

The Antibodies Titer of Anti-*Serratia marcescens* Protease by Passive Hemagglutination

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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 (30 µg), 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 simple 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. The result of passive haemagglutination indicated that the optimum concentration of Protease was (7.5) µg/ ml which gave positive reaction with a highest dilution of serum in (1/ 320).

الخلاصة

تم عزل بكتريا *Serratia marcescens* من عينات مرضى مصابين بالتهاب المجاري البولية أخذت من مختبر الصحة المركزي . بين فحص الحساسية للمضادات الحيوية مقاومة هذه البكتريا للتراساكلين ٣٠ ميكروغرام ،والاموكسيسيلين ٢٥ ميكروغرام ،والامبيسلين ١٠ ميكروغرام ،والكاناميسين ٣٠ ميكروغرام . لكنها حساسة للسبروفلاكسيسيلين ٥ ميكروغرام تم استخلاص البروتين الخارج في وسط LB وتنقيته بخطوتين تتضمن الترسيب بكريات الامونيوم المشبعة (٣٠-٥٥%) ، ثم عملية ديلزة وعملية تبادل أيوني ب-EDTA-سليولوز . كان منحنى واحد يمثل النشاط الانزيمي وكانت قمة النقاوة عند ٢٢,٦ مع ٣٧,٩٢ أسترجاع . نتيجة التكتل الدموي المنفصل بينت أن أفضل تركيز للبروتين كان ٧,٥ ميكروغرام/مل حيث أعطى نتيجة موجبة مع أعلى تخفيف من المصل (١/٣٢٠).

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 (Hejazi and Falkiner, 1997). Monospecific rabbit antisera were raised against the purified proteases vaccine and agglutination antibody titers of the sera from control animal and from the animals vaccinated with the protease can be determined by passive hemagglutination method . various investigators have indirectly demonstrated vaccination against the *serratia* protease elicited active immunization against experimental *serratia* , these findings strongly support the idea that *serratia* protease is involved in the pathogenesis of *serratia* (David and Arnold, 1983) .

The aim of this research is to determine the antibodies titer in rabbit sera and for the determination of the optimal antigen (protease) concentration.

Materials and Methods

Bacterial strains: *S. marcescens* in 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 (Holt *et al.*, 1994; Collee *et al.*, 1996) and API- system.

Sensitivity test:

This done according to the method of Kirby-Bauer that mentioned in (Prescott *et al.*, 1990). The isolates were cultured on Mueller – Hinton agar with the following antibiotics Tetracyclin (30 µg), Amoxicillin (25 µg), 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 NCCI,2002 (NCCLS,2002).

Detection of isolate ability to produce exoenzyme (Protease) and Determination of protease activity:

This was done according to (Castelli *et al.*,2008 ; Murachi, 1970 ; Shimogaki *et al.*,1991 and Mao-Hua *et al.*,2010).

Determination of protein concentration and specific activity:

This was done according to (Whitaker and Granum, 1980) .

Purification of Protease:

1- Extraction of protease:

The enzyme was extracted from the local isolate after growing the isolate in optimum conditions (0.5 % peptone, 10^6 cell/ ml of media, pH= 7.5) for 48 hours. Then the harvesting of the cells was done by centrifugation at 3000 xg for 15 minutes. The supernatant was taken to purify the enzyme (Al-Bayati,2004).

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 (Lyerly and Kreger ,1979).

3- Ion exchange chromatography:

The 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.(Lyerly and Kreger ,1979).

Passive Heamagglutination:

2 Rabbits, weighing (2.7-3) kg were vaccinated intradermaly For 2 week on (0 ,4 ,9,14) days for a total of four vaccinations with 0.5 ml of injections containing 1 mg of purified protease isolated from serratia for each one. Control animal was vaccinated with phosphate-buffered saline (PBS, pH 7.4).

Rabbits were bled 7 to 10 days after the last vaccinations and agglutination antibody titers of the sera from control animal and from the animals vaccinated with the protease were determined by passive hemagglutination method(Harbert,1978).

A . Collection of R.B.C. :

Alsever's solution used to collect SR.B.Cs which prepared according to Herbert (1978) by dissolving 2gm of dextrose ,0.8 gm of sodium citric acid in small volume of D.W. ,and then sterilized by autoclaving for 10 minutes.

B . Preparing of S.B.Cs :

S.R.B.Cs were separated from plasma and Alsever's solution by centrifuged at (3000 rpm) for 10 minutes . The supernatant was removed and sediment cells were washed three times with PBS, the R.B.Cs were suspended in 10% PBS.

C. Inactivation of serum :

The serum of the rabbits was inactivated by heating at 56° C for 30 minutes to destroy the complement .

D. Adsorption of serum :

This was done to remove the natural antibody which may be found in the serum of rabbits against sheep R.B.Cs (anti-sheep) ,which it lead to non-specific reaction ,the adsorption of serum must be done by making a reaction between inactivated serum and sheep RBCs suspension .

Adsorption of serum was done by :

-The serum was diluted by transferring (1)ml of serum with 9 ml of PBS to obtain dilution (1/10).

-(1)ml of diluted was mixed with (0.1 ml) of SR.B.Cs suspension ,then the tube was put in water bath at 37° C for 10 minutes.

-The serum (supernatant) was collected in sterile tubes.

-The reaction between adsorption serum and SR.B.Cs suspension was done.

E. Determination correct quantity Of protease to adsorb on surface of SR.B.Cs :

In order to determine the correct quantity of protease adsorb on surface of SR.B.Cs the concentration (0.25 ,0.5 ,0.75 ,1.0) μ g /ml prepared by using PBS .(1)ml of each concentration was added in sterile tubes and mixed with (1)ml of SR.B.Cs suspension then incubated at 37° C for (1)hr in a water bath . The tubes must be shaken gently once or twice during this period SR.B.Cs which coated with antigen were precipitated by centrifuge at (3000)rpm for 10 minutes with . The supernatant was removed and the sediment cells were washed two times with PBS, then content of each tube was resuspend with 0.5% PBS .

F. Titration of antibody with coated R.B.Cs :

Serial of 2 fold dilutions (1/10 ,1/20 ,1/40 ,----1/640) of serum (inactivated and adsorbed) were prepared by using PBS . (0.5)ml of each dilution was added in the individual well of coated SR.B.Cs was added .The plate was gently agitated and incubated at 37° C for 1hr and then left in the refrigerator overnight .

Three controls were prepared :

- First control consist of (0.5)ml SR.B.Cs (uncoated cells) with (0.5)ml of serum (heat inactivated and adsorbed) .

-Second control consist of (0.5)ml SR.B.Cs (uncoated cells) with (0.5)ml of PBS .

-Third control consist of (0.5)ml of SR.B.Cs coated with antigen with (0.5)ml of PBS .

Results and Discussion

Sensitivity test:

The results showed that the strain of *Serratia marcescens* isolated from urine infection was resistant to the following antibiotics: Tetracyclin (30 μ g), Amoxicillin (25 μ g), Gentamycin(30 μ g), ampicillin (10 μ g), and Kanamycin (30 μ g), but it was sensitive to Ciprofloxacin (5 μ g) only according to the NCCL 2002(10) .

These findings correspond to previous reports concerning the efficacy-of these antibiotics(Lyerly and Kreger ,1979; Herbert,1978)

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 (Cooksey *et al.*, 1975).

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 1205 unit/mg and the purification fold was 23 times with 22.5 % recovery (Table 1).

This result may indicate that *Serratia marcescens* produce only one type of protease.

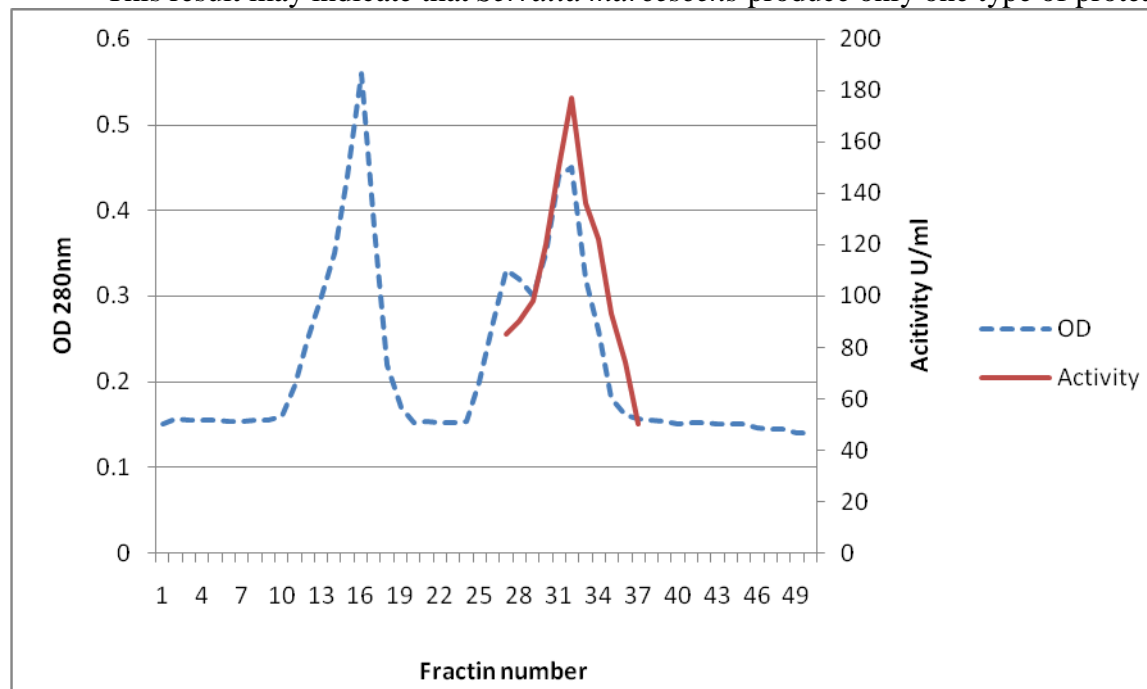


Figure 1: Ion exchange for purification of proteases produced by *Serratia marcescens* 4 by DEAE – Cellulose column (2.6 by 96 cm), the gel was equilibrated with phosphate buffer (0.02 M, pH= 7), the flow rate was 2 ml/ minutes, the fractions volume was 5 ml.

It was observed in (table1) 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	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) ₂ SO 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 appeared 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) that showed 5.98 and 19.87 respectively, whereas Loomes and his co-workers used 30% 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 (Haddix *et al.*,2000), also one peak was appeared in Phenyl-Sepharose gel filtration contain protease activity belong to metalloprotease from other strain of *P.mirabilis* BB2000 (Mlynarczyk *et al.*,2007). 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, and the recovery was 23.75% (Whitaker and Granum, 1980).The study of Al-Rubai, 2009 on *Proteus mirabilis* metalloprotease showed 23 purification fold, 22.5% recovery and 1205 U/mg of enzyme activity(Loomes,1990).

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 (Wassif *et al.*,1995),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 (Al-Rubai,2009),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 (Kothary *et al.*,2007), whereas the zinc metalloprotease of *Vibrio anguillarum* was precipitated by treated with 80 % saturation of ammonium sulfate and SephadexG-200 was used for gel filtration chromatography (Najafi *et al.*,2005).

Passive haemagglutination:

This test was used to determine the titration of anti- protease (antibodies) for protease of *S. marcescens* in serum of rabbits (control and experimental). Agglutination was indicated by a complete carper of cell covering the bottom of wells of microtiter plate wheras non- agglutinated cells from a compact button or small ring at the center of the wells (Ling *et al.*, 2001) .

The result by using this test indicated that the optimum concentration of Protease was (7.5) µg/ ml which gave positive reaction with a highest dilution of serum in (1/320) as shown

in figure (2), while the serum of the control animal gave a negative result (non- agglutination) because the control animal was injected with a phosphate buffer saline so that there is no antibody in the serum for protease as shown in figure (2).

Other local studies showed that the antigens (glucan binding protein (GBP)) of *Streptococcus mutans* and the LPS of *Pseudomonas aeruginosa* were 50µg/ml and 10µg/ ml that gave positive reaction with highest dilution of serum in 1/320 and 1/160 respectively (Yang *et al.*,2007 ; Venkatish *et al.*, 2006).

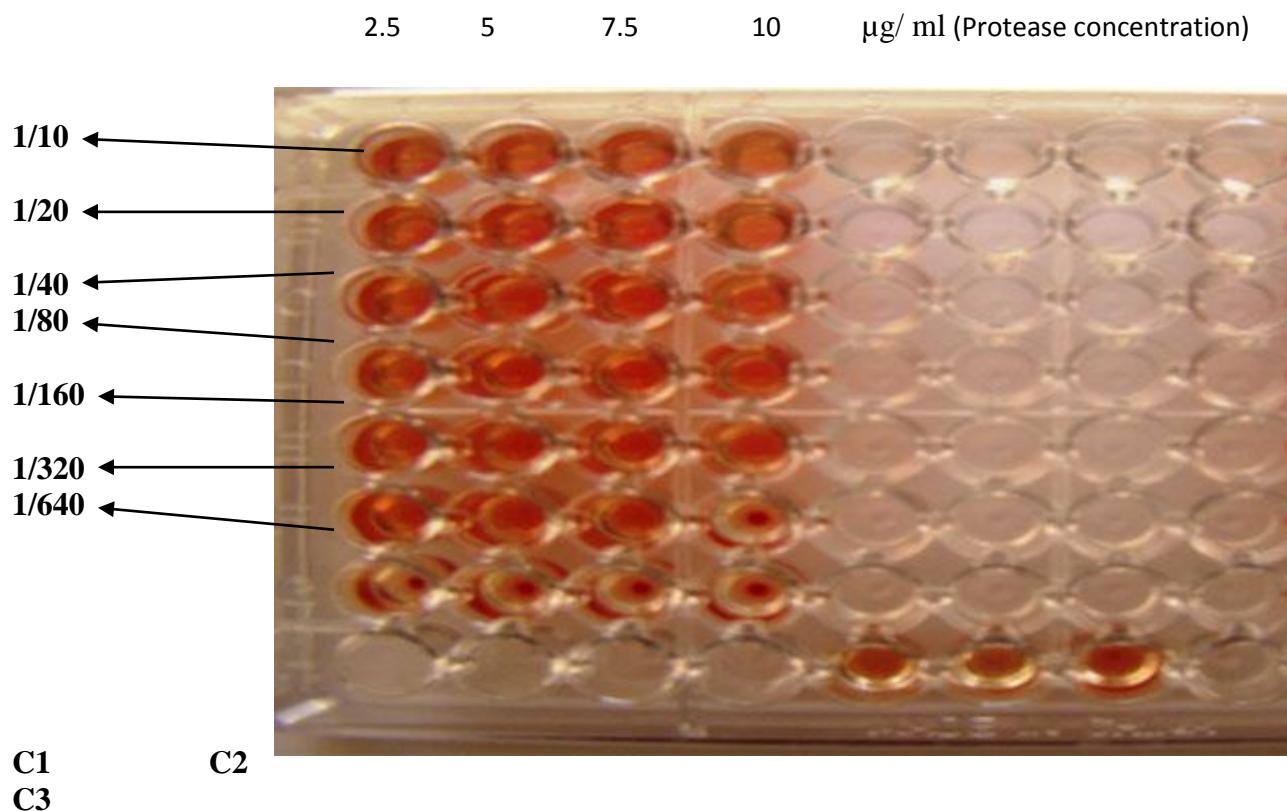


figure (2): Passive haemagglutination test –optimum concentration of protease was (7.5) µg/ml which gave positive reaction with highest dilution 1/320 of serum , control showed negative result .

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