

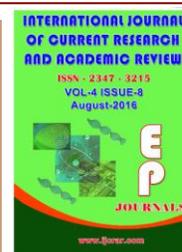


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Purification and Properties of β -lactamase from *Escherichia coli* and *Klebsiella pneumoniae*

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A B S T R A C T

β -lactamase was purified from a β -lactam antibiotic resistant strain of *Escherichia coli* and *Klebsiella pneumoniae*. The purified enzyme of *Escherichia coli* and *Klebsiella pneumoniae* by ammonium sulfate fractionation followed by Sephadex G75 gel filtration, resulted in an overall recovery of 30% with purification fold of 63 (*E.coli*) and of 23% with purification fold of 33 (*K.pneumoniae*). The molecular weight of the native enzyme as determined by gel filtration was 32000 dalton (*E.coli*) and 35000 dalton (*K.pneumoniae*). The enzyme showed, a unique substrate profile by hydrolysing most of the Cephalosporines at a high rate.

Introduction

β -Lactamases (Penicillin amino hydrolase, EC 3.5.2.6) are designated as enzymes which catalysing the hydrolysis of the beta lactam ring at 4 C position in penicillins and cephalosporins (Agbor and Opajobi, 2011; Cartwright and Waley, 1984). These enzymes are the major cause of bacterial resistance to β -lactam antimicrobial agents and have been the subject of excellent microbiological, biochemical and genetic investigations (Takeda, 2007).

Many genera of gram negative bacteria are usually very susceptible to extended spectrum cephalosporins (Spanu, 2002). However, not surprisingly resistance to these expanded spectrum β -Lactam antibiotics

are considered to be due to production of an extended spectrum β -Lactamase (ESBLs). We described here, the existence and characterization of the crude and purified β -Lactamase enzyme produced by the highly resistant strains of clinical isolates of *E. coli* and *K. pneumoniae*.

Materials and Methods

Bacterial strains

The isolates of *E. coli* and *K. pneumoniae* were obtained from clinical specimens of hospitalised patients urine in Kirkuk city. Isolates were identified by using the API 20 E system (BioMerieux).

Susceptibility testing: Antimicrobial susceptibility testing was carried out with the disc diffusion method using current NCCLS recommendation (Kadhim *et al.*, 2010; CLSI, 2007).

Commercially available antibiotic discs (Oxoid, Basingstok, UK) were used. The antimicrobial susceptibility profiles against ampicillin, cephalothin, amoxicillin, cefixime, cefotaxime, amoxicillin-clavulanic acid, ceftazidime, ceftriaxone, meropenem, imipenem and piperacillin were studied. The MICs were determined by the NCCLS broth microdilution method in cation – adjusted Mueller-Hinton broth (Becton, Dickinson) using inocula of 10⁵, 10⁶ and 5x10⁷ CFU / ml and drug concentrations from 0.12 to 128 µg /ml (Schwaber *et al.*, 2004)

β- Lactamases: Irrespective of their antimicrobial susceptibility profile all isolates of *E. coli* and *K. pneumoniae* were tested for ESBL production using ceftazidime (30 µg) and cefotaxime (30 µg) disk and clavulanic acid (10 µg/disk) as recommended by the NCCLS (Balasubramaniam and Parasakthi, 2001).

Increase in zone diameter (≥ 5mm) for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was a positive test for ESBL producers.

β- Lactamase purification: β- Lactamase was purified from 2-4 liter cultures grown overnight at 37°C in tryptic soy broth (Difco) containing 100 µg of penicillin G per ml. Cells were harvested by centrifugation, washed thrice and resuspended in 50 mM phosphate buffer, pH 7.0.

Cell free crude extracts were obtained by seven freeze thaw cycles. Cell debris was

eliminated by centrifugation at 120000 x g for 1 hr at 4°C and the supernatant produced was brought to 80% saturation with ammonium sulphate and centrifuged at 10000 x g at 4°C for 10 min.

Then the precipitates were collected and dissolved in a 50 mM phosphate buffer, pH 7.0 and dialysed overnight at 4°C against 50 mM phosphate buffer, pH 7.0. The dialysed extract was loaded on to a Sephadex G-75 gel filtration column and eluted in 50 mM phosphate buffer pH 7.0. The fractions (3ml) were collected at a flow rate 3 ml/min and assayed for protein at 280 nm as well as for enzyme activity. The active fractions were pooled, dialysed against 50 mM phosphate buffer pH 7.0 and concentrated.

Determination of molecular weight: The molecular weight of the purified enzyme was estimated by gel filtration on a Sephadex G-75 column by the method of (Andrews, 1962) with bovine serum albumin (mol.wt. 68000) ovalbumin (mol.wt. 45000), Chymotrypsinogen (mol.wt. 25000) and Cytochrome C (mol.wt. 12500) as molecular weight standards.

Assay of beta-lactamase. The activity of β-Lactamase was assayed log a modification of the (Manchanda and Singh, 2003) method with penicillins as substrates. One unit of the enzyme activity was defined as the amount of enzyme which hydrolysed 1µmol of a substrate per min at 37°C.

Results and Discussion

Both *E. coli* and *K. pneumoniae* were represented in all clinical isolates as being showing pinpoint pink colonies on MacConkys agar, indicating their Lactose fermentative nature. By using different biochemical tests (Table 1) all clinical isolates were also confirmed to be both *E. coli* and *K. pneumoniae*.

The current NCCLS protocol indicates that *E. coli* (Table 2) and *K. pneumoniae* (Table 3) strains with cefotaxime, ceftazidime, ceftriaxone or aztreonam MIC of ($\geq 2\mu\text{g/ml}$) are to be identified as possible ESBL producers and undergo confirmation testing. In addition to their ability to hydrolyze extended spectrum cephalosporins ESBLs are identified in confirmation testing by their inhibition by clavulanic acid in combination with both cefotaxime and ceftazidime.

Purification of β -lactamase: The results of purification are summarised in Tables 4 and 5. The β -lactamase from *E. coli* (Table 4) was purified over 62 fold from crude extract with a yield of 30%. The purified enzyme elutes from Sephadex G.75 gel filtration column as a single peak with specific activity of 64000 $\mu\text{mole/min/mg}$ protein. On the other hand, the purified β -lactamase from *K. pneumoniae* (table 5) yielded two peaks

in Sephadex G-75gel filtration. The specific activity of the major peak (peak I) was 16000 $\mu\text{mole/min/mg}$ protein with a yield of 23% over 33 fold purification while the peak II show specific activity of 10909 $\mu\text{mole/min/mg}$ protein with a yield of 10% over 23 fold purification. The approximate molecular weight of the β -lactamase was determined to be 32000 dalton for *E. coli* and 35000 dalton for *K. pneumoniae* as measured by gel filtration through a Sephadex G 75 column (Fig. 1).The pH activity curve for the β -lactamase with penicillin G as a substrate was determined for a range of pHs from 5.0 to 10.0 in sodium acetate - Tris –hydrochloride buffer. The optimal pH was about 7.0 for both *E. coli* and *K. pneumoniae* enzyme. A temperature of 37°C was optimal for β -lactamase activity when penicillin G was used as a substrate for *E. coli* and *K. pneumoniae* enzyme.

Table.1

Table 1: Results of biochemical tests

Test	Bacterial Isolates	
	<i>E. coli</i>	<i>K. pneumoniae</i>
Oxidase Test	-	-
Catalase Test	+	+
Indole Test	+	-
Methyl Red Test	+	-
Voges –Proskaur test	-	+
Citrate Utilization test	-	+
KIA		
Suger fermentation	A/A	Alk/A
Gas-H ₂ S	+ -	+ -
Motiliy Test	+	-
Growth in 42C	x	x

Positive (-) Negative (x) No test, KIA= Kliglers Iron Agar) + (A/A) Acid/ Acid,(Alk/A) Alkaline/ Acid, (NC/NC) No change/ No change

Table.2 The percentage of resistant bacteria *E. coli* beta-lactam antibiotics

Antibiotic	Code	Concentration µg/disk	Sensitivity	Intermediate	Resistance
Ampicillin	Am	10	(%3)1	(%3)1	(%94) 30
Cephalothin	KF	30	(%6)2	(%0)0	(%94) 30
Amoxicillin	Ax	25	(%3)1	(%0)0	(%97) 31
Cephalexin	Cl	30	(%18)6	(%0)0	(%82) 26
Cefixime	CFM	5	(%0)0	(%0)0	(%100)32
Cefotaxime	CTX	30	(%6)2	(%0)0	(%94) 30
Amoxicillin-Clavulanic acid	AMC	20/10	(%0)0	(%0)0	(%100)32
Ceftazidime	CAZ	30	(%44)14	(%34)11	(%22)7
Ceftriaxone	CRO	30	(%0)0	(%9)3	(%91)29
Aztreonam	ATM	30	(%12)4	(%44)14	(%44)14
Meropenem	MEM	10	(%100)32	(%0)0	(%0)0
Imipenem	IPM	10	(%94) 30	(%0)0	(%6)2
Piperacillin	PRL	100	(%0)0	(%0)0	(%100)32

Table.3 The percentage of resistant bacteria *K. pneumoniae* beta-lactam antibiotics

Antibiotic	Code	Concentration µg/disk	Sensitivity	Intermediate	Resistance
Ampicillin	Am	10	(%100)19	(%0)0	(%0)0
Cephalothin	KF	30	(%100)19	(%0)0	(%0)0
Amoxicillin	Ax	25	(%100)19	(%0)0	(%0)0
Cephalexin	Cl	30	(%100)19	(%0)0	(%0)0
Cefixime	CFM	5	(%100)19	(%0)0	(%0)0
Cefotaxime	CTX	30	(%89.50)17	(%5.25)1	(%5.25)1
Amoxicillin-Clavulanic acid	AMC	20/10	(%100)19	(%0)0	(%0)0
Ceftazidime	CAZ	30	(%42)8	(%16)3	(%42)8
Ceftriaxone	CRO	30	(%94.75)18	(%0)0	(%5.25)1
Aztreonam	ATM	30	(%79)15	(%10.5)2	(%10.5)2
Meropenem	MEM	10	(%0)0	(%0)0	(%100)19
Imipenem	IPM	10	(%0)0	(%5.25)1	(%94.75)18
Piperacillin	PRL	100	(%73.7)14	(%5.25)1	(%21.05)4

Table.4 Purification of the β-lactamase from *E. coli*

Purification steps	Volume (ml)	Activity Unit / ml	Total protein (mg)	Total activity (Unit)	Specific activity Unit / mg protein	Purification fold	% Yield
Crude extract	23	23.8	53.9	54740	1016	-	100
Supernatant	20	22.6	32.6	45200	1387	1.36	83
Ammonium sulfate precipitation	15.5	20.8	18.3	32240	1762	1.73	59
Dialysis	14.5	18.5	14.5	26825	1850	1.82	49
Sephadex G -75	12	16	0.3	19200	64000	62.9	30

Table.5 Purification of the β -lactamase from *K.pneumoniae*

Purification steps	Volume (ml)	Activity Unit / ml	Total protein (mg)	Total activity (Unit)	Specific activity Unit / mg protein	Purification fold	% Yield
Crude extract	21	11	49	23100	471	-	100
Supernatant	19	9	35	17150	489	1.03	74
Ammonium sulfate precipitation	15	7	17	10500	617	1.3	45
Dialysis	14	6	13	8400	646	1.37	36
Sephadex G -75							
Peak I	12	4	0.3	4800	16000	33.9	23
Peak II	12	2	0.22	2400	10909	23.1	10

Table.6 Purified lactamase activity with different substrate

Antibiotic	Enzyme activity (nmol / min)		
	<i>E.coli</i>	<i>K. pneumoniae</i> (peak I)	<i>K. pneumoniae</i> (peak II)
Penicillin G	25.14	29.30	10.98
Ampicillin	32.89	128.28	69.07
Cefotaxime	8.96	13.45	8.96
Ceftazidime	15.58	10.39	5.77
Cloxacillin	628.09	280.99	528.92

Table.7 Effect of divalent metal ions on the activity of purified lactamase in *E.coli* and *K. pneumoniae*

Divalent ions	Concentration(mM)	% inhibition	
		<i>E.coli</i>	<i>K. pneumoniae</i> (peak 1)
CuCl ₂	5	85	81
	10	87	86
FeCl ₂	5	91	87
	10	95	91
CaCl ₂	5	70	73
	10	79	76

Table.8 Effect of inhibitors on the activity of purified β -lactamase from *E.coli* and *K. pneumoniae* using Pencillin G as substrate

inhibition	Concentration (mg / ml)	% Inhibition		
		<i>K. pneumoniae</i> (Peak I)	<i>K. pneumoniae</i> (Peak I)	<i>E.coli</i>
EDTA	0.05	-	-	-
	5	-	-	63.61
	10	93.75	93.75	92.73
AMC	0.05	-	-	-
	5	43.75	43.75	-
	10	62.5	62.5	-
Cloxacillin	0.05	-	-	-
	5	43.75	43.75	-
	10	62.5	62.5	12.61

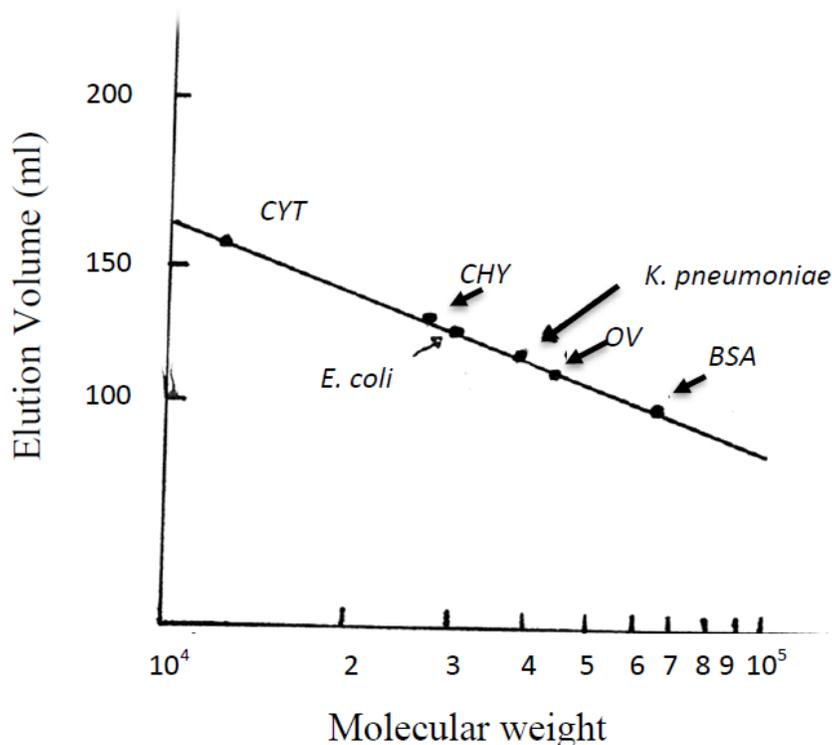
Table.9 Effect of inhibitors on the activity of purified β -lactamase from *E.coli* and *K. pneumoniae* using Ceftazidime as substrate

inhibition	Concentration (mg / ml)	% Inhibition		
		<i>K. pneumoniae</i> (Peak I)	<i>K. pneumoniae</i> (Peak I)	<i>E.coli</i>
EDTA	0.05	31.1	-	-
	5	83.22	4	42.27
	10	91	64	42.27
AMC	0.05	66.7	22	-
	5	74.5	62	-
	10	75.5	64	-
Cloxacillin	0.05	52.3	32	-
	5	60	78	-
	10	90	88	-

Table.10 Effect of inhibitors on the activity of purified β -lactamase from *E.coli* and *K. pneumoniae* using Cefotaxime as substrate

inhibition	Concentration (mg / ml)	% Inhibition		
		<i>K. pneumoniae</i> (Peak I)	<i>K. pneumoniae</i> (Peak I)	<i>E.coli</i>
EDTA	0.05	52.2	33.3	73.74
	5	78	66.7	93.75
	10	80	70	95
AMC	0.05	13.2	60	85
	5	50	66.7	87.5
	10	92.3	80	95
Cloxacillin	0.05	43.4	60	-
	5	64	66.7	12.5
	10	82.3	76.7	66.33

Fig.1 Sephadex G 75 gel filtration by molecular weight markers : Bovine Serum albumin: BSA 68000; Ovalbumin: OV 45000; Chymotrypsinogine: CHY 25000; Cytochrome C: CYT 12500



The Michaelis constant (K_m) determined for penicillin G by Lineweaver –Burk plots was 0.45 mM and 0.52mM for *E.coli* and *K. pneumoniae* enzymes respectively.

Ampicillin, Cefotaxime, Cefotazidime and Cloxacillin were hydrolysed by the lactamase of both organisms, although ampicillin and cloxacillin had a high affinity for the enzyme.

A number of enzyme inhibitors (divalent ions, EDTA, cloxacillin and amoxicillin – Clavulanic acid) were tested for their inhibitory effect on the activity of the purified enzyme. The enzymes were preincubated with each of the inhibitors at indicated concentrations for 10 min at 37° C, and then the remaining enzyme activity was assayed with penicillin G or with

cefotaxime or ceftazidime as substrate. The enzyme activity of both organisms was almost inhibited by divalent metal ions by 76-95% inhibition at a concentration of 10 mM (Table 7).

The activity of the β -lactamase from *E.coli* and *K. pneumoniae* was also found to be inhibited by EDTA, amoxicillin and cloxacillin when penicillin G, ceftazidime and cefotaxime were used as substrates as shown in Table (8-10).

Susceptibility testing is designed to identify resistant bacteria and to guide physicians in choosing the most effective antibiotic regimen for the treatment of infection. Because the effectiveness of the extended – spectrum cephalosporins is compromised by the activity of ESBLs (Saeed *et al.*, 2015;

Moussa, 2010) NCCLS guidelines have been developed to identify and conform ESBL-producing isolated of *E. coli* and *K. pneumoniae* (CLSI, 2007; Schwaber *et al.*, 2004). To test the accuracy of the procedures, we measured MICs at inocula within 0.5 log unit of standard NCCLS inoculum, using clinical isolates with ESBL and AmpC β -lactamases. These limits represent the range of inocula that might be encountered in routine testing.

The sequence of purification steps effectively removes proteins with contaminate enzyme and purifies β -lactamase to apparent homogeneity as shown by gel filtration chromatography. The molecular weight of β -lactamase of both organisms was similar to those of penicillinases mediated by various resistant factors (Cartwright and Waley, 1984) but smaller than those of typical cephalosporinase purified from *Pseudomonas aeruginosa* (Takeda *et al.*, 2007) and *Proteus morgani* (Spanu *et al.*, 2002). The purified β -lactamase of *E. coli* and *K. pneumoniae* showed a unique substrate profile by hydrolysing most Cephalosporines and penicillines including cefotaxime and cloxacillin, which have been reported to be resistant to hydrolysis by β -lactamases of gram negative bacteria (Sirot *et al.*, 1991).

In conclusion, *E.coli* and *K.pneumoniae* contains β -lactamases with molecular weight, reaction kinetics and sensitivity to inhibitors quite similar to those shown by other β -lactamases of gram negative bacteria. It should be interesting to design a specific inhibitor against its activity. This inhibitor could be a useful chemotherapeutic agent effective in the elimination of gram negative bacteria without causing any ill effects to the human.

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