



Isolation, purification and characterization of β -1,3-glucan binding protein from the plasma of marine mussel *Perna viridis*

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Abstract A β -1,3-glucan binding protein (β GBP) specific for laminarin (a β -1,3-glucan) was detected for the first time in a mollusc, *Perna viridis*. β GBP was isolated and purified from the plasma using laminarin precipitation and affinity chromatography on laminarin-Sepharose 6B, respectively. It agglutinated bakers yeast, bacteria, and erythrocytes and enhanced prophenoloxidase (proPO) activity of the plasma in a dose-dependent manner. The purified β GBP appeared as a single band in native-PAGE and the purity was conformed by HPLC. The protein has a molecular weight estimate of 510 kDa as determined by SDS-PAGE and in isoelectric focusing the purified β GBP was focused as a single band at pI 5.3. β GBP was found to possess inherent serine protease activity but lacked β -1,3-glucanase activity and all these results suggest that plasma β GBP of *P. viridis* functions as a recognition molecule for β -1,3-glucan on the surface of microbial cell walls. This recognition and binding lead to the activation of the prophenoloxidase cascade mediated by the inherent serine protease activity of β GBP. Presence of agglutinating activity and serine protease activity shows that β GBP is a bifunctional protein. The findings are discussed in light of the importance of this protein in the innate immune response of *P. viridis*, and they implicate evolutionary link with similar proteins found in other invertebrates.

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Introduction

Invertebrates constitute a diverse assemblage of animals that have evolved a variety of efficient and sometimes

unique host defence strategies by which they protect themselves against the threat of microbial invasion or disturbance of homeostatic integrity [1]. Thus, the ability to distinguish non-self is a fundamental aspect of immunity.

Such non-self recognition involves pattern recognition proteins (PRPs) that recognize and respond to microbial invaders by the presence of signature molecules on the surface of the invaders. They lack the specificity of antibodies and bind to certain classes of polysaccharides, such as LPS, lipoteichoic acid, peptidoglycan and β -1,3-glucans.

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Some of them, namely, agglutinins [2], LPS [3] and β -1,3-glucan binding protein [4,5] appear to interact with haemocytes and facilitate cellular immune reactions. Among these β -1,3-glucans are known to induce synthesis of phytoalexins and defense molecules [6] as well as modulators of mammalian and other vertebrate immunity by activating alternative complement pathway [7], macrophages and monocytes [8]. In invertebrates, β -1,3-glucans have been used as an effective immunostimulant [9–11]. Apparently, this effect is mediated by potentiation/activation of circulating hemocytes [12] and induction of hemolymph proteins [13–15]. Although the mechanisms by which invertebrates recognize glucans are still not completely understood, Toll-like receptors, proteases and immunity transcription factors have been implicated [16,17]. Thus it is obvious that such immune processes require availability of appropriate receptors, for β -1,3-glucans, on the surface of immunocompetent cells or binding proteins in plasma.

β -1,3-Glucan binding protein (β GBP) has been detected in many invertebrate groups and subsequently purified [18–23]. Söderhäll et al. [24] and Ochiai and Ashida [25] were probably the first to attempt partial purification of these proteins from the plasma of insects, *Blaberus craniifer* and *Bombyx mori*, respectively. β GBP has been implicated in a variety of functions in invertebrates, that includes their role in non-self recognition [19,22,26], activation of prophenoloxidase cascade involving proteases or peptidases [21,25,27–30], activation of coagulation cascade [22] and hemocyte degranulation leading to the release of a variety of cytotoxic/cytolytic/antibacterial factors [5,18,31]. Roux [32] has shown upregulation of β GBP gene in response to infection of shrimps by white spot syndrome virus. Quite recently, Wang [33] has shown the importance of r β GBP raised in *Escherichia coli* BL21 in cellular encapsulation responses in the mosquito *Armigeres subalbatus*.

Although many earlier studies have shown the presence of opsonins or lectins in bivalve molluscs, there are no reports on the presence or functional characterization of β GBP from molluscs. In this connection, earlier work on *Perna viridis* in our laboratory revealed that laminarin mediated the activation of proPO system in plasma and hemocytes [34] and such activation facilitated the phagocytic response of hemocytes [35]. Furthermore, we have also shown that β -1,3-glucans induced the mussel hemocytes, *in vitro*, to synthesize and release exocytotically immune factors such as proPO system, thereby indicating the possible presence of β GBP in the hemolymph of *P. viridis*.

In this paper, we describe the purification and functional characterization of β -1,3-glucan binding protein from the marine mussel, *P. viridis*. We show that this β GBP can trigger activation of the proPO pathway through serine proteases upon binding to laminarin and can agglutinate yeast and microbes, thus serving as non-self recognition molecule in the molluscan immune system.

Material and methods

Experimental animals

Perna viridis was collected from the coast of Royapuram, Chennai, India and maintained in laboratory in large plastic

troughs containing seawater (30‰; $26 \pm 2^\circ\text{C}$) with continuous aeration. Animals were acclimated to laboratory conditions for 24 h prior to use.

Purification

Preparation of plasma and isolation of β GBP

Hemolymph of *P. viridis* does not clot and 3–7 ml was withdrawn from the posterior adductor muscle of individual mussel with a sterile plastic syringe (10 ml) and the plasma was obtained following Asokan et al. [34]. β -1,3-Glucan binding protein was isolated from the plasma by laminarin precipitation following the method described previously [36]. Briefly, plasma was centrifuged ($25,000 \times g$, 15 min, 4°C) and 20 ml of the supernatant (clarified plasma) was mixed with 10 mg of laminarin (Sigma), stirred (4 h at 4°C) and centrifuged ($25,000 \times g$, 15 min, 4°C). The pellet (laminarin-precipitable substances) was dissolved in tris-buffered saline (TBS-I: 10 mM tris-HCl, 50 mM KCl, 100 mM NaCl pH 8.0, 300 mOsm) and both the supernatant and the dissolved pellet were dialysed (M_w exclusion limit $<14,000$ Da) extensively against the same buffer. All these fractions were subsequently tested for hemagglutination, yeast and bacterial agglutination, and PO enhancing activity.

Purification of β -1,3-glucan binding protein

The plasma was diluted with equal volume TBS-II (50 mM tris-HCl, 100 mM NaCl, 10 mM CaCl_2 ; pH 7.5, 300 mOsm) to an agglutination titre of 16 against trypsinized yeast cells. This dilute plasma was then centrifuged ($10,000 \times g$, 30 min, 4°C) and 110 ml of supernatant was passed through laminarin-Sepharose CL 6B column (0.8×1.6 cm) at a flow rate of 3 ml h^{-1} . Unbound plasma components were removed from the column by washing (3 ml h^{-1}) with TBS-II until the absorbance of the effluent at 280 nm was 0, followed by re-equilibration in TBS-II. β GBP bound to the affinity matrix was eluted (4 ml h^{-1}) with 10 ml of 0.1 M acetate buffer (pH 4.5) containing 2.5 M MgCl_2 and 1 M D-glucose and the fractions that showed absorbance at 280 nm were collected, wherever necessary pooled and dialysed extensively (M_w exclusion limit $<14,000$ Da) against TBS-II at 10°C . The dialysate obtained was checked for purity and homogeneity by native-PAGE, SDS-PAGE and HPLC.

Electrophoretic analyses

Samples of purified β -1,3-glucan binding protein eluted from affinity matrix were electrophoresed in discontinuous PAGE under non-denaturing condition (native-PAGE) using 3% stacking gel (pH 6.5) and 4.5% separating gel (pH 8.3) following Maurer [37]. SDS-PAGE was performed as described previously [38], in discontinuous buffer system using 3% stacking gel (pH 6.8) and 10% separating gel (pH 8.8) in tris-glycine buffer (pH 8.3). Prior to SDS electrophoresis, β GBP (50 μg) was mixed with an equal volume of sample buffer (60 mM tris-HCl buffer, 4% SDS, pH 6.8) that did or did not contain 5% β -mercaptoethanol (β -ME). A mixture of standard marker proteins (GENEI, Bangalore, India) containing 17 μg protein in SDS and β -ME was also prepared identically. All samples were boiled in a water bath for

10 min and cooled to room temperature. Both native and SDS-PAGE were run at a constant current (4 mA/well) in a slab gel measuring $170 \times 150 \times 1.5$ mm. The proteins were stained with silver nitrate [39].

HPLC and isoelectric focusing

The homogeneity of affinity-purified β GBP was checked in its native form by HPLC using a reversed phase C_{18} column ($7.8 \text{ mm} \times 30 \text{ cm}$) previously equilibrated with TBS-II at a flow rate of 0.8 ml min^{-1} . HPLC system (Waters, Model: SPD 10A UV-Visible Shimadzu) equipped with 2414 RI detector, was used for the analysis. Isoelectric focusing of purified β GBP on a gel (Ampholine-PAGE plates, pH 3.5–9.5) was performed in an LKB 2117 multiphore apparatus with an LKB 2197 constant power supply. Focussed gels were fixed for 1 h in 12% TCA and stained with 0.1% Coomassie Brilliant Blue R-250 in methanol:acetic acid:water (9:2:9 by volume).

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were obtained using a JASCO j-715 spectropolarimeter. The spectrum obtained was an average of 3 seconds. Each measurement was performed in two separate occasions and the mean deviation was less than 2%. The CD instrument was calibrated with ammonium d-10-camphor sulphonic acid (0.06% water solution). The sample temperature was 25°C . The protein concentration and the light path length in UV region were about 0.5 mg ml^{-1} and 0.1 cm, respectively. CD spectra of purified β GBP were obtained in a solution of TBS-II.

Protein determination

Total protein concentration was determined following the method of Lowry et al. [40] using bovine serum albumin (BSA) as a standard.

Functional analyses assays

Assay of phenoloxidase-enhancing activity of β -1,3-glucan binding protein

An aliquot (200 μl) of supernatant of plasma (without β GBP) and effluent from affinity column were mixed with 100 μl of laminarin-precipitable substances (5, 10, 15 or 20 μg) or 100 μl of purified β GBP (5, 10, 15 or 20 μg), respectively. To these samples, 100 μl of 0.1% laminarin were added and incubated for 10 min at 25°C . In controls, laminarin was replaced with buffer. After incubation, 2 ml of 5 mM L-DOPA (Sigma) were added and further incubated for 5 min at 25°C . The activity of phenoloxidase was measured photometrically at 480 nm and expressed as units $\text{min}^{-1} \text{ mg protein}^{-1}$.

Assay for serine protease activity

The serine protease activity in the purified β GBP was determined following the method of Perazzolo & Barracco [41]. A mixture of 300 μl of purified β GBP ($53 \text{ } \mu\text{g ml}^{-1}$) and 100 μl of laminarin (1 mg ml^{-1}) was incubated at 25°C for 15 min. To this 200 μl of 0.25 M BAPNA was added and incubated at 37°C for 30 min and the reaction terminated by the addition of 100 μl of 50% acetic acid. In controls, laminarin was replaced by tris-HCl buffer (0.25 M, pH 8.0). The released para-nitroanilide from the substrate in the sample was measured spectrophotometrically at 405 nm using Shimadzu (UV-

160A) spectrophotometer. Enzyme activity was expressed as the change in absorbance at $405 \text{ nm min}^{-1} \text{ mg protein}^{-1}$.

Assay for β -1,3-glucanase activity

A mixture of 900 μl of laminarin (1 mg ml^{-1}) and 100 μl of β GBP ($53 \text{ } \mu\text{g ml}^{-1}$) was incubated at 37°C for 15 min. After incubation, a known aliquot of reaction mixture (100 μl) was diluted with 900 μl of glass distilled water. To this 4 ml of copper reagent and 4 ml of neo-cuproine reagent were added, mixed and kept in a boiling water bath for 8 min, then cooled and again diluted using 25 ml glass distilled water [42]. Absorbency of the samples were taken at 450 nm in a spectrophotometer. Controls for the assay consisted of heat inactivated β GBP. The results are expressed optical density (O.D.) $\text{min}^{-1} \text{ mg protein}^{-1}$.

Agglutination assays

Hemagglutination, yeast and bacterial agglutination assays were performed for plasma, laminarin-precipitable substances (pellet) and affinity-purified β GBP ($53 \text{ } \mu\text{g ml}^{-1}$ initial concentration). Agglutination assays were performed in microtitre plates by 2-fold serial dilutions of test substances in TBS-II. Fresh mammalian RBCs (see Results), native and trypsinized yeast cells as well as eight bacterial species were used as indicator cells.

Agglutination-inhibition assay

The inhibitor to be tested (25 μl) was 2-fold serially diluted in the microtitre plates with an equal volume of diluted plasma ($452 \text{ } \mu\text{g ml}^{-1}$) or purified β GBP ($53 \text{ } \mu\text{g ml}^{-1}$) and incubated for 1 h at RT. Trypsinized yeast cells (25 μl) was added to each well and further incubated for 3 h at RT. Mono-, di- and polysaccharides were used as inhibitors.

Table 1 Agglutinating activity profile of various plasma fractions of *Perna viridis* against mammalian RBC types and yeast cells

RBC types and yeast cells tested	Agglutinating activity (titre)		
	Whole plasma	Supernatant ^a	Pellet ^b
Buffalo	32	0	4
Human A	16	8	0
Human B	8	0	2
Human O	4	4	0
Mice	16	8	2
Rat	16	4	0
Rabbit	16	8	0
Sheep	16	8	0
Goat	2	2	0
Ox	0	0	0
Yeast			
Native	16	0	8
Trypsinized	64	0	32

Data represent median values from 10 determinations for each RBC type and yeast cells.

^a Supernatant obtained from the clarified plasma previously incubated with laminarin.

^b Pellet (dissolved and dialysed against TBS-I) was collected after centrifugation ($25,000 \times g$, 15 min, 4°C) of supernatant previously incubated with laminarin.

Table 2 Phenoloxidase activity in different plasma fractions of *Perna viridis*

Fractions tested	Phenoloxidase activity (units min ⁻¹ mg protein ⁻¹)	
	Control ^a	Experimental ^b
Whole plasma	0.016 ± 0.002	0.022 ± 0.002
Supernatant I ^c	0.010 ± 0.002	0.014 ± 0.001
Supernatant II ^d	0.007 ± 0.001	0.012 ± 0.002
Supernatant II + pellet ^e	0.019 ± 0.001	0.026 ± 0.001

Values are given as mean ± SD from five determinations using samples from different preparations.

^a Control data were obtained after incubating plasma fractions with an equal volume of tris buffer (0.25 M, pH 6.5).

^b Experimental data were obtained after incubating plasma fractions with an equal volume of tris buffer (0.25 M, pH 6.5) containing 100 µg laminarin.

^c Supernatant I was collected after centrifugation (25,000 × g, 15 min, 4 °C) of plasma samples.

^d Supernatant II was collected after centrifugation (25,000 × g, 15 min, 4 °C) of supernatant I previously incubated with laminarin (0.1% w/v) for 4 h at 4 °C, and dialysed against TBS-I.

^e Pellet was collected after centrifugation (25,000 × g, 15 min, 4 °C) of supernatant I previously incubated with laminarin (0.1% w/v) for 4 h at 4 °C, and then dissolved and dialysed against TBS-I. The increase in PO activity in different fractions compared to control values is statistically significant ($p < 0.001$).

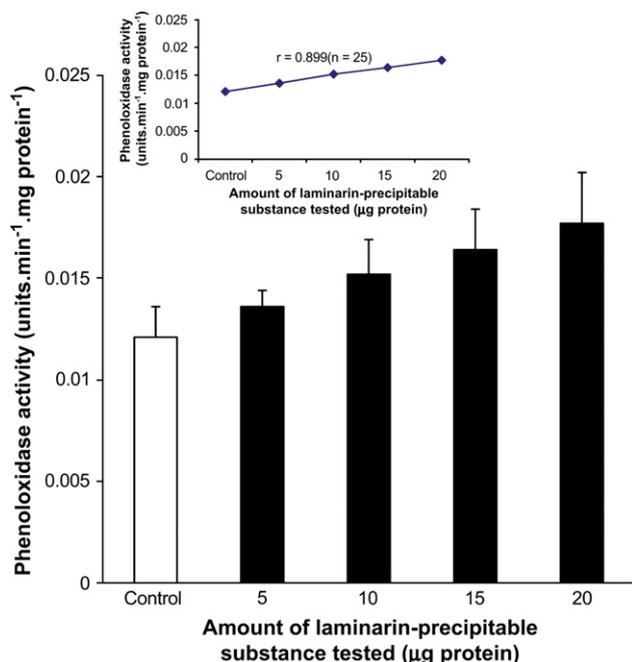


Figure 1 Dose-dependent laminarin-precipitable substance mediated enhancement of PO activity in a plasma fraction (supernatant II) of *Perna viridis*. Activation of phenoloxidase in plasma fraction (supernatant II) of *P. viridis* in response to various concentration of laminarin-precipitable substance. Vertical bars represent mean ± SD of five determinations for each using samples from different preparations.

Table 3 Inhibition of agglutinating activity (titre = 4) of plasma from the marine mussel *Perna viridis* by various carbohydrates

Carbohydrates tested	Maximum concentration tested (mM)	Minimum inhibitory concentration (mM) ^a
<i>Monosaccharides</i>		
Simple sugars		
D-Glucose	200	100
D-Mannose	200	100
Deoxy sugars		
2-Deoxy-D-glucose	200	100
Amino sugars		
D-Glucosamine (GlcN)	200	50
Mannosamine (ManN)	200	100
<i>N-acetyl sugars</i>		
N-acetyl-D-glucosamine (GlcNAc)	200	50
N-acetyl-D-mannosamine (ManNAc)	200	50
<i>Glycosides</i>		
Methyl- α -D-glucopyranoside	200	100
Methyl- β -D-glucopyranoside	200	50
Methyl- α -D-mannopyranoside	100	50
p-Nitrophenyl- α -D-glucopyranoside	50	25
p-Nitrophenyl- β -D-glucopyranoside	100	50
<i>Disaccharides</i>		
D-Maltose (glc α 1 → 4 glc)	200	100
D-Cellobiose (glc β 1 → 4 glc)	200	100
β -D-Gentiobiose (glc β 1 → 6 glc)	200	50
Sucrose (glc α 1 → 2 fru)	200	100

The following carbohydrates did not inhibit the agglutinating activity and unless otherwise stated, all carbohydrates were tested at concentrations up to 200 mM: D-galactose, L-sorbose (100 mM), β -allose, D-fructose (100 mM), D-fucose (100 mM), L-fucose, 2-deoxy-D-glucose, 2-deoxy-D-galactose, D-glucuronic acid, D-galacturonic acid, D-galactosamine (GalN), N-acetyl-D-galactosamine (GalNAc) 100 mM, methyl- α -D-galactopyranoside, methyl- β -D-galactopyranoside (100 mM), p-nitrophenyl- α -D-galactopyranoside (50 mM), D-trehalose (glc α 1 → 1 glc), palatinose (glc α 1 → 6 fru), D-melibiose (gal α 1 → 6 glc), and D-lactose (gal β 1 → 4 glc).

^a The assay was repeated five times for each carbohydrate with identical results.

Statistical analysis

Each experiment was performed 3 to 10 times using samples from different preparations. Differences between control and experimental values were statistically analysed for significance by the paired-sample Student *t*-test.

In dose response study, the relationship between different concentrations of β GBP and the level of L-DOPA oxidation by effluent from affinity column was tested by Karl Pearson's correlation coefficient (r). All these statistical analyses were performed following Ref. [43].

Results

Isolation and purification of β GBP

Isolation of β GBP

The β -1,3-glucan binding protein was isolated from mussel plasma by two different methods. The first method was to precipitate β GBP using laminarin. This affinity-precipitated protein in the pellet, unlike plasma, did not agglutinate most of the RBCs tested, but by contrast it could agglutinate yeast at a titre of 8 (native yeast) and 32 (trypsinized yeast). This yeast agglutination reaction of affinity-precipitated protein was comparable to plasma (Table 1). Interestingly, this affinity-precipitated protein led to a significant increase ($p < 0.001$) in PO activity of supernatant II obtained after laminarin affinity precipitation (Table 2). As shown in Fig. 1, an increase in the protein concentration of laminarin-precipitable substances from 5 to 20 μ g, resulted in a linear increase ($r = 0.899$) in PO activity of supernatant. These results suggest the possible presence of β -1,3-glucan binding protein in laminarin affinity precipitable substances present in the mussel plasma. The yeast agglutination-inhibition assays performed with 34 carbohydrates (Table 3), showed that many diverse types of carbohydrates could inhibit yeast agglutinating activity of plasma at concentrations ranging from 25 to 200 mM. On other hand among the six polysaccharides tested (Table 4), only laminarin effectively inhibit yeast-agglutinating activity of plasma at a concentration as low as 0.0625 mg ml⁻¹.

Purification of β GBP

In a second method, β -1,3-glucan binding protein was purified from 110 ml of mussel plasma by affinity chromatography on laminarin-Sepharose 6B (Fig. 2). The protein bound to the matrix was eluted using 10 ml acetate buffer (pH 4.5) containing 2.5 M MgCl₂ and 1 M D-glucose which emerged as a sharp symmetrical peak with absorbance at 280 nm as well as a coincident peak of agglutinating activity

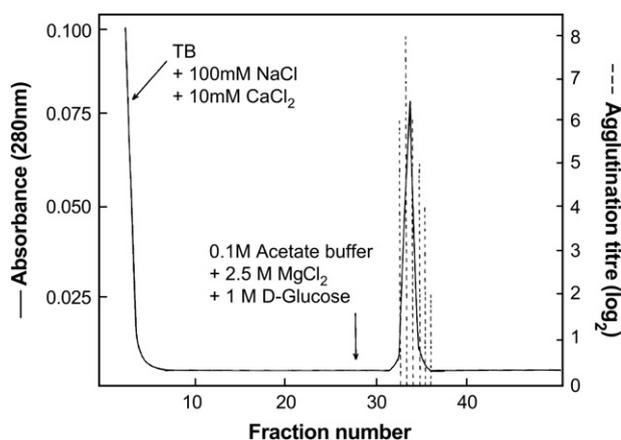


Figure 2 Purification of *Perna viridis* plasma β GBP by affinity chromatography. Dilute plasma (110 ml with a titre of 16 against trypsinized yeast cells) was applied to laminarin-Sepharose 6B column (0.8×1.6 cm). After reequilibrating the column with TBS-II, the bound protein was eluted with acetate buffer (0.1 M, pH 4.5) containing 2.5 M MgCl₂ and 1 M glucose. The absorbency of the effluent was continuously monitored at 280 nm and 1 ml fractions were collected, dialysed and assayed for agglutinating activity against trypsinized yeast cells.

(titre = 256) against trypsinized yeast cells. As summarized (Table 5), the chromatographic separation of β GBP yielded a 90-fold increase in specific yeast-agglutinating activity compared to the initial sample.

Electrophoretic and HPLC analyses

Purified β -1,3-glucan binding protein migrated as a single band in native PAGE (Fig. 3) and this homogeneity was confirmed using reversed phase HPLC with C₁₈ column, wherein the purified protein emerged as a single prominent symmetric peak with a retention time of 27.93 min (Fig. 4). Under reducing conditions purified β GBP dissociated into nine protein bands with a total subunit molecular mass estimate of 510 kDa (Fig. 3). In isoelectric focusing, the purified protein preparation focused as a single band at pI corresponding to 5.3 (Fig. 5).

Circular dichroism assay

In order to understand the secondary structure of the purified β GBP, circular dichroism spectra were recorded

Table 4 Agglutination-inhibition of *Perna viridis* plasma (agglutination titre = 4) by polysaccharides against trypsinized yeast cells

Polysaccharides tested	Maximum concentration tested (mg ml ⁻¹)	Structural linkages	Minimum inhibitory concentration (mg ml ⁻¹) ^a
Laminarin	1	β -1,3 Homopolymer of glucose	0.0625
Mannan	1	α -1,6 Homopolymer of mannose	0.125
Dextran T ₇₀	2	α -1,6,3,2 Homopolymer of glucose	0.5
Dextran T ₅₀₀	2	α -1,6,3,2 Homopolymer of glucose	0.5
Inulin	3	α -2,6 Homopolymer of fructose	—
Colomonic acid	5	α -2,8 Homopolymer of Neu5Ac	—

— Indicates no inhibition.

^a The assay was repeated three times for each polysaccharide with identical results using samples from different preparations.

Table 5 Summary of purification of β -1,3-glucan binding protein from the plasma of *Perna viridis*

Sample	Volume (ml)	Agglutination titre	Total protein (mg)	Total activity (YA units)	Specific activity (YA units mg protein ⁻¹)	Purification (-fold)
Diluted plasma	110	16	32.45	70,400	2.1×10^3	1
Eluate from affinity chromatography on laminarin-Sepharose 6B	6	256	0.318	61,449	1.9×10^5	90

Data represent the mean values from 30 preparations and represented as median titre values. One unit of activity is defined as the minimum amount of protein required to give one well agglutination of trypsinized yeast cells YA = yeast agglutination.

in TBS-II (pH 7.5) in the range from 195 to 300 nm. The spectrum showed a broad negative minimum at ~ 225 nm and a cross-over at ~ 205 nm. The low positive ellipticity values below 205 nm clearly suggest the presence of unordered segments in the protein. The broad negative minimum extending from 217 to 227 nm can be attributed to the presence of β -sheet structure (Fig. 6).

Functional analyses

proPhenoloxidase activity of β GBP

Purified β -1,3-glucan binding protein significantly ($p < 0.001$) enhanced the PO activity of effluent (flow-through plasma fractions from affinity, chromatography) compared to effluent alone (Table 6). In addition, increase in concentration of purified β GBP from 5 to 20 μ g led to a corresponding

increase in PO activity of effluent containing laminarin ($r = 0.789$; Fig. 7).

Agglutinating assays

Hemagglutinating activity revealed that unlike plasma, β GBP at a concentration of 53 μ g ml⁻¹ could agglutinate buffalo, human B, mice and rat RBC only, whereas it failed to show any agglutination of other mammalian RBC types including human A, human O, rabbit, sheep, goat and ox RBC. On the other hand, purified β GBP (53 μ g ml⁻¹) gave a high agglutination titre against both native (64) and trypsinized yeast (256) cells. Interestingly, purified β GBP was found to agglutinate five species of bacteria as against eight with whole plasma (Table 7).

Agglutination-inhibition assays

Yeast agglutination-inhibition assays performed with 34 carbohydrates revealed that none of them could inhibit yeast agglutinating activity of purified β GBP even at concentrations up to 200 mM (data not shown). On the other hand, among the six polysaccharides tested (Table 8) only laminarin, a β -1,3-glucan, inhibited the yeast agglutinating activity of purified β GBP at a concentration as low as 0.0312 mg ml⁻¹.

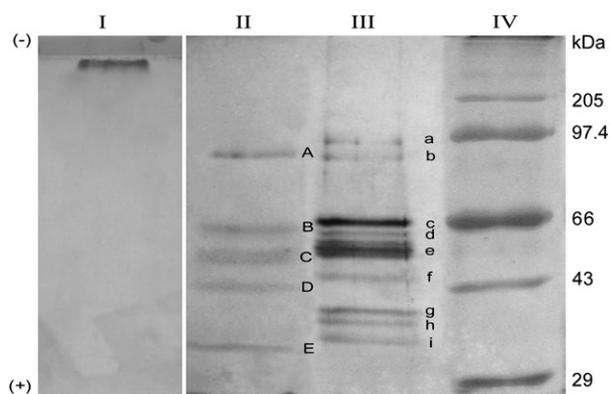


Figure 3 Analysis of β GBP isolated from the plasma of *Perna viridis* on discontinuous polyacrylamide gel electrophoresis (PAGE) under non-denaturing as well as denaturing and reducing conditions. Lane I: Protein profile of β GBP (25 μ g) in native-PAGE. Lane II: Analysis of β GBP (50 μ g protein) isolated from the plasma of *P. viridis* in SDS-PAGE. Upon treatment with SDS alone (lane II), the β GBP appeared as five protein bands (A–E) with a total molecular mass estimate of 270 kDa. Lane III: The β GBP (50 μ g protein) was denatured and reduced with 2% SDS and 2.5% β -mercaptoethanol (final concentrations), respectively. The β GBP, after treatment with SDS and β -mercaptoethanol (lane III), disassociated into nine protein bands (a–i) with a total molecular mass estimate of 510 kDa. Position of molecular weight markers is shown in lane IV. Myosin from rabbit muscle (205 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa). All proteins were stained with silver nitrate.

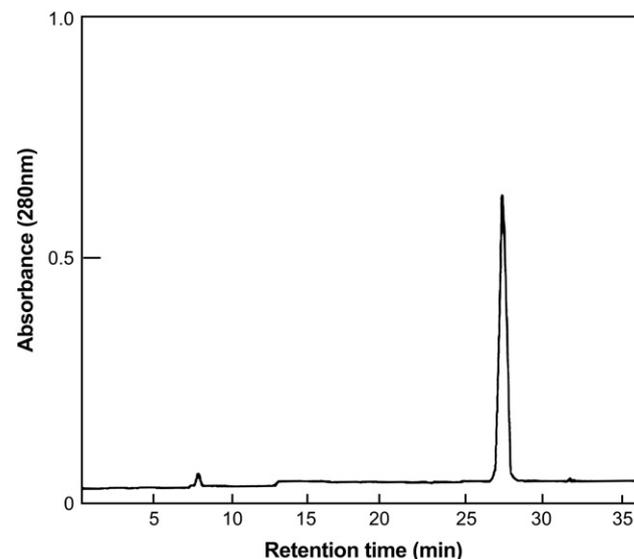


Figure 4 HPLC analysis of β GBP isolated from the plasma of *Perna viridis*. The isolated β GBP was analysed using a reversed phase C₁₈ column and the isolated protein emerged as a single peak with a retention time of 27.93 min.

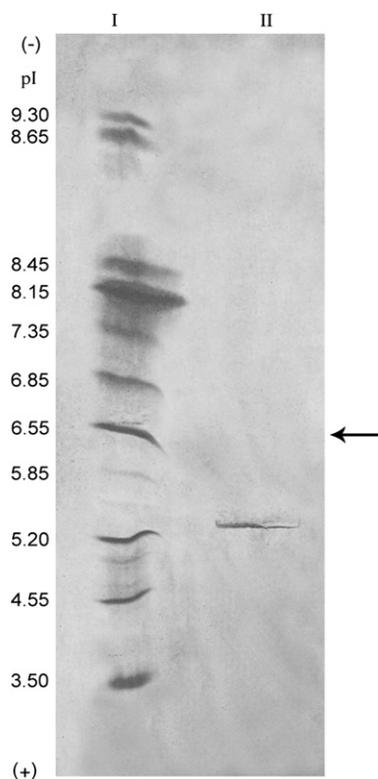


Figure 5 Analytical isoelectric focusing of purified β GBP of *Perna viridis*. Arrow indicates the point of application of standards (each 7 μ l). Focussing was done for 2.30 h on a pH gradient gel. Lane I: Ampholine standard mixture containing the following marker proteins. Amyloglucosidase (pI 3.50), soybean trypsin inhibitor (4.55), β -lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin acidic band (6.85), horse myoglobin basic band (7.35), lentil lectin-acetic bands (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65), and trypsinogen (9.30). Lane II: Purified β GBP (1.4 mg ml⁻¹).

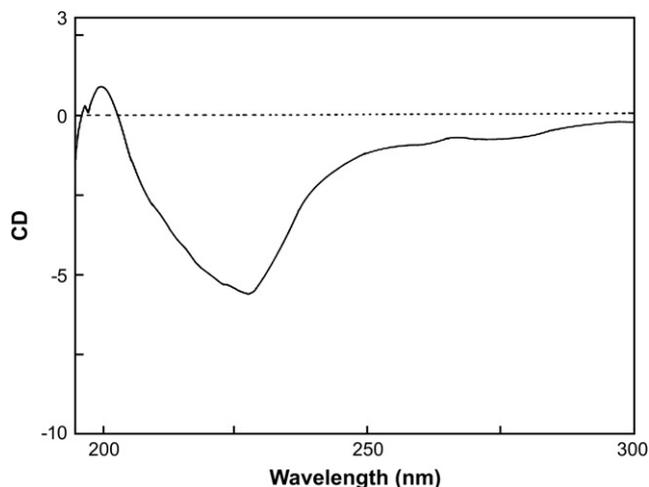


Figure 6 Circular dichroism spectroscopy of β GBP. CD spectra were obtained for β GBP using a Jasco J-720 spectropolarimeter.

Table 6 Effect of purified β GBP (53 μ g ml⁻¹) on the activation of proPO in the effluent of *Perna viridis*

Fractions tested	Phenoloxidase activity (units min ⁻¹ mg protein ⁻¹)	
	Control ^a	Experimental ^b
Effluent	0.038 \pm 0.013	0.056 \pm 0.017 ^c
Effluent + β GBP	0.066 \pm 0.021	0.123 \pm 0.024 ^c

Values are given as mean \pm SD from five determinations using samples from different preparations.

^a Control data were obtained after pre-incubating effluent (flow-through fractions of dilute plasma collected during affinity purification of β GBP) with an equal volume of tris-HCl buffer (pH 6.5).

^b Experimental data were obtained after pre-incubating effluent with an equal volume of tris buffer (pH 6.5) containing laminarin (100 μ g).

^c The increase in PO activity compared to control values is statistically significant ($p < 0.001$).

Serine protease and β -glucanase activity

Serine protease activity of purified β GBP preincubated with laminarin increased by 45-fold ($p < 0.001$) compared to control (Table 9). On the other hand, neither β GBP nor the effluent showed β -1,3-glucanase activity (Table 10).

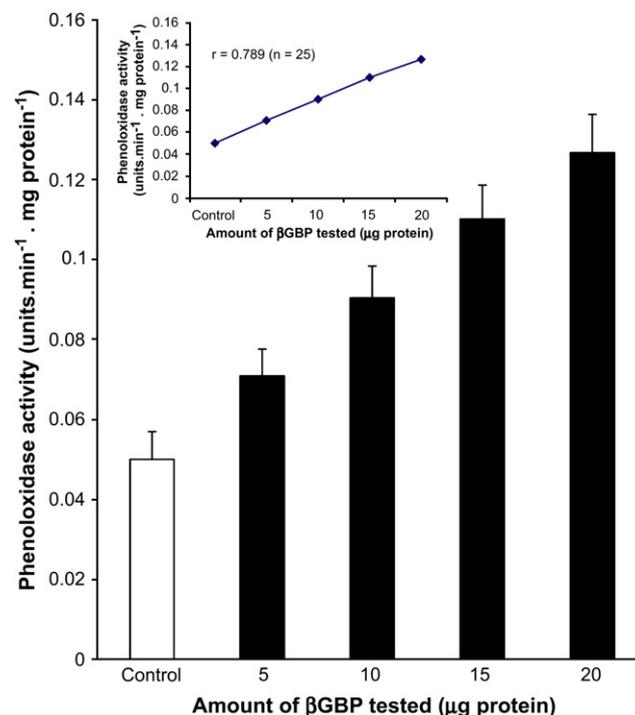


Figure 7 Effect of different concentrations of purified β GBP on the enhancement of phenoloxidase activity in the effluent of *Perna viridis*. The phenoloxidase activity in effluent (200 μ l) was tested by the addition of different concentrations of isolated β GBP 100 μ l, dissolved in TBS-II and 100 μ l laminarin (100 μ g) and incubated for 5 min at 25 $^{\circ}$ C. Effluent represents fractions without β GBP. Vertical bars represent mean \pm SD of five determinations for each test using samples from different preparations.

Table 7 Agglutination of various bacterial species by the plasma and purified β GBP (53 $\mu\text{g ml}^{-1}$) of *Perna viridis*

Bacterial species tested	Agglutination titre ^a	
	Plasma	Purified β GBP
<i>Vibrio fluvialis</i>	8	4
<i>Vibrio alginolyticus</i>	4	2
<i>Vibrio parahaemolyticus</i>	4	2
<i>Vibrio vulnificus</i>	4	2
<i>Vibrio anguillarum</i>	2	0
<i>Vibrio mimicus</i>	2	0
<i>Pseudomonas</i> sp.	2	0
<i>Escherichia coli</i>	4	2

^a The assay was repeated five times for each bacterial species with identical results using samples from different preparations.

Discussion

This study reports for the first time in a bivalve mollusc, the occurrence, purification and functional characterization of β -1,3-glucan binding protein in the plasma of *P. viridis* and this protein after binding to β -1,3-glucans mediates different defense reactions in this mussel. Studies conducted previously in our laboratory have shown that the plasma of *P. viridis* contains PO in its inactive form (proPO), which could be activated by laminarin (a polymer of β -1,3-glucan) through serine protease in a dose-dependent manner, thereby indicating the possible presence of β -1,3-glucan binding substances in the plasma of *P. viridis* [34]. Hence in the present study, laminarin-precipitable substance isolated from the plasma was tested for its ability to mediate the laminarin-stimulated activation of proPO system in plasma. Indeed, these substances enhance the laminarin-activable proPO activity by ~116% in the supernatant in a dose-dependent manner. These results clearly show the presence of β -1,3-glucan binding proteins in *P. viridis* plasma, and the laminarin-stimulated activation of proPO system appears to involve binding of laminarin with β GBP, a pre-requisite for activation of non-self mediated proPO system, thereby it serves as a natural defense mechanism against invading non-self as demonstrated in a few crustaceans and insects [12,21,25,27,29].

Table 9 Serine protease activity in the purified β GBP from plasma of *Perna viridis*

Sample	Serine protease activity (OD min ⁻¹ mg protein ⁻¹ × 10 ³)
Control ^a	2.60 ± 1.16
Experimental ^b	93.62 ± 4.87

Values are given as mean ± SD from five determinations using samples from different preparations.

^a Control samples consisted of the substrate (BAPNA: 33 μg), tris-HCl buffer (pH 8.0) and purified β GBP (16 μg).

^b Experimental samples consisted of the substrate (BAPNA: 33 μg), laminarin (100 μg) and purified β GBP (16 μg). The increase in serine protease activity in different fractions compared to control values are statistically significant ($p < 0.001$).

The laminarin-precipitable substances in the mussel plasma were tested for yeast agglutinating activity, since a few earlier studies have shown the presence of yeast agglutinating molecules in the blood of insects and crustaceans by affinity precipitation technique using laminarin [5,25,36] or curdlan [29,44]. Our results with laminarin-affinity precipitation showed enrichment of yeast agglutinating activity in the pellet. In addition, the substances obtained from the pellet failed to show any agglutinating activity against most of the mammalian RBC types tested. Thus, there exists an interrelationship between two humoral components of the defense system of molluscs, namely, proPO system and yeast agglutinating molecules and this has been shown in a few previous studies [28,36].

A number of earlier studies have adopted multi-step chromatographic procedure to isolate β GBP from diverse invertebrate animals [18,20,21,24,27,36,45]. In the present study, we have successfully isolated, for the first time, β GBP from the plasma of *P. viridis* by a single step affinity chromatography using laminarin-Sepharose 6B as affinity matrix, and it resulted in high purity and homogeneity of the isolated β GBP as revealed by electrophoretic and HPLC analyses. The success of this procedure was mainly due to the empirically formulated elution strategy which yielded β GBP not only with the desired purity but also 90-fold purification and 87% recovery from the starting plasma sample. As observed with the whole plasma, the purified β GBP showed a strong yeast agglutinating activity, thereby

Table 8 Agglutination-inhibition of purified β GBP from plasma of *Perna viridis* (agglutination titre = 4) by polysaccharides against trypsinized yeast cells

Polysaccharides tested	Maximum concentration tested (mg ml ⁻¹)	Structural linkages	Minimum inhibitory concentration (mg ml ⁻¹) ^a
Laminarin	1	β -1,3 Homopolymer of glucose	0.0312
Mannan	1	α -1,6 Homopolymer of mannose	—
Dextran T ₇₀	2	α -1,6,3,2 Homopolymer of glucose	—
Dextran T ₅₀₀	2	α -1,6,3,2 Homopolymer of glucose	—
Inulin	3	α -2,6 Homopolymer of fructose	—
Colominic acid	5	α -2,8 Homopolymer of Neu5Ac	—

— Indicates no inhibition.

^a The assay was repeated three times for each polysaccharide with identical results using samples from different preparations.

Table 10 β -1,3-Glucanase activity in the purified β GBP from the plasma of *Perna viridis*

Sample	β -1,3-Glucanase activity (OD min ⁻¹ mg protein ⁻¹)	
	Control ^a	Experimental ^b
Isolated β GBP	2.345 \pm 0.408	2.431 \pm 0.408
Effluent	0.330 \pm 0.010	0.460 \pm 0.003

Values are given as mean \pm SD from five determinations using samples from different preparations.

^a In controls, heat-treated (100 °C, 5 min) β GBP samples were used.

^b The experimental sample consisted of incubation of β GBP (5.3 μ g) with substrate (laminarin, 900 μ g) for 15 min at 37 °C. The reaction was stopped by heating the mixture (100 °C, 5 min) and cooled. To 0.1 ml of this aliquot copper–neocuprine reagent was added and β -1,3-glucanase activities were measured. The increase in β -1,3-glucanase activity in different fractions compared to control values are statistically significant ($p < 0.001$).

representing almost the entire humoral yeast agglutinin of the bivalve mollusc *P. viridis*.

The purified β GBP under denaturing conditions was found to be composed of nine protein subunits, with total molecular mass estimate of 510 kDa and with a *pI* of 5.3. Although the native molecular mass of mussel β GBP is quite different from those isolated from most other invertebrates [22,25,44], its close similarity to the β GBP of an insect, *Blaberus discoidalis*, with a molecular mass of 520 kDa [27] could only be ascertained by comparison of amino acid composition and sequence analyses. Our preliminary studies on the CD spectra of purified β GBP, suggest the presence of predominantly β -sheets in the protein structure [46].

The β -1,3-glucan binding protein was found to strongly enhance activation of prophenoloxidase from the effluent of the affinity column in the presence of laminarin in a dose-dependent manner. Such an enhancement of proPO activation by purified β GBP has been reported in other invertebrates too [12,24,25,27,28,36] and similar effect has also been shown with homologous lectins in insects [47]. The mussel β GBP specifically binds to only laminarin, as revealed by inhibition of yeast agglutinating activity by various carbohydrates, and this property was also supported by a strong yeast agglutinating activity of purified β GBP. Thus the present study reports, for the first time, a strong yeast agglutinating activity of purified β GBP in a higher invertebrate animal.

The conversion of inactive proPO to active PO by non-self molecule is mediated by serine protease that causes limited proteolysis of the inactive zymogen [48]. Serine protease activity for isolated β GBP has been reported in chelicerates, crustacean and insects [20,36,40,47,49]. In the present study the purified β GBP was found to contain inherent serine protease activity that catalyzes the proteolytic conversion of proPO to PO upon binding to non-self molecules such as laminarin. Presence of yeast agglutinating activity and PO enhancing activity in β GBP suggests that this protein is bifunctional. By contrast, purified β GBP was found to lack β -1,3-glucanase activity,

while this activity detectable in whole plasma. Thus, all these observations clearly show the functional significance of the presence of β GBP as a component of the humoral immune system of molluscs.

Plasma from *P. viridis* was found to agglutinate all the eight bacterial species tested, whereas purified β GBP agglutinated only five species of bacteria. This differential profile of bacterial agglutinating activity of plasma and purified β GBP shows the possible presence of other bacterial agglutinins in the hemolymphatic system of *P. viridis*. The reactivity of the mussel β GBP with restricted specificity as observed in agglutination of bacterial species suggests that it may function as a pattern recognition molecule [26,29].

In conclusion, our study demonstrates that plasma β GBP may be a bifunctional molecule capable of agglutinating selective microorganisms and activating plasma proPO system. Thus β GBP from the plasma of *P. viridis* appears to contain at least two functional domains, whose physico-chemical and functional characteristics need further elucidation. This study also envisages that activation of proPO by purified β GBP closely resembles the complement activation in vertebrates through mannan binding lectin pathway [50,51]. This implicates, in spite of phylogenetic non-relatedness, the evolutionary link between two such systems as found in invertebrates and vertebrates.

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