

Assessment of antigen preparation methods for polyclonal antibody production against *Streptomyces* spp.

Kusavadee Sangdee^{1*}, Petcharat Thummabenjapone², Aphidech Sangdee³

¹ Preclinical Division, Faculty of Medicine, Mahasarakham University, Thailand 44150

² Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University, Thailand 40002

³ Department of Biology, Faculty of Science, Mahasarakham University, Thailand 44150

ABSTRACT

Aims: To determine the optimum antigen preparation method for producing specific polyclonal antibody specific for *Streptomyces* species.

Study design: Cross-sectional study.

Place and Duration of Study: Preclinical Division, Faculty of Medicine, Mahasarakham University, Mahasarakham Province between May 2011 and December 2011.

Methodology: Two *Streptomyces* isolates were used for antisera production. The sonication method was chosen for antigen preparation. Antigen suspensions were emulsified with incomplete Freund's adjuvant and 1 ml was injected into rabbit thigh muscle for the first, second and third immunization. The fourth and fifth immunizations were injected intravenously. Antibody titer and specificity were measured using indirect-ELISA. *Streptomyces* antigen mixed with soil was investigated.

Results: The 5 min sonication method gave a higher resolution protein profile than other test methods, so this protocol was chosen for all subsequent work. The sonicated *Streptomyces* antigen was used for polyclonal antibody production. At week 7, the rabbit anti-*Streptomyces* sp. isolate STPR78 and anti-*Streptomyces* sp. isolate STPR84 gave maximum titers at 1:64000 and 1:32000, respectively. The sensitivity detection limit of both antibodies was in the range 2.5 to 0.3125 µg. Anti-*Streptomyces* sp. isolate STPR78 and anti-*Streptomyces* sp. isolate STPR84 were also found to have good specificity. Minimal cross-reactivity was detected with antigens from other test bacteria. Regrettably however, neither antibody was capable of detecting *Streptomyces* spp. STPR78 or STPR84 in inoculated soil.

Conclusion: A specific and high titer of polyclonal antibody was produced using sonicated antigen.

Keywords: polyclonal antibody, *Streptomyces* spp., antigen preparation, sonication method

1. INTRODUCTION

Streptomyces is a genus of Gram positive bacteria found predominantly in soil and decaying vegetation. These bacteria produce many clinically useful antibiotics (Atta et al., 2009;

* Tel.: +66 43 754121; fax: +66 43 754121.

E-mail address: kusavadee@gmail.com.

Deboer and Dietz, 1976; Chater, 2006) and enzymes used in industry (Chellapandi and Jani, 2008; Jayasree et al., 2009; Vishnupriya et al., 2010). *Streptomyces* species are also important in controlling soil-borne pathogens by antibiosis (Shimizu et al., 2009; Rothrock and Gottlieb, 1981; Yuan and Crawford, 1995). Detection, identification and monitoring of these organisms in their natural habitats, which contain many other soil microorganisms, requires multiple time-consuming and labor-intensive procedures.

An alternative strategy for detection would be to use immunological methods. Immunodiffusion, indirect-ELISA, immunofluorescence, and the immunomagnetic capture technique have all successfully been used for the identification, isolation or tracking of specific microorganisms in the environment (Cross and Spooner, 1963; Kirby and Rybicki, 1986; Schlöter et al., 1995). Cross and Spooner (1963) used a serological technique to identify and compare antibiotic producing isolates of *Streptomyces*. Kirby (1986) used indirect-ELISA to determine the relationships between *Streptomyces* spp. Wipat et al. (1994) produced monoclonal antibody against *Streptomyces lividans* spore surface antigens and employed this monoclonal antibody for isolating *Streptomyces* from soil using the immunocapture technique. Also, Bartosch et al. (2002) used a monoclonal antibody specific to a nitrite oxidation enzyme for the detection of *Nitrospira*-like bacteria in various soils. In the present study, we sought to determine the optimum antigen preparation method for producing polyclonal antibody specific for *Streptomyces* spp. and apply these antibodies to monitor the bacteria in soil using indirect-ELISA.

2. MATERIAL AND METHODS

2.1 Test strains

Streptomyces sp. isolate STPR78 and *Streptomyces* sp. isolate STPR84 were used for antisera production. Twenty two isolates of unknown *Streptomyces* spp., *Pseudomonas aeruginosa* PDMZnCd 2003, *Pseudomonas aeruginosa* (isolated from environment) *Bacillus cereus* ATCC11778 and a *Enterobacter* sp. (isolated from papaya wilt) were used for testing antibody specificity.

2.2 *Streptomyces* protein preparation

Streptomyces sp. isolate STPR78 and *Streptomyces* sp. isolate STPR84 were inoculated into 100 ml arginine glycerol mineral salt broth and incubated for 3-5 days at 30°C on a rotary shaker at 150 rpm. The cells were harvested by centrifugation at 10,000 rpm for 15 min. The pellet was washed three times with phosphate-buffer saline (PBS; pH 7.4). Three antigen preparation methods, boiling at 100°C for 5 and 10 min, autoclaving at 121°C for 5 and 10 min, and sonication for 1 and 5 min using a High Intensity Ultrasonic Processor (Model VCX 750, USA), were used to prepare *Streptomyces* protein antigen. The protein solutions were adjusted to equal concentrations before measuring on 12% SDS-PAGE.

2.3 Antigen preparation for immunization

The 5 min sonication method was chosen for antigen preparation. The *Streptomyces* pellets were resuspended in PBS (pH 7.4), followed by sonication with a microprobe. This was done on ice for a total of 5 min in 10s bursts with 2s gaps for cooling. The sonicated solutions were centrifuged at 10,000 rpm for 5 min and filtered through a 0.2 µm filter. Protein in the sample was measured using a Micro-Volume UV-Vis Spectrophotometer (Nanodrop 2000, USA) at optical density 280 nm. The samples were diluted to 0.5 mg/ml with PBS for antibody production.

2.4 Polyclonal Antibody Production

Antigen suspension (0.5 mg/ml) was emulsified as a 1:1 (v/v) mixture with incomplete Freund's adjuvant (Difco Laboratories, USA), and 1 ml was injected into the thigh muscle of a New Zealand white rabbit on days 0, 7 and 14 respectively. Rabbits were injected intravenously at the marginal ear with 1 ml of antigen suspension (0.5 mg/ml) on days 21 and 28. Blood (10 ml) was taken from the central ear vein one week before immunization and weekly intervals after the fourth immunization. Blood continued to be collected until 5 weeks after the last immunization. Animal experimental procedures were performed with approval of the Animal Ethics Research Committee of Mahasarakham University (MSU-0013/2554).

2.5 Measurement of rabbit anti-*Streptomyces* titer using Indirect-ELISA

Polystyrene microtitre plates (SPL life sciences, Korea) were coated with twofold dilutions of the antigen samples in coating buffer for 16-18 h, the dilution ranging from 0 to 5 µg. The plates were washed three times using 0.05% (v/v) Tween 20 in PBS. Nonspecific binding was blocked using 3% (w/v) bovine serum albumin in PBS (BSA-PBS) for 2 h at 37°C. After washing, twofold dilutions of test serum in BSA-PBS (ranging from 1:1000 to 1:64000) were added in duplicate and incubated for 1 h at 37°C. Following washing, goat anti-rabbit IgG antibody conjugated with alkaline phosphatase diluted in BSA-PBS (dilution 1: 20000) was added and incubated for 1 h at 37°C. The substrate *p*-nitrophenyl phosphate (1mg/ml) in 10% (w/v) diethanolamine, pH 9.8 was added and incubated at room temperature for 45 min to 1 h. The reaction was stopped using 50 µl 3M KOH. The *p*-nitrophenol released was measured by optical density at 405 nm using an ELISA reader (Opsys MRTM Microplate Reader, USA).

2.6 Antibody specificity test

Antibody specificity was evaluated by testing the 22 isolates of *Streptomyces* spp., *Pseudomonas aeruginosa* PDMZnCd 2003, *Pseudomonas aeruginosa*, *Bacillus cereus* ATCC11778 and the *Enterobacter* sp. *Streptomyces* spp. were cultured on half PDA at 30°C for 5-7 days, while the other test species of bacteria were grown on nutrient agar at 37°C for 24 h. One loopful of test bacteria was mixed with 500 µl of coating buffer, and then sonicated using a High Intensity Ultrasonic Processor (Model VCX 750, USA) fitted with a microprobe. This was done on ice for a total of 5 min in 10s bursts with 2s gaps for cooling. The sonicated solution was centrifuged at 10,000 rpm for 5 min. The supernatant was used as protein antigen for indirect-ELISA detection.

2.7 Detection of *Streptomyces* antigen in soil by Indirect-ELISA

Antigen of *Streptomyces* sp. isolate STPR 78 and *Streptomyces* sp. isolate STPR 84 were each mixed with a 1:1 (v/v) mixture of soil suspension in coating buffer. These were then serially diluted from 25 to 3.125 µg using twofold dilution intervals. Indirect-ELISA was performed as described above.

3. RESULTS AND DISCUSSION

3.1 *Streptomyces* protein preparation

Three *Streptomyces* protein preparation methods were tested to determine the optimum procedure for antibody production. Protein profiles were observed using protein generated from the autoclaving and sonication method, but not the boiling method. Moreover, the 5 min sonication method gave a higher resolution protein profile than the 1 min sonication method, or either of the autoclaving methods (Fig. 1). Therefore, the 5 min sonication method was chosen for *Streptomyces* antigen preparation. This technique resembles one previously reported. Kirby and Rybicky (1986) successfully produced specific polyclonal antibodies from sonicated antigen of *Streptomyces*. Moreover, this sonication method is widely used to prepare antigen for antibody production from other microorganisms such as *Mycobacterium bovis* (Wood et al., 1988), *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* (Hampl et al., 1981). Also, the sonication method is good for antigen preparation in diagnosis. Mallqui et al. (2000) demonstrated that antibody against *Bartonella bacilliformis* gave higher specific reactivity to sonicated antigen than antigen preparation by the glycine extraction method.

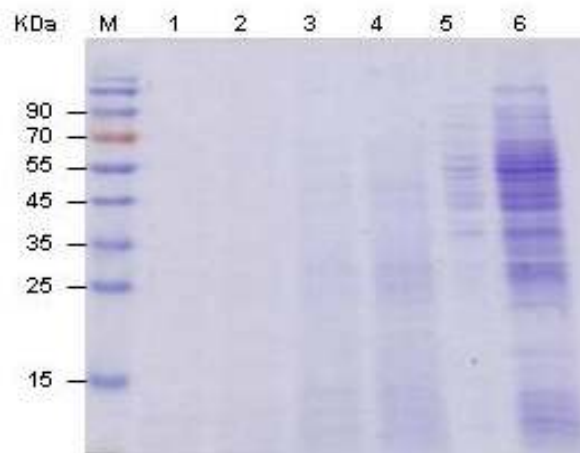


Fig. 1. Antigen protein profile for three antigen preparation methods as determined by 12% polyacrylamide gel electrophoresis; broad range protein marker (M), antigen boiled for 5 min (1) and 10 min (2), antigen autoclaved for 5 min (3) and 10 min (4), and antigen sonicated for 1 min (5) and 5 min (6)

3.2 Measurement of rabbit anti-*Streptomyces* titer using Indirect-ELISA

Rabbit anti-*Streptomyces* sp. isolate STPR78 and anti-*Streptomyces* sp. isolate STPR84 titers were measured by reaction with whole cell sonicated proteins of *Streptomyces* spp. antigen at antigen concentrations ranging from 5 to 0 µg. Antibody titers sharply increased (week 5 and 6), both types giving a maximum titer at week 7. The highest titer of anti-*Streptomyces* sp. isolate STPR78 was 1:64000, while the highest titer of anti-*Streptomyces* sp. isolate STPR84 was 1:32000. Antibody titer dropped rapidly 3-5 weeks following the last immunization. Sensitivity detection limit, which in general agrees with antibody titer, was 1.25 to 0.3125 µg and 2.5 to 0.3125 µg for anti-*Streptomyces* sp. isolate STPR78 and anti-*Streptomyces* sp. isolate STPR84, respectively. The highest sensitivity detection limit occurred in week 7 (Table 1).

Table 1. Antibody titer of anti-*Streptomyces* sp. isolate STPR78 and STPR84 after the fourth immunization

	Antigen concentration (μg)				
	5	2.5	1.25	0.625	0.3125
Anti-<i>Streptomyces</i> sp. isolate STPR78					
Serum 1 (Week 5)	8,000	4,000	1,000	<1,000	<1,000
Serum 2 (Week 6)	16,000	8,000	4,000	2,000	<1,000
Serum 3 (Week 7)	64,000	32,000	16,000	8,000	4,000
Serum 4 (Week 8)	16,000	8,000	4,000	2,000	<1,000
Serum 5 (Week 9)	ND*	4,000	2,000	1,000	<1,000
Serum 6 (Week 10)	ND*	4,000	2,000	1,000	<1,000
Anti-<i>Streptomyces</i> sp. isolate STPR84					
Serum 1 (Week 5)	8,000	2,000	<1,000	<1,000	<1,000
Serum 2 (Week 6)	16,000	8,000	2,000	1,000	<1,000
Serum 3 (Week 7)	32,000	16,000	8,000	4,000	1,000
Serum 4 (Week 8)	16,000	8,000	4,000	2,000	1,000
Serum 5 (Week 9)	ND*	4,000	1,000	<1,000	<1,000
Serum 6 (Week 10)	ND*	4,000	1,000	<1,000	<1,000

* ND = Not determined

3.3 Antibody specificity test

Anti-*Streptomyces* sp. isolate STPR78 and anti-*Streptomyces* sp. isolate STPR84 both showed good specificity. No cross-reactivity was detected with *Pseudomonas aeruginosa* PDMZnCd 2003, *Pseudomonas aeruginosa*, *Bacillus cereus* ATCC11778, *Enterobacter* sp. or 17 of the 22 *Streptomyces* spp. In the case of anti-*Streptomyces* sp. isolate STPR78, this did cross-react with the *Streptomyces* isolates SRF-3, SRF-4, SRF-5, SRF-12 and SRF-13. A similar result was obtained for anti-*Streptomyces* sp. isolate STPR84, which cross-reacted with the *Streptomyces* isolates SRF-3, SRF-4, SRF-12 and SRF-13 (Fig. 2). This cross-

reaction suggests the environmental *Streptomyces* isolates are closely related to the *Streptomyces* strains used for immunization. This problem could potentially be resolved by using isolated cell wall antigens, since different *Streptomyces* strains have different cell wall compositions (Cummins, 1962). Whole cell sonicates, by contrast, contain identical cytoplasmic antigen (Kwapinski, 1963).

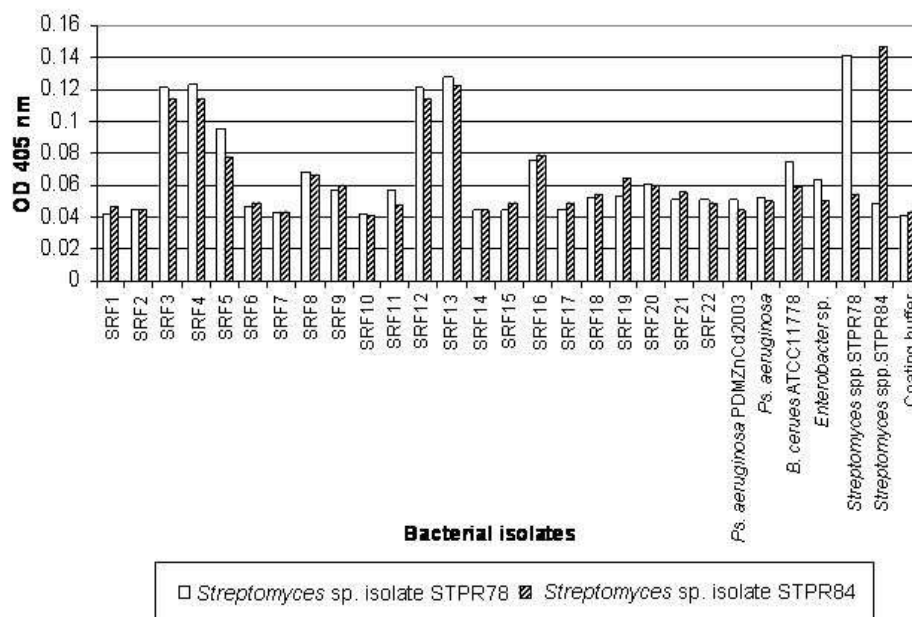


Fig. 2. Antibody specificity of anti-*Streptomyces* sp. isolate STPR78 and anti-*Streptomyces* sp. isolate STPR84 as determined by Indirect-ELISA.

3.4 Detection of *Streptomyces* antigen in soil by Indirect-ELISA

Tests were performed to assess the feasibility of using the rabbit antibodies for monitoring *Streptomyces* spp. from the soil by indirect-ELISA. Whole cell sonicated protein antigens were mixed with soil suspension before coating on microtiter plates. None of the antigen concentrations were detected by either of the rabbit antibodies. Antigen at a concentration of 5 μ g was used as a positive control. One possible explanation for this result is the presence of inhibitors in the soil. Alternatively, it may be that the antigen was trapped by competitive molecules. Our results resemble those of Wipat et al. (1994), who found that naturally occurring *Streptomyces* reduce the sensitivity of monoclonal antibody specific for *Streptomyces lividans*. To increase the sensitivity of this detection technique, enrichment of

the microorganism may be required. For example, Bartosch et al. (2002) successfully detected *Nitrospira*-like bacteria in soil samples through enrichment of the bacterial population using an immunoblot technique.

4. CONCLUSION

The present study describes a means of generating *Streptomyces* antigen that elicits high titers of specific polyclonal antibody. Our method involves sonicating bacterial cells in 10s bursts (with 2s gaps for cooling) for a total of 5 min. This method is more rapid than others currently available. Whilst the antibodies are not suitable for soil microbiology, they could be used with serological techniques such as double immunodiffusion and ELISA for other purposes.

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