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Full Length Research Paper

Antibacterial substance produced by *Streptomyces* sp. No. 87

Kanlayani Charoensopharat[1], Petcharat Thummabenjapone[2], Pisan Sirithorn[2] and Sompong Thammasirirak[1*]

[1]Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand.
[2]Department of Plant Pathology, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand.

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An antimicrobial substance produced by *Streptomyces* sp. No. 87 was partially purified and studied for its antibacterial characteristics using the swab paper disc technique. The cell-free culture filtrate showed antibacterial activity against several species of pathogens including Gram-positive bacteria, i.e. *Bacillus cereus* ATCC 11778, *B. subtilis* TISTR 008, *B. megaterium*, *Staphylococcus aureus, S. aureus* ATCC 25923, and *S. epidermidis* and Gram-negative bacteria, i.e *Klebsiella pneumoniae* ATCC 27736, *K. pneumoniae*, *Salmonella typhi* ATCC 5784, *Vibrio cholerae* and *Xanthomonas* sp. 60% ammonium sulfate precipitation of the culture supernatant shows markedly antibacterial activity against *B. cereus* ATCC 11778. Then supernatant was purified by gel filtration chromatography with sephadex G-25 resin. Five peaks namely P1, P2, P3, P4 and P5 were obtained. Results indicate that P3 is the only peak possessing the antibacterial activity, therefore, the final purification of P3 was conducted using FPLC with a superdex 30 column. Only one peak, namely P3-1 retained the antibacterial activity. P3-1 showed that its activity was insensitive to proteolytic enzymes such as trypsin, pepsin and proteinase K. In addition, the activity of P3-1 could be observed temperatures of 50 -121^{o}C and no protein or polypeptide band was seen when P3-1 was analyzed by SDS-PAGE. These results suggest that P3-1 might not be proteinacious in nature.

Key words: Antibacterial substance, characterization, purification, *Streptomyces*.

INTRODUCTION

Antibiotic resistant pathogens have been widely and continuously reported. In consequence, novel antibiotics have been investigated intensively. At present, 4,000 antibiotic substances obtained from bacteria and fungi have been applied in medicine, out of which about 75% are produced from Gram-positive actinomycetes bacteria such as *Streptomyces* sp. (Miyadoh, 1993). Various antimicrobial substances from *Streptomyces* sp. and actinomycetes bacteria have been isolated and characterized including aminoglycosides, anthracyclins, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyester, polyketides, actinomycins and tetracyclines (Goodfellow et al., 1988; Okami and Hotta, 1988; Baltz, 1998). Most of the antibiotics are extracellular-secondary metabolites which are normally secreted in culture media and serve as intermediates from primary metabolisms as precursors for their bio-synthetic process (Vilches et al., 1990) and they have been used as herbicides, anticancer agents, drugs, immunoregulators and antiparasitic agents (Thomson and Bialphos, 1955). For instance, the culture super-natant of *Streptomyces* sp. No. 87 previously isolated from agricultural soil from Sakonnakhon Province, Thailand, was partially purified and characterized. Its antimicrobial activity against the various plant pathogens has been investigated and presented. The results show that the active substance from *Streptomyces* sp. No. 87 is not proteinacious and greatly inhibits growth of the plant pathogens including Gram-negative bacteria. This compound might be useful for use as biocontrol in plants (Purichinawut et. al., 2004). Therefore, this study aimed to assess the antimicrobial capability of the active sub-

*Corresponding author. E-mail: somkly@kku.ac.th. Tel: +66-43-342911. Fax: +66-43-342911.

stance from *Streptomyces* sp. No. 87 against human pathogenic bacteria. In order to obtain the active antimicrobial substance from the cultured broth of bacteria, a combination of various purification steps such as chemical precipitation, ion-exchange chromatography and liquid chromatography techniques are generally performed (Mellouli et al., 2003).

In the present study, the culture filtrate of *Streptomyces* sp. No. 87 previously isolated from agricultural soil at Sakonnakhon Province, Thailand, was partially purified and characterized. Its antibacterial activity against the indicator pathogenic microorganisms was investigated and presented. This report shows broad spectrum antibiosis of *Streptomyces* sp. culture filtrate against several Gram-positive and Gram-negative bacteria. The fractions of partially purified culture filtrate by column chromatography were characterized for their antibiosis activity and some chemical properties. *Streptomyces* sp. has been previously reported against both plant pathogenic bacteria and fungi causal agents of economic diseases in Cucurbitaceae and Solanaceae crops (Thummabenjapone and Pachinburavan, 2002; Purichinawut and Thummabenjapone, 2004; Purichinawut et al., 2004; Thummabenjapone and Pachinburavan, 2005).

MATERIALS AND METHODS

Preparation of the tested microorganisms

The nineteen microorganism strains used as indicators for antibacterial activity consisted of 7 strains of Gram-positive bacteria, i.e. *Bacillus cereus* ATCC 11778, *B. subtilis* TISTR 008, *B. megaterium*, *S. aureus, S. aureus* ATCC 25923, *S. pneumoniae* DMS 5851 and *S. epidermidis*, and 11 strains of Gram-negative bacteria, i.e *Klebsiella pneumoniae* ATCC 27736, *K. pneumoniae*, *S. typhi* ATCC 5784, *V. cholerae*, *Xanthomonas, Eschorichia coli* O157:H7*, E. coli, Pseudomonas aeruginosa* ATCC 27853, *Salmonella paratyphi* B*, S. typhi* and *S. typhi* B. These microorganisms were cultured in BHI medium at 37^{o}C for 24 h before adding 30% glycerol and storing at -70^{o}C for further studies.

The *Streptomyces* sp. No. 87 used in this study, kindly provided by the Department of Plant Pathology, Faculty of Agriculture, Khon Kaen University, was grown in arginine glycerol mineral salt broth at room temperature (28-32^{o}C) for 7 days with shaking at 130 rpm before using in all the further experiments.

Streptomyces sp. No. 87 culture filtrate preparation

The culture filtrate was prepared by culturing *Streptomyces* sp. No. 87 in 10 L of arginine glycerol mineral salt broth for 7 days at room temperature (28-32^{o}C) with shaking at 130 rpm. The cultured broth was then centrifuged at 4^{o}C, 12,000 g for 10 min. The supernatant was filtrated through 0.45 ∝m filter membrane and concentrated by evaporator at 55^{o}C to obtain the final volume of evaporated culture filtrate of 100 ml prior to usage in further experiments.

Antimicrobial activity

Antibacterial activity was tested by the swab paper disc technique. A paper disc was impregnated with 30 ∝l of the concentrated evaporated culture filtrate and then placed on the surface of an agar plate that was previously swabbed with the particular bacterial strain to test. After 6 h of incubation at 37^{o}C, clear zones of inhibition appeared, and were measured for their diameter. The Streptomycin (Oxoid) and BHI (Himedia) broth were used as positive and negative controls, respectively. It was obvious that the inhibition zone against *B. cereus* ATCC 11778 was larger when compared to the other test strains, and therefore this strain was selected to use as test strain for further experiments.

Ammonium sulfate precipitation and gel filtration chromatography

The evaporated culture filtrate was precipitated by salting out with ammonium sulfate. Firstly, ammonium sulfate was added to 30% saturation and stirred overnight at 4^{o}C. The precipitate was centrifuged at 12,000 g at 4^{o}C and the pellet was re-suspended in distilled water. The supernatant was further purified by precipitation with 60% ammonium sulfate with overnight stirring at 4^{o}C. The precipitate was centrifuged at 12,000 g at 4^{o}C and the pellet was re-suspended in distilled water. The precipitate and supernatant parts obtained after precipitation with 30 and 60% ammonium sulfate, respectively, were tested for their antibacterial activity against *B. cereus* ATCC 11778. The fractions with highest antibacterial activity were pooled and further purified by gel filtration chromatography with a sephadex G-25 resin column. Gel filtration chromatography was performed by applying into the gel filtration column sephadex G-25 and eluted with 200 ml of 0.02 M pH 7.0 Tris-HCl buffer at a flow-rate of 0.5 ml/min. Each 2 ml fraction of eluate was pooled and tested for optical density at 220 nm. The pooled fractions from active peaks were checked for their antibacterial inhibitory activity.

Fast performance liquid chromatography (FPLC)

The antibacterial activity fractions from gel filtration chromatography were diluted with buffer A (25 mM pH 8.1 Tris-HCl buffer) (1:5, v/v). The diluent was filtrated through a 0.45 ∝m filter membrane and fractionated by FPLC (BioLogic Duo flow, Bio-rad, USA) with a superdex 30 column (4.6 x 250 mm). The pooled fractions which peaked at 280 nm were concentrated by evaporator at 55°C and then tested for inhibitory activity against *B. cereus* ATCC 11778 by the swab paper disc technique.

Effect of enzymes and heat treatment

The sensitivity of the active substance in the evaporated high peak from the FPLC fraction was investigated against proteolytic enzymes and heat treatment was used to characterize its proteinous nature. The pooled fraction was mixed with trypsin, pepsin and proteinase K (Sigma) at final concentrations of 0.5, 2 and 10 mg/ml, respectively, before incubating at 37^{o}C for 24 h. The sensitivity to heat was examined by boiling the supernatant to 50, 60, 70, 80 and 90^{o}C for 60 min and autoclaving at 121^{o}C for 30 min. Then the treated culture filtrates were tested against *B. cereus* ATCC 11778 to determine their antibacterial activity by the swab paper disc technique. After incubation for 8 h at 37^{o}C, the inhibition zone was measured and calculated.

RESULTS

Determination of antibacterial activity of *Streptomyces* sp. No. 87 culture filtrate against indicator microorganisms

An antibacterial substance produced by *Streptomyces* sp.

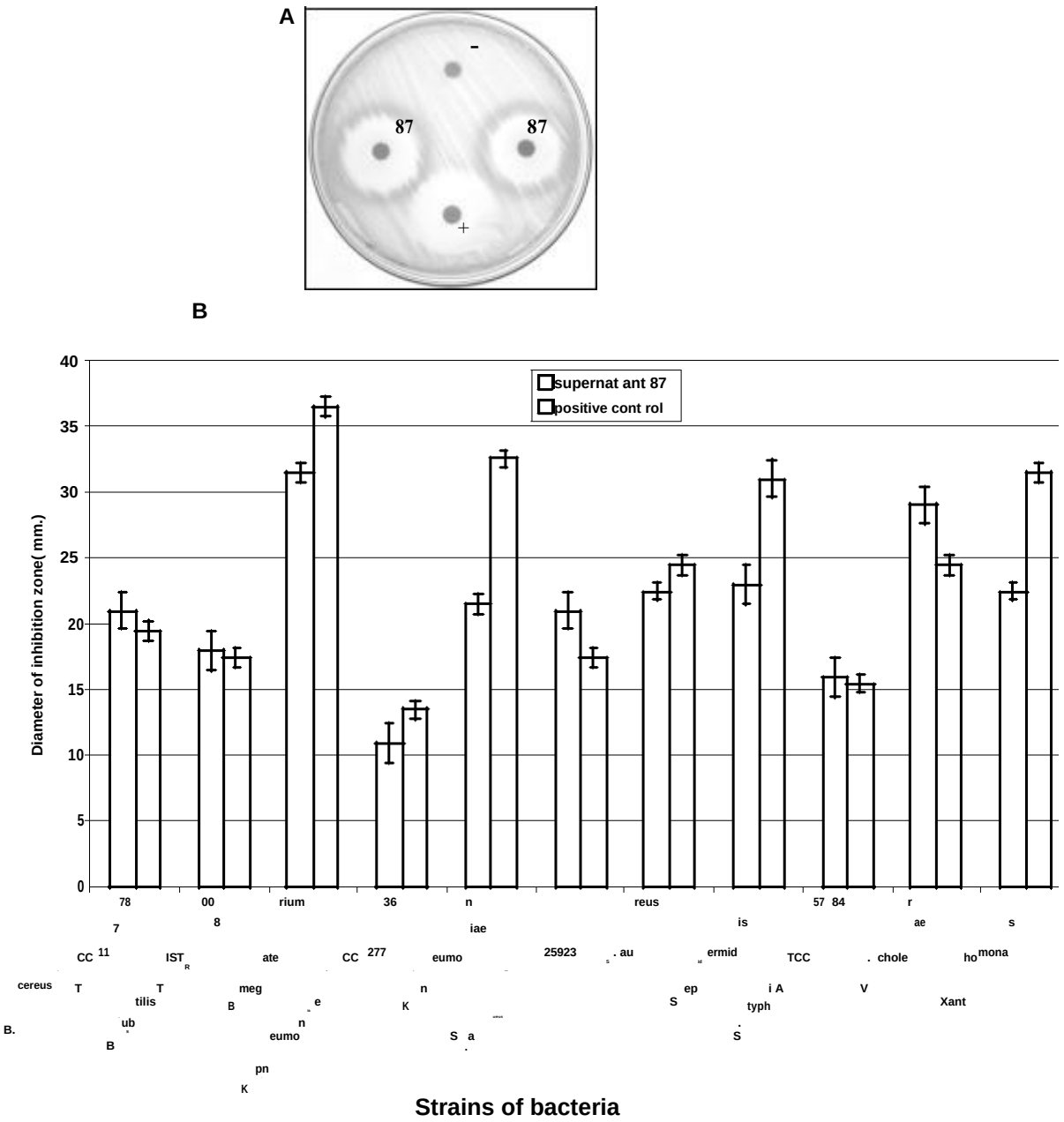

Figure 1. Antibacterial activity of evaporated culture filtrate from *Streptomyces* sp. No. 87 against human and plant pathogenic bacteria. (A) Inhibition zone on agar plate tested by swab paper disc technique, culture supernatant (87), positive control (0.428 mg/ml streptomycin) (+) and negative control (BHI broth) (-). (B) Comparison of inhibitory efficiency between *Streptomyces* sp. No. 87's culture filtrate and streptomycin as positive control.

No. 87 at protein concentration of 0.12 mg/ml (Bradford method) was studied for its antibacterial characteristic by the swab paper disc technique. Streptomycin at a concentration of 0.428 mg/ml and BHI broth were used as positive and negative controls, respectively. The antibacterial activity was observed as an inhibition zone against the test strains used in this study (Figures 1A and 1B). Results indicate that the cell-free culture filtrate has antibacterial activity against several species of pathogens including Gram-positive bacteria, i.e. *B. cereus* ATCC 11778, *B. subtilis* TISTR 008, *B. megaterium*, *S. aureus, S. aureus* ATCC 25923, and *S. epidermidis* and Gram-negative bacteria, i.e *K. pneumoniae* ATCC 27736, *K. pneumoniae*, *S. typhi* ATCC 5784, *V. cholerae* and *Xanthomonas* sp. However its activity was not evident against *E. coli* O157:H7*, E. coli, P. aeruginosa* ATCC 27853*, S. pneumoniae* DMS 5851*, S. paratyphi* B, *S. typhi* and *S. typhi* B. The inhibition zone against *B.*

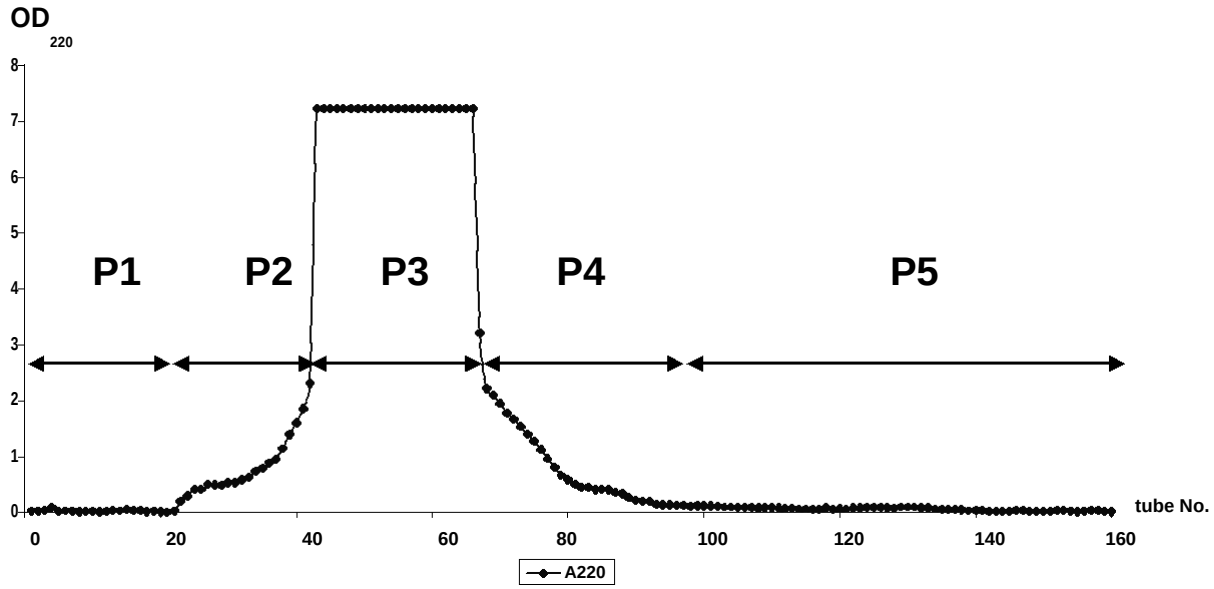

Figure 2. Elution profile of evaporated culture filtrate from *Streptomyces* sp. No. 87 after purification by gel filtration chromatography with a sephadex G-25 resin column at 220 nm. The chromatogram at the absorbance of 220 nm (A220) was obtained after purification by gel filtration chromatography with a sephadex G-25 resin column. Flow rate was 1.00 ml/min and 5 ml/fraction was used.

cereus ATCC 11778 was larger as compared to the other tested strains, and therefore this strain was selected to be used as a test strain for further experiments.

Ammonium sulfate precipitation and gel filtration chromatography

The culture filtrate of *Streptomyces* sp. No. 87 was first purified by precipitation with 30 and 60% ammonium sulfate. The precipitate and supernatant after precipitation were examined for their antibacterial capability characteristic against *B. cereus* ATCC 11778. Results reveal that both precipitate and supernatant parts of 30 and 60% ammonium sulfate exhibited an inhibition zone against *B. cereus*. The supernatant part of 60% ammonium sulfate showed the greatest antibacterial activity. This part was then purified by gel filtration chromatography with a sephadex G-25 resin column. The absorbance at 220 nm was measured. Five peaks, P1, P2, P3, P4 and P5 were obtained (Figure 2). The antimicrobial activity of the pooled fraction of each peak was tested against *B. cereus*. Results indicate that P3 was the only peak possessing antibacterial activity with the protein concentration of 0.09 mg/ml determined by using the Bradford method.

Fast performance liquid chromatography (FPLC)

The final purification step of P3 was conducted using fast performance liquid chromatography (FPLC) with a superdex 30 column. Only one peak, P3-1, observed at absorbance of 280 nm was obtained from FPLC as a profile indicates in Figure 3. The pooled fraction of P3-1 containing protein at concentration of 0.07 mg/ml had antibacterial activity against *B. cereus*.

Enzymatic effect and heat treatment

The effect of various proteolytic enzymes, i.e. trypsin, pepsin and proteinase K, on the inhibitory substance in P3-1on target bacteria was investigated. Results reveal that its activity was insensitive to all tested proteolytic enzymes. An inhibition zone against *B. cereus* was noted after treatment for 8 h at 37^{O}C (Figure 4). The antibacterial activity of P3-1 against *B. cereus* was also tested after treating at 50, 60, 70, 80 and 90^{O}C for 60 min and 121^{O}C for 30 min. It is found that the activity of P3-1 was retained at around 95% for all treated temperature ranges (Figure 5). In addition, no protein or polypeptide band was seen when P3-1 was analyzed by SDS-PAGE technique. It is concluded that P3-1 might not be proteinacious in nature.

DISCUSSION

Our results coincided with the findings of Singh and Gurusiddaiah (1984) in which growth of *B. subtilis,* and *S. aureus* could be inhibited by a substance produced from *S. lydicus.* Several species of *Streptomyces* from different soil and water samples are a virtually unlimited

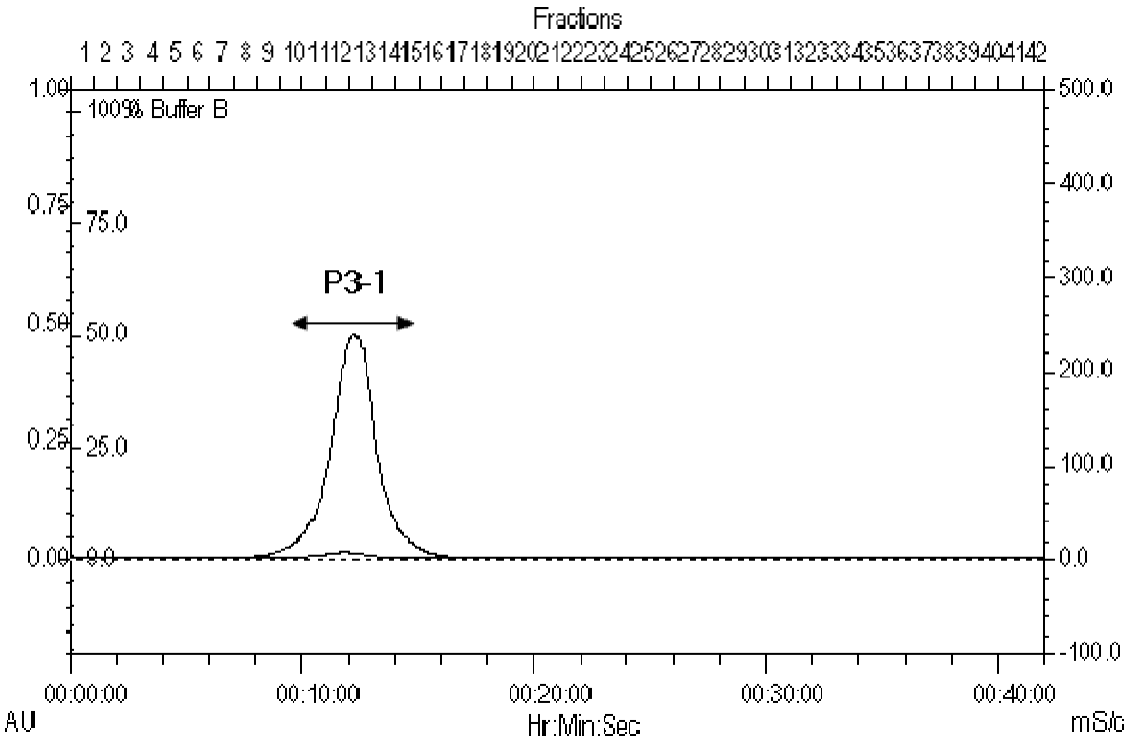

Figure 3. The elution profile of P3-1 from evaporated culture filtrate *Streptomyces* sp. No. 87 after purification through fast performance liquid chromatography (FPLC) with a superdex 30 column. The antimicrobial activity peaks from gel filtration chromatography were diluted with buffer A (25 mM pH 8.1 Tris-HCl buffer) (1:5, v/v). The diluent was filtrated through a 0.45 µm filter membrane and fractionated by FPLC with a sephadex 30 column. The pooled fractions which peaked at 280 nm were concentrated by evaporator.

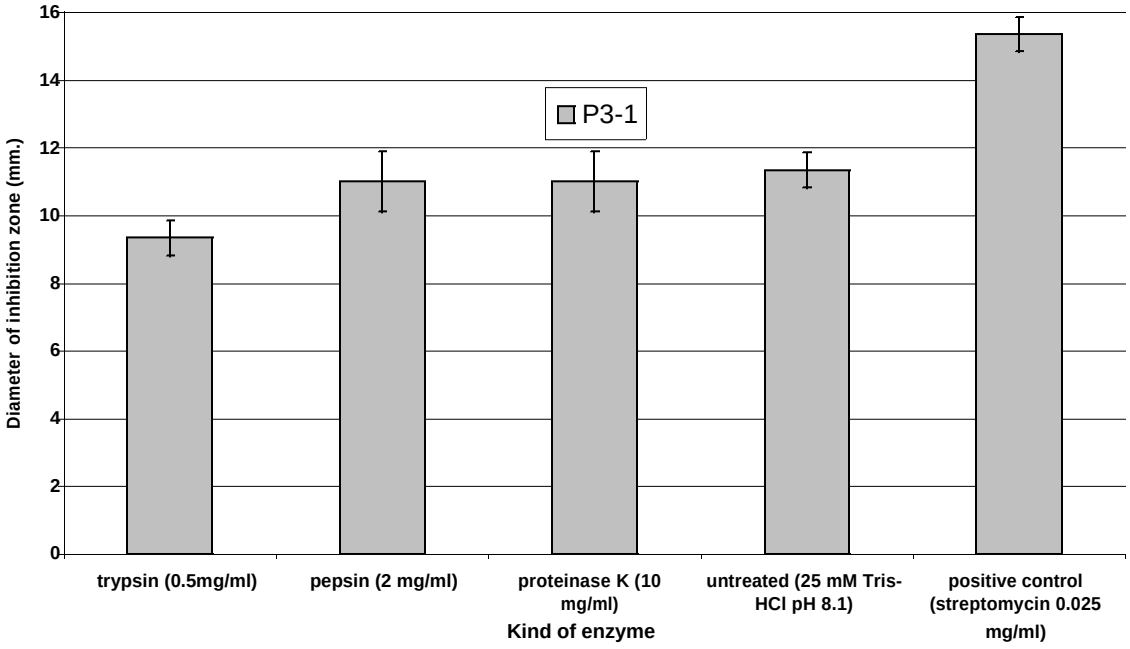

Figure 4. Inhibition zone against *B. cereus* ATCC 11778 of fraction P3-1 after treatment with various enzymes compared with commercial chemicals. The enzymes consisted of 0.025 mg/ml trypsin, 2 mg/ml pepsin, 10 mg/ml proteinase K, 0.025 mg/ml streptomycin, 25 mM pH 8.1 Tris-HCl buffer and untreated. The plates were cultivated on BHI agar at 37°C for 8 h. The clear zones of inhibition were measured for their diameter. Values represent the mean ± SD for three replications.

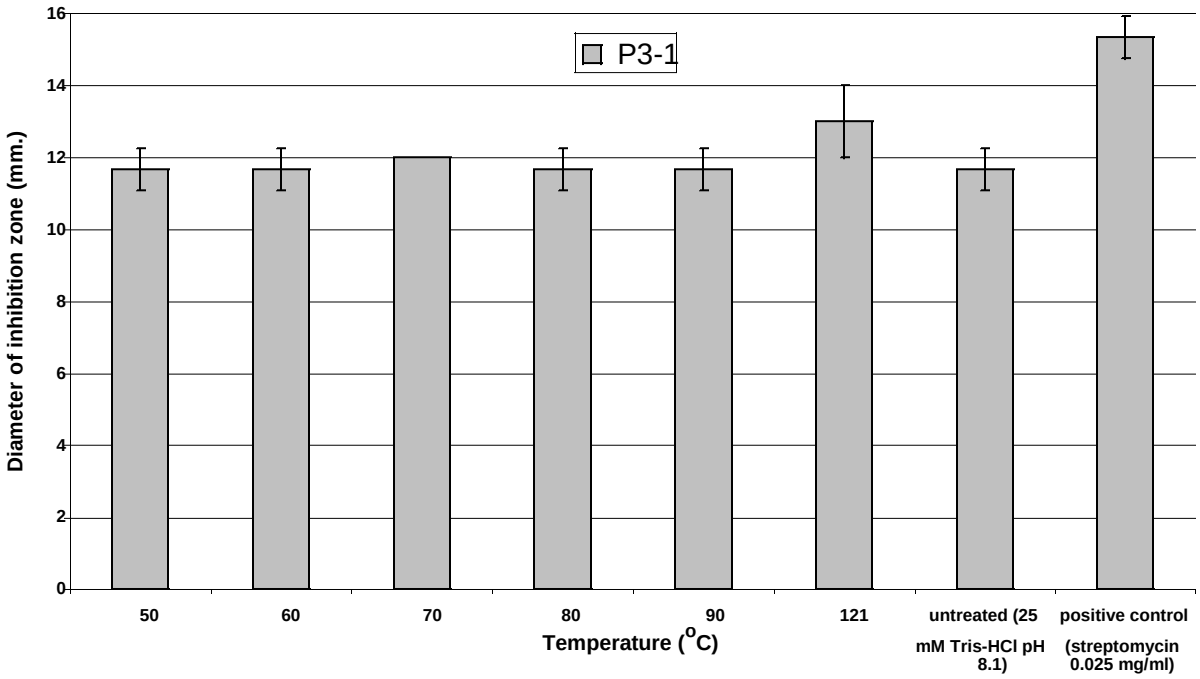

Figure 5. Inhibition zone against *B. cereus* ATCC 11778 of P3-1 after treatment with high temperatures compared with untreated protein. The antimicrobial activity of P3-1 against *B. cereus* ATCC 11778 was tested after treating P1 at high temperatures of 50, 60, 70, 80 and 90^{o}C for 60 min and 121^{o}C for 30 min. The clear zones of inhibition were measured for their diameter. Values represent the mean ± SD for three replicates.

source of natural secondary metabolites, many kinds of which are used as pharmaceutical and agrochemical products (El-Naggar et al., 2003; Pamboukain and Facciotti, 2004; Ben-Fguira et al., 2005) and they have a wide variety of chemical structures, including tetracyclines, macrolides, quinocyclines and meroparamycin. These antibiotics show antibacterial activity against Gram-positive bacteria and Gram-negative bacteria (Stryzhkova et al., 2002; White et al., 2001; Furumai et al., 2002; El-Nagger et al., 2006). In contrast however to this study, *E. coli* ATCC 8739 could be inhibited by the active substance produced from *Streptomyces* sp. (Mellouli et al., 2004; Singh and Gurusiddaiah, 1984). Due to their structure or active sites, protein substances are normally destroyed at high temperature. This finding implies that P3-1 might not be proteinacious in nature. Other enzyme tests against antimicrobial activity of P3-1 such as amylases, neuraminidases and lipases should be conducted in order to investigate its characteristic. In addition, the antibiotics from *S. lydicus* were seen to inhibit the growth of *P. aeruginosa* (Singh and Gurusiddaiah, 1984).

Conclusion

The antimicrobial substance produced by *Streptomyces* sp. No. 87 was partial purified and its antibacterial characteristics were investigated in this study. The crude culture supernatant showed antibacterial activity against several species of human pathogens including both Gram-positive and Gram-negative bacteria. These findings indicated that our produced substance might be the alternative antimicrobial substance as a tool for controlling human diseases. The activity of the inhibitory substance in partially purified supernatant P3-1 was insensitive to proteolytic enzymes and stable at high temperature. In addition, no protein or proteolytic band was observed when it was analyzed by SDS-PAGE technique. These results indicate that the antimicrobial substance produced by *Streptomyces* sp. No. 87 might not be proteinacious in nature.

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