academicJournals

Vol. 7(25), pp. 3262-3268, 18 June, 2013
DOI: 10.5897/AJMR2012.2299
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

In vitro inhibition of aminoglycosides-resistant *Staphylococcus aureus* by modifying enzyme inhibitory protein of *Pseudomonas aeruginosa*

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Accepted 29 May, 2013

Eighty clinical bacterial isolates were collected from Sayed Galal hospital in Cairo. Minimum inhibitory concentrations (MICs) were determined for five aminoglycoside antibiotics: netilmycin, sissomycin, spectinomycin, gentamicin and neomycin. The most potent gentamicin-resistant bacterial isolate (IS-20) was identified as *Staphylococcus aureus*. On the other hand, the same eighty clinical bacterial isolates were screened to produce modifying enzyme-inhibitory protein against the same five antibiotics mentioned above. Only two bacterial isolates gave activity: IS-37 and IS-46. However, IS-46 isolate higher activity than IS-37 isolate with all five antibiotics, especially with gentamicin. Bacterial isolate IS-46 was identified as *Pseudomonas aeruginosa*. Optimization was studied to obtain the maximum yield of gentamicin modifying-enzyme inhibitory protein. Gentamicin modifying-enzyme inhibitory protein was precipitated at 50% saturated ammonium sulfate, and then purified using ion exchange (DEAE-cellulose) and gel filtration column chromatography (Sephadex G-200). The purified inhibitory protein was electrically separated at 32 KDa. Amino acids sequence and concentration were determined by HPLC. Gentamicin modifying-enzyme inhibitory protein was combined at 128 mg.L^{-1} with gentamicin antibiotic at 128 µg.ml^{-1} to inhibit the growth of *S. aureus*.

Key words: Antimicrobial resistance, aminoglycosides, enzyme inhibitors, protein purification.

INTRODUCTION

The resistance to antimicrobial agents is an increasingly global problem worldwide, especially among nosocomial pathogens. Staphylococci have become one of the most common causes of nosocomial infections. Multidrug-resistant staphylococci pose a growing problem for human health. The rise of drug-resistant virulent strains of *Staphylococcus aureus*, particularly methicillin-resistant *S. aureus* (MRSA) is a serious problem in the treatment and control of staphylococcal infections (Livermore, 2000; Zapun et al., 2008).

Methicillin-resistant staphylococci (MRS) cause hard-to-treat infections because these are resistant to most of the antibiotics such as beta-lactams, aminoglycosides and macrolides. The most important mechanism of resistance to penicillin is production of beta-lactamase which inactivates penicillin by hydrolysis of its beta-lactam ring. Another mechanism is associated with penicillin-binding protein 2a (PBP2a), encoded by *mecA*2. Another gene

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involved in penicillin resistance in staphylococci is *blaZ* which encodes β-lactamase (Zapun et al., 2008).

The most important mechanism of aminoglycoside resistance in staphylococci is drug inactivation by AMEs like aminoglycoside nucleotidyltransferases (APHs). AMEs can be plasmid or chromosome encoded. In staphylococcal strains, the most commonly found AME is *aac(6')/aph(2")*. The bifunctional enzyme *aac(6')/aph(2")* is encoded by the *aac(6')/aph(2")* gene. In addition, APH (3')-III is encoded by *aph(3')- IIIa* gene and the ANT(4')-I by *ant(4')-Ia* gene, are also found in staphylococcal isolates (Yadegar et al., 2009; Sekiguchi et al., 2004).

Methicillin resistant MRSA is a highly infectious strain of the ordinary *S. aureus* bacteria that is able to withstand the curative powers of ordinary antibiotics. *S. aureus* was the most common cause of hospital acquired infections reported in 1990 to 1996. MRSA is found worldwide with an estimated colonization rate ranging from 11 to 40% in specific populations with more than 50% of these estimated to develop infection. However, it is very common to find a high rate of nasal carriage of MRSA in health care workers where MRSA is endemic (Boyce, 1994). *S. aureus* is still one of the five most common causes of hospital acquired infections and because it's primary habitat is moist squamous epithelium of the anterior nares. Most invasive *S. aureus* infections are assumed to arise from nasal carriage (Duran et al., 2006). The incidence of hospital-acquired *S. aureus* infections has been rising with increasing emergence of MRSA (Emori and Gaynes, 1993; Steinberg et al., 1996; Fluit, 2001; Ryan and Ray, 2004; Deresinski, 2005).

The aim of this study was to obtain purified aminoglycoside modifying enzyme inhibitory protein from pathogenic *Pseudomonas aeruginosa*, which could inhibite the growth of pyogenic common bacteria known as *S. aureus*.

MATERIALS AND METHODS

Aminoglycoside antibiotics

Streptomycin (Medeva pharma Ltd), Gentamicin (Aventis pharma), Neomycin (SIGMA), Kanamycin (Sanofi Winthrop), Netilmycin (Schering Plough), Spectinomycin (Pharmacia & Upjohn Ltd), Sissomycin (SIGMA), Tobramycin (Eli Lilly & Co Ltd) and Amikacin (Bristol Myers Squibb) were used.

Microorganisms

All bacteria used in this study were isolated from Sayed Galal hospital, Cairo, Egypt. There were two bacteria used: *P. aeruginosa* and *S. aureus*.

Determination of MIC

The minimum inhibitory concentration (MIC) was determined using McFarland method (Jennifer, 2001).

Preparation of the McFarland standard

Firstly, 0.5 ml of 0.048 M $BaCl_2$ (1.17 % w/v $BaCl_2.2H_2O$) was added to 99.5 ml of 0.18 M H_2SO_4 (1 % v/v) with constant stirring. The standard was distributed into screw cap tubes of the same size and with the same volume as those used in growing the broth cultures. The tubes were sealed tightly to prevent loss by evaporation. They were stored and protected from light at room temperature. The turbidity was agitated vigorously on a vortex mixer before use. Standards were stored for up to 6 months, after which time they were discarded. Alternatively, prepared standards can be purchased (Leslie et al., 1990).

Growth method

P. aeruginosa growth was transferred into ISB or equivalent that has been shown not to affect the performance of the test, and incubate broth with shaking at 35 to 37°C until the visible turbidity is equal to or greater than the 0.5 McFarland standard. Alternatively, an overnight broth culture can be used (Leslie et al., 1990).

Identification of bacteria

Identification of bacteria was carried out according to Bergey's Manual of Determinative Bacteriology (1989). Analysis Profile Index (API) was used as confirmatory test.

Rapid PCR identification

The bacterial sample IS-46 was identified by rapid PCR technique according to Luiz et al. (1999).

Optimizations of enzyme inhibitor

Determination of optimum factors affecting the maximum production of modifying-enzyme inhibitor was carried out with *P. aeruginosa*, against gentamicin-resistant *S. aureus* (Vanderzant and Don, 1992).

Effect of incubation period

Erlenmeyer flasks (250 ml) containing 100 ml sterile nutrient broth, each was inoculated with *P. aeruginosa* and incubated at 35 ± 2°C for various incubation periods (6 to 28 h). At each incubation period, 5 ml of bacterial suspension were then taken aseptically and the antibacterial activity was measured using the inhibition zone method described earlier. Three plates were used within each incubation period.

Effect of incubation temperature

Erlenmeyer flasks (250 ml) containing 100 ml sterile nutrient broth, each was inoculated with *P. aeruginosa* and incubated at different temperatures (20 to 44°C). For each, 5 ml of bacterial suspension were then taken aseptically and the antibacterial activity was measured using the inhibition zone method described earlier. Three plates were used for each incubation temperature.

Effect of pH

Erlenmeyer flasks (250 ml) containing 100 ml sterile nutrient broth, each was adjusted at certain value of pH (5 to 9) using a phosphate buffer before the sterilization and then inoculated with *P. aeruginosa* and incubated at the optimum incubation period (24 h) and optimum temperature (30°C). For each, 5 ml of the culture filtrate were then taken aseptically and the antibacterial activity was measured using the inhibition zone method described earlier. Three plates were used for each pH value.

Preparation of cell free extract

The nutrient broth medium was inoculated by *P. aeruginosa* and then incubated at 30°C as a best incubation temperature for 24 h as a best incubation period. After incubation period, the suspension was centrifuged at 10,000 g for 10 min for precipitation of the bacterial cells. The supernatant (cell free extract) which contains aminoglycoside modifying enzyme inhibitory protein was taken and subjected to precipitation by saturated ammonium sulphate.

Precipitation by ammonium sulphate

A range of ammonium sulphate was used (10 to 90%). In each step, with at definite concentration the mixture was left for 2 h at 4°C and was followed by centrifugation at 8000 g for 20 min at 4°C. The obtained precipitate was dissolved in 10 ml phosphate buffer (pH 7.5), the buffered extract was heated at 70°C for 10 min, cooled immediately and centrifuged at 8000 g for 20 min at 4°C. The resulting supernatant was brought to definite concentration saturation with ammonium sulphate. Further centrifugation and the obtained precipitate was dissolved in 10 ml phosphate buffer (pH 7.5).

Quantitative estimation of total protein content

Firstly, 1 ml of the protein containing fraction was pipette then 5 ml of working alkaline copper reagent was added, standing was allowed at room temperature for 15 min (Lowery et al., 1951). Immediately, 0.5 ml of diluted folin reagent was mixed and allowed for standing at room temperature for 30 min. The color intensity of the sample was measured by spectrophotometer at 280 nm. Blank is 5 ml of copper reagent with 0.5 ml of diluted folin reagent.

Ion-exchange chromatography

Only 100 g of DEAE-cellulose was placed in 1 L conical flask and then washed with distilled water followed by using 1 N HCl and water till the pH of the suspension was about 6.5. It was then washed several times with 0.5 M NaOH until no more color was removed after the last alkaline wash, the resin was rinsed with distilled water until it was free from alkalinity. The washed resin was then suspended in about three volume of the phosphate buffer at pH 7.5. At this stage, the supernatant fluid was almost clear indicating the removal of the fine particles; this precipitate was used for column packing.

Gel filtration

Ten grams of sephadex G-200 were dissolved in 400 ml phosphate buffer at pH 7.5, boiled in water bath for 6 h, then cold to 50°C and packed in column (2.5 × 20 cm). Gel filtration was done basically according to Andrews (1969). Active fractions were pooled and dialyzed from ion-exchange column were applied to a sephadex G-200 column which was pre-equilibrated with phosphate buffer pH 7.5, at a flow rate of 5 ml/25 min.

Separation of gentamicin modifying-enzyme inhibitor by SDS-PAGE

The aminoglycoside modifying enzyme inhibitory protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, which was carried out with 10% polyacrylamide gel as described by Blackshear (1984), in the presence of 0.1% sodium dodecyl sulphate at pH 8.8 and 1 mM dithiothreitol. Gel and gel buffers were prepared as described by Laemmli (1970) and See and Jackowski (1990). Gel was stained to show the formed bands clearly by Coomassie Blue dye as described by Fairbanks et al. (1971).

Amino acids analysis by HPLC

The apparatus used was Spectra-Physics Analytical, Inc. A0099-600 with spectra focus optical scanning detector and spectra system UV 2000 detector and ultra sphere C_{18} Beckman column. The analysis was carried out using a gradient of Pico-Tag solvent A & B at 40°C and flow rate of 1 ml/minute. Detection of the separated Pico-Tag amino acids was at 254 nm wavelength. Before injecting the sample, it was calibrated by two injections of the standards (Steven et al., 1989).

Susceptibility test

The suitable range of gentamicin concentrations against *S. aureus* (32to 512 $\mu g.ml^{-1}$) was chosen, stock solutions was prepared using the formula W = C × V × 1000 / P; where W is the weight of gentamicin (mg), C is the concentration of gentamicin ($mg.L^{-1}$) multiply by 1000, V is the volume of distilled water as a solvent (ml) and P is the potency of gentamicin which equal 600 $\mu g.mg^{-1}$ (Michael et al., 1993). The suitable range of gentamicin-modifying enzyme inhibitor concentrations (0.015 to 128 $mg.L^{-1}$) was chosen, stock solutions were prepared using the same formula mentioned above. The nutrient agar medium was prepared, after cooling at 50°C certain gentamicin concentration (32-512 $\mu g.ml^{-1}$) were added with each concentration of gentamicin-modifying enzyme inhibitor (0.015 to 128 $mg.L^{-1}$). Then, plates were poured (each one contains 20 ml) and left at solidification, inoculated by *S, aureus*, and incubated at 30°C for 24 h.

RESULTS AND DISCUSSION

We chose Sayed Galal Hospital (Bab Al-Sharia Area, Cairo) for the collection of the clinical bacterial isolates, because it is considered as one of the free and largest hospitals in Cairo, which receives many cases daily. So, it gave us the opportunity to collect a variety of clinical bacterial isolates within six months (February to July). There were eighty clinical bacteria isolated from infected urine, sputum and blood. The bacterial isolates were treated by five aminoglycoside antibiotics mentioned above (30 $\mu g.ml^{-1}$). These antibiotics were chosen according to many reasons: belong to different generations of aminoglycosides, use widely worldwide and prescribed in many indications and serious cases of bacterial infections. The minimum inhibitory concentrations (MICs) were determined for all five antibiotics against each resistant bacteria isolate. Therefore, the most resistant bacteria isolate for each antibiotic was determined. Bolaji et al. (2011) reported that, many pathogenic bacteria isolates were isolated from hospitals. These bacteria had become resistant to all the tested antibiotics including streptomycin (30 $\mu g.ml^{-1}$). With unprecedented trial, the same eighty clinical bacterial isolates were screened against five antibiotics to produce modifying-enzyme inhibitory protein. Only two isolates (IS-46 and IS-37) had given activity; however, the former had higher activity than the latter against all antibiotics, especially with gentamicin (Figure 1A and B). This means, the two isolates IS-46 and IS-37 played two

Figure 1. (A) Antibacterial activity of bacterial isolate IS-46, (B) Antibacterial activity of bacterial isolate IS-37 against gentamicin-resistant *S. aureus.*

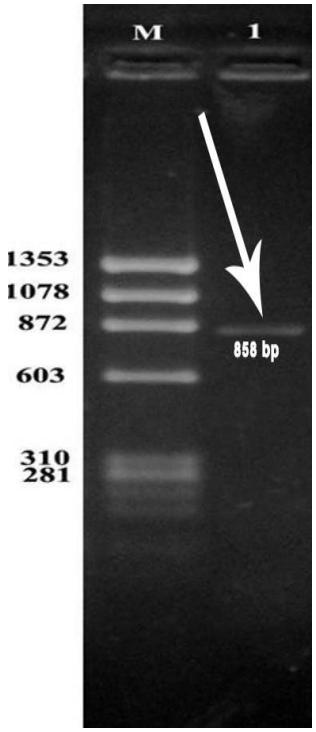

Figure 2. Agarose gel electrophoresis of PCR products for *P. aeruginosa*.

important and opposite roles; production of modifying-enzyme and modifying-enzyme inhibitory protein but not in the same time. Modifying-enzyme was produced in the presence of gentamicin only, while modifying-enzyme inhibitory protein was produced in the presence of gentamicin and *S. aureus*.

Bacterial isolate IS-46 was identified as *P. aeruginosa* using traditional methods, 20-NE API kits and rapid PCR technique using *algD* mannose dehydrogenase gene which had 858 bp (Figure 2). The *algZ* controls *P. aeruginosa* alginate synthesis by activating *algD*, yet *algZ* expression is not detectable in nonmucoid strains. Mobility shift and Western blot assays revealed that *algZ* expression requires the sigma factor *algT*. The mapped *algZ* transcription start site revealed a consensus *algT*-dependent promoter that, when mutated, substantially reduced *algZ* transcription (Daniel et al., 2003).

Gentamicin-resistant isolate IS-20 was identified as *S. aureus* using traditional methods and STAPH API kits. Optimum environmental and nutritional factors were studied to maximize the yield of gentamicin modifying-enzyme inhibitory protein. The maximum yield of gentamicin modifying-enzyme inhibitory protein was observed at 200 µl (8 $\times 10^7$ CFU), incubation for 24 h at 30°C and pH 7.5. Another study stated that, the members of gentamicin family presented the highest *Kt* values at pH 7.5 (Culebras, et al., 1996). D-glucose and L-asparagine were the sole carbon and nitrogen sources, respectively.

Gentamicin modifying-enzyme inhibitory protein was precipitated at three concentrations of saturated ammonium sulphate (30 to 50%). However, the highest activity of inhibitory protein and specific activity were observed at 50% (Table 1). The emerging structural data now has the potential to be exploited in the design of specific inhibitors of enzyme activity. For example, the similarity between APH (3$^{\prime}$)-III-a and protein kinases inspired a study in which known inhibitors of protein kinases were assessed for the capacity to inhibit APHs (Daigle et al., 1997; Roestamadji et al., 1995; Roestamadji and Mobashery, 1998; Yang et al., 1998). The aminoglycoside kinase inactivators did not reverse antibiotic resistance, a alternative approach, the synthesis of aminoglycoside molecules that are antibiotics but not substrates for modifying-enzymes (McKay and Wright, 1995), and potent antibacterial agents in their own way (Umezawa et al., 1971a; Umezawa et al., 1971b). Unfortunately, these compounds are substrates for other aminoglycoside kinases such as

Table 1. Precipitation of Gentamicin-modifying enzyme inhibitory protein by saturated ammonium sulphate.

$(NH4)_2SO_4$ concentration	Activity	Total activity of GMEI (u)	Total protein content (mg)	Specific activity (u/mg)	Fold purification	Yield (%)
Control	40.0 ± 1.1	200.0	180.0	1.1	1.0	100.0
10.0	0.0	0.0	4.0	0.0	0.0	0.0
20.0	0.0	0.0	6.0	0.0	0.0	0.0
30.0	20.0 ± 1.0	170.0	6.8	25.0	22.7	85
40.0	25.0 ± 0.57	185.0	7.2	25.7	23.4	92.5
50.0	30.0 ± 1.0	195.0	7.5	26.0	23.7	97.5
60.0	0.0	0.0	6.3	0.0	0.0	0.0
70.0	0.0	0.0	5.8	0.0	0.0	0.0
80.0	0.0	0.0	5.3	0.0	0.0	0.0
90.0	0.0	0.0	4.2	0.0	0.0	0.0

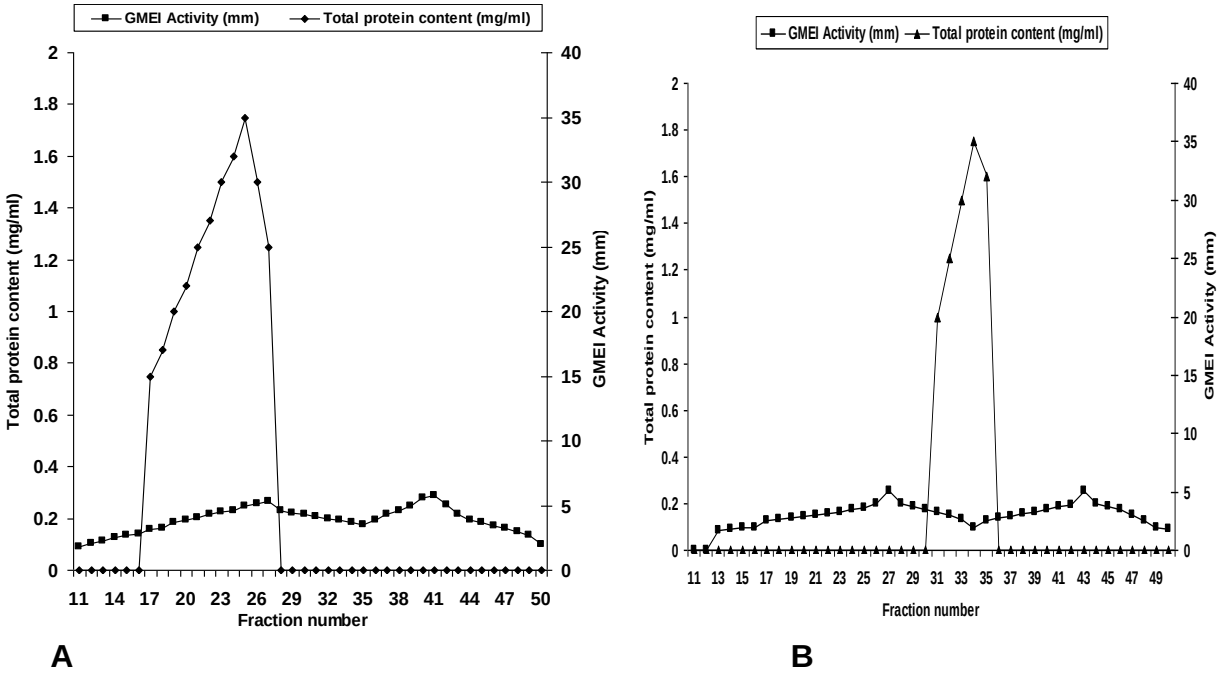

Figure 3. Purification of gentamicin modifying-enzyme inhibitory protein by (A) ion exchange and (B) gel filtration column chromatography.

APH ($2^{\prime\prime}$), which is frequently found in Gram-positive organisms as part of a bifunctional enzyme with acetyltransferase activity (Daigle et al., 1999; Gerard, 1999; Roestamadji et al., 1995).

Gentamicin modifying-enzyme inhibitory protein was purified by using ion exchange (DEAE-cellulose) and gel filtration (sephadex G-200) column chromatography (Figure 3A and B). Purified gentamicin modifying-enzyme inhibitory protein was separated at 32 KDa by SDS-PAGE and stained by Coomassie blue dye (Figure 4). In another work, the modifying enzymes were extracted and purified after the final step of DEAE-Sephadex A-50 chromatography. AAC (6') and APH (2") activities were observed as a single peak when GM was the substrate, and the position corresponded to one of the protein peaks. In the purification of AAC(6') and APH(2") from extracts of MS353(pTU068), specific activity after DEAE-Sephadex chromatography was 6.0 $U.mg^{-1}$. The molecular weight of APH(3')-III was 31 KDa and that of AAD(4',4") was 36 KDa. The bifunctional enzyme with AAC(6') and APH(2") activity had a molecular weight of 54 KDa (Kimiko et al., 1984). Amino acids of gentamicin-modifying enzyme inhibitory protein were determined by using HPLC. Gentamicin-modifying enzyme inhibitory protein composed of 17 amino acids at different concentrations. Susceptibility test was conducted according to Checkerboard method using different concentrations of gentamicin (512 to 32 $\mu g.ml^{-1}$) and gentamicin-modifying

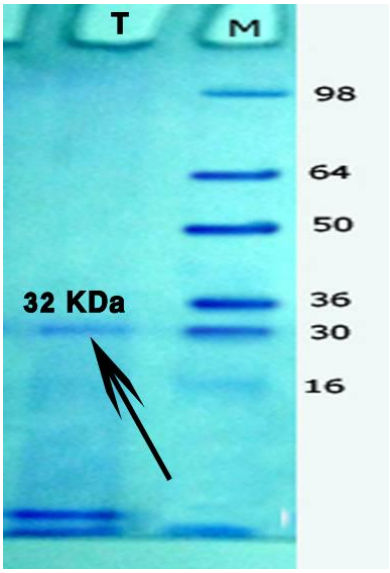

Figure 4. SDS-PAGE of purified Gentamicin modifying-enzyme inhibitor.

enzyme inhibitory protein (128 to 0.015 $mg.L^{-1}$). Combination of gentamicin-modifying enzyme inhibitory protein at 128 $mg.L^{-1}$ and gentamicin antibiotic at 128 $\mu g.ml^{-1}$ led to complete inhibition for the growth of gentamicin-resistant *S. aureus*.

ACKNOWLEDGEMENTS

Authors thank Scientific Research Deanship, King Saud University, Saudi Arabia for the fund of this work. The authors also thank head and members of Botany and Microbiology Department, Faculty of Science (Boys), Al-Azhar University as well as head and members of Microbiology Department, National Organization for Drug Control and Research (NODCAR). They are greatly indebted and sincerely thankful to the members of Sayed Galal Hospital who facilitated the clinical samples collection.

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