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Characterization of β-Lactamase from Two Pathogenic Bacteria

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A B S T R A C T

Beta-lactamase (EC 3.5.2.6) was isolated and purified from two clinical isolates of *Staphylococcus sciuri* and *Klebsiella pneumoniae* by several steps included precipitation with ammonium sulphate at 80% saturation, DEAE-Cellulose and gel filtration on Sephadex G-200 column. The characterization of the purified *β*-lactamase showed that the molecular weight was 30 KDa for *S. sciuri β*-lactamase, and 28 KDa for purified *K. pneumoniae β*-lactamase as estimated by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The purified enzyme from *S. sciuri* and *K. pneumonae* has an optimal temperatures of 35°C and 40°C, respectively. The enzyme from *S. sciuri* was more stable than that of *K. pneumoniae*. The optimal pH value were 7.0 and 6.0 from *S. sciuri* and *K. pneumoniae*, respectively. The best concentrations of penicillin G were 400 µg ml- and 500 µg ml-1 for the enzyme from *S. sciuri* and *K. pneumoniae*. The increase in the enzyme concentration resulted in continuous increase in its activity from both bacteria.

Introduction

Enzymes occur in all living cells, hence in all microorganisms. Each single strain of organism produces a large number of enzymes, oxidizing, hydrolyzing or reducing and metabolic in nature (El-Shora and Ashour, 1993; El-Shora and Metwally, 2008).

The increase in antimicrobial resistance for pathogenic bacteria is represents major problem over the last decade (Gniadkowski, 2001). Among the multidrug resistant pathogens are *Klebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter baumannii* and *Staphylococcus aureus* represents an example of methicillin-resistant. *Streptococcus pneumoniae* is penicillin-resistant and vancomycin-resistant *Enterococcus*. However, *Mycobacterium tuberculosis* is extensively drug-resistant (Alekshun and Levy, 2007).

Beta-lactamases production is an important mechanism of bacterial resistance to β-lactam antibiotics.β-lactam drugs inhibited the last sage of bacterial cell wall synthesis and they are the largest family of antimicrobial agents (Suarez and Gudiol, 2009). β-lactamases destroyed the utility of benzyl penicillin against *Staphylococci*. New enzymes and new

modes of production of old enzymes now threaten the value of cephalosporins against *Enterobacter* (Livermore, 1995). Since cephalosporins, penicillins, and carbapenems are included in the preferred treatment regimens for many infectious diseases, the presence and characteristics of these enzymes play a critical role in the selection of appropriate therapy (Bush and Jacoby, 2010).

There are three major groups of the above enzymes. They are class C cephalosporinase (AmpC), extended-spectrumβ-lactamases (ESBL) and different types of β-lactamases with carbapenemase activity of which so called metal lo-β-lactamases (MBLs), are of great concern (Helfaut and Bonomo, 2005).

The aim of the present work to purify and characterize β-lactamase from two clinical isolates: Gram-positive *S. sciuri* and Gram-negative *K. pneumoniae*.

Materials and Methods

Bacterial isolates

The two bacterial isolates (Gram-positive and Gram- negative) used in the present investigation were obtained from laboratory of clinical microbiology of the Faculty of Medicine at Mansoura University from clinical specimens of patients. The two bacterial isolates were subjected to screening tests forβ-lactamase production by phenotypic methods (iodometric method and acidimetric method) according to Livermore and Brown (2001) and were given a fast positive results (immediately result within 20-30 seconds).

The identification of the two isolates was carried out using Microscan Walk A way system (2013 Siemens Healthcare Diagnostics Inc., UK) using dried Gram-positive and Gram-negative panels which designed for use in identification to the species level and antimicrobial susceptibility testing by determining Minimum Inhibitory Concentration (MIC). This diagnosis was carried out in microbial laboratory of Mansoura University hospital for children, and the results proved two isolates *Staphylococcus sciuri* and *Klebsiella pneumoniae*. Other detection methods ofβ-lactamase were applied on *S. sciuri* and *K. pneumoniae* to make sure that these isolates producing β-lactamase which include: antibiotic susceptibility test and molecular detection of β-lactamase encoding genes by polymerase chain reaction (PCR) for both bacteria.

Isolation of crude β-lactamase

β-lactamase was isolated from two clinical isolates *S. sciuri* and *K. pneumoniae*. The isolation was carried out according to Hedberg *et al.,* (1995) with slight modification. Bacterial isolates were grown overnight in 100 ml brain heart infusion (BHI) broth at 37ºC then diluted 10-fold with the fresh brain heart infusion broth.

The culture was incubated with shaking at 37ºC. After 1.5 h of incubation, the penicillin G was added to final concentration of 100 µg ml^{-1} for enzyme induction. The incubation was continued for 4 h. The bacterial cells were collected by centrifugation at 5000 g for 15 min at 4ºC, washed twice with 50 mM Na_2HPO_4 /KH_2PO_4, (pH 7.0), and suspended the same buffer. The suspension was disrupted by ultra-sonicater in an ice-water bathfor 15 min. The disrupted cell suspension was centrifuged at 5000 g at 4ºC for 15 min.The resulting supernatant represents the crude enzyme extract which was stored at -20ºC until use.

Beta-lactamase purification

The purification of the crude enzyme extracts

of *S. sciuri* and *K. pneumoniae* was carried out at 4ºC.

Ammonium sulphate precipitation

Partial purification of the crude β-lactamase was carried out by adding of ammonium sulphate up to 80% saturation at 4ºC. The mixture was stored at 4ºC overnight followed by centrifuging under cooling at 5000 g for 15 min. The precipitated protein was dissolved in a 50 mM phosphate buffer (pH 7.0) and stored for further purification at 4ºC.

DEAE-cellulose chromatography

The enzyme from the above step was applied to DEAE-Cellulose column (2.5×20 cm) that was pre-equilibrated with 50 mM phosphate buffer (pH 7.0). The dialyzed fraction was layered carefully on the top of gel under cooling condition. The protein elution was done with the same buffer at a flow rate of 2 ml/1 min. The fractions were collected and the active fractions were pooled and concentrated by dialysis using 50 mM phosphate buffer (pH 7.0).

Gel –filtration chromatography

The concentrated DEAE-Cellulose dialyzed sample was applied to Sephadex G-200 column (2.5 x 20 cm) at 4ºC, equilibrated and eluted with 50 mM phosphate buffer (pH 7.0). Fractions were collected and analyzed for protein estimation at 280 nm and *β* -lactamase activity at 620 nm.

Estimation molecular weight of β-lactamase

The purity and the molecular weight of *β*-lactamase preparation following gel filtration chromatography were estimated by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970).

Effect of different pH values on β-lactamase activity

This experiment was carried out at various pH values (3, 4, 5, 6, 7, 8, 9 and 10). The enzyme solution was adjusted using sodium acetate buffer (pH 4 to 6), phosphate buffer (pH 7), and Tris-buffer (pH 8, 9, 10). The enzyme activity was measured at 620 nm. The relation between pH values and enzyme activity was plotted.

Heat stability of β-lactamase

One ml of enzyme solution was added in test tubes and incubated in water-bath at different temperatures (from 20°C to 90°C in the scale of 5 degree) for 15 min. The test tubes were then cooled directly in ice-bath, and the remaining activity was determined. The relation between temperature and the percentage of remaining enzyme activity was plotted.

Effect of incubation time on stability of β-lactamase

The enzyme solution was incubated at 45 ºC for different time intervals (10, 20, 30, 40, 50, 60 and 120 min), and cooled directly in ice -bath. The remaining enzyme activity was determined and the relation between different time intervals and the enzyme activity was plotted.

Effect of different substrate concentrations on β-lactamaseactivity

Substrate concentration was tested at a range of 100-500 µg ml^{-1}. The assay mixture contained 2.91 ml of 50 mM phosphate buffer (pH 7.0), 40 µl of penicillin G (100-500 µg ml^{-1}) and 50 µl of enzyme. The decrease in absorption at 620 nm against a reference containing only the enzyme in the buffer was recorded.

Determination of the kinetic parameters of β-lactamase (Km, Vmax)

The kinetic values of the free *β*-lactamase were calculated from Lineweaver – Burk plots

(Palmer, 1995). The Michaelis-Menten's constant (K_m) and the maximum attainable velocity (V_{Max}) were determined by investigating the effect of different substrate concentrations on enzyme activity. Enzyme activity was determined at different substrate (S) concentrations. The Lineweaver-Burk plot (1/V vs 1/S, where V is the reaction velocity) was then constructed, and from this graph, the K_m and V_{max} were determined for *β*-lactamase.

Effect of different enzyme concentrations on β-lactamase activity

The assay mixture contained (2.91 ml of 50 mM phosphate buffer (pH7.0), 40 µl of penicillin G, and different volumes of enzyme (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 µg ml^{-1}). The decrease in absorption at 620 nm against a reference containing only the substrate in the buffer was recorded.

Storage stability of *β-lactamase*

This experiment was carried out by storing the purified enzyme solution at 4°Cand -20°C for 7, 14, 21, 28, 35 and 42 days. The residual enzyme activity was determined after each period.

Results and Discussion

Beta-lactamase Purification

β-lactamase of *S. sciuri* and *K. pneumoniae* were purified using schedule including ammonium sulfate precipitation, DEAE-Cellulose and Sephadex G-200. The results of purification are shown in Tables 1 and 2. The specific activities were 70 and 100 units mg^{-1} protein for the enzyme from *S. sciuri and K. pneumoniae*.

Elution profile of β-lactamase

The profile of purification contained 17 fractions and in each fraction the enzyme activity and the protein concentration were determined as in Figs. 1 and 2. It was observed that the fraction number 11 expressed the highest activity and the highest protein content.

Estimation molecular weight of β-lactamase

The purity of *β*-lactamase was examined and the molecular weight was determined from both *S. sciuri* and *K. pneumonae* using SDS-PAGE and the results demonstrated the presence of a single protein band for both bacteria (Fig. 3). The molecular weight of the purified *β*-lactamase was 30 KDa for *S. sciuri β*-lactamase and 28 KDa for *K. pneumonae* enzyme (Fig. 3).

Effect of different pH values on β-lactamase activity

The results in Fig. 4 showed that there was a gradually increase in *β*-lactamase activity with increasing pH values up to pH 7.0 and pH 6.0 for *β*-lactamase from *S. sciuri* and *K. pneumonae*, respectively which seem likely to be the optimum values after which there was a gradual decline in the enzyme activity of both bacteria.

Effect of temperature on β-lactamase activity

The results in Fig. 5 showed that by increasing the incubation temperature there was a corresponding increase in *β*-lactamase activity up to 35 ºC and 40 ºC for the enzyme from *S. sciuri* and *K. pneumonae*, respectively.

Heat stability of β-lactamase

The results in Fig. 6 indicate that the enzyme activity at ºC was reduced gradually through 100 min but the enzyme from *S. sciuri* was more stable than that from *K. pneumoniae.*

Effect of incubation time on stability of β-lactamase

The results in Fig. 7 indicate that the activity decreased gradually with increasing the incubation time from 10 min to 120 min. This was observed for both types of bacteria under the same experimental conditions.

Effect of different substrate concentrations on β-lactamase activity

The results in Fig. 8 indicated that the increase in the substrate concentration led to a corresponding increase in *β*-lactamase activity up to 400 µg ml-1 for *S. sciuri β*-lactamase and 500 µg ml-1 for *K. pneumoniae β*-lactamase.

Determination of Km and V_{max}

The initial velocity of *β*-lactamase reaction was measured as a function of substrate concentration and plotted as double reciprocal plot with substrate concentration in accordance with the Lineweaver-Burk analysis. The Km values were 175.43 and 222.22 µg ml-1 for the enzyme from *S. sciuri and K. pneumoniae*, respectively. V_{max} values were 7.69 and 8.33 units mg-1 protein for both bacteria in the same order (Figs. 9 and 10).

Effect of different enzyme concentrations on β-lactamase activity

The results in Fig. 11 showed that increasing the enzyme concentration resulted in continuous increase in the enzyme activity for both bacteria.

Storage stability of β-lactamase

The results shown in Fig. 12 demonstrated that the *S. sciuri β*-lactamase retained 73.2% of its activity when stored at -20º C for a period of 28 days, compared to 21.3% at 4ºC for the same period. However, the enzyme lost its activity after 42 days at 4ºC and retained 31.5% when stored at -20ºC for the same period. However, the remaining activity of *K. pneumoniae β*-lactamase (Fig. 13) was 46% at -20ºC for a period of 28 days compared to 12 % at 4ºC for the same period, but after 42 days it is inhibited competly at 4ºC and retained 17% of its activity on storing at -20ºC for the same period.

Table.1 Summary of the purification of *S. sciuri β*-lactamase

Purification	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Purification fold	Yield (%)
Crude enzyme	1400	2100	1.5	1.0	100
Ammonium sulfate (75%)	540	1400	2.6	1.7	66.7
DEAE-Cellulose	130	1000	7.7	5.1	47.6
SephadexG-200	10	700	70	46.7	33.3

Table.2 Summary of the purification of *K. pneumoniae* β-lactamase

Purification	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Purification fold	Yield (%)
Crude enzyme	1320	1600	1.2	1.0	100
Ammonium sulfate (75%)	340	1200	3.5	2.9	75
DEAE-Cellulose	80	850	10.6	8.8	53
Sephadex G-200	5	500	100	83.3	31

Fig.1 Fractions of β-lactamase from *S. sciuri*

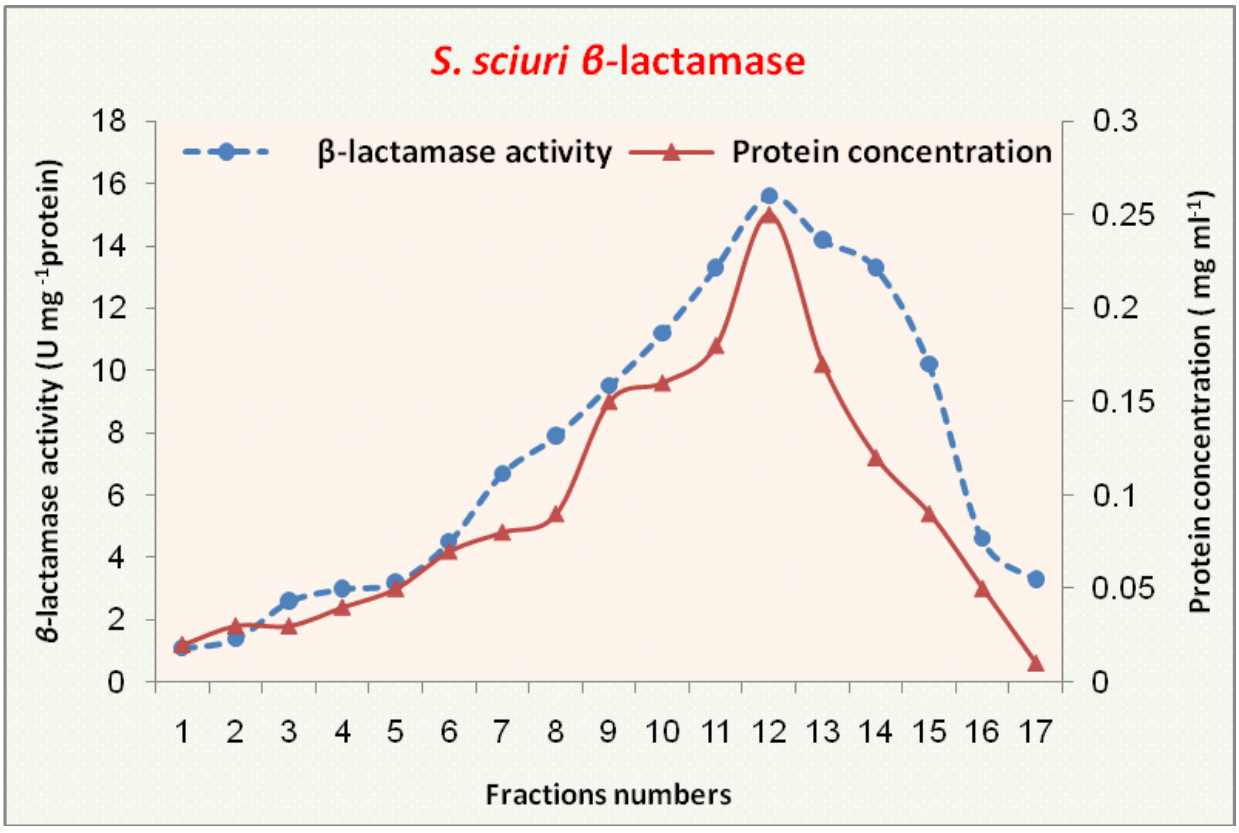


Fig.2 Fractions of *β*-lactamase from *K. pneumoniae*

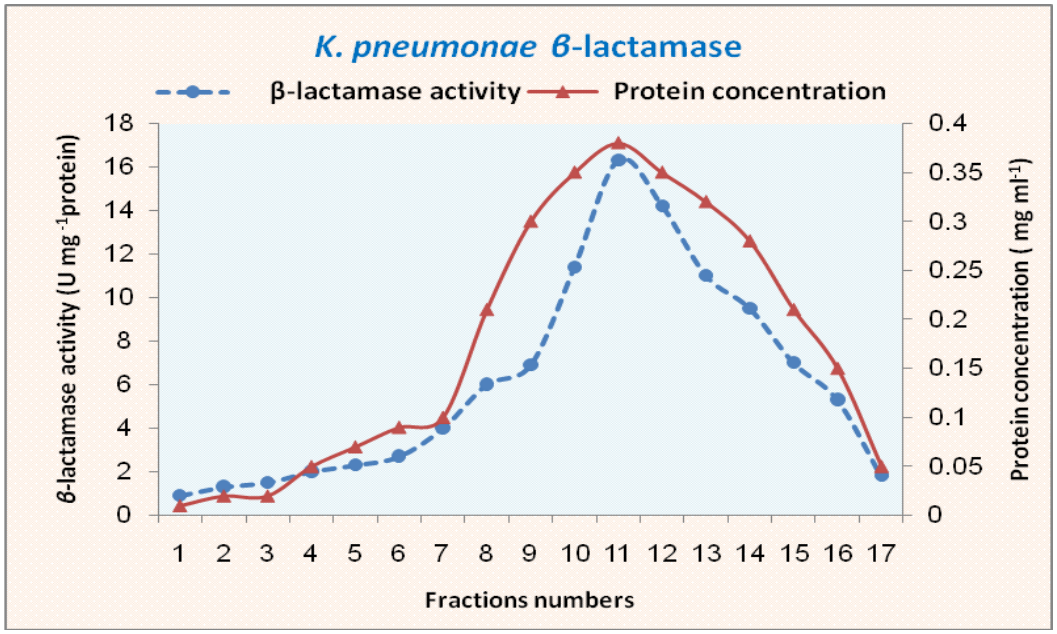


Fig.3 SDS-PAGE profiling of the purified *S. sciuri* and *K. pneumoniae β*-lactamase. M= Protein markers (in kilo daltons; molecular weight standards), Lane 1= purified *S. sciuri β*-lactamase; lane 2= purified *K. pneumoniae β*-lactamase

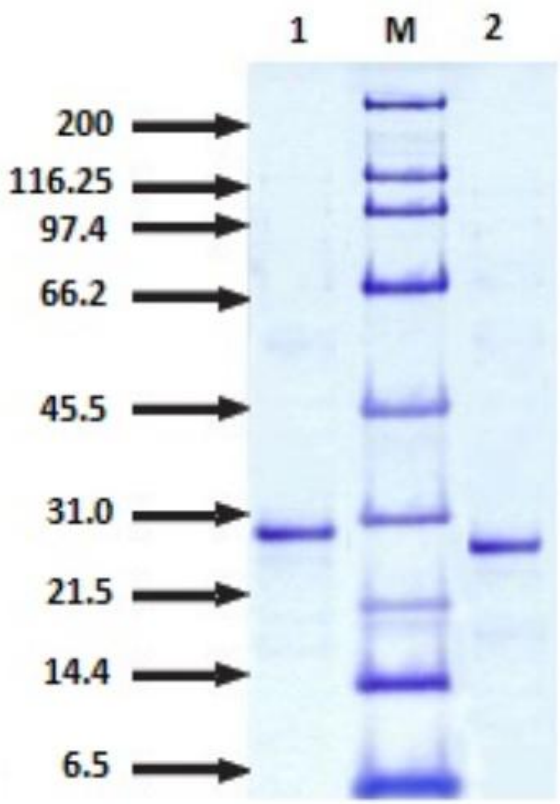


Fig.4 Effect of different pH values on *S. sciuri* and *K. pneumonia* β-lactamase activity

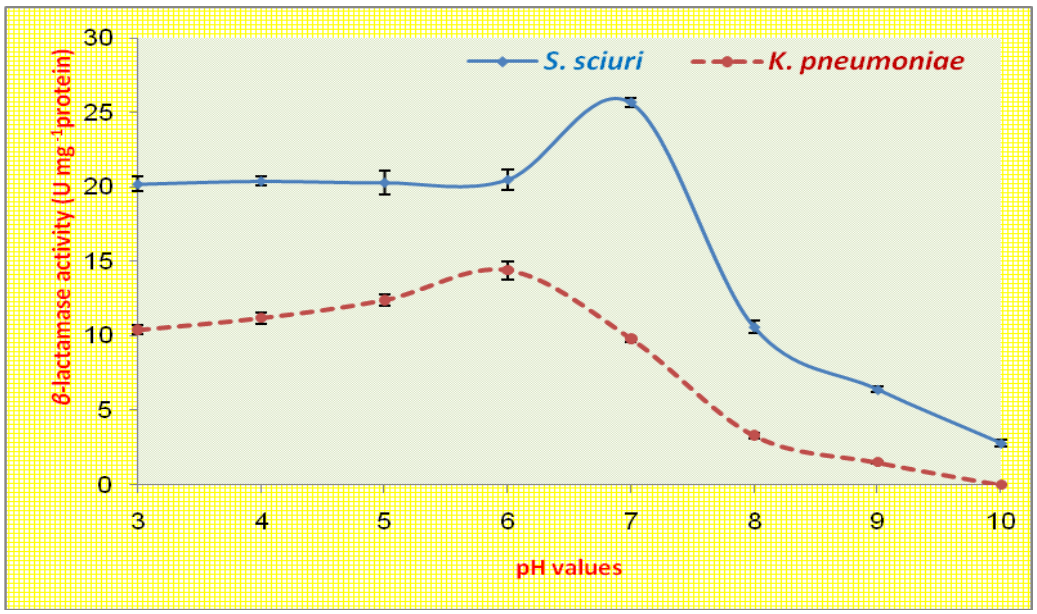


Fig.5 Effect of temperature on *S. sciuri* and *K. pneumonia* β-lactamase

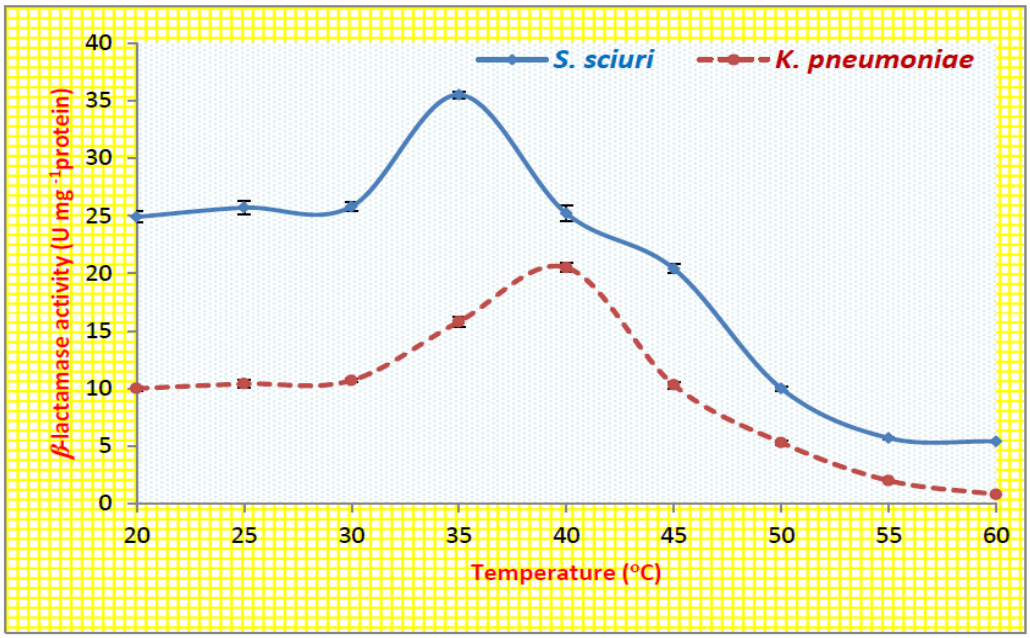


Fig.6 Thermostability of *S. sciuri* and *K. pneumoniae* β-lactamase at 45 ºC.

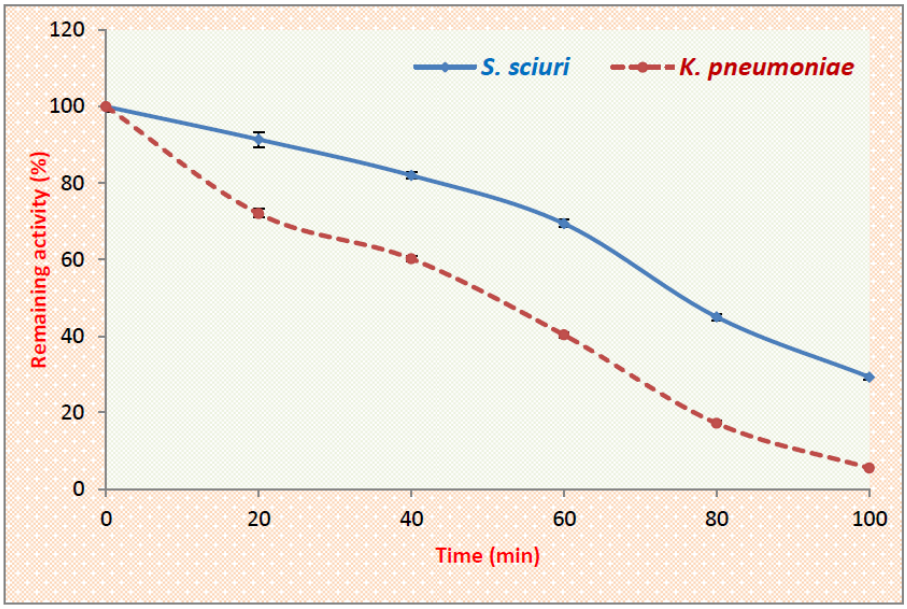


Fig.7 Effect of incubation time on *S. sciuri* and *K. pneumonia β*-lactamase activity

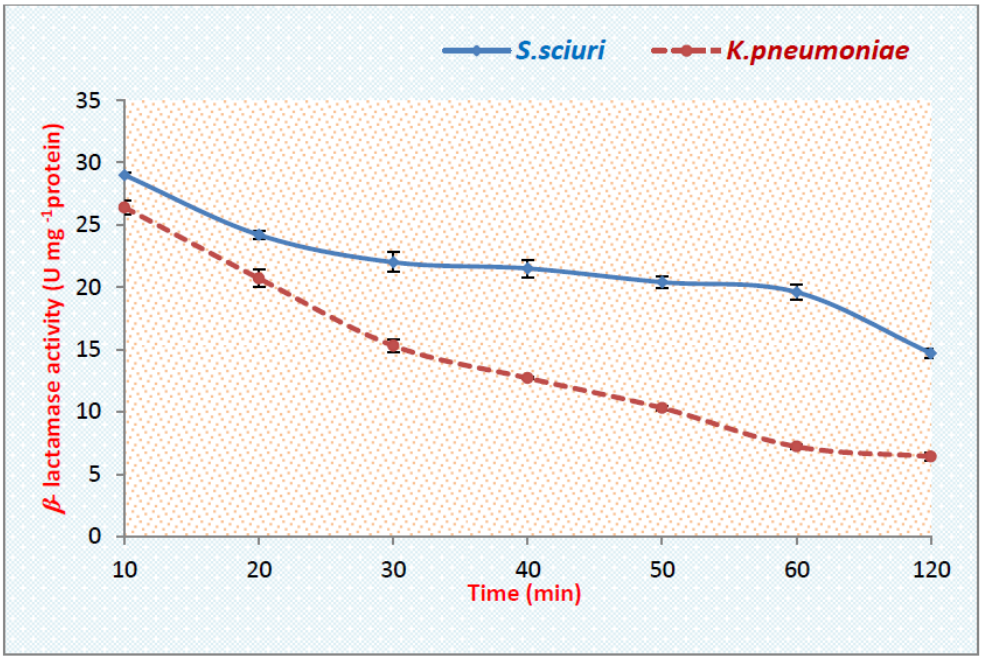


Fig.8 Effect of different substrate concentrations on *S. sciuri* and *K. pneumoniae* *β*-lactamase activity

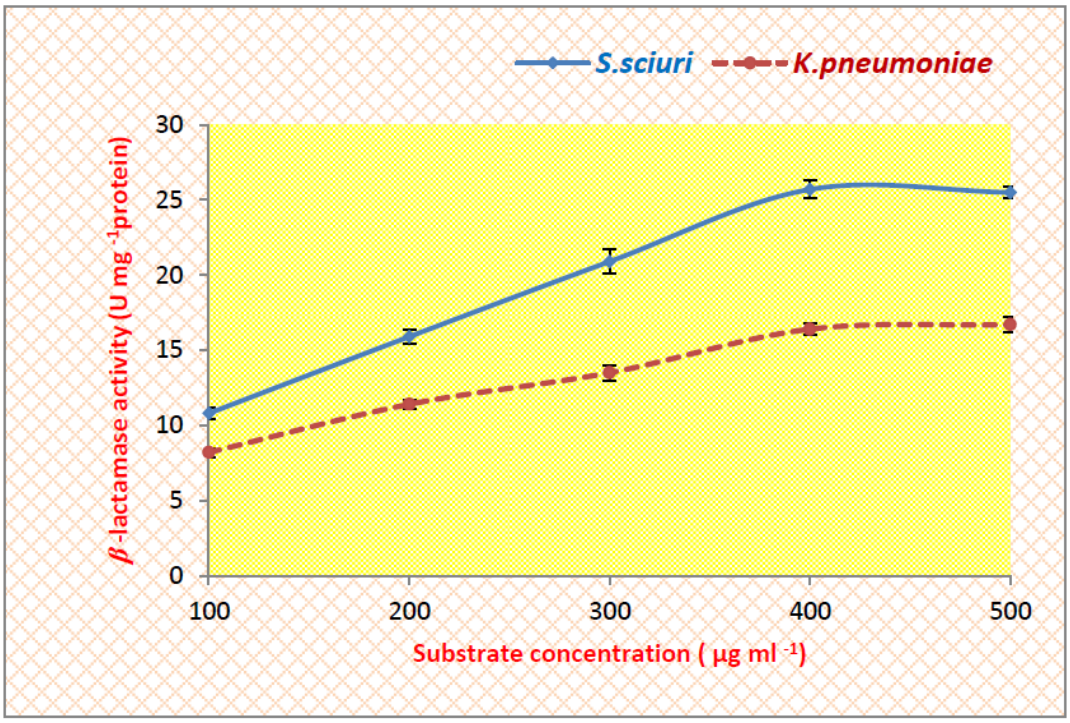


Fig.9 Reciprocal of V against reciprocal of S for β-lactamase from *S. sciuri* (Lineweaver-Burk plot).

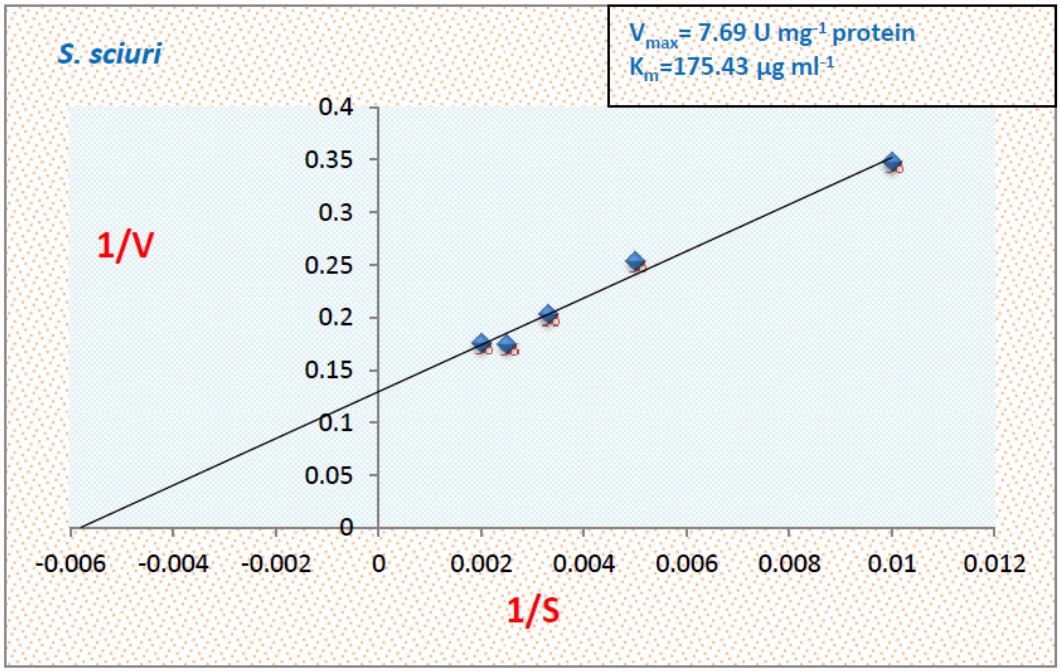


Fig.10 Reciprocal of V against reciprocal of S for β-lactamase from *K. pneumoniae* (Lineweaver-Burk plot).)

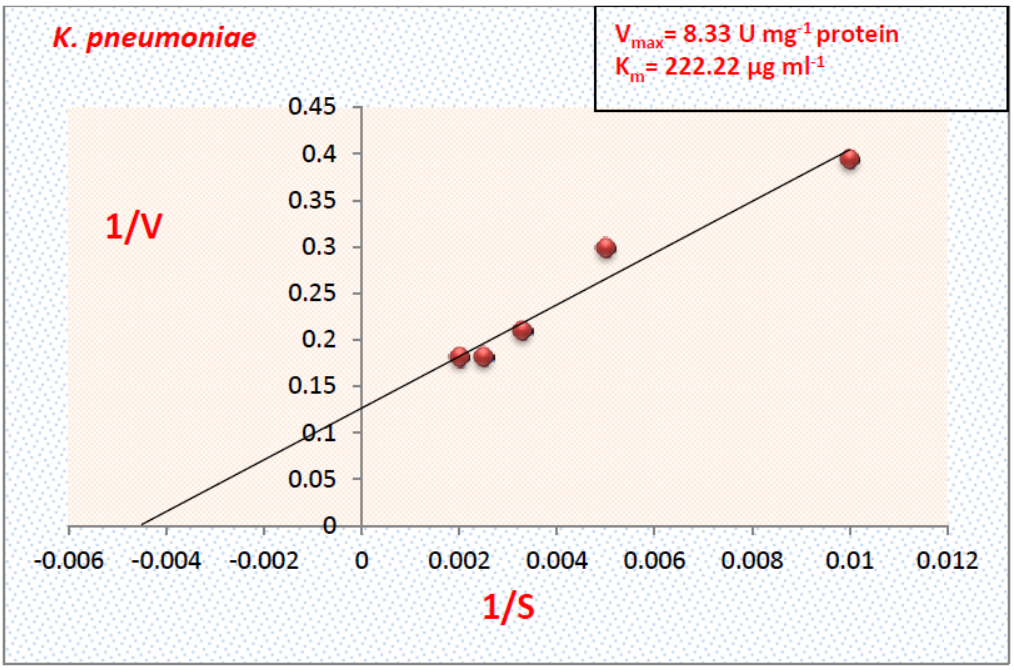


Fig.11 Effect of different enzyme concentrations on *S. sciuri* and *K. pneumoniae* β-lactamase activity

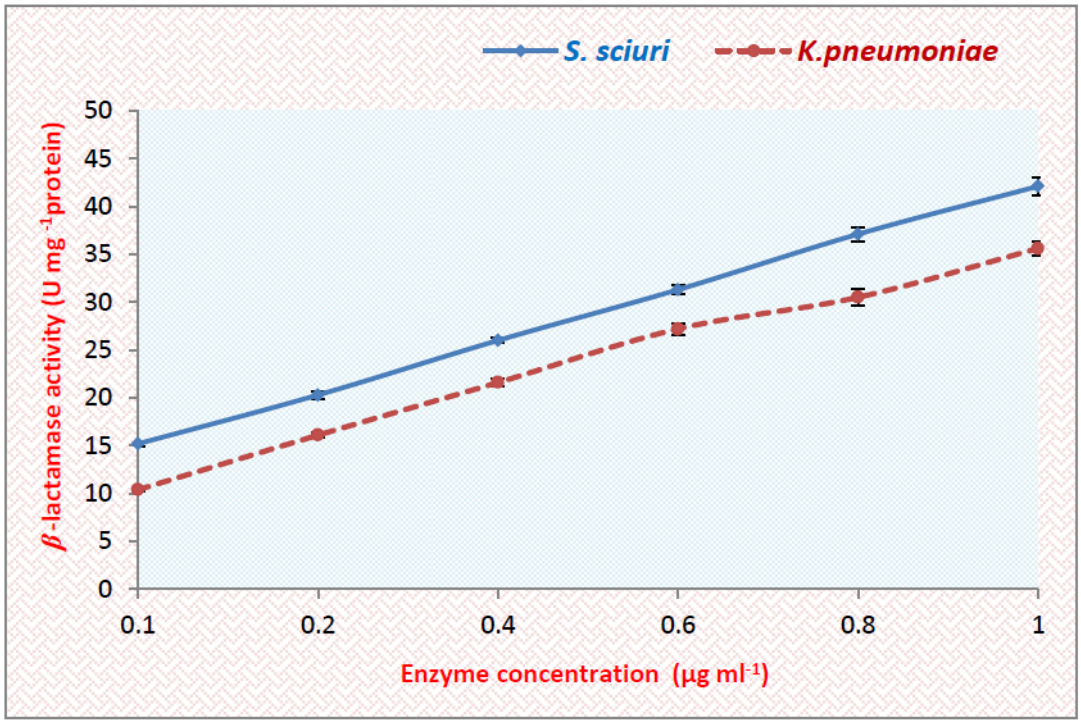


Fig.12 Effect of storage period on *S. sciuri* β-lactamase activity.

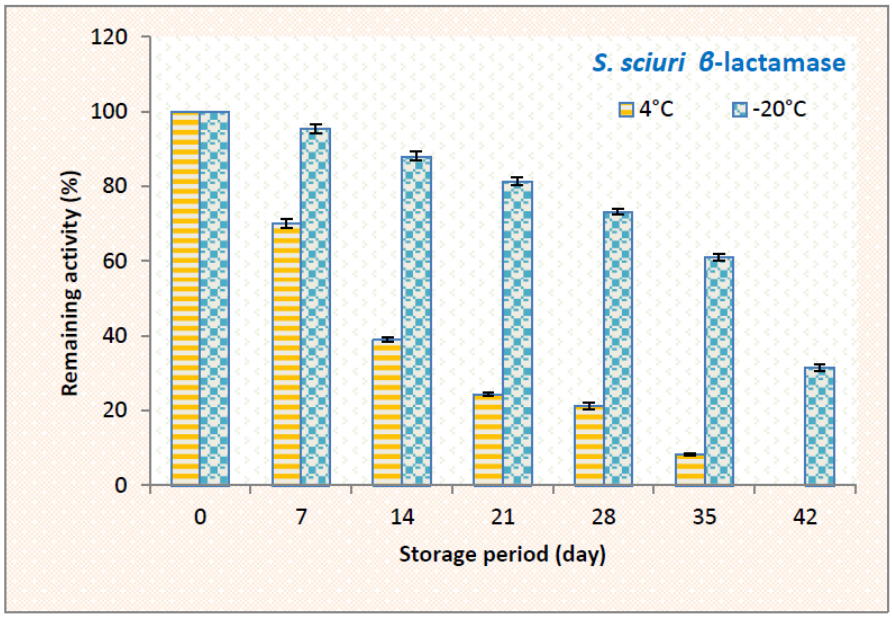
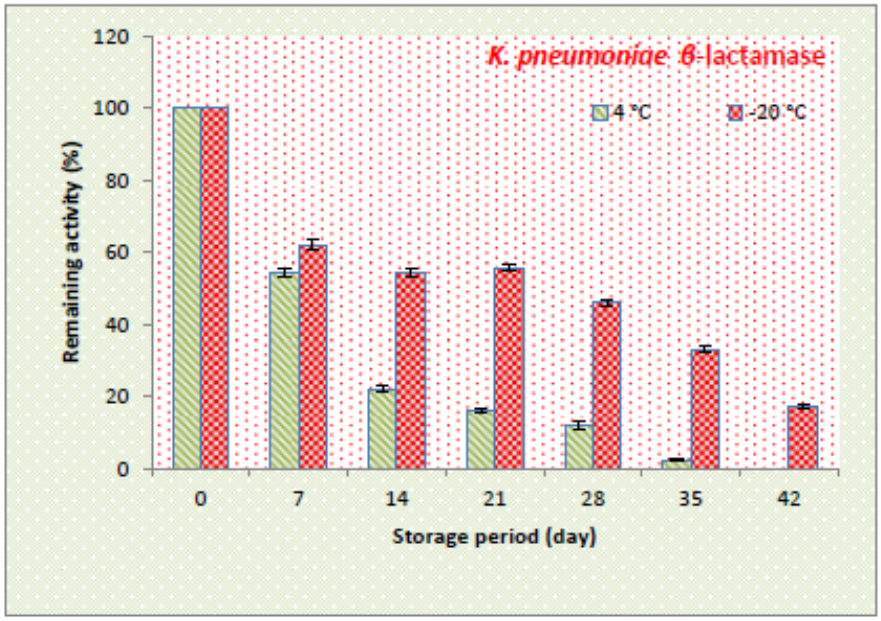


Fig.13 Effect of storage period on *K. pneumoniae* β-lactamase activity



The purified enzyme is required for detailed biochemical and kinetic analysis to allow a deeper understanding of the mechanism of enzyme interaction. One of the principles for purification of an enzyme is to find a source of large quantities of the enzyme in a soluble form (El-Shora and Khalaf, 2008; El-Shora *et al.*, 2008).

One of the aims of the present investigation was to purify the *β*-lactamase enzyme from *S. sciuri* and *K. pneumoniae.* Many procedures

have been reported for the purification of *β*-lactamase (Ranade *et al*., 2013; Omeiri *et al*., 2014).

The enzyme was purified using ammonium sulfate, DEAE-Cellulose and Sephadex G-200 with specific activity of 70 U mg-1protein and purification-fold of 46.7 of *S. sciuri β*-lactamase. In case of *K. pneumoniae β*-lactamase the specific activity of 100 U mg-1protein and purification-fold of 83.3.

The specific activity of *β*-lactamase from *S. sciuri* was 23.8 U mg-1protein from *K. pneumoniae* with purification fold of 32.7 and yield of 47.04% (Al-Jumaily *et al*., 2009). Omeiri *et al*. (2014) reported a specific activity of 24.1 mg-1protein from *S. aureus* with purification fold of 102.3 and yield of 58.74%. De Castillo *et al*. (2001) recorded specific activity of 13.7 U mg-1protein for the enzyme from *Neisseria gonorrhoeae.*

SDS-PAGE showed that the molecular weight of *S. sciuri β*-lactamase was 30 KDa whereas that from *K. pneumonae* was 28 KDa. Issa *et al*. (2010) recorded a molecular weight for *S. aureus* enzyme. However, the molecular weight of 35 kDa was reported for *N. gonorrhoeae β*-lactamase by De Castillo *et al*. (2001). Al-Taai (2005) reported molecular weight of 35.5 kDa for the enzyme from *Proteus mirabilis.* Furthermore, Al-Jumaily *et al*. (2009) recoded a molecular weight of 40 kDa for *K. pneumoniae β*-lactamase. Ranade *et al*. (2013) reported a higher molecular weight between 100 to 150 kDa for *E.coli β*-lactamase.

The optimal pH values for purified *β*-lactamase activity were 7 and 6 from *S. sciuri* and *K. pneumoniae,* respectively. These results agree with those reported by Livermore and Corkill (1992) and Ranade *et al.* (2013) who found that the optimal pH values were 6-8 for *E. coli*. The optimum pH was 7.0-7.2 for purified *Neisseria gonorrhoeae β*-lactamase (De Castillo *et al*., 2001). Al-Jumaily *et al*. (2009) found that the optimal pH of *β*-lactamase activity from *K. pneumoniae* was 7.0. Issa *et al*. (2010) and Omeiri *et al*. (2014) reported optimal pH of 6-7 for *S. aureus β*-lactamase.

The optimal temperatures were 35 ºC and 40ºC for purified *β*-lactamase activity from *S. sciuri* and *K. pneumoniae,* respectively. The enzyme from *K. pneumoniae* was more stable than that from *S. sciuri* at 45 ºC. These results agree with those reported by Al-Jumaily *et al*. (2009) and Ranade *et al*. (2013) who found that the optimal temperature of *β*-lactamase activity from *K. pneumoniae* was 35ºC. However, Issa *et al*. (2010) and Omeiri *et al*. (2014) reported optimal temperature range of 25-35ºC for *S. aureus β*-lactamase activity. De Castillo *et al*. (1998) found optimal temperature of 37ºC for *Neisseria gonorrhoeae β*-lactamase activity. It has been reported that *β*-lactamases are thermolabile proteins which inactivate rapidly by heat (De Castillo *et al*., 2001).

There is a continuous increase in the enzyme activity with the increase of penicillin G as a substrate. The reaction of the enzyme will continue to increase continuously as long as some of the active sites of the enzymes are still able to breakdown the substrate. However, when all the active sites of the enzyme are full occupied then the rate of the enzyme reaction will reach the maximum rate (Vmax) and not well be affected by further increase of substrate concentration.

The results show Km value of 175.43 µg ml-1 and Vmax of 7.69 U mg-1 protein for *S. sciuri β*-lactamase. On the other hand, a Km value of 222.22 µg ml-1 and Vmax of 8.33 U mg-1 protein were recorded for *K. pneumoniae β*-lactamase. von Tigerstrom and Boras (1990) reported that Km for *β*-lactamase of *Lysobacter enzymogenes* was of 116 µg ml-1.

Also, Omeiri *et al.* (2014) reported that the Km and Vmax values of 111 µg ml-1 and 16.66 U mg-1 protein for *S. aureus* *β*-lactamase.

The enzyme from both bacteria expressed appreciable storage stability at -20 ºC compared to that at 4 ºC. In conclusion, this study showed several characteristics of *β*-lactamase from the two pathogenic bacteria which can be useful for controlling the enzyme activity.

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