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Microbial transformation of neomycin by a mutant of neomycin-producing *Streptomyces fradiae*

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Utilizing a mutant of neomycin-producing *Streptomyces fradiae* mutagenized with neutron radiation, biotransformation of neomycin into modified compounds was studied. The biotransformation products were isolated by ion exchange chromatography and monitored by thin layer chromatography bioautography (TLCB). Antibacterial activity of biotransformation products against ten species of bacteria including four plant pathogens was tested qualitatively by TLCB and detected quantitatively by Oxford cup method. The minimal inhibitory concentration (MIC) of biotransformation products was tested by agar diffusion method. Three isolated transformation products had obvious antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus vulgaris* and *Pseudomonas solanacarum*. At the concentration of 50 µg/ml, the transformation product X had a similar antibacterial effect with neomycin but the transformation product Y and Z showed a decreased effect compared to neomycin except for *P. vulgaris* and *P. solanacarum*. However, the results from MIC analysis demonstrated that only the transformation product X maintained the same inhibitory effect with neomycin.

Key words: Neomycin, biotransformation, *Streptomyces fradiae*, mutant, neutron radiation.

INTRODUCTION

Neomycin produced from the fermentation of the actinomycete *Streptomyces fradiae* is a broad spectrum water-soluble aminoglycoside antibiotic. Neomycin possesses excellent effect on streptomycin-resistant bacteria (Waksman and Lechevalier, 1949), and was once used as a therapeutic agent in clinics for applications including ophthalmic, topical and oral administrations (Church, 1954; Nichols et al., 1973; Poth et al., 1950). However, because of the ototoxicity, nephrotoxicity and neuro-muscular block (Masur et al., 1976; Vital and Prado-Franceschi, 1969), the clinical employment of neomycin through intravenous administrations was limited extremely. Neomycin was also used early in veterinary medicine

(Konde and Monroe, 1955; Tucker and Johnson, 1953) and in animal nutrition (Luecke et al., 1951).

Over time, there had been a few reports on controlling plant diseases (Hagborg, 1956; Katznelson and Sutton, 1951) using neomycin. Recently, we studied the effect of neomycin sulfate against four species of plant pathogen by pot experiments, and it indicated that the control efficiency of neomycin was found to be equivalent to streptomycin (He et al., 2008). We further explored the action mechanism of neomycin sulfate on *Erwinia carotovora*, a plant pathogen causing soft rot of cabbage (Cui et al., 2009).

Biotransformation of aminoglycosides antibiotics such as gentamicin (Testa and Tilley, 1976), ribostamycin (Kojima et al., 1973), kanamycin (Cappelletti and Spagnoli, 1983), sisomicin (Lee et al., 1977) and arbekacin (Hotta et al., 1996) has been reported previously. The newly modified compounds were not only transformed from the antibiotic itself by producing strain (Kojima et al., 1973), but also obtained by mutasynthesis (Cappelletti and

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Spagnoli, 1983; Testa and Tilley, 1976; Testa et al., 1974). Apart from direct fermentation using micro-organism, the resting cells (Kase et al., 1982), cell free extract (Hotta et al., 1996) and enzyme (Haas et al., 1976) were also employed for biotransformation. Many reports about the chemical conversion of neomycin have been published in the past decades (Fridman et al., 2003; Hainrichson et al., 2005; Hanessian et al., 1978; Toda et al., 1983), but only a few papers reported the mutasy-nthesis of neomycin. Shier et al. (1969) reported the semi-synthesis of neomycin from the related aminoc-yclitols streptamine and 2-epistreptamine transformed by a mutant of *S. fradiae* and thus four new antibiotics was obtained. Soon after, Shier et al. (1973) further studied the conversion of some analogs of 2-deoxystreptamine to antibiotics by 2-deoxystreptamine-negative mutants of *S. fradiae*. In addition, the conversion from three phosphoamido-neomycins to neomycin was also reported by Majumdar and Majumdar, (1970). However, up till now, no information about the direct biotransformation from neomycin by its producer or mutant of *S. fradiae* is available. In the present study, a mutant of *S. fradiae* mutagenized with neutron radiation was used for conver-sion and neomycin itself was used as the substrate. Our aim is to obtain the new derivatives of neomycin with antibacterial activity so as to provide more candidate compounds for medical or agricultural application.

MATERIALS AND METHODS

Isolation of *S. fradiae* mutant mutagenized with neutron radiation

Three growth culture of 7-day-old *S. fradiae* spores grown on Gause No.1 agar medium slant were washed with 30 ml phosphate buffer (0.1 mol/L, pH 7.0) and incubated for 30 min with reciprocal shaking at 150 rpm. After centrifuging at 4000 rpm and discarding the supernatant, the spores were suspended with 2 ml sterile water and added to 8 ml Gause's liquid medium. The suspension was incubated for 3 h until proper germination of the spores. An aliquot of spore suspension (0.5 ml) was transferred into test tube (1.0 × 7.5 cm) and radiated at the dose of 400 Gy for 4 h with neutron accelerator in 720 Institute of Sichuan University, China. Four replicates of radiation treatment were applied. The radiated spore suspension was diluted with 4.5 ml sterile water. Petri plates containing Gause No.1 agar were inoculated with survivors of the radiation treatment, incubated for 3 days at 28°C until individual colony occurred, and the spore suspension without radiation treatment was served as the control. The individual colonies were re-inoculated to fresh Gause No.1 agar plates for another 3 days incubation. The occurred colonies punched with a puncher (6 mm diameter) were placed on the Gause agar bioassay plates seeded *Staphylococcus aureus* and incubated at 28°C for 18 h. The plates were screened for a colony that showed no inhibition zone against *S. aureus*. The procedures were repeated more than three times until the stable mutant of *S. fradiae* was obtained.

Culture conditions of the mutant

The mutant of *S. fradiae* which was mutagenized by neutron

radiation was maintained on a Gause's No.1 slant at 4°C and was subcultured at monthly intervals. A well sporulated slant culture (7 days old) was grown in a seed medium consisting (g/L) of: Soluble starch, 20 g; KNO$_3$, 1 g; K$_2$HPO$_4$, 0.5 g; MgSO$_4$, 0.5 g; NaCl, 0.5 g; FeSO$_4$, 0.01 g; for 3 days at 36°C on a reciprocal shaker. The growth-well seed culture in the above medium was used for transformation fermentation.

Detection of transformation products from small-scale fermentation by RP-HPLC

Transformation fermentation

Inocula prepared in seed culture were used at 2.5% (v/v) for fermentations in a 100 ml Erlenmeyer flask containing 30 ml of Gause medium as described above. Neomycin sulfate used for biotransformation was supplied by Sichuan Long March Pharma-ceutical Corporation, Chengdu, China. Neomycin was added at 600 μg/ml after 72 h of fermentation. Four treatments including neomycin aqueous solution, the medium without neomycin and inoculum, the medium only with inoculum and the medium only with neomycin were used as the controls. The equivalent of neomycin or inoculum was applied at each treatment as the transformation fermentation. The transformation fermentation together with other four controls was simultaneously carried out on a reciprocal shaker at 150 rpm for 120 h at 36°C.

Derivatization reagent, potassium borate buffer (0.2 mol/L) was prepared by dissolving 12.37 g boric acid in 980 ml of deionized water and adjusting the pH to 10.4 with 10 mol/L potassium hydroxide solution, followed by dilution to 1 L. OPA reagent was prepared by dissolving o-phthalaldehyde (100 mg) in 2 ml of methanol, followed by addition of 2-mercaptoethanol (1 ml). The solution was gently mixed until complete decolorization occurred. Potassium borate buffer (100 ml) was then added with vigorous stirring. The solution was placed in brown bottle at 4°C, and used within 5 days.

Samples preparation and pre-column derivatization

The fermentation broth of different treatment was centrifuged at 10000 rpm, 10°C for 10 min. The supernatant was filtered to remove debris through reduced pressure. The resulting solution and neomycin aqueous solution were stored at 4°C for less than 2 days before analysis by reversed-phase high-performance liquid chromatographic (RP-HPLC). A 0.2 ml aliquot of each resulting solution or neomycin aqueous solution was added to 0.2 ml of OPA reagent. The mixture was shaken sufficiently and allowed to react for 10 min at 30°C water bath. After the reaction, the mixture was added to 0.8 ml isopropanol and the resulting solution was shaken several times. Each derivative sample was filtered through a 0.2 μm membrane filter.

Chromatography analysis

Chromatography was carried out on Shimadzu LC system and SPD-M20A diode array detector (Shimadzu Corporation, Kyoto, Japan). Data integration and processing were performed with LCSolution software (Shimadzu, Japan). The HPLC column used was an ODS-SP packed C$_{18}$ (4.6 mm i.d. × 150 mm length, 5 μm, Inerstil, Tokyo, Japan). The mobile phase was methanol-water (65:35, v/v). This mobile phase was filtered through a 0.45 μm membrane filter and degassed prior to use. The flow rate was performed at 1 ml/min and the UV detector set at a wavelength of 334 nm. Aliquots of 10 μl of each derivative sample were injected into the HPLC system.

Large scale fermentation and isolation of biotransformation products

Inocula prepared in seed culture were used at 5% (v/v) for fermentations in a 500 ml Erlenmeyer flask containing 200 ml of a medium with the following composition: Peptone, 6 g; casein, 4 g; yeast extract powder, 3 g; beef powder, 1 g; and glucose, 2 g. Neomycin was added at 600 µg/ml after 72 h of fermentation. The transformation fermentations were carried out on a reciprocal shaker at 150 rpm for 120 h at 36°C. Occasional checking of the flask to drop adhering cells into medium was necessary during the whole process.

The fermentation broth (3 L) was centrifuged and filtered as above. The supernatant was adjusted to pH 7.5 with aqueous ammonia and passed through a column (3.5 × 40 cm) containing 100 ml of Amberlite FPC-3500 cation exchange resin in NH_4^+ form (Rohm and Haas Co., Philadelphia, Pennsylvania, USA). After washing with adequate deionized water, the resin was eluted with 2 mol/L ammonium hydroxide. The eluates were concentrated to dryness under reduced pressure at 50°C. The dried material was dissolved in deionized water to a desired concentration and applied to anion exchange resin Amberlite FPA-90Cl (Rohm and Haas Co., USA) for decolorization. The decolored eluates were passed through a column (2.0 × 30 cm) containing 50 ml of Amberlite CG-50 cation exchange resin (type I, 100-200 mesh, Rohm and Haas Co., USA) and washed with deionized water. The CG-50 resin was eluted with a linear gradient of 0.05 to 0.5 mol/L ammonium hydroxide. Fractions were collected with 15 ml in each tube. Each fraction was concentrated to a range of 0.5 - 1.0 ml and then monitored using thin layer chromatography bioautography (TLCB). Similar fractions were combined and evaporated under reduced pressure to yield crude preparations. The crude preparations were further chromatographically separated on a smaller CG-50 resin column (1.0 × 30 cm) and eluted with a linear gradient of 0.01 to 0.2 mol/L ammonium hydroxide. Fractions were collected with 5 ml in each tube and monitored as above.

Bioautography of fractions from ion exchange chromatography

The high-performance thin layer chromatography (HPTLC) plates (5.0 × 6.5 cm, cut from 5.0 × 20 cm precoated silica gel plates) were pre-developed to the top with chloroform-methanol-25% ammonium hydroxide (2:3:2) as developing solvent which was an improvement based on the published report (Baud et al., 1977; Testa and Tilley, 1975). The pre-developed plates were dried completely at 100°C and then spotted with fractions eluted from Amberlite FPC-3500 or Amberlite CG-50 resin column. Simultaneously, neomycin sulfate diluted solution was served as the control. The spotted plates were dried naturally and redeveloped with the same developing solvent up to the height of 5.5 cm. The developed plates were dried at 60°C for 1 h and placed in 90 mm Petri dishes with face upward. After sterilizing under UV light for 20 min, the plates in Petri dishes were covered with 20 ml of the sterilized beef extract peptone agar medium (beef extract 0.03%, peptone 1%, NaCl 0.05% and agar 2%) mixed S. aureus, a sensitive Gram-positive bacterium to neomycin. The plates were incubated overnight at 36°C and the surface of the medium was then sprayed with triphenyltetrazolium chloride (TTC) solution (0.2%). The plates were incubated for 30 min. White inhibition zones were visible against a red background.

Antibacterial activity of biotransformation products by bioautography

Antibacterial activity of three purified biotransformation products against 10 species of bacteria including S. aureus was tested by bioautography method as above. The tested bacteria including S. aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Enterobacte cloacae (kindly provided by Sichuan Industrial Institute of Antibiotic, China) and four phytopathogenic bacteria (Xanthomonas oryzae, Xanthomonas axonopodis, Erwinia carotovora and Pseudomonas solanacarum, kindly provided by Institute of Plant Protection, Sichuan Academy of Agricultural Science, China).

Antibacterial activity of biotransformation products by Oxford cup method

The antibacterial activity of transformation products of neomycin sulfate against 10 bacteria was evaluated by the modified Oxford cup method (Carlson and Douglas, 1948) and the inhibition zone diameters were measured. Beef extract peptone agar media was used for bacterial culture. One milliliter (1 ml) of an 18 h old culture was added to 60 ml of the medium, and 20 ml of this culture was shaked and poured into sterile Petri dishes. After the solidification of the agar medium, the sterile stainless steel cylinders (6 × 10 mm) were placed on the surface of the seeded agar and filled with 100 µl of the tested samples at 50 µg/ml. The plates were incubated at 36°C for 24 h.

Minimal inhibitory concentration (MIC) of biotransformation products

The agar diffusion method was used to evaluate the MIC of tested materials. An overnight culture grown in beef extract peptone broth was diluted to approximately 10^5 CFU/ml in sterile water. The diluted bacterial suspension was inoculated onto agar plates containing serial two-fold dilutions of neomycin and its transformation products at a final concentration ranging from 16 to 0.0156 µg/ml (Dienstag and Neu, 1972). The MIC was defined as the lowest concentration of antibiotic preventing visible organism growth after incubation for 16 to 18 h at 36°C.

RESULTS AND DISCUSSION

HPLC analysis of transformation product

Directed analysis of biotransformation fermentation broth by reversed-phase high performance liquid chromate-graphy (RP-HPLC) showed the mutant of S. fradiae had the ability to convert neomycin to new derivative of neomycin (Figure 1). In the medium control (Figure 1A) and the control of medium only with inoculum (Figure 1B) no distinct peak was found except the reagent peaks. The peak area and peak height of neomycin (I) in aqueous solution (Figure 1C) and in medium without inoculum (Figure 1D) were similar and the retention time was at 15.073 and 14.944 min, respectively. In transfor-mation treatment (Figure 1E), the area and height of neomycin peak (I) decreased obviously compared with two controls added with neomycin. Another new small peak (II) at the retention time 21.485 was detected, but no corresponding peak was found for the other four controls. This result implies that neomycin may be degrades mostly due to providing C, N and energy for
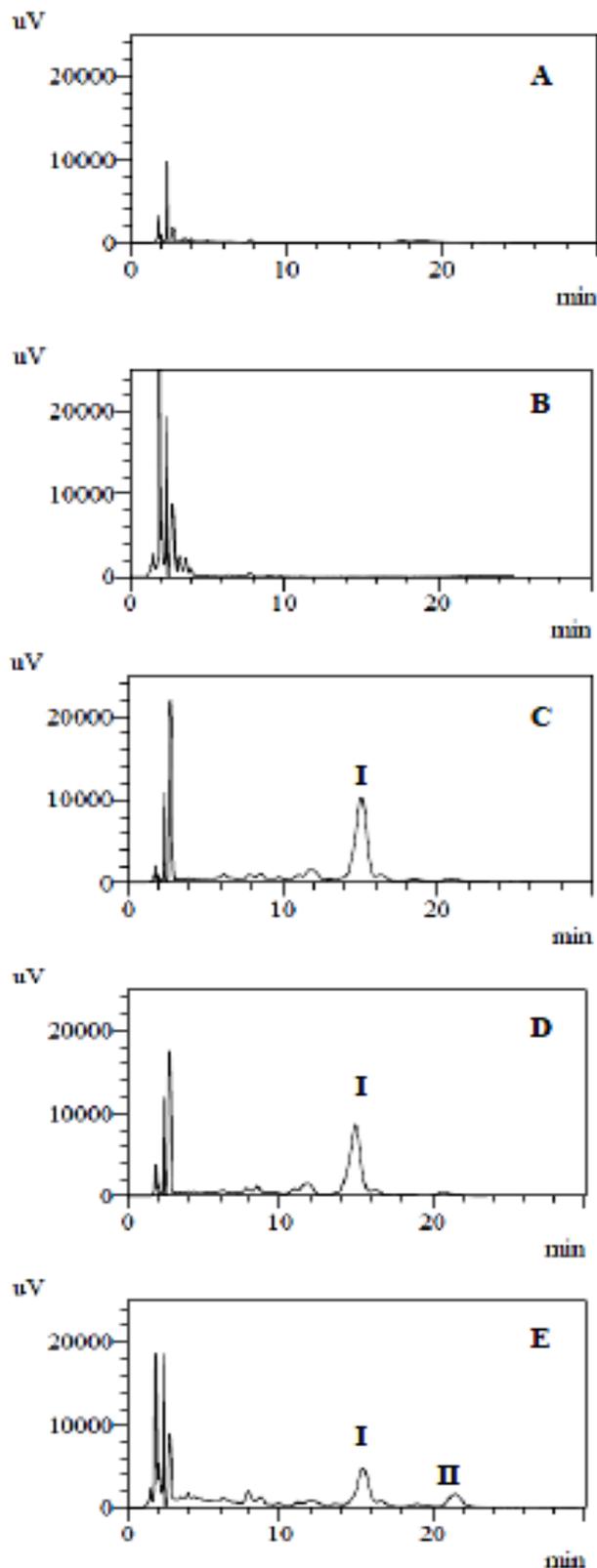
Figure 1. RP-HPLC of the transformation product from culture broth of *Streptomyces fradiae* mutant. A. medium; B. medium with inoculation; C. neomycin in aqueous solution; D. medium with addition of neomycin but no inoculation, E. transformation of neomycin. I. neomycin peak, II. possible biotransformation peak.

growth (Perlman and Sebek, 1971), and transformation of neomycin to its analog also occurs.

Detection of biotransformation products of aminoglycoside antibiotics from culture broth by HPLC was seldom reported previously (Cappelletti and Spagnoli, 1983). Although thin layer chromatography (TLC) and high-voltage paper electrophoresis were also used in monitoring modification product of arbekacin from reaction mixture (Hotta et al., 1996), in most cases, detection of biotransformation products were performed after isolating and purifying the fermentation broth by ion exchange chromatography. The usual methods for identifying transformation products includes: TLC (Cappelletti and Spagnoli, 1983; Kojima and Satoh, 1973; Takeda et al., 1978; Testa and Tilley, 1975, 1976), paper chromatography (Majumdar and Majumdar, 1970; Shier et al., 1973) and preparative paper chromatography (Kojima et al., 1975, Shier et al., 1969, 1973).

HPLC analysis of culture broth could provide a rapid detection for determining whether the transformation occurs or not. However, we found the pre-column derivation reaction of neomycin with OPA plus 2-mercaptoethanol was significant interfered by culture broth. When organic ingredients such as peptone, beef extract and yeast powders were supplemented to the culture medium, the neomycin peak could not be monitored by HPLC (data not shown). It is not clear whether other derivation reagents such as 2,4,6-trinitrobenzenesulfonic acid could be affected by organic ingredients (Cappelletti and Spagnoli, 1983). When Gause's No. 1 medium was used for transformation of neomycin, the derivation reaction of culture broth with OPA was normal and stable. However, Gause's No. 1 medium can only provide limited nutrients for growth. Thus nutrient medium which contained natural organic substance was applied for a large scale transformation.

Isolation and identification of modified neomycin

In order to isolate the modified neomycin compounds, a large scale transformation culture was carried out and followed by a column chromatography using Amberlite FPC-3500 (NH_4^+ type, former IRC-50) and Amberlite CG-50 (NH_4^+ type). From 3 L preparative transformation of neomycin (1.8 g substrate), 1.59 g of crude product was isolated after being chromatographed by Amberlite FPC-3500 resin and decolored by Amberlite FPA-90 (Cl^- type) resin. The result of bioautography of partial fractions collected from chromatography by Amberlite CG-50 resin (1.59 g crude product was loaded on 2.0 × 30 cm column) is shown in Figure 2. Fractions 16 - 25 were eluted with 0.05 mol/L ammonium hydroxide. Two distinctive transformation product spots were observed at fractions 18 - 20 (Rf 0.72) and fractions 21 - 23 (Rf 0.49) respectively (Figures 2A and B), despite incomplete isolation with neomycin substrate (Rf 0.37). Among fractions 26 - 35 eluted with 0.1 mol/L ammonium
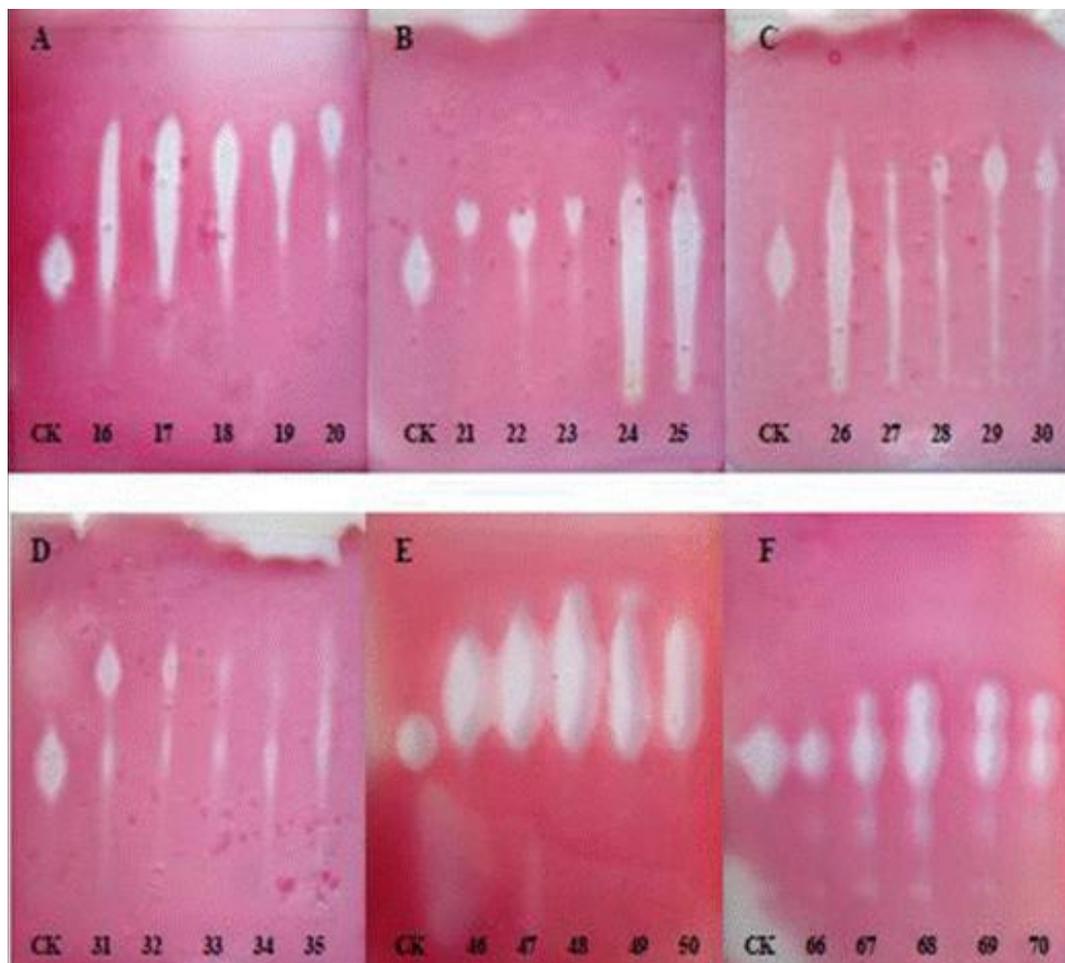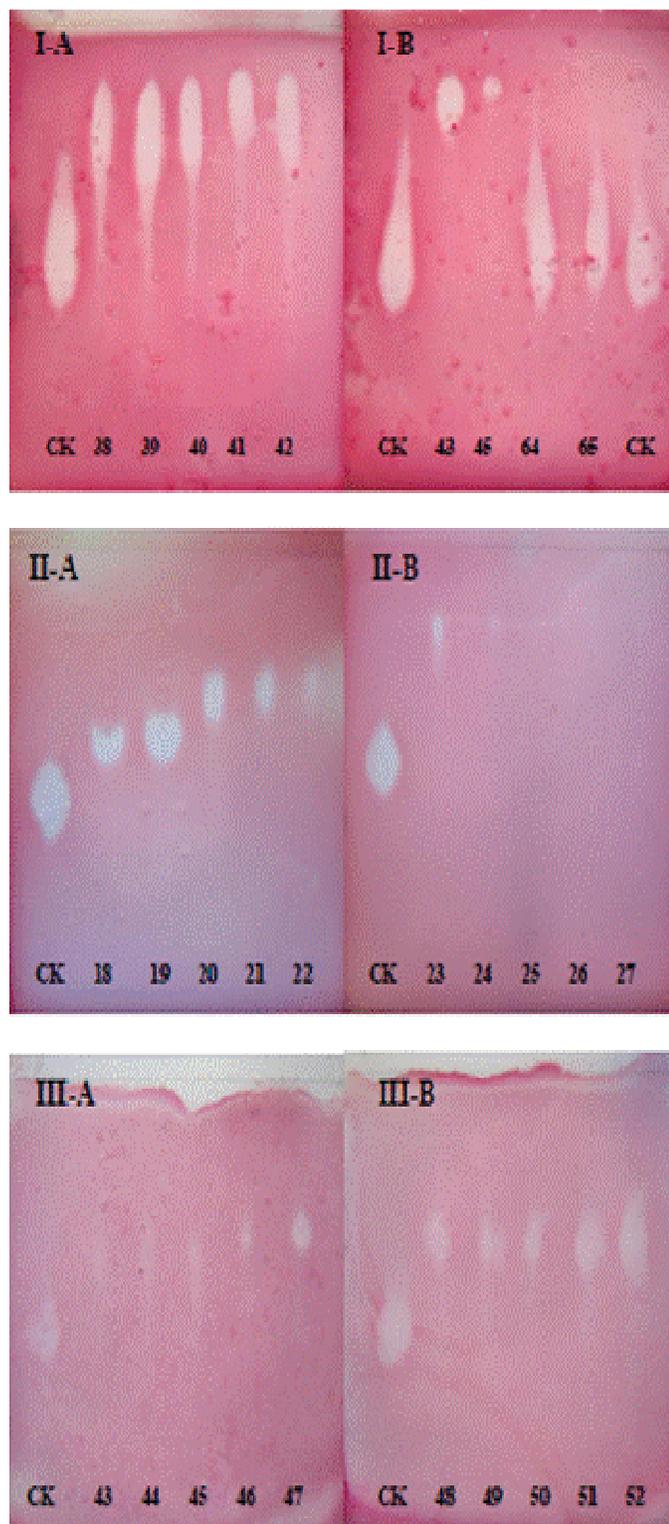
Figure 2. Bioautography of neomycin (CK) and the fractions isolated form Amberlite CG-50 cation ion exchange chromatography (2.0 × 30 cm) against *Staphylococcus aureus.* A. fractions 16 - 20; B. fractions 21 - 25; C. fractions 26 - 30; D. fractions 31 - 35; E. fractions 46 - 50; F. fractions 66 – 70.

hydroxide, fractions 28 - 32 (Rf 0.60) also showed a clear new spot which was different from authentic neomycin (Figure 2C and D). Fractions 46 - 50 and 66 - 70 were eluted with 0.15 and 0.2 mol/L ammonium hydroxide, respectively. Fractions 46 - 50 were obviously different from the authentic neomycin but could not be considered a single compound. Fractions 66 - 70 showed a higher polar spot than neomycin (short movement) except for another less polar spot (long movement). To obtain purified transformation products, the pooled fractions of 16 - 19 (I), 20 - 23 (II) and 24 - 35 (III) from Amberlite CG-50 column (2.0 × 30 cm) were rechromatographed by Amberlite CG-50 column (1.0 × 30 cm) and monitored by bioautograph (Figure 3). From the mixture I (16 - 19), the fractions 38 - 45 showed similar movement, so these fractions were combined and concentrated to dry (17.8 mg, designated compound X). From the mixture II (20 - 23), the fraction 18 and 19 were combined (4.3 mg, designated compound Y) and the pooled fractions 46 - 52 from the mixture III (24 - 35) were obtained and

designated compound Z (15.6 mg). The lower biotrans-formation rate of neomycin is similar to the result of transformation of arbekacin reported by Hotta et al. (1996). In addition, Perlman and Sebek, (1971) had pointed that the nearly complete degradation of anti-biotics by microbial attack in some cases could lead extreme low transformation to modified compound.

Column chromatography with weakly acidic ion-exchange resin was a common method used for isolating amino-glycoside antibiotics (Awata et al., 1983; Claes et al., 1974; Majumdar and Majumdar, 1970). Moreover, the strongly basic anion exchange resin (Inouye and Ogawa, 1964; Maehr and Schaffner, 1964), dextran gels (Berdy et al., 1977), cellulose powder (Rinehart et al., 1960) and silica gel (Lee et al., 1977) were also applied for column chromatography. In the present study, cation exchange resin Amberlite FPC-3500 (former IRC-50) was used to isolate fermentation broth. After elution by 2 mol/L ammonium hydroxide, the eluted solution contained pigment heavily, and so we loaded the colored crude

Figure 3. Bioautography of neomycin (CK) and the fractions isolated from Amberlite CG-50 cation ion exchange chromatography (1.0 × 30 cm) against *Staphylococcus aureus.* I-A, fractions 38 - 42 and I-B, fractions 43 - 65: isolated from the pooled fractions 16 - 19 (see Figure 2). II-A, fractions 18 - 22 and II-B, fractions 23 - 27: isolated from the pooled fractions 20 - 23 (see Figure 2). III-A, fractions 43-47 and III-B, fractions 48-52: isolated from the pooled fractions 24 - 35 (see Figure 2).

product on Amberlite FPA-90 (Cl⁻ type) column for decolorization through which the decolored rate could reach 90%. Leach et al. (1951) have reported the decolorization with carbon chromatography (Darco G-60) for commercial preparation neomycin sulfate, but the yield over the carbon column was only 57%. When the decolored crude product was applied on Amberlite CG - 50 column, the concentration of eluted solvent (ammonium hydroxide) was very important for good separation. According to previous published reports (Hotta et al., 1996; Kojima et al., 1973, 1975), we carried out two step isolation with Amberlite CG-50 resin at different eluted gradient and acquired an excellent separation of transformation products.

Antibacterial activity of transformation products

Thin-layer chromatography bioautography (TLCB) is quite simple, inexpensive and a reliable method for identification of active compounds; it is especially suitable for sensitive biological detection (Ramírez et al., 2003; Rosner and Aviv, 1980; Salisbury et al., 1989). We modified the method described by Salisbury et al. (1989), not placing TLC plate face down on the surface of agar for 15 min and then removing TLC plate, but directly distributing the agar mixed inoculum over TLC plate. Our method is more simple and easy to operate.

Antibacterial activity against six human pathogen bacteria and four plant pathogen bacteria of neomycin's biotransformation products (compound X, Y and Z) were tested by TLCB. The results showed *E. coli*, *P. aeruginosa*, *E. cloacae*, *X. oryzae*, *X. axonopodis* and *E. carotovora* were not sensitive to neomycin and its transformation products, but *S. aureus*, *B. subtilis*, *P. vulgaris* and *P. solanacarum* were observed to be inhibited (Figure 4). At the condition of same sample application, the inhibitory spots of neomycin and its transformation products against *P. solanacarum* were more obvious than against the other three plant pathogen bacteria (Figure 4D).

Quantitative detection of antibacterial activity was carried out by Oxford cup method and the result was showed in Table 1. Compound X displayed a similar antibacterial activity with neomycin sulfate. The difference of the diameter of inhibitory zone between compound X and neomycin sulfate was not significant except for *E. coli*. The antibacterial activity of compound Y and Z against *S. aureus*, *B. subtilis* and *E. coli* was lower than neomycin sulfate, but no evident difference was observed among the tested materials against *P. vulgaris*. *E. cloacae* and *P. aeruginosa* showed insensitive to neomycin and its transformation products at the test concentration (50 µg/ml). For plant pathogens, three transformation products and neomycin sulfate had the similar inhibitory effect against *P. solanacarum* at the test concentration, but compound Y and Z showed no inhibitory effect against *X. oryzae, X. axonopodis* and *E. carotovora*. The present work merely tested the anti-
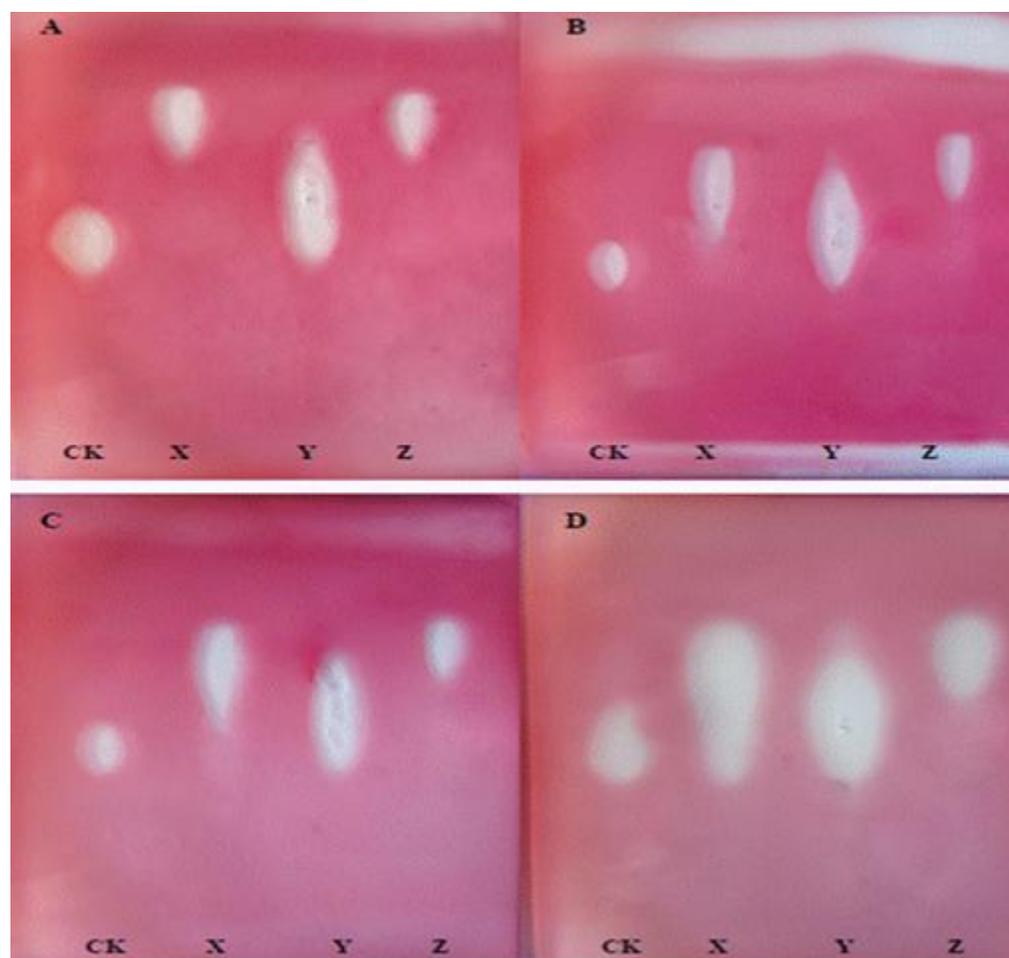
Figure 4. Bioautography of neomycin (CK) and transformation products (X, Y and Z) against *Staphylococcus aureus* (A); *Bacillus subtilis* (B); *Proteus vulgaris* (C) and *Pseudomonas solanacarum* (D).

Table 1. Antibacterial activity of neomycin and its biotransformation products by Oxford cup method.

Bacteria	Diameter of inhibitory zone (mm)			
	Neomycin sulfate [a]	Compound X [a]	Compound Y [a]	Compound Z [a]
Human pathogen				
Staphylococcus aureus	15.3 ± 0.6 a	15.0 ± 1.0 a	11.3 ± 0.6 b	8.8 ± 1.0 c
Bacillus subtilis	16.3 ± 1.2 a	17.3 ± 1.2 a	12.2 ± 0.8 b	10.3 ± 0.6 c
Escherichia coli	10.3 ± 0.6 a	9.2 ± 0.3 b	8.7 ± 0.6 b	7.3 ± 0.6 c
Proteus vulgaris	14.0 ± 1.0 a	14.8 ± 1.2 a	14.5 ± 0.9 a	14.5 ± 1.3 a
Enterobacter cloacae	- [b]	-	-	-
Pseudomonas aeruginosa	-	-	-	-
Plant pathogens				
Pseudomonas solanacarum	14.0 ± 1.0 a	15.2 ± 0.8 a	14.8 ± 1.2 a	14.5 ± 1.3 a
Xanthomonas oryzae	8.3 ± 0.6 a	8.7 ± 1.2 a	-	-
Xanthomonas axonopodis	9.3 ± 0.6 a	8.7 ± 0.6 a	-	-
Erwinia carotovora	12.2 ± 1.8 a	11.0 ± 1.3 a	-	-

The data was showed as mean ± SD with triplicate at each treatment. Values followed by the same letter in the same row were not significant according to the Least Significant Difference Test at 5% level. [a] The concentration of tested materials was 50 µg/ml and [b] No inhibitory zone.

Table 2. Minimal inhibitory concentration (MIC) of biotransformation products of neomycin by agar diffusion method.

Bacteria	MIC (µg/ml)			
	Neomycin sulfate	Compound X	Compound Y	Compound Z
Human pathogens				
Staphylococcus aureus	0.25	0.25	4	4
Bacillus subtilis	0.25	0.25	2	2
Escherichia coli	1	1	8	8
Proteus vulgaris	0.25	0.25	4	4
Enterobacter cloacae	> 16	> 16	> 16	> 16
Pseudomonas aeruginosa	> 16	> 16	> 16	> 16
Plant pathogens				
Pseudomonas solanacarum	2	2	16	16
Erwinia carotovora	4	4	> 16	> 16
Xanthomonas oryzae	> 16	> 16	> 16	> 16
Xanthomonas axonopodis	> 16	> 16	> 16	> 16

bacterial activity against 10 strains which was not sufficient for reflecting the biological activity of transformation products. Accordingly, more pathogen bacteria need to be detected further.

The difference of antibacterial activity between neomycin and the transformation products was detected further by MIC analysis. The results of Table 2 showed that the transformation product X still had the same antibacterial activity with neomycin, but the transformation product Y and Z were found a lower inhibitory effect than neomycin against all tested bacteria. Our previous study in pot experiment showed that the highest control efficacy of neomycin was observed at *X. axonopodis* (He et al., 2008). However, from *in vitro* antibacterial experiment in the present study, *P. solanacarum* was the most sensitive plant pathogen bacteria and *E. carotovora* was the next. This difference is most likely related to the absorption and translocation of neomycin in plant (Pramer, 1953) because of different application method in pot experiment. We found that the MIC of neomycin and transformation product X against *E. carotovora* was 4 µg/ml, which is in agreement with our previous study (Cui et al., 2009). From antibacterial activity analysis, it is suggested that the transformation product X deserves further research such as structure analysis through spectroscopy detection. More detailed analysis studies are underway.

ACKNOWLEDGEMENTS

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