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Antimicrobial activity of coelomic fluid and coelomic fluid lectin from starfish *Protoreaster linckii* (Blainville, 1830)

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ABSTRACT

Lectin from the coelomic fluid of the starfish, *Protoreaster linckii* was purified by affinity chromatography using PSM (Porcine Stomach Mucin). The purified lectin has the capacity to bind to fungal cell structures that express sugars like galactosamine, glucose and fucose on the surface thus paving way for lectin targeted therapy. Antimicrobial potential of solvent extracts of coelomic fluid and crude coelomic fluid of starfish, *Protoreaster linckii* was assessed against five human pathogenic bacteria, two fungal pathogens and five fish pathogenic bacteria. Only fish pathogenic bacteria, *Staphylococcus aureus* and human fungal pathogens *Aspergillus niger* and *Aspergillus flavus* were sensitive to crude coelomic fluid. Hence it was attempted to compare the crude coelomic fluid with the purified coelomic fluid lectin. It was noted that the lyophilized crude coelomic fluid and purified lectin was efficient in inhibiting the growth of the human fungal pathogens *A. flavus* and *A. niger*. From our studies it is clear that *Protoreaster linckii* lectin is capable of specifically recognizing the sugar moieties present in the cell wall of both the fungi. Hence this could be in future used as an anti-fungal agent.

KEY WORDS

Coelomic fluid, affinity chromatography, lectin, antimicrobial potential, antimicrobial assay

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INTRODUCTION

Echinoderms appear as untapped source in the pursuit of the identification of new and useful products[1]. Echinoderms are rich in antibiotic compounds. Some bioactive antiviral, antitumor, antimicrobial or generally cytotoxic compounds have been extracted from species of starfish[2].

Coelomocytes mediate the cellular responses to immune challenges through phagocytosis, encapsulation, cytotoxicity and the production of antimicrobial agents. In addition, a variety of humoral factors found in coelomic fluid, including lectins, agglutinins, lysins, acute phase proteins and antimicrobial factors[3, 4] are important in host defense against pathogens and other foreign substances.

Invertebrates lack an adaptive immune system and lectins play an important role in their innate immune systems by recognizing invading microbes or pathogens[5]. Although invertebrate lectins are mainly involved in immune system response[6], lectins have been retrieved in almost all forms of life and animal lectins, though fulfilling a variety of functions, have been implicated in defense against pathogens, immune regulation and prevention of autoimmunity[7].

Marine invertebrates are reported to possess ligand-binding lectins which may act in defense reactions such as opsonisation or phagocytosis. This has been demonstrated in shellfish lectins[8], the lectin from the serum of *Crassostrea virginica*[9] and a sialic acid binding lectin from horse mussel *Modiolus modiolus*[10]. Achatinin, a lectin from the hemolymph of *Achatina fulica* snail has also bacteriostatic effect on the gram-negative bacteria, *E. coli*[11]. T-antigen binding lectin from sea cucumber *Holothuria scabra*, exhibited strong antibacterial activity against both gram positive and gram negative bacteria[12].

Since echinoderms and echinoderm lectins are known to possess antimicrobial effect, an attempt was undertaken to study the antimicrobial activity of coelomic fluid extracts, crude coelomic fluid and coelomic fluid lectin of the starfish, *Protoreaster linckii*.

MATERIALS AND METHODS

Animal collection

Starfish were collected by dip net fishing from Kadiyapattanam, Muttom, Kesavanputhenthurai and Pallam coasts, Kanyakumari District, Tamilnadu, India. Animals were maintained in sea water and transported to the laboratory.

Collection of sample

Coelomic fluid of the starfish *P. linckii* was collected by cutting off the tip of the arm and draining the fluid into the centrifuge tubes.

Immediately after collection, the coelomic fluid was centrifuged for the collection of serum. The serum was aliquoted and stored at -20^{o} C.

Purification of lectin from the coelomic fluid of Protoreaster linckii

The lectin was purified by affinity purification as reported elsewhere[13]. Briefly, affinity purification of the coelomic fluid lectin of *Protoreaster linckii* was performed by clarified coelomic fluid (20 ml) was applied to 3.5 ml of PSM-agarose in an econo-column (Bio-Rad) previously equilibrated with TBS at 4°C. The eluant was collected at a rate of 0.6 ml/ min. The column was washed with HSB until the A_{280} of the effluent was < 0.002. The column was further washed with LSB at 4°C until the A_{280} of the effluent was < 0.002, and it was washed with warm LSB (32°C) until the A_{280} of the effluent was < 0.002. This step eluted additional inert proteins and was necessary for obtaining homogeneous lectin. In all these steps, the buffers contained the calcium required for binding of lectin to PSM-agarose. The elution of lectin was done with elution buffer (EB) that contained 5 mM trisodium citrate and the eluant was collected as 1 ml fractions on ice in polypropylene tubes containing 10 µl of 10 mM calcium chloride at the rate of 0.3 µl/ minute. The fractions were vortexed immediately after collection and stored at 4°C. The effluents (fractions) collected during adsorption, washing, re-equilibration and elution were tested for HA activity with 1.5% suspension of rabbit erythrocytes to determine the presence of agglutinin. The eluted fractions with trisodium citrate that contained significant amount of lectin were pooled on the same day, dialysed against 10 mM $CaCl_2$, at 4°C for 5 minutes and the dialysate was then aliquoted, lyophilized and stored at -20°C.

Preparation of extracts for antimicrobial activity

Starfish coelomic fluid extracts were prepared following the method of Karthikeyan with slight modification[14]. 1 ml of coelomic fluid was mixed in 5 ml of 70% methanol, acetone, chloroform, butanol and ethyl acetate and kept for three days at room temperature. The extracts was concentrated by evaporating in room temperature to give a gummy mass and used for the antimicrobial agar disc diffusion assay.

Preparation of lyophilized coelomic fluid and P. linckii purified lectin for antimicrobial assay

Different concentrations of lyophilized coelomic fluid and purified lectin (PLL) (5 µg, 10 µg, 25 µg and 50 µg) were prepared by dissolving in distilled water and were used for antimicrobial activity.

Test microorganisms and culture media

Test microorganisms, fish pathogenic Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Vibrio harveyi*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Proteus mirabilis*) were obtained from Inbiotics, Nagercoil. Human pathogenic Gram positive bacteria *Staphylococcus aureus* (MTCC 3160), Gram negative bacteria [*Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 424), *Klebsiella pneumoniae* (MTCC 3040), *Proteus vulgaris* (MTCC 744)] and human fungal pathogens *Aspergillus niger* (MTCC 1344) and *Aspergillus flavus* (MTCC 7589) were purchased from Microbial Type Culture Collection and Gene Bank, IMTECH, Chandigarh and were used for antimicrobial assay.

Fish pathogenic bacteria (*Staphylococcus aureus*) and human fungal pathogens (*Aspergillus niger* and *Aspergillus flavus*) were used to assess the antimicrobial property of lyophilized coelomic fluid and purified lectin.

Bacterial strains were cultured in Muller Hinton Agar and fungal strains were cultured using Sabouraud Dextrose Agar.

Antimicrobial assay

In vitro antimicrobial assay was carried out by disc diffusion technique[15]. Solvent extracts of coelomic fluid of starfish *Protoreaster linckii* was used for the assay. Sterile discs of 6 mm were impregnated with extracts of 50 µl and allowed to dry at room temperature and extract loaded discs were placed on agar plates seeded with microorganisms and incubated at 37°C for 24 hours. The susceptibility of the test organisms were determined by the diameter of the zone of inhibition and then measured in mm. The amikacin discs were used as a positive control for bacteria, flucanazole for fungi and solvent discs were used as a negative control. All the extracts were tested in triplicate and the results were expressed as mean ± SD of three independent values.

RESULTS

Purification of Protoreaster linckii lectin by affinity column

The PSM affinity column purification of the lectin gave a 3354.76 fold increase in the specific activity of the clarified serum. 30 ml of clarified coelomic fluid was applied in the affinity column and 9 ml of purified lectin was obtained. The lectin was lyophilized and final amount of lectin yielded 108 mg.

Antimicrobial activity

Antibacterial and antifungal activity of crude coelomic fluid and coelomic fluid extracts of starfish *P. linckii* was screened against five human pathogenic bacteria and two fungi and five fish pathogenic bacteria.

All the tested human pathogenic bacteria were sensitive to butanol extract of starfish coelomic fluid. Similarly, the coelomic fluid extract of starfish inhibited the growth of the fish pathogenic bacteria except *K. pneumoniae*. Antimicrobial activity was observed in the coelomic fluid extracts against two human fungal pathogens *A. niger* and *A. flavus*. The ethyl acetate treated coelomic fluid showed high activity only with fish pathogenic bacteria *P. aeruginosa* (14 mm). The chloroform, acetone and methanol treated coelomic fluid failed to inhibit all the tested pathogens.

The crude coelomic fluid inhibited only the fish pathogenic bacteria *Staphylococcus aureus* (13 mm) and human fungal pathogens *A. niger* (13 mm) and *A. flavus* (15 mm)(Table 1).

Gram positive *Staphylococcus aureus* was resistant to lyophilized samples of both coelomic fluid and purified lectin. Human fungal pathogens *A. niger* and *A. flavus* were sensitive to 25 µg and 50 µg of the lyophilized crude coelomic fluid as well as the purified lectin suggesting the PLL as a potential antifungal agent(Table 2 and Figure 1).

Table 1: Antimicrobial activity of crude coelomic fluid and coelomic fluid extracts of starfish, *P. linckii*

Microbes		Zone of inhibition (mm) (Mean ± SD)							
		CF	CF-B	CF-EA	CF-C	CF-A	CF-M	Positive	Negative
Bacteria (human pathogens)	*S. aureus*	_	12 ± 1	_	_	_	_	23 ± 0.5	-
	E. coli	_	8 ± 1.5	_	_	_	_	15 ± 1	-
	P. aeruginosa	_	10 ± 1.1	_	_	_	_	20 ± 1.1	-
	K. pneumoniae	-	13 ± 0.5	_	_	_	_	30 ± 1	-
	P. vulgaris	_	8 ± 1	_	_	_	_	22 ± 0.5	-
Bacteria (fish pathogens)	*S. aureus*	13 ± 1	12 ± 0.5	_	_	_	_	28 ± 0.5	-
	V. harveyi	-	-	_	_	_	_	27 ± 0.5	-
	A. hydrophila	-	11 ± 1.5	_	_	_	_	26 ± 1	-
	P. mirabilis	-	11 ± 1	_	_	_	_	30 ± 1.1	-
	P. aeruginosa	-	15 ± 1	14 ±1.1	-	-	-	21 ± 1	-
Fungi (human pathogens)	*A. niger*	13 ± 1	10 ± 0.5	_	_	_	_	20 ± 1.1	-
	A. flavus	15 ± 1	10 ± 0.5	_	_	_	_	21 ± 1	-

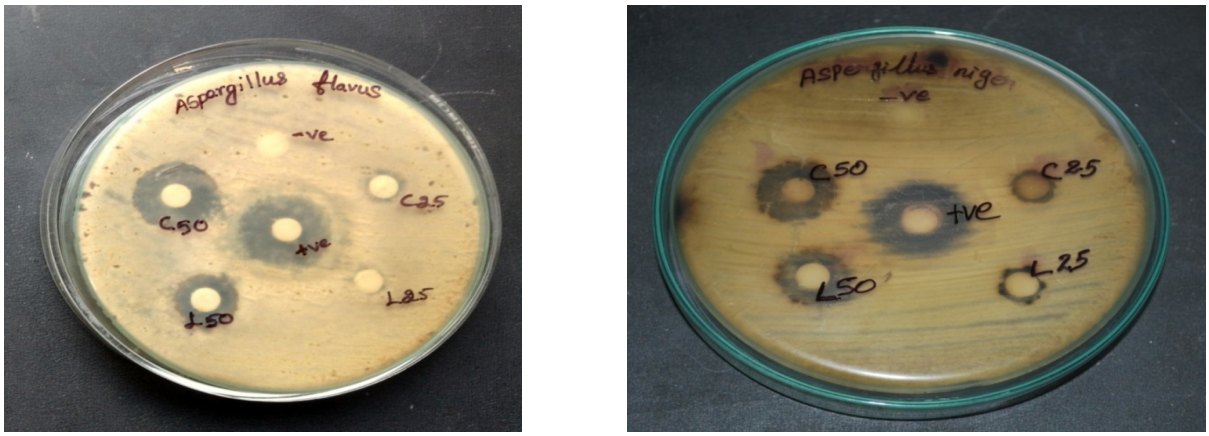
CF = crude coelomic fluid, CF-B = coelomic fluid butanol treated, CF-EA = coelomic fluid ethyl acetate treated, CF-C = coelomic fluid chloroform treated, CF-A = coelomic fluid acetone treated, CF-M = coelomic fluid methanol treated

Table 2: Antimicrobial activity of different concentrations of crude coelomic fluid and purified lectin

Microbes		Zone of inhibition (mm)									
		C5	C10	C25	C50	L5	L10	L25	L50	Positive	Negative
Fish pathogenic bacteria	*S. aureus*	-	-	-	-	-	-	-	-	29	0
Human pathogenic fungi	*A. niger*	-	-	10	16	-	-	8	13	28	0
	A. flavus	-	-	10	18	-	-	7	14	25	0

C = Lyophilized crude coelomic fluid, L = Lyophilized purified lectin (PLL)

Aspergillus niger ***Aspergillus flavus***



C = Crude Coelomic Fluid, L = Purified Lectin (PLL)

Figure 1: Antifungal activity of different concentrations of crude and purified coelomic fluid lectin of starfish *P. linckii*

DISCUSSION

The result of purification, strongly suggest that *P. linckii* lectin (PLL) from the coelomic fluid can be a powerful tool to study pathological process.

Antimicrobial efficacy of the *P. linckii* coelomic fluid extracts, lyophilized coelomic fluid and purified lectin (PLL) were compared. The coelomic fluid extracts were treated with different solvents while the lyophilized samples were dissolved only in distilled water.

Among the crude coelomic fluid and coelomic fluid extracts tested, crude coelomic fluid exhibited antimicrobial activity only against fish pathogenic bacteria *Staphylococcus aureus* and human fungal pathogens *A. niger* and *A. flavus.* Hence, it was decided to assess the antimicrobial activity of the lyophilized crude coelomic fluid as well as the purified lectin against fish pathogenic bacteria, *Staphylococcus aureus* and human fungal pathogens *Aspergillus niger* and *Aspergillus flavus.* Absence of antimicrobial activity against fish pathogenic bacteria *Staphylococcus aureus* could possibly mean that the isolated lectin do not possess biological activity against the test organism. Similar studies were also reported in the lectin from *Holothuria scabra*[16] and sialic acid binding lectin from the body walls of brown sea cucumber[17] which also does not possess antimicrobial potential.

Antifungal assay demonstrated a maximum zone of inhibition against the human fungal pathogens *A. niger* and *A. flavus* with higher concentrations (25 and 50 µg) of the coelomic fluid and purified lectin. *Aspergillus* species are ubiquitous fungi which can cause a variety of clinical syndrome, especially in immunosuppressed patients and as agents of foetal systemic infections and have therefore gained considerable public health importance[18]. Analysis of cell wall composition of *A. niger* showed the presence of six sugars, glucose, galactose, mannose, arabinose, glucosamine and galactosamine[19]. According to Pinto the cell wall of *Aspergillus* species is composed of a number of unique interconnected polysaccharides, including chitin and a variety of glucans[20]. Results of Leal showed there was an expression of N-acetyl-D-glucosamine, L-fucose and D-galactose on the cell wall surface of *A. flavus* and *A. niger*[21]. PLL is a lectin that is highly specific to N-acetyl-D-galactosamine and D-galactosamine and has affinity also towards glucose and fucose. It may possess the ability to recognize the saccharide moieties present on the cell wall and may cause fungal cell wall polymer degradation or damage to cellular ribosomes or may inhibit the cell cycle[22]. Thus our results indicate PLL as an antifungal agent.

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