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Research Paper

Colicin type 7 produced by majority of *Shigella sonnei* isolated from Thai patients with diarrhoea

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Abstract

Thirty one out of 153 strains of *Shigella sonnei* isolated from Thai patients with diarrhoea showed antibacterial activity against *S. sonnei* by agar well diffusion method. All of them harbor plasmids with the genetic determination of *colicin type 7* (Js) gene but without *colicin E* and *colicin U* gene. The PCR product obtained from strain 35/44 was shown to be the gene for colicin type 7 lytic protein (*cja*). The partially purified bacteriocin (PPB) containing colicin type 7 of strain 35/44 was prepared and used for characterization. The antibacterial activity of PPB against a total of 17 selected Gram-positive and Gram-negative bacteria was tested. It was found that PPB of strain 35/44 was active against *E. coli* O157, *S. sonnei* and *S. boydii*. The sensitivity of PPB from this strain to proteinase K, trypsin and α-chymotrypsin suggests the proteinaceous nature of these antimicrobial substances. Therefore, this isolated bacterium can be regarded as bacteriocin producing bacteria. The bacteriocin produced by this isolated *S. sonnei* was heat stable as evidenced by its ability to maintain the activity at 80 °C for 60 min. In addition, it was stable within a wide range of pH (3-9). The molecular weight of colicin type 7 from isolated *S. sonnei* strain 35/44 analyzed by SDS-PAGE was 54.4 kDa composing of at least five subunits. It is to our knowledge; the first report of Thai patients with diarrhoea that *S. sonnei* isolated from them contained colicin type 7.

Key words: *Shigella sonnei*, colicin type 7, diarrhoea.

Introduction

Many Gram-negative bacteria such as *Shigella*, *E. coli*, *Salmonella* and *Vibrio* are common causes of foodborne diseases. *Shigella* is one of the most common bacterial agents of acute diarrhoea spread through the faecal-oral route, and as few as 100 bacterial cells can be enough to cause an infection. It has been estimated that nearly 165 million of *Shigella* cases occurred throughout the world with 1.1 million deaths mainly in developing countries (Kotloff *et al.*, 1999). The major cause could be the contamination of *Shigella* in fresh and processed food. The development of natural food preservatives to be used instead of chemical preservatives in food products has dramatically increased in recent years. One way is the utilization of bacteriocins produced by bacteria.

Bacteriocins are ribosomally synthesized antimicrobial proteinaceous compounds that are produced by bacteria that usually inhibit closely related species (Klaenhammer 1988). The most extensively studied Gram-negative bacteriocins are colicins from *E. coli* which also serve as a model for ecology, evolution, function, structure and genetic organization of bacteriocin from Gram-negative bacteria nowadays (Cascales *et al.*, 2007). Colicins are high molecular weight toxic proteins that are produced by colicinogenic strains of *E. coli* and some related species of the family Enterobacteriaceae (Smajs and Weinstock 2001). They are usually encoded by colicinogenic plasmids and

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kill other cells by a variety of mechanisms such as membrane permeabilization, nucleic acid degradation or protein synthesis inhibition (Padilla *et al.*, 2006). There are at least 23 colicin types have been described in detail (Smajs and Weinstock 2001). It has been reported that *S. sonnei*, *S. flexneri*, and *S. boydii* produce colicin (Horak 1994; Padilla *et al.*, 2006; Smajs and Weinstock 2001; Smajs *et al.*, 1997; Smarda *et al.*, 1987; Sousa *et al.*, 2010); however, there are only a few reports on the characterization of bacteriocin from *S. sonnei* (Smarda *et al.*, 1987; Sousa *et al.*, 2010; Tigyi 2005). The aim of this study was to determine the colicin production by *S. sonnei* strain isolated from Thai patients with diarrhoea and to characterize its properties.

Materials and Methods

Bacterial strains and culture conditions

One hundred and fifty three *S. sonnei* strains were isolated from patients with shigellosis in the Kaeng-Khoi district of Saraburi province during May 2000 to April 2003 (Na-ubol *et al.*, 2006). Bacteriocin activity was screened for antibacterial activity against the indicator strain, *S. sonnei*. It was grown in Tryptic soy broth (TSB) at 37 °C under shaking (200 rpm). Agar was added when needed.

Antibacterial activity assays

The antibacterial activity of 153 *S. sonnei* isolated strains was determined by agar well diffusion method (Millette *et al.*, 2007). A 5 mL nutrient broth was inoculated with single colony of an overnight culture of each isolate and incubated at 37 °C for 16-18 h with shaking (200 rpm). Fifty μL of cell-free culture supernatant (CFS) obtained through centrifugation at 8,000 x g for 20 min (Sorvall Biofuge, Mandel Scientific, Canada) and sterile by filtration was dropped in a soft agar (1.2% agar) inoculated with the cell suspension of the indicator strain at a final concentration of ca. 10^5 cfu/mL. The plates were then incubated at 37 °C for 16-18 h and the appearance of clear zones showing the antagonistic activity was observed. The assay for each sample was done in triplicate.

Isolation, purification and analysis of plasmid DNA

Plasmid DNA from thirty one bacteriocinogenic *S. sonnei* was isolated using GeneJET Plasmid Miniprep Kit (Fermentas). DNA was visualized following electrophoresis in 0.7% (w/v) agarose gel in TBE buffer by staining with ethidium bromide (0.5 μg/mL).

PCR Amplification of colicin gene

Detection of colicin structural genes (*colicin 7*, *colicin E1* and *colicin U*) was performed by PCR. The plasmid DNA from forty bacteriocinogenic *S. sonnei* was used as a template. Gene specific primer sequences were designed based on the published *colicin* gene sequences (Higashi *et al.*, 1986; Smajs and Weinstock 2001; Smajs *et al.*, 1997). The primer sequences for amplification of *colicin 7* gene were, forward: 5'-TCT CAA AAT GTT TGG GCT CC-3', and reverse: 5'-CCC TGT CCC ACT GAC ACT TT-3'. The primer sequences for amplification of *colicin E1* were, forward: 5'-GGC GGT GGT GGT GGA ACT GG-3', and reverse: 5'-ACA GCC CGG GCC TCT TCA CT-3'. The primer sequences for amplification of *colicin U* were, forward: 5'-ATG CGC TGC AGG CAC AGG TT-3', and reverse: 5'-GCA TCA GCG GCC CCC AGT TT-3'. Each PCR reaction was performed in a 50 μL reaction volume containing 0.2 mM dNTPs (dATP, dGTP, dCTP and dTTP), 20 μM of individual primers, 50 ng of DNA template, 5μL of 10 X PCR buffer and 0.5 U of *Taq* polymerase enzyme. The final volume was adjusted by adding sterilized distilled water to 50 μL. The PCR condition was as follow; initial denaturation at 94 °C for 2 min; followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and polymerization at 72 °C for 1 min; and a final extension at 72 °C for 5 min. PCR products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide (0.5 μg/mL). The expected PCR product size of colicin 7, *colicin E1* and *colicin U* gene are 249, 467 and 570 bp, respectively. The nucleotide sequence of PCR products were sequenced and analyzed with GenBank nucleotide database using Blastn search (http://www.ncbi.nlm.nih.gov/blastn).

Preparation of partially purified bacteriocin (PPB)

One of the isolated *S. sonnei* strains 35/44 was selected for further study. Two hundred mL TSB medium was inoculated with 1% (10^6 cfu/mL) of an overnight culture of each isolate and incubated at 37 °C for 16-18 h with shaking. Following cultivation, cell-free culture supernatant was obtained through centrifugation at 8,000 x g for 20 min (Sorvall Biofuge, Mandel Scientific, Canada) followed by sterile filtration. Ammonium sulfate (103.2 g) was added to the supernatant while stirring to reach 80% saturation and left overnight at 4 °C. The sample was centrifuged at 8000 x g for 50 min. Then the supernatant was discarded and the precipitate was dissolved in 5 mL of sterile distilled water and dialyzed against 1.5 L of sterile distilled water for 16-18 h. The active supernatant was designated as partially purified bacteriocin or PPB.

Spectrum of inhibitory activity

PPB was used to assess the antibacterial activity against a total of 17 selected Gram-positive and Gram-negative test bacteria (Table 1) by the agar-well diffusion method (Millette *et al.*, 2007). Equal volume of sterile distilled water was used as control solution. The appearance of the inhibition zone was determined after 18 h of incubation.

Enzyme sensitivity, heat and pH stability

The PPB was treated at 37 °C for 1 h with 1 mg/mL final concentration of the following enzymes: trypsin, α-chymotrypsin and proteinase K (Sigma-Aldrich, USA). After incubation, the reaction mixtures were boiled for 10 min to inactivate the enzymes and the residual antibacterial activity was measured by agar-well diffusion (Millette *et al.*, 2007). Thermal stability of bacteriocin was investigated by determination of the residual antibacterial activity after incubation of PPB at different temperature ranging from 40-100 °C for 30 and 60 min, and at 121 °C for 15 min. To investigate the effect of pH, antibacterial activity was measured following the pH adjustment of the bacteriocin with 0.1 N NaOH or 0.1 N HCl and incubation at 4 °C for 1 h.

Molecular weight determination

The molecular weight of bacteriocin from S. sonnei strain 35/44 were determined by glycine SDS-PAGE with 5% stacking gel and 12% separating gel. The first half of the gel (protein gel) was stained with PageBlue Protein staining solution (Fermentas, USA) whereas another half of the gel (activity gel) was washed in sterile distilled water for 30 min and overlaid with TSBYE (0.8% agar) seeded with 0.1% (v/v) *S. sonnei* strain 44/44 (0.5 McFarland) and incubated at 37 °C for 16-18 h. The formation of clear halo or inhibition zone was observed and compared with protein gel.

Results

Among 153 strains of *S. sonnei* isolated from Thai patients, thirty one strains showed antibacterial activity against *S. sonnei* by agar well diffusion method. It was found that plasmid extracted from all of isolated strains of bacteriocinogenic *S. sonnei* gave PCR product with the expected size of 249 bp with primer specific to colicin type 7 (Js) gene (Figure 1). By DNA sequencing, the PCR product obtained from strain 35/44 showed 99% identity to complete sequence plasmid ColJs, plasmid pScol 7 and complete codons of colicin type 7 (*cja*) lytic protein of *S. sonnei*. There were no PCR products obtained from primer specific to *colicin E* and *colicin U* (Figure 1). The antimicrobial activity spectrum of partially purified bacteriocin (PPB) from isolated *S. sonnei* strain 35/44 against 17 test microorganisms was examined by agar diffusion method. It showed antibacterial activity against only Gram-negative test bacteria (Table 1). The PPB prepared from two isolated strains was tested for sensitivity to various proteolytic enzymes (trypsin, α-chymotrypsin and proteinase K), a key criterion for bacteriocin characterization. The complete inactivation was observed after treatment with all proteolytic enzymes (Table 2). Temperature stability experiment revealed that PPB from isolated *S. sonnei* strain 35/44 was completely stable at temperature up to 80 °C for 60 min (Table 2). With regard to pH stability, antibacterial activity

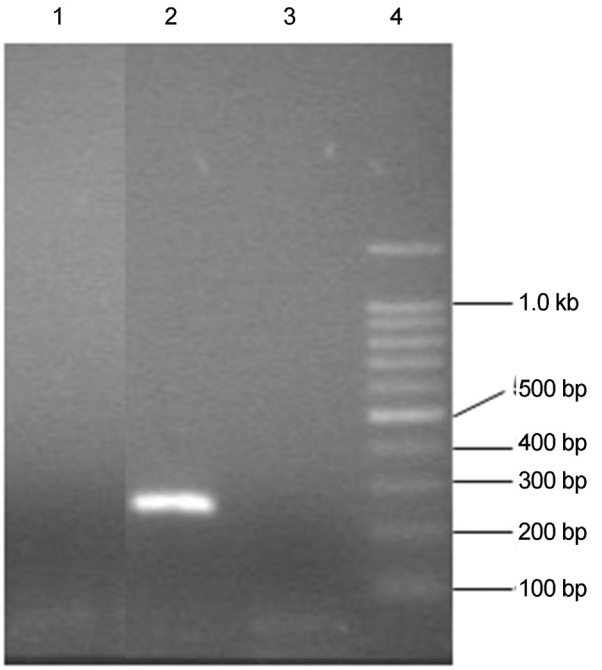

Figure 1 - PCR products obtained from *S. sonnei* strain 35/44. Lane 1: amplified product of *colicin U* gene; Lane 2: amplified product of *colicin 7* gene; Lane 3: amplified product of *colicin E* gene; Lane 4: 100 bp DNA marker.

of PPB of strain 35/44 was maintained more than 90% residual activity within the pH range of 3.0-9.0 (Table 2). The molecular weight of bacteriocin from isolated *S. sonnei* was determined by SDS-PAGE analysis of PPB. As shown in Figure 2, a single protein band with clear halo revealed a bacteriocin activity. The molecular weight of bacteriocin from isolated *S. sonnei* strain 35/44 was determined as 54.4 kDa.

Discussion

A variety of bacteriocins have attracted attention for their potential applications as natural and safe food preservatives due to their specificity and sensitivity. In this study, thirty one out of 153 strains of *S. sonnei* isolated from Thai patients with diarrhoea showed antibacterial activity against *S. sonnei* by agar well diffusion method. Only the *S. flexneri* strains isolated from dysenteric diarrhoea produced bacteriocin active against several *E. coli* and non-bacteriocin producing *S. flexneri* strains (Padilla *et al.*, 2006). Bacteriocins have been reported to be produced by several enteric bacteria including *S. sonnei*, *S. flexneri*, and *S. boydii*. They are usually designated as colicins (Horak 1994; Padilla *et al.*, 2006; Smajs and Weinstock 2001; Smajs *et al.*, 1997; Smarda *et al.*, 1987; Sousa *et al.*, 2010). Colicin genes are found mostly in colicinogenic plasmids of Gram-negative *E. coli* (Cascales *et al.*, 2007). It was demonstrated that all of thirty one isolated strains of bacteriocinogenic *S. sonnei* harbor plasmids with the genetic

Table 1 - Inhibitory spectrum of PPB from isolated *S. sonnei* strain 35/44.

Strain	Source*	Growth medium**	Incubation temperature	Inhibitiory activity
Gram-positive				
Bacillus cereus	MT	TSA	37 °C	
Bacillus pumilus	WAPB4	TSA	37 °C	-
Bacillus subtilis	ATCC6633	TSA	37 °C	-
Bacillus sphearicus	SOPB1	TSA	37 °C	-
Enterococcus sp.	MT	BHI	37 °C	-
Vancomycin resistant *Enterococcus* (VRE)	DMST4737	BHI	37 °C	-
Staphylococcus aureus	MT	TSA	37 °C	-
Methicillin resistant	DMST5199	TSA	37 °C	-
S. aureus (MRSA)			37 °C	
Listeria monocytogenes	MT	MRS	37 °C	-
Gram-negative				
Escherichia coli	O157 SW4	TSA	37 °C	++
Ampicillin resistant *E.coli* (ARE)	DMST19374	TSA	37 °C	-
Salmonella typhi	MT	TSA	37 °C	-
Salmonella typhimurium	MT	TSA	37 °C	-
Shigella dysenteriae	MT	TSA	37 °C	-
Shigella flexneri	MT	TSA	37 °C	-
Shigella boydii	MT	TSA	37 °C	+
Shigella sonnei	MT	TSA	37 °C	++

*ATCC, American Type Culture Collection; MT, Department of Medical Technology, Thammasat University, Thailand; DMST, Department of Medical Sciences, Ministry of Public Health Thailand.
**TSA, Tryptic Soy Agar; MRS, de Man, Rogosa and Sharpe; BHI, Brain Heart Infusion.
(-) no inhibition, (+) mild inhibition (1-5 mm of inhibition zone), (++) strong inhibition (more than 5 mm of inhibition zone).

determination of *colicin type 7* gene but without *colicin E* and *colicin U* gene. The PCR product obtained from strain 35/44 was shown to be the gene for colicin type 7 (*cja*) lytic protein. The presence of a DTLSN pentapeptide motive in translated polypeptide of strain 35/44 suggests that it could be imported to sensitive cells via the TonB transport system (Tigyi *et al.*, 2005). The 5.2-kb ColJs plasmid of a colicinogenic strain of *S. sonnei* (colicin type 7) contained three genes encoding 10.4 kDa Colicin Js activity polypeptide, 14.3 kDa Colicin Js immunity peptide and 7.5 kDa Colicin Js releasing peptide (Smajs and Weinstock 2001). Colicin gene clusters are usually composed of a colicin gene, which encodes the toxin; an immunity gene, which encodes a protein conferring specific immunity to the producer cell; and a lysis gene, which encodes a protein involved in colicin release through lysis of the producer cell (Riley and Wertz 2002). The absence of neither *colicin E* nor *colicin U* among these isolated strains indicated that the bacteriocin produced by *S. sonnei* strain 35/44 is colicin type 7. Therefore *S. sonnei* isolated strain 35/44 can be regarded as colicin type 7 producing bacteria.

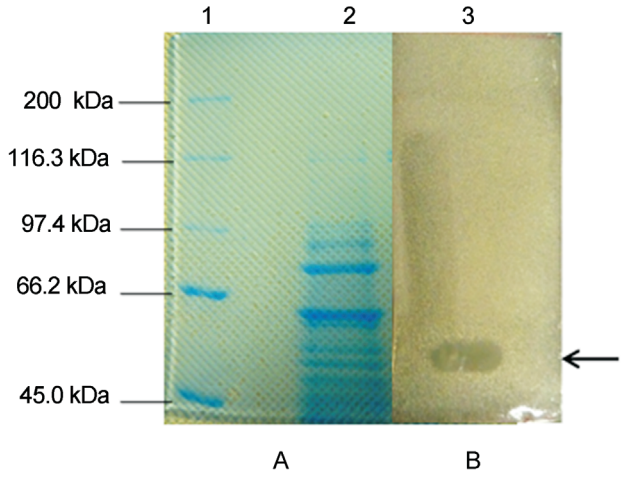

Figure 2 - SDS PAGE analysis of PPB prepared from *S. sonnei* strain 35/44 (A) Coomassie brilliant blue stained gel (B) The activity gel shows the clear zone (arrow) after overlaid with TSA (0.8% agar) seeded with *S. sonnei* and incubated overnight. Lane 1: Broad range protein molecular weight marker (Biorad); lane 2 and 3: PPB of strain 35/44.

The partially purified bacteriocin (PPB) prepared from *S. sonnei* strain 35/44 was active against *E. coli* O157, *S. sonnei* and *S. boydii*. The narrow target range of colicins was due to the presence of specific receptors at the surface of the sensitive strains (Cascales *et al.*, 2007). Nine out of 16 *S. sonnei* strains isolated from children with diarrhoea exhibited isoantagonism and heteroantagonism against *S. flexneri* and diarrhoeagenic *E. coli* by the overlay method

Table 2 - Effect of enzymes, temperature and pH on PPB from *S. sonnei* strain 35/44.

Treatments and conditions	Residual activity (%)
None (control)	100
Enzyme treatment	
Trypsin	0
α-chymotrypsin	0
Proteinase K	0
Temperature	
40 °C, 30 min	100
40 °C, 60 min	92
60 °C, 30 min	95
60 °C, 60 min	83
80 °C, 30 min	83
80 °C, 60 min	66
100 °C, 30 min	0
100 °C, 60 min	0
121 °C, 15 min	0
pH	
3.0	100
4.0	100
5.0	100
6.0	100
7.0	92
8.0	92
9.0	92

(Sousa *et al.*, 2010). *S. sonnei* colicin 7 (Scol7) is a bacteriocin acting only on certain dysentery-causing bacteria, like enteroinvasive *Escherichia coli*, *S. sonnei* or *S. boydii* (Tigyi *et al.*, 2005).

The sensitivity of PPB from strain 35/44 to proteinase K, trypsin and α-chymotrypsin suggests the proteinaceous nature of these antimicrobial substances. Therefore, this isolated bacterium can be regarded as bacteriocin producing bacteria. The colicin from this isolated *S. sonnei* was heat stable as evidenced by its ability to reserve the activity at 80 °C for 60 min. In addition, it was stable within a wide range of pH (3-9). The 46 kDa colicin Js produced by *S. sonnei* is unstable at pH 8.0 and at the temperature of 70 °C (Smarda *et al.*, 1987). The heat stable property was normally observed in bacteriocin from Gram-positive *Bacillus* sp. such as pumilicin 4 from *B. pumilus* (Aunpad and Na-Bangchang 2007) but rarely found in Gram-negative bacteria. The stability of bacteriocin from this isolated *S. sonnei* at high temperature and over a wide range of pH indicates the potential application in agro-industries as it could preserve its activity at extreme conditions.

The molecular mass of colicin from isolated *S. sonnei* strain 35/44 was 54.4 kDa. Colicins have been purified and found to be proteins of high molecular mass ranging from 40 to 80 kDa (Cascales *et al.*, 2007). There are only two reports on molecular mass determination of colicin from *S. sonnei* which are 10.4 kDa colicin Js (Smajs and Weinstock 2001) and 11.2 kDa colicin 7 (Tigyi *et al.*, 2005). These are in good agreement with previous estimations for their subunit but the molecular filtration experiments suggest a multimeric structure of at least 50 kDa (Tigyi *et al.*, 2005). The colicin type 7 in *S. sonnei* strain 35/44 might be composed of at least five subunits. It is to our knowledge; the first report of Thai patients with diarrhoea that *S. sonnei* isolated from them contained colicin type 7.

Acknowledgments

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