THE ACETYLATION OF 6′-AMINO GROUP OF AMIKACIN BY A NEW ENZYME PREPARED FROM *SERRATIA* SP.

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It was found that *Serratia marcescens* 43, *Serratia proteamaculans* 48 and *Serratia* sp. 45, all of which were clinically isolated, produced a new type of aminoglycoside acetyltransferase which acetylated amikacin at the 6′-amino group. 1-*N*-[(*S*)-3-Amino-2-hydroxypropionyl]-gentamicin B (HAPA-B, SCH 21420) and gentamicin C_2 were hardly inactivated by the enzymes and had effective antimicrobial activities against these strains both *in vitro* and *in vivo*. This kind of aminoglycoside acetyltransferase should be classified into a new group other than previously reported AAC(6′) enzymes.

Many papers have revealed that bacterial strains resistant to certain aminoglycosides inactivated these antibiotics by acetylation at the 6′-amino group of the 6-aminoglucosyl moiety of the molecule[1~4]. Thus kanamycin (KM) and gentamicin C_{1a} (GM-C_{1a}) were acetylated at the 6′-amino group as well as the 3-amino group of the cyclitol moiety. Recently we have found a few clinical isolates of *Serratia* sp. resistant to amikacin (AMK), tobramycin (TOB), dibekacin (DKB) and KM, but susceptible to GM-C_2, gentamicin B (GM-B), and HAPA-B [1-*N*-[(*S*)-3-amino-2-hydroxypropionyl]gentamicin B, SCH 21420] suggesting the presence of a new enzyme system for inactivation of aminoglycosides.

This paper deals with the inactivation mechanism of AMK by the above strains, the chemical structure of the inactivated AMK and also the protective effects of some antibiotics on mice experimentally infected by the strains producing such enzymes.

Materials and Methods

Bacterial Strains

Serratia marcescens, *Serratia proteamaculans* and *Serratia* sp. strains were isolated from clinical sources. *Bacillus subtilis* ATCC 6633 was used as a test organism for the microbial assay procedure.

Chemicals

HAPA-B and SM were supplied by Toyo Jozo Co., Ltd., all other antibiotics used were commercially available ones, KM and DKB by Meiji Seika Co., Ltd., TOB and GM by Shionogi Seiyaku Co., Ltd., AMK by Banyu Seiyaku Co., Ltd. respectively. Adenosine 5′-triphosphate, acetyl CoA were also commercially available. GM-C_{1a} and GM-C_2 were prepared in our laboratories.

Media

Mueller-Hinton broth and Mueller-Hinton agar (Difco) were used for bacterial culture.

Determination of Antibacterial Activity

An overnight culture of each bacterial strain was diluted 100-fold with the same broth and a

loopful of the diluted culture was spotted onto each of the agar plate containing serial two-fold dilutions of a given drug. After incubation for 20 hours, the minimal inhibitory concentration (MIC) of the drug for each strain was examined.

Preparation of the Cell Free Extracts

The bacterial cells growing exponentially in 100 ml culture were harvested by centrifugation at $8,000 \times g$ for 15 minutes and washed with Tris-HCl buffer (0.1 M, pH 7.5, with 1 mM 2-mercaptoethanol). The washed cells were suspended into 5.0 ml of the same buffer solution and sonicated for 5 minutes. The supernatant fluid obtained by centrifugation at $30,000 \times g$ for 2 hours was further centrifuged at $105,000 \times g$ for 2 hours to prepare the final supernatant, designated as the cell free extract.

Inactivation of Antibiotics by the Cell Free Extract

A reaction mixture containing the solution, 0.1 ml of the cell free extract, 0.05 ml of 2.0 mM acetyl CoA, 0.05 ml of 160 mM adenosine 5'-triphosphate, 0.05 ml of 20 mM magnesium chloride solution, 0.05 ml of a 250 μg/ml solution of the test antibiotic and 0.2 ml of 0.1 M Tris-HCl buffer solution was incubated at 37°C for 2 hours. After heated at 100°C for 2 minutes, the residual antibacterial activity was determined using *B. subtilis* ATCC 6633 as the test organism.

Isolation of Inactivated AMK[3~5]

AMK (100 mg) was inactivated in a medium containing 100 ml of the cell free extract (20 mg of protein per ml), 882 mg of disodium ATP, 162 mg of acetyl CoA, 958 mg of magnesium chloride and 140 mg of AMK at 37°C for 6 hours. The mixture was heated over a boiling water bath for 5 minutes to stop further reaction. The supernatant obtained by centrifugation at $30,000 \times g$ for 30 minutes was subjected to Amberlite IRC-50 (NH_4^+ form, 100 ml) column chromatography. After washing with 1,000 ml of distilled water, the inactivated AMK was eluted with 1.0 N NH_4OH. The eluate was adjusted to pH 7.0 and subjected to CM-Sephadex C-25 (NH_4^+ form, 50 ml) column chromatography for further purification. After washing the column with 1,000 ml of distilled water, elution was carried out with a linear gradient of ammonium hydroxide (0~0.1 N NH_4OH). Ninhydrin-positive fractions were collected and lyophilized to yield 40 mg of product.

Thin-layer Chromatography and NMR Analysis

The enzymatically inactivated AMK was primarily investigated by TLC, in which the absorbent was DL-Plastikfolien Kiesel gel 60 F_{254} (Merck), the developing solvent systems were 10% CH_3COONH_4-CH_3OH (1:1), CH_3CN - CH_3OH - EtOAc - NH_4OH (4:2:1:5) and $CHCl_3$ - CH_3OH - NH_4OH (2:3:2). The detecting reagent was ninhydrin. The chemical structure of the inactivated AMK was confirmed by mass spectrum, ^1H NMR and ^{13}C NMR spectroscopy.

Experimental Infection of Mice

The *in vivo* antibacterial activities of HAPA-B, AMK and GM were determined in mice. Four-week-old male Slc: ICR mice, weighing 18~20 g, were infected intraperitoneally with *S. marcescens* 43 or *S. proteamaculans* 48, suspended in 0.5 ml of 5% mucin (Difco). The challenge dose was approximately 10^7 cells/mouse. Groups of 10 mice were treated intramuscularly with 0.2 ml of the test antibiotic.

The 50% effective dose (ED_{50}, mg/kg) was calculated in accordance with the LITCHFIELD-WILCOXON method from the survival rates recorded 7 days after the infection.

Results

Resistant Levels of Aminoglycoside Antibiotics

Five strains of *Serratia* sp., *S. marcescens* and *S. proteamaculans* were selected from the stock cultures. The minimal inhibitory concentration (MIC) of several drugs against these strains are shown in Table 1. Most strains were susceptible to HAPA-B. It should be noted, however, that all the strains were resistant to AMK, TOB and DKB, but susceptible to GM-C_2 and GM-B.

Inactivation of Aminoglycoside Antibiotics by Cell Free Extract Prepared from Resistant Strains

The cell free extract from strains *Serratia* sp. 45, *S. proteamaculans* 48 and *S. marcescens* 43 were prepared and the biochemical mechanism of resistance to aminoglycoside antibiotics was investigated. The inactivation reaction did not take place without acetyl CoA. The rates of the inactivation of AMK, HAPA-B, GM-C_{1a}, GM-C_2 and DKB by the extracts of these strains are summarized in Table 2. The extracts of strains 43, 45 and 48, being resistant to AMK and DKB but susceptible to HAPA-B, GM-C_2 and GM-B inactivated both AMK and DKB but not GM-C_2. HAPA-B was slightly inactivated, 75 to 80% of the activity remaining after 2-hour incubation.

Purification and Properties of Inactivating Enzyme

The supernatant fluid by ultracentrifugation from *Serratia* sp. 45 was passed through a KM-Sepharose 4B column[4] and eluted by a linear gradient elution with NaCl from 0 to 0.5 M in 50 mM phosphate buffer (pH 7.2) containing 2-mercaptoethanol. The active fractions which inactivated AMK were applied to Sephacryl S-300, then the active enzyme fractions were collected. The optimal pH for the inactivation of AMK was in a range between 7.5 and 9.0. Enzymatic activity was decreased at pH lower than 6.5 or higher than 9.0. The optimal temperature was in a range from 37°C to 42°C and the inactivating activity was drastically decreased at a temperature below 30°C or over 50°C. Isoelectric focusing was carried out with LKB Ampholine (column volume 110 ml) at 4°C and 700 V for 36 hours. The isoelectric point of this enzyme was 4.60, and the molecular weight was 58,000 by Sephadex G-75 (superfine) gel filtration.

This enzyme is clearly different from AAC(6′)-4, produced by *Pseudomonas aeruginosa*[3,4].

Table 1. Minimal inhibitory concentrations (MIC) of aminoglycoside antibiotics against *Serratia* sp., *S. marcescens* and *S. proteamaculans*.

Strain	MIC (μg/ml) 10^6 cells/ml						
	AMK	HAPA-B	GM-C_{1a}	GM-C_2	GM-B	TOB	DKB
Serratia sp. 40	12.5	1.56	6.25	1.56	3.13	25	50
S. proteamaculans 41	12.5	1.56	6.25	0.39	1.56	25	25
S. marcescens 43	6.25	0.78	6.25	1.56	1.56	25	25
Serratia sp. 45	25	1.56	6.25	0.78	3.13	25	50
S. proteamaculans 48	25	3.13	6.25	0.39	3.13	50	50
S. marcescens TL-1[a]	3.13	1.56	1.56	0.78	1.56	6.25	6.25

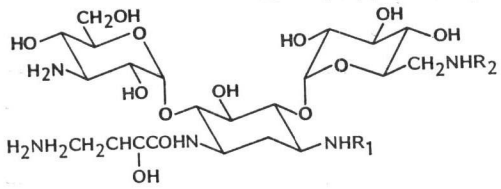
[a] This strain was used as a standard strain of *S. marcescens*.

Table 2. Inactivation of HAPA-B, AMK, GM-C_{1a}, GM-C_2 and DKB with enzyme.[a]

Strain	Inactivation (%)				
	AMK	HAPA-B	GM-C_{1a}	GM-C_2	DKB
S. marcescens 43	100	15	65	0	75
Serratia sp. 45	100	20	75	0	80
S. proteamaculans 48	100	25	75	0	85

[a] The cell free extracts were prepared from each strain as described in Materials and Methods. After incubation for 90 minutes at 37°C, the residual antibiotic activity in the reaction mixture was determined by bioassay. The cell free extracts used were at concentration of 630 μg protein per ml.

Fig. 1. Chemical structure of acetylated AMK.



	R_1	R_2
AMK	H	H
Inactivated AMK	H	$-\overset{O}{\overset{\|\|}{C}}CH_3$

Table 3. Protective effects of HAPA-B, AMK and GM on experimental systemic infection of mice with *S. marcescens* 43 and *S. proteamaculans* 48.

Organism	Challenge dose	Compound	ED_{50} (mg/kg)
S. marcescens 43	10^7	HAPA-B	8.4
		AMK	47
		GM	3.5
S. proteamaculans 48	10^7	HAPA-B	10
		AMK	75
		GM	2.6

Identification of the Acetylated Product

On TLC chromatography with the solvent of 10% CH_3COONH_4 - CH_3OH (1: 1), Rf values of the inactivated AMK and the intact AMK were 0.26 and 0.04, respectively.

The ^1H NMR spectrum of the inactivated AMK suggested the existence of a newly formed *N*-acetyl group (2.20 ppm singlet) and in the ^{13}C NMR spectrum of the same product, two other signals consistent with the presence of an acetate group were observed. The inactivated product melted at 167 to 170°C and $[\alpha]_D^{28}$ +63° (*c* 0.990, H_2O) (Ref. 3, 4: mp 168~173°C, $[\alpha]_D^{23}$ +69° (*c* 0.5, H_2O)). In view of the above experimental findings and the molecular ion peak in mass spectrum, the chemical structure of the inactivated AMK was 6′-*N*-acetyl-AMK. (Fig. 1)

Experimental Infection in Mice

The protective effects of HAPA-B in mice infected intraperitoneally with *S. marcescens* 43 or *S. proteamaculans* 48 were compared with those of AMK and GM. The protective effect of HAPA-B appeared stronger than AMK, but weaker than GM (Table 3).

Discussion

The enzymatic inactivation of aminoglycoside antibiotics has been reported to be one of the main mechanisms of resistance both in Gram-positive and Gram-negative organisms. The inactivation enzymes have been known as aminoglycoside phosphotransferase, aminoglycoside adenylyltransferase and aminoglycoside acetyltransferase[6,7].

Aminoglycoside 6′-acetyltransferases, *i.e.*, AAC(6′), have been further classified into four groups according to their substrate profiles and susceptibilities to drugs, namely AAC(6′)-1,2,3 and -4[8].

The enzyme AAC(6′)-1 acetylates KM, bekanamycin and neomycin (NM), but does not affect to other aminoglycoside antibiotics such GM-C_{1a}, DKB or AMK.

The enzyme AAC(6′)-2 inactivates KM, bekanamycin, NM, GM-C_{1a} and GM-C_2 but does not acetylate AMK.

The enzyme AAC(6′)-3 inactivates KM, bekanamycin, NM, GM-C_{1a} and DKB but does not acetylate AMK.

The enzyme AAC(6′)-4 acetylates KM, bekanamycin, GM-C_{1a}, DKB and AMK.

The enzyme which we have found from *Serratia* sp. 45, *S. proteamaculans* 48 or *S. marcescens* 43 hardly inactivates GM-C_2 and HAPA-B but inactivates AMK and DKB. The substrate profile and susceptibility of the enzyme suggest that the enzyme is a new type not belonging to any of above four categories.

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