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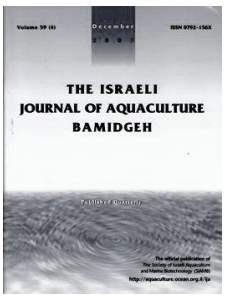

Partial Purification and Characterization of the Inhibitory Substance of *Bacillus subtilis* against Common Bacterial Fish Pathogens

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Abstract

The present investigation was carried out to study the active component of *Bacillus subtilis*, a gastrointestinal bacterium of mrigal (*Cirrhinus mrigala*) that shows inhibitory activity against a wide range of fish pathogens. The bacterium was found to inhibit all the fish pathogens and an extra cellular protein fraction (ECP) of 5-10 kD was found to be the active component responsible for inhibitory activity. The component was heat stable (60°C for 1 h) and a change in pH had no effect on the antibacterial activity. The protein fraction was sensitive to trypsin, chymotrypsin, lysozyme, and proteinase K at 2.5 mg/ml and resistant at 0.1 and 0.5 mg/ml. The present investigation confirmed that the active component of the *B. subtilis* responsible for antibacterial activities against common fish pathogens is a low molecular weight protein fraction (5-10 kD). A protein or peptide with such a low molecular weight has the potential to serve as an alternative health management strategy for combating disease in aquaculture.

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Introduction

The emergence of new pathogens and antibiotic resistance among pathogens threatens all forms of life including aquatic organisms. Therefore, much attention is being paid to new sources of anti-microbial and therapeutic agents (Clardy et al., 2006). Being a natural source of anti-microbial agents, microorganisms are often exploited to control infectious agents. Bacteria normally inhibit other bacteria by producing a variety of bactericidal and/or bacteriostatic metabolites. These compounds are mainly antibiotics, small peptides, metabolites such as alehydes, hydrogen peroxide, carbon dioxide, or lytic agents such as lysozyme (Tagg et al., 1976; Ray, 1992; Jack et al., 1995; Hutter et al., 2004).

Among the bacterial genera with potent antimicrobial agents, the gram-positive *Bacillus* species have the potential to produce many kinds of antimicrobial compounds including bacitracin, pumulin, and gramicidin (Todar, 2005). *Bacillus subtilis* has received particular attention due to its nonpathogenic nature and ability to produce antimicrobial peptides (Korenblum et al., 2005; Huang et al., 2006; El-hamshary and Khattab 2008).

A wide range of antibiotics, drugs, and chemicals are routinely used to control diseases in aquaculture. The indiscriminate use of such agents often leads to the development of resistance to antibiotics. The recent concept of disease management is to replace antibiotic therapy with probiotics or new compounds capable of inhibiting a wide range of pathogens (Ramakrishnan et al., 2008; Ganguly et al., 2010). The present investigation was carried out to partially purify and characterize the inhibitory substance of *Bacillus subtilis*, a gastrointestinal bacterium of mrigal (*Cirrhinus mrigala*), that possesses immunomodulatory (Nayak et al., 2007) and antibacterial activities against common fish pathogens.

Materials and Methods

Bacillus subtilis, a gastrointestinal bacterium isolated from mrigal (*C. mrigala*) along with five bacterial pathogens (*Aeromonas hydrophila, Edwardsiella tarda*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Flavobacterium branchiophilum*) and four opportunistic pathogens (*Salmonella*, *Shigella*, *Escherichia coli*, *Staphylococcus*) were obtained from the Fish Health Management Division of the Central Institute of Freshwater Aquaculture in Orissa, India, and screened.

The inhibitory activity of the bacteria was screened *in vitro* in an inhibition test on solid medium as per the double layer method of Dopazo et al. (1988) with slight modifications. The bacterium was first grown in tryptone soya broth (TSB) at 30°C for 24 h, then cultures were spot-inoculated into tryptone soya agar plates and incubated at 30°C for 120 h to create a macro colony. After incubation the cells were killed with chloroform vapor (15-20 min) and an overlay containing pathogenic strains was poured over the plates (5 ml/plate). The plates were incubated up to 120 h at 30°C and the zones of inhibitions around the macro colony were recorded.

A well diffusion assay was carried out as follows: tryptone soya agar (TSA) plates were prepared and wells (6 mm diameter) were made by puncture. The lower end of the plates was sealed with molten agar and the pathogens were swabbed on the plates. About 100 ml of the test sample was poured into the wells which were incubated 24-48 h at 30°C. Inhibition zones were recorded up to 120 h.

The inhibitory activity of the bacteria was further evaluated by growing the bacteria in brain heart infusion agar (BHIA), diagnostic sensitive agar (DSA), nutrient agar (NA), and TSA, procured from Hi-media, India. Next, the effects of temperature (22-37°C), pH (5.5-8.5), and salt concentration (0-10%) on inhibitory activity were studied on TSA.

To screen the inhibitory activity of the extra cellular product (ECP) of *B. subtilis*, the bacteria was grown in TSB for 72 h at 30°C, the supernatant was collected by centrifuging the culture at 10,000 × *g* for 10 min at 4°C, and the supernatant was filter sterilized in 0.22 nm membrane filters (Satorius, Germany) and serially passed through protein filters with cut-off values of 5, 10, 20, and 100 kD (Satorius, Germany). Finally, the inhibitory activity of the crude ECP, along with individual fractions of the ECP component, was tested at different temperatures and pH by the well diffusion method

described above. ECP samples were heat-inactivated in water baths at 60°C for 15 min, 60°C for 30 min, 60°C for 1 h, 100°C for 15 min, or 121°C for 15 min and the activity was tested by well diffusion assay. ECP samples were kept at -20°C, 4°C, and room temperature (27-34°C) for six months and the inhibitory activity was checked every 15 days by well diffusion assay.

The enzymes trypsin, chymotrypsin, lysozyme, proteinase K, lipase, DNase, and RNase (Sigma, USA) were added to the active fraction at 0.1, 0.5, and 2.5 mg/ml, the mixtures were incubated 1 h at 37°C, the enzymes were inactivated at 56°C for 30 min, and the antibacterial activity of the mixtures as well as the pure enzymes was tested by the disc diffusion method.

Results

The bacteria produced inhibitory activity against all the tested fish pathogens and secondary pathogens in all the media. The inhibition was significantly greater in the TSA and BHIA media than in the NA and DSA media and greatest when the bacterium was grown in TSA with a 1% salt concentration, pH 7, and 30°C. The least inhibitions were recorded at pH 5.5 and 8.5, with no significant difference in zone size.

The crude ECP component of *B. subtilis* inhibited all the tested pathogens. Formost pathogens, inhibitory activity was significantly better at 30°C and pH 7 (Table 1). The protein fractions <5, 20, and >100 kD showed no inhibitory activity against any tested primary or secondary pathogen, however, the 5-10 kD fraction inhibited all. Crude and 5-10 kD protein activity was recorded at 60°C/30 min and 60°C/1 h, however, no inhibitory zones were detected at 100°C/15 min or 121°C/15 min.

Table 1. Zones of inhibition (mean±SD) exerted by the ECP component of *Bacillus subtilis* on various fish pathogens at different temperatures and pH.

*Strain**	*37°C (pH)*			*30°C (pH)*			*22°C (pH)*		
	5.5	*7*	*8.5*	*5.5*	*7*	*8.5*	*5.5*	*7*	*8.5*
Ah1	1.17 ± 0.17^{b}	1.52 ± 0.09^{c}	0.87 ± 0.22^{a}	1.0 ± 0.08^{ab}	1.62 ± 0.05^{c}	0.92 ± 0.18^{a}	0.92 ± 0.12^{a}	1.20 ± 0.08^{b}	0.80 ± 0.18^{a}
Ah2	1.07 ± 0.17^{b}	1.82 ± 0.12^{c}	0.92 ± 0.09^{b}	1.02 ± 0.09^{b}	1.8 ± 0.14^{c}	0.92 ± 0.09^{b}	0.65 ± 0.10^{a}	1.60 ± 0.08^{c}	0.67 ± 0.05^{a}
Ah3	0.77 ± 0.05^{a}	1.45 ± 0.1^{cd}	1.07 ± 0.15^{b}	0.97 ± 0.17^{b}	1.47 ± 0.12^{d}	1.00 ± 0.14^{b}	0.75 ± 0.23^{a}	1.20 ± 0.05^{c}	1.05 ± 0.05^{bc}
Ah4	1.15 ± 0.1^{bc}	1.62 ± 0.12^{e}	1.17 ± 0.17^{bc}	1.32 ± 0.12^{cd}	1.80 ± 0.16^{e}	1.15 ± 0.1^{bc}	0.85 ± 0.12^{a}	1.40 ± 0.05^{d}	1.12 ± 0.09^{b}
Et	1.15 ± 0.1^{b}	1.47 ± 0.05^{c}	1.17 ± 0.12^{b}	1.20 ± 0.01^{b}	1.52 ± 0.09^{c}	1.20 ± 0.08^{b}	0.92 ± 0.09^{a}	1.15 ± 0.05^{b}	0.87 ± 0.12^{a}
Pa	1.1 ± 0.08^{bc}	1.32 ± 0.05^{d}	1.12 ± 0.12^{bc}	1.02 ± 0.09^{b}	1.42 ± 0.05^{d}	1.12 ± 0.12^{bc}	0.77 ± 0.05^{a}	1.17 ± 0.05^{c}	0.82 ± 0.09^{a}
Pf	1.17 ± 0.05^{c}	1.32 ± 0.09^{d}	1.12 ± 0.05^{c}	1.12 ± 0.05^{c}	1.45 ± 0.05^{e}	1.10 ± 0.01^{c}	0.97 ± 0.09^{b}	0.95 ± 0.1^{b}	0.77 ± 0.05^{a}
Fbr	1.17 ± 0.18^{c}	1.82 ± 0.2^{d}	0.87 ± 0.09^{ab}	1.10 ± 0.18^{c}	1.75 ± 0.12^{d}	1.12 ± 0.15^{c}	1.02 ± 0.09^{bc}	1.65 ± 0.12^{d}	0.80 ± 0.08^{a}

Different superscripts in a row indicate significant differences.

* Ah1, Ah2, Ah3, Ah4 = four strains of *Aeromonas hydrophila*; Et = *Edwardsiella tarda*; Pa = *Pseudomonas aeruginosa*; Pf = *Pseudomonas fluorescens*; Fbr = *Flavobacterium branchiophilum*

Inhibitory activity was observed in the crude ECP and 5-10 kD protein fraction after six months in storage at -20°C, 4°C, and room temperature (27-34°C). The best activity was recorded in samples kept at room temperature while the least, but not significantly least, activity was in samples stored at -20°C for six months for both crude *B. subtilis* and its 5-10 kD protein ECP component.

Inhibitory activity of the ECP component was detected when treated with trypsin, chymotrysin, lysozyme, and proteinase K at 0.1 and 0.5 mg/ml, but no activity was recorded when treated with these enzymes at 2.5 mg/ml (Table 2). Activity was detected in both crude *B. subtilis* and the pure ECP component when treated with lipase, RNAase, and DNAase irrespective of the enzyme concentration.

Table 2. The effect of different enzymes on the antibacterial activity of the 5-10 kD protein fraction of the ECP component of *Bacillus subtilis.*

	Enzyme concentration (mg/ml)					
Enzyme	*0.1 and 0.5*			*2.5*		
	Enzyme only	*ECP only*	*Both*	*Enzyme only*	*ECP only*	*Both*
Trypsin	+	+	+	+	+	-
Chymotrypsin	+	+	+	+	+	-
Lysozyme	+	+	+	+	+	-
Proteinase K	+	+	+	+	+	-
RNAsae	-	+	+	+	+	+
DNAse	-	+	+	+	+	+
Lipase	-	+	+	+	+	+

Discussion

Bacillus subtilis is one of the dominant producers of antibiotics and anti-microbial compounds of the *Bacillus* genus. The broad spectrum inhibitory activity of *B. subtilis* has been reported against pathogens of humans and animals, including fish and shellfish. During, the present investigation *B. subtilis* inhibited all the tested pathogens and secondary pathogens. The 5-10 kD protein fraction of the ECP component was responsible for the inhibitory activity. Similarly, a heat labile sideraphore of less than 5 kD was the active component of a *Bacillus* species isolated from fish (Sugita et al. (1998). The component was soluble, extracellular and diffusible, in accordance with the findings of Fiddaman and Rossal (1993).

Various proteolytic enzymes such as proteinase K, trypsin, α-chymotrypsin (type II), and protease (type XIV), when treated at 0.1 mg/ml, had no effect on the antibacterial activity in the culture supernatant of *Bacillus* species (Sugita et al., 1998). In the present investigation, the ECP component of *B. subtilis* resisted these enzymes up to 0.5 mg/ml but not at 2.5 mg/ml. This characteristic is typical of gram-positive bacteria (Ray, 1992; Jack et al., 1995).

The present investigation confirms that the active component responsible for the antibacterial activity of the bacterium *B. subtilis* against common fish pathogens is a low molecular weight ECP protein fraction (5-10 kD). This low molecular weight protein/peptide has potential as an alternative health management practice for combating disease in aquaculture. However, *in vivo* studies, especially with respect to dose, route of supplementation, and efficacy of such compound(s) in a piscine system, are warranted.

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