (the remaining activity has approached 30% and 12% respectively) and ion copper in two concentrations 5and 10mM (the remaining activity has approached 18% and 15% respectively).

This result is coincided with other local study that showed decreased activity in the presence of mercury and copper in 5 and 10mM (42%, 39%, 29% and 25% respectively) (16). The enzyme activity was increased in the presence of Mg^{2+} ion table(2) that reaches to 115% and 110% in concentration of 5mM and 10mM respectively.

Other study revealed that the activity of the protease was decreased in the presence of 1mM and 5mM of copper and zinc (42%,16%,67% and 10% respectively).While the nickel showed increment of activity to 116% and 115% respectively (14).

Table(2): Effects of metal ions on the activity of the purified protease.

Metal ion	Remainig activity (%)	
	5 mM	10mM
None	100	100
Hg^{2+}	30	12
Mg^{2+}	115	110
Zn^{2+}	120	82
Ca^{2+}	98	95
Cu^{2+}	18	15
Mn^{2+}	90	85
Co^{2+}	90	79
Ba^{2+}	86	71

*Each value represents the mean of triplicate measurements.
The individual values did not vary from the mean by more than 10%.

The purified protease was inhibited by metal chelators such as EDTA (5mM). This result suggests that the protease from this strain belongs to the metalloproteinase family. Enzyme inhibition experiments showed that PMSF(5mM), a serine protease inhibitor, had minor effects on the protease. Nonionic surfactants such as Tween 80 (1%(v/v)) slightly enhanced the protease activity, whereas 1%(v/v) solutions of the anionic surfactant SDS strongly inhibited the protease activity. Sulfhydryl reagents such as β-mercaptoethanol has no effect on the protease. Urea at 5.0mM concentration showed no effect on the protease table(3).

Table(3):Effects of inhibitors and surfactants on the activity of the purified protease.

Reagent	Concentration	Remaining activity
None	-	100
EDTA	5mM	32
PMSF	5mM	75
Urea	5mM	65
ß- Mercaptoethanol	5mM	82
SDS	1%	10
Tween-80	1%	102
Triton X-100	1%	70

*Each value represents the mean of triplicate measurements.
The individual values did not vary from the mean by more than 10%.

   This result is in agreement with other local study(32); also with Mao-Hua *et al*., 2010.other study used 1,10 phenanthrolin showed decreased in total activity of protease (84%) (23). The protease from *S. marcescens* was purified by a two-step purification procedure. The protease was moderately inhibited by the sulfhydryl reagents β-mercaptoethanol, suggesting that there are disulfide bonds. Nonionic surfactants significantly enhanced the protease activity, whereas ionic surfactants such as SDS (anionic) was strong inhibitors of the protease, which suggested that there are a few ionic residues on the surface of the protease and these can easily be paired. Anionic and cationic surfactants are known to denature enzymes through interactions between the surfactants and the surface charge of the protein (33,34). The partial purified protease was inhibited by metal-chelating reagents such as EDTA, indicating that this protease is a metalloproteinase.

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