The Isolated and Purified β-lactamase from Local Isolate of Staphylococcus aureus

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Abstract

β-lactamase was isolated from local isolate of *Staph. aureus*, which elicited its resistance to penicillin–G, and rapid β-lactamase production. β-lactamase has been purified by using gel-filtration chromatography in sephadex G–100 column. The molecular weight of purified β-lactamase was estimated by SDS-poly acryl amide gel electrophoresis and shown one band protein with molecular weight of 30 kDa. Antiserum was prepared for purified β-lactamase in rabbits, the measurements of antibodies titer were done by using passive haemagglutination test and it equaled to 40960, which indicated the immunogenicity of purified β-lactamase. The neutralization of β-lactamase activity by antiserum was performed *in vitro*, so *Staph. aureus* isolates regained their sensitivity to penicillin–G.

Introduction

β-lactamase is a type of enzymes, produced by some bacteria, and it is responsible of their resistance to β-lactam antibiotics like penicillins, cephalosporins, monobactams, and carbapenems. The β-lactamase enzymes break the β-lactam ring, deactivating molecules antibacterial properties (Oefner *et al.*, 1990; Jacoby and Munoz, 2005).β-lactamases is the most prevalent mechanism of bacterial resistance to the β-lactam family of antibiotics (Hedberg and Nord,1996; Rice and Bonomo,2000 ;Shoichet Lab., 2003). These enzymes protect bacteria from the lethal effect of β-lactam antibiotics, and are therefore of considerable clinical importance (Herzberg and Moult, 1987). Their occurrence in many bacterial pathogens poses a threat to public health and a challenge to medicinal chemists when developing new and more effective β-lactam antibiotics (Nord and Hedberg, 1990; Rupp and Fey, 2003).Many strains of *Staph. aureus* produce an inducible β-lactamase (penicillin amido-β-lactam-hydrolyse, EC 3.5.2.6.). In exponentially growing cultures, much of the enzyme is released into the culture medium, and this exoenzyme can readily be purified in large amounts (Richmond, 1963). Enzymes with slightly different chemical and enzymatic properties have been found in different isolates of *Staph. aureus* (Richmond, 1965). Four wild-type variants of *Staph. aureus* β-lactamases, designated A,B,C and D, have been identified. Although kinetically distinguishable, they differ in the primary structure by only a few amino acids (Voladri *et al.*, 1996), and have been identified by serologic (Richmond, 1965) and kinetic (Kernodle *et al.*, 1990) methods. These variants were designated as types A, B, C, and D.Each of the four recognized types of *Staph. aureus* β-lactamases (A,B,C, and D) is a class A β-lactamase with a serine active site. The mature form of the enzymes has a molecular mass of 30 kDa, contains 257 amino acids, and is excreted extra cellulary (Ambler, 1980).Four variants of *Staph. aureus* β-lactamases can be distinguished by serotype (Richmond, 1965; Rosdahl, 1973), and kinetic attributes (Kernodle *et al.*, 1980; Kernodle *et al.*, 1990; Zygmunt *et al.*, 1992).

Materials and Methods

Isolation and purification of β-lactamase

Isolation of crude β-lactamase

β-lactamase was isolated from local isolate of *Staph. aureus* obtained from (Al-Shalal, 2006), briefly *Staph. aureus* was

isolated in 100 ml nutrient broth at 37ºC, diluted 10 fold with the fresh nutrient broth; the culture was incubated with shaking at 37ºC. After 1.5 hr. of incubation, the penicillin-G was added to the final concentration of (6g/l) as the inducing agent, the incubation continued for 3 hr. The bacterial cells were harvested by centrifugation at 10000 g for 30 min at 4ºC, washed once with (0.05M) Na_2HPO_4/KH_2PO_4,(pH 7.0), suspended in 4 ml of the same buffer, and disrupted by ultra-sonicater (Labsonic 2000) for 30 min in an ice-water bath. The disrupted cells suspension was centrifuged at 10000g for 15 min at 4ºC, and the crude enzyme extract (the supernatant) was lyophilized by the freeze dryer type (Lab.Con Co.), then stored at -20ºC until use.

Assay of β-lactamase

β-lactamase activity was determined by a micro-iodometric assay according to (Novick, 1962; WHO, 1978), described as follows:

Solutions

a- Iodine solution

(2.03 g) iodine, and 5.32 g potassium iodide were dissolved in 100 ml of distilled water, and stored in a brown bottle in 4ºC.

b- Starch solution

(2 g) soluble starch was suspended in 100 ml of distilled water, incubated in a water bath at 100 ºC for 10 min, until clear, then cooled to room temperature.

1- (0.2 M) Na_2HPO_4 solution

Prepared by dissolving 28.39 g from Na_2HPO_4 in 900 ml distilled water, then volume is completed to liter.

2- (0.2 M) NaH_2PO_4 solution

Prepared by dissolving 31.2 g from NaH_2PO_4 in 900 ml distilled water, then volume is completed to liter.

c- phosphate buffer solution

prepared by mixing 92 ml of solution No.1 with 8 ml of solution No.2, volume was completed to 200 ml to get (0.1 M) phosphate buffer solution at pH 7.0, then diluted with 100 ml distilled water to get (0.05 M) at pH 7.0

d- Starch-Iodine reagent

(0.3 ml) of Iodine-potassium iodide was mixed with 180 ml of the phosphate buffer solution, and 20 ml of 2% starch solution was added slowly with stirring, then stored in a brown bottle in 4ºC.

e- Penicillin-G solution

Prepared at moment, by dissolving 0.0089 g from penicillin-G antibiotic powder in 10 ml of phosphate buffer solution, kept on ice.(1ml) of starch-iodine reagent was added to:

Penicillin-G	0.025 ml
Crude or pure enzyme	1 ml
Starch solution	0.2 ml

These components were mixed in small test tubes, blue colour developed immediately due to the reaction of iodine with starch. Rapid decolourization indicated β-lactamase production.

Control solution was made up by replacing the enzyme in phosphate buffer solution, and the absorbance was read spectrophotometrically at 620 nm.

Protein determination

Protein concentration was carried out spectrophotometrically in absorbance at (280, 260) nm, by the method of (Hudson and Hay, 1989) according to the equation:

Protein concentration mg/ ml = $1.55\times A_{280} - 0.77\times A_{260}$

β-lactamase purified

Purification of crude β-lactamase was performed according to (Ambler, 1975 by the development of the method of Richmond, 1963).

Dialysis

The crude β-lactamase was subjected to dialysis against 3 liters of (0.05M) Na_2HPO_4/KH_2PO_4 phosphate buffer solution at pH 7.0 to remove salts, then lyophilized.

Gel –Filtration chromatography

Gel–Filtration chromatography was employed by dissolving 0.5g of the crude β-lactamase in 5 ml of (0.05 M) Na_2HPO_4/KH_2PO_4 phosphate buffer solution

at pH 7.0 then, after filtration it was subjected to a sephadex G-100, applied on a column (80 by 2 cm), equilibrated and eluted with the same phosphate buffer solution pH 7.0 at a flow rate of 12 ml/hr., and 3 ml fractions were collected during elution. Column fractions were monitored for protein concentration by measuring the absorbance at (280, 260) nm.

Estimation molecular weight of β-lactamase

The purity and the molecular weight of β-lactamase preparation following gel-filtration chromatography were estimated by disc sodium dodecyl sulphate–poly acrylamide gel electophoresis according to the method of (Laemmli, 1970).

Preparation of anti-β-lactamase antibodies

An antiserum production was performed according to (Fujii *et al.*, 1987), where rabbits are injected with 1 mg of β-lactamase protein, which is dissolved in 0.5 ml of saline, emulsified with 0.5 ml Aluminum potassium sulphate (Alum), an amount of 0.5 ml was injected between the shoulder blades of the rabbits. The injection was repeated to one week intervals. A booster injection containing 0.5 mg of β-lactamase protein in 0.5 ml saline was administrated intravenously two weeks after the second injection, antiserum was collected two weeks after the last injection.

Passive haemagglutination test

The titer of β-lactamase antibodies was determined by passive haemagglutination test according to (Harbert, 1973).

Neutralization of β-lactamase activity by anti-β-lactamase antibodies

It was performed according to (Fujii *et al.*, 1987). Mueller-Hinton agar medium was prepared and distributed in 2 ml amount into clean test tubes, sterilized in autoclave at 121ºC/15pound/inch^2 for 15 min, cooled to 45ºC, then antiserum was added in (50, 100, 150) µl volumes and mixed well with the medium, poured in plates (4mm diameter Petri dish), then inoculated with a 24 hr. nutrient broth culture of *Staph. aureus* containing 10^6CFU/ ml (according to McFarland standard scale), then ten units of penicillin-G disc was placed in the centre of each plate to detect the inhibition zones. Control plates were employed without adding antiserum.

Results and discussion

The determination of β-lactamase production may be achieved by a biochemical tests for the enzyme presence by measuring the production of penicilloic acid, which is produced when β-lactamase hydrolyzes penicillin–G, the acid production has been detected by two ways:

a. measuring the change in pH with an indicator dye (acidometric method)

b. exploiting the ability of penicilloic acid to reduce iodine and reverse the formation of the blue colour when the latter complexes with starch (iodometric method), (Miles and Amyes, 1996; Lianes *et al.*, 2003) .The inducible nature of staphylococcal β-lactamase may explain the presence of the enzyme in all isolates subjected to the iodometric method in the current study. Novick, (1962) clarified that the continuous presence of the inducer is required for the induction of staphylococcal β-lactamase, the induction of the enzyme by several new penicillins, and its activity towards them are presented. The inducible formation of β-lactamase is of special importance in clinical medicine and development of new β-lactam antibiotics (Minami *et al.*, 1980).

Isolation and purification of β-lactamase

The isolate selection

The typical local isolate of *Staph. aureus* was selected from other β-lactamase producing isolates for its potent production of the enzyme, indicated by:

a- rapid decolourization of iodine in iodometric method.

b- noticeable resistance to penicillin-G (giving 5mm inhibition diameter) indicated by the penicillin sensitivity test.

The iodometric method revealed that all of *Staph. aureus* isolates were β-lactamase producers demonstrated through the rapid decolourization of iodine, although the isolates showed differences in the time of decolourization from one isolate to another with notification that isolates which exhibit less inhibition diameters in penicillin sensitivity test than the other, will be the faster in the reduction of iodine. These results agreed with (Rennie, 1999; Lianes *et al.*, 2003). This finding indicated the resistance of these isolates to penicillin, and also mean resistance of *Staph. aureus* to penicillin was β-lactamase mediated.

Assay of crude β-lactamase

The supernatant that the enzyme isolation yielded was assayed to detect β-lactamase presence. Rapid decolourization was noticed just after the reaction of iodine with starch, which indicates β-lactamase presence. This is compatible with the results reported by (Novick, 1962; Sargent, 1968).The staphylococcal β-lactamase has so great an affinity for its substrate (Novick, 1962), and its activity can be reliably estimated iodometrically (Perret, 1954), manometrically (Henry and House wright, 1947), and alkalimetrically (Wise and Twigg, 1950), spectrophotometrically (Waley, 1974; Samuni, 1975).The micro-iodometric assay was a sensitive method for measuring the rate of hydrolysis of penicillin to penicilloic acid by β-lactamase. It depended upon the reduction of iodine by penicilloic acid but not by penicillin, and it was carried out by measuring the rate of decolourization of the dark-blue starch–iodine complex when the enzyme and substrate react in the presence of the starch–iodine (Novick, 1962).

β-lactamase purification by Gel–filtration chromatography

The result recovered from gel-filtration chromatography revealed one peak at 280 nm, which was obtained from 5 tubes (15, 16, 17, 18, 19) Fig. (1). This result established the purity of the enzyme from contaminants and it agrees with studies performed by (Richmond, 1963; Robson and Pain, 1976) on the purification of staphylococcal β-lactamase.

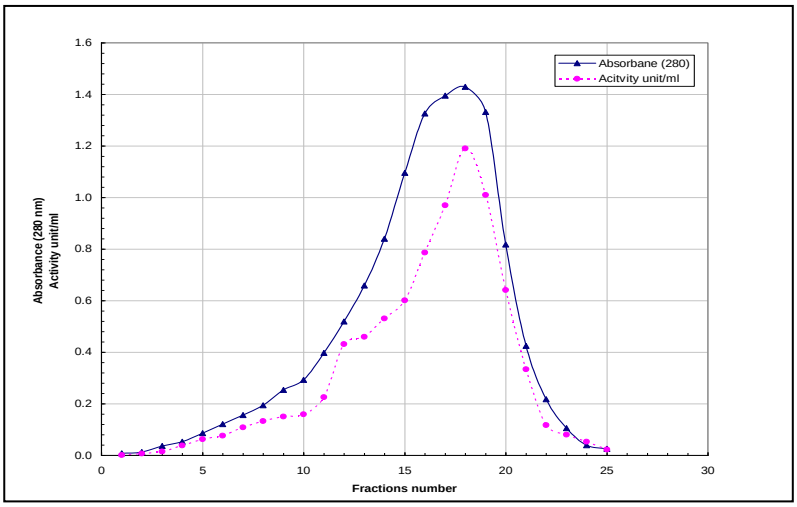

Fig. (1) Absorbance and activity values of protein concentration of purified β-lactamase by gel-filtration chromatography.

Using of gel–filtration chromatography in staphylococcal β-lactamase purification as in the researches of Ambler, (1975) and Moult *et al.*, (1985), indicated the presence of an accurate and facile process in the purification of such proteins and also in purification of β-lactamase enzymes produced by another species like in the work

of Kajsa *et al.*, (1985); Okonogi *et al.*, (1985); and Sawai *et al.*, (1973).

Protein concentration

The protein concentration was 1.047mg / ml according to the following equation:

$$1.55 \times 1.315 - 0.77 \times 1.287 = 1.047 mg/ml$$

Purity and estimation of molecular weight

The purity of β-lactamase preparation was subjected to SDS-Poly acryl amide gel electrophoresis and it was demonstrated by the presence of a single protein band which has a β-lactamase activity Fig. (2). This result is compatible with the results obtained by (Richmond, 1963; Kernodle *et al.*, 1990; Zygmunt *et al.*, 1992).

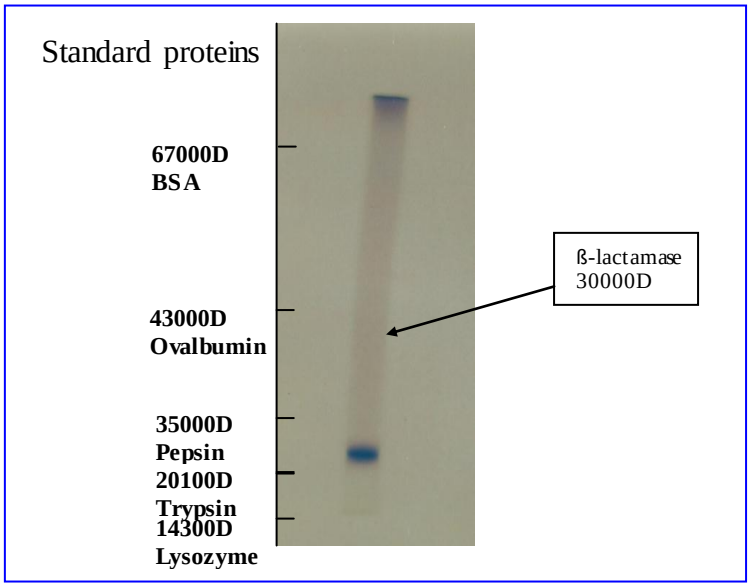

Fig. (2) SDS-poly acrylamide gel electrophoresis of purified staphylococcal β-lactamase.

The molecular weight of the purified enzyme was found to be 30 kDa, Fig.(3) This result agrees with the results reported by (Richmond, 1963; Zygmunt *et al.*, 1992; Voladri *et al.*, 1996).

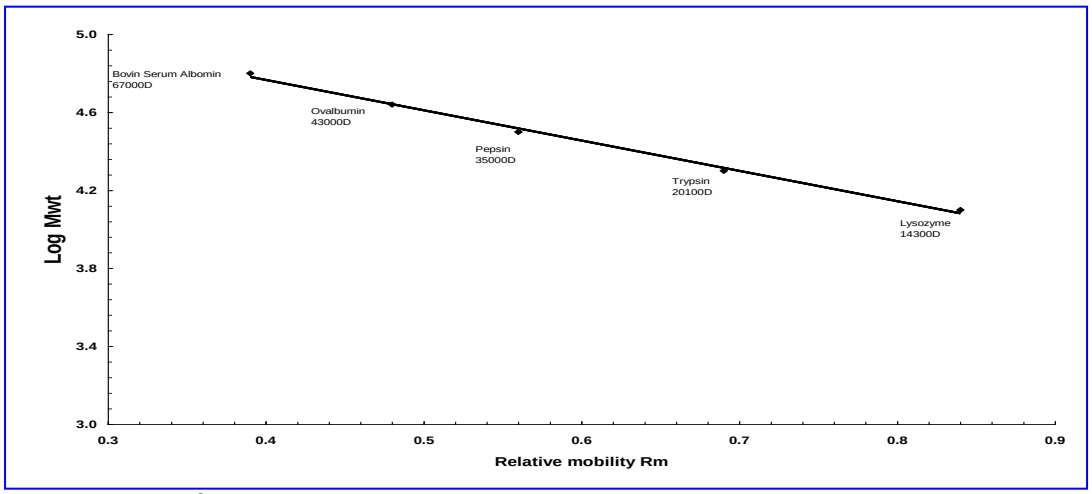

Fig. (3) Curve of standard proteins and a relationship between log M.W. and relative mobility.

The molecular weight of β-lactamases was determined by two different ways, at first, gel exclusion chromatography was used, but soon replaced by sodium dodecyl sulphate–poly acryl amide gel electrophoresis (Bush, 1989). The replacement occurred because the latter revealed other useful data that were not obtainable by the gel exclusion chromatography (Herzberg, 1991).

The immunological properties

The results obtained from passive haemagglutination test confirmed β-lactamase immunogenicity, and the antibodies titer was evaluated, it will be 40960. β-lactamase stimulated active immune response through raising the immunoglobulins value in the sera recovered from immunized rabbits with the enzyme protein. This finding is close to results reported in (Richmond, 1963; Conrath *et al.*, 2001), shown in Fig. (4).

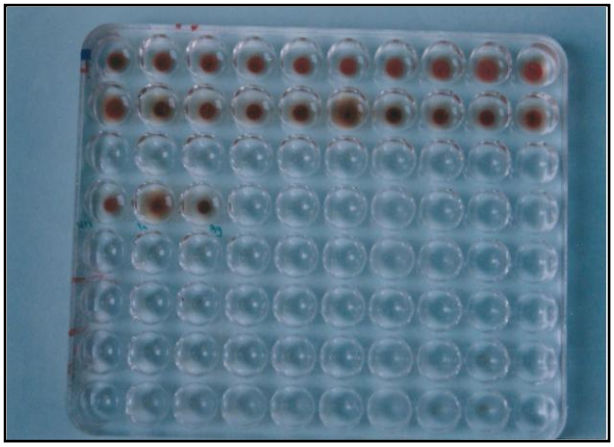

Fig. (4) Antibodies titer of anti-β-lactamase by Passive Haemagglutination Test.

The results recovered from the neutralization of β-lactamase activity by anti-β-lactamase antibodies showed that the isolates exhibited distinguishable clear inhibition zones around penicillin–G disc without a secondary growth inside the inhibition zones or cliff of colonies around it as presented in penicillin sensitivity test. A good result was seen in 50 µl volume of antiserum and this is meeting with the study of (Fujii *et al.*, 1987).

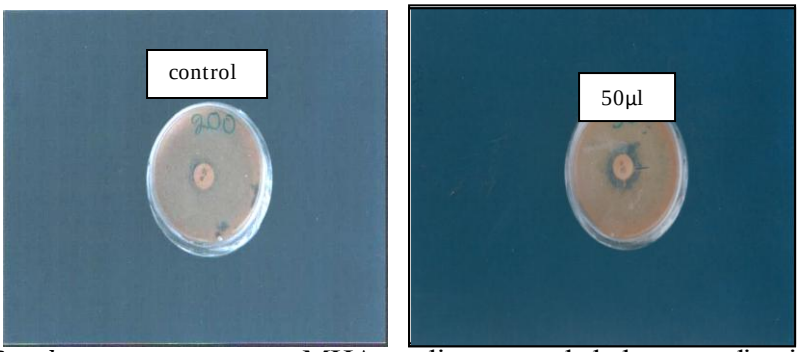

Fig. (5) *Staph. aureus* grown on MHA medium revealed the neutralization of β-lactamase activity by anti-β-lactamase antibodies.

The explanation of these results may rely on the counteracted function of β-lactamase by antiserum that gave back *Staph. aureus* its sensitivity to penicillin. On the other hand, the differences noticed in inhibition diameters may be attributed to the variation in staphylococcal β-lactamases since *Staph. aureus* produces four types of β-lactamases (A,B,C,and D)with different properties (Zygmunt *et al.*, 1992).

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عزل وتنقية انزيم البيتالاكتاميز من عزلة محلية لجرثومة المكورات العنقودية الذهبية

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الخلاصة

تم عزل انزيم البيتالاكتاميزمن عزلة محلية لجرثومة المكورات العنقودية الذهبية اظهرت مقاومتها للبنسلين –جي وأنتاجها السريع لأنزيم البيتالاكتاميز بالاعتماد على طريقة اليود، استخدمت طريقة الترشيح الهلامي في عملية التنقية بأستخدام سيفادكس جي – ١٠٠ و حسب الوزن الجزيئي للأنزيم بطريقة الترحيل الكهربائي بأستخدام هلام البولي اكريل امايد وظهرت حزمة بروتينية واحدة وبوزن جزيئي ٣٠٠٠٠ دالتون. حضرت الامصال المضادة لأنزيم البيتالاكتاميز المنقى في الارانب وتم قياس المعيار الحجمي للاضداد المتكونة بأستخدام اختبار التلازن الدموي غير المباشر وكان الانزيم مولد مناعي قوي أذ اظهر معيار حجمي يعادل 40960. استعادت جميع عزلات المكورات العنقودية الذهبية حساسيتها للبنسلين – جي بعد معادلة فعل انزيم البيتالاكتاميز بالمصل المضاد له خارج الجسم الحي.