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A LIPOPOLYSACCHARIDE-BINDING AGGLUTININ ISOLATED FROM BROWN SHRIMP (*PENAEUS CALIFORNIENSIS* HOLMES) HAEMOLYMPH

FRANCISCO VARGAS-ALBORES, MARÍA-ANTONIA GUZMÁN and JOSÉ-LUÍS OCHOA
Center of Biological Research, Division of Experimental Biology, P.O. Box 128, La Paz, B.C.S. 23000, México (Tel. 682 5-3633; Fax 682 5-3625)

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Abstract—1. Haemolymph from the brown shrimp (*Penaeus californiensis* Holmes) agglutinates erythrocytes of different vertebrate species.

2. A fraction containing haemagglutinating activity was isolated by affinity chromatography. The corresponding protein showed a molecular weight of 175 kDa, formed by four subunits.

3. The haemagglutinin is inhibited by GalNAc, NANA, fetuin and bacterial lipopolysaccharide.

4. Here we demonstrate the ability of this agglutinin to react with different *Vibrio* strains, and the inhibitory capacity of LPS in this reaction.

INTRODUCTION

Many humoral and cellular proteins with haemagglutinating activity have been isolated from various animals (Marchalonis and Edelman, 1968; Hammarström and Kabat, 1969; Baldo *et al.*, 1978; Komano *et al.*, 1980; Yeaton, 1981; Ashwell and Harford, 1982; Barondes, 1984; Umetsu *et al.*, 1984; Ravindranath *et al.*, 1985; Giga *et al.*, 1985; Qu *et al.*, 1987; Drickamer, 1988; Richards and Renwrantz, 1991). Most of these proteins are carbohydrate-binding proteins and, as such, can be defined as animal lectins. These proteins are usually detected by their ability to agglutinate untreated and enzyme-treated vertebrate erythrocytes yet, so far, their biological role has not been determined.

As invertebrates do not synthesize specific antibodies (Lackie, 1980), and since a number of haemagglutinins have been isolated from their haemolymph and haemocytes, these proteins are presumed to participate in their defence against invading microorganisms or infections. Several haemagglutinins have been detected on the haemocyte surface (Amirante and Mazzalai, 1978; Vasta *et al.*, 1984; Cassels *et al.*, 1986; Parrinello and Arizza, 1989; Azumi *et al.*, 1991; Richards and Renwrantz, 1991) suggesting that they act as receptors which bind directly to the surface sugars of foreign particles.

The interaction of invertebrate agglutinins with bacteria has been poorly established. Renwrantz (1986) indicated that although the precipitation of bacteria occurs under experimental conditions, it is difficult to explain how infection leads to sufficient bacterial density required for agglutination under natural conditions. Thus, it has been proposed that the most important role of invertebrate agglutinins in bacterial elimination is phagocytosis, rather than agglutination or precipitation. Hence these proteins seem to have an important role in self/non-self recognition, independent of any elimination mechanism in which agglutinin participates.

Most invertebrate agglutinins have been detected and identified using the haemagglutination assay but, this does not define the role of these proteins in the defence against bacteria. Research into how these agglutinins react with bacteria or bacterial surface components could provide more information about their physiological role.

In this paper, we report on the isolation of an agglutinin from the brown shrimp (*P. californiensis*) cell-free haemolymph, which can bind bacterial lipopolysaccharide (LPS). In addition, we describe its main molecular properties, its interaction with bacteria, and its inhibition by sugars, glycoproteins and bacterial LPS.

MATERIALS AND METHODS

Haemolymph extraction

Shrimp (*Penaeus californiensis*) of both sexes were collected from San Carlos Bay, BCS, México, and maintained in an aquarium (24 ± 4°C, salinity 36‰). Only the haemolymph from intermoult, apparently healthy, shrimps was used. To avoid coagulation, 2–3 parts of anticoagulant solution (450 mM NaCl, 10 mM KCl, 10 mM EDTA Na_2, 10 mM HEPES, pH 7.3, 850 mOsm/kg) were used. In this solution, the pH, osmolality, sodium and potassium concentrations were equivalent to shrimp haemolymph (Vargas-Albores and Ochoa, 1992). The haemolymph (50–200 μl) was obtained as previously described (Vargas-Albores and Ochoa, 1992) from the pleopod base of the first abdominal segment. The pooled samples were centrifuged for 10 min (10°C) at 2000 ***g***.

The cell pellet was discarded, and the supernatant (plasma) was frozen until use.

Haemagglutinating assay

The human and animal blood samples (except from the mouse) were obtained by venous puncture, then collected and stored in sterile Alsever's solution. The mouse blood was obtained by cardiac puncture and stored in the same way. Before use, the cells were washed twice by centrifugation (800 *g*, 10°C, 10 min) with saline solution (NaCl 0.15 M) and twice with TBS–Ca (Tris buffered saline: 50 mM Tris–HCl, 100 mM NaCl, pH 7.5, plus 10 mM $CaCl_2$). Finally, the red blood cells (RBC) were suspended to 2% (v/v) in TBS–Ca. The haemagglutination assays were performed on U-plates (Falcon). Two-fold serial dilutions of the shrimp serum were made in TBS–Ca then an equal volume (25 μl) of a 2% suspension of erythrocytes was added. The plates were incubated at room temperature (26 $\pm$ 2°C) for 1 hr. The control was the substitution of shrimp serum by TBS–Ca. The agglutination titre was recorded as the reciprocal of the last dilution, giving evidence of agglutination at 1 hr of incubation. The haemagglutinating unit is referred to as the minimum protein concentration (mg/ml) required for positive haemagglutination.

Preparation of asialofetuin

A solution (10 mg/ml) of fetuin was hydrolysed with 0.1 N H_2SO_4 in 0.15 M NaCl, at 80°C for 1 hr, according to Spiro (1960). The asialofetuin was extensively dialysed against TBS–Ca.

Isolation of the haemagglutinin

The shrimp serum was dialysed overnight against TBS containing 1 mM $CaCl_2$, and clarified by centrifugation at 40,000 *g* (20 min, 10°C). Eight millilitres of serum were applied to a column (5 ml) of *N*-acetyl-galactosamine–agarose (Pierce) previously stabilized with TBS + 1 mM $CaCl_2$. The column was washed with the same buffer until the optical density was zero. The retained material was eluted with TBS–EDTA (Tris-buffered saline plus 5 mM EDTA Na_2). One millilitre fractions were collected and analysed by optical density at 280 mm, and by haemagglutinin activity against both human and mouse erythrocytes. The fractions with anti-human activity were pooled and concentrated by lyophilization and applied to a column (1 × 56 cm) of Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden). The gel-filtration was run at 5 ml/hr, and fractions of 0.5 ml were collected. The fractions were analysed for haemagglutinating activity and pooled.

Effect of calcium

The purified haemagglutinin was dialysed overnight (at 4°C) against TBS–EDTA. Next, the sample was completely re-dialysed against TBS (without Ca^{2+} or EDTA). The remaining haemagglutinating activity was then determined in the presence or absence of 10 mM $CaCl_2$.

Molecular weight determination

The apparent molecular weight of the native protein was estimated by gel-filtration performed at 18°C on a column (1 × 56 cm) of Sepharose 6B. Polyacrylamide gel electrophoresis (PAGE) was used to estimate the molecular weight of the native and denatured (Weber and Osborne, 1969) protein. Protein bands were developed with a silver-stain kit (Sigma).

Chemical analysis

The total protein content was measured according to Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

Inhibition of the haemagglutination

The haemagglutination inhibition assay was done as follows. Twenty-five microlitres of inhibitor solution (in TBS–Ca) were incubated for 1 hr at room temperature with an equal volume of the agglutinin solution having a haemagglutination titre of 1:4. Then, 50 μl of a 2% human erythrocyte were added. The inhibitory capacity was given in terms of the concentration needed to decrease the haemagglutination titre to zero. The following sugars were tested: L-fucose (Fuc), D-galactose (Gal), *N*-acetyl D-galactosamine (GalNAc), D-glucose (Glc), *N*-acetyl D-glucosamine (GlcNAc), D-lactose (Lac), D-mannose (Man), and *N*-acetyl neuraminic acid (NANA). The glycoproteins tested were: fetuin, asialofetuin, porcine, mucin, bovine submaxillary mucin, orosomucoid, and human transferrin. The inhibitory capacity of the lipopolysaccharide from *E. coli* (Sigma) was also assayed.

Bacteria

Three bacterial strains were used in this experiment: *Vibrio parahaemolyticus* (ATCC 13456), *V. vulnificus* (ATCC 45671) and *V. fisheri* (ATCC 2987). In all experiments, the bacteria were grown in marine medium 2216 (Difco, Detroit, MI) for 18 hr at 28°C, 120 rpm. To maintain homogeneity in the cultures, the inoculum was one-tenth of the total culture volume of a bacterial suspension with an optical density of 1 at 580 nm. The bacteria were washed three times by centrifugation (30,000 *g*, 10°C, 30 min) using MSS (Marine Salt Solution: NaCl 450 mM, HEPES 10 mM, pH 7.3). After this, the bacterial pellet was suspended in MSS containing 2.5% (v/v) of glutaraldehyde and incubated under agitation for 3 hr. The reaction was suspended by washing the bacteria with MSS, and the residual aldehyde was blocked by incubating overnight with glycine (1 M). No viable bacteria were observed after this treatment. Before using, the bacteria were washed with MSS and their concentration adjusted (1.0 A_{580} unit).

Table 1. Haemagglutinating activity of the brown shrimp haemolyph against human and other animals erythrocytes

Erthrocytes	Titre	R.H.A
Human A-type	1:16	1.00
Human B-type	1:16	1.00
Human O-type	1:16	1.00
Cow	1:2	0.25
Duck	1:2	0.25
Horse	0	0.0
Mouse	1:256	16.00
Rabbit	1:8	0.50
Rat	1:2	0.25
Sheep	1:2	0.25

Titre = inverse of maximum dilution which gives positive agglutination; RHA = relative haemagglutinating activity (human = 1.0).

Sedimentation of bacteria

The ability of the isolated agglutinin to interact with bacteria was tested by determining the rate of sedimentation. One-millilitre cuvettes were filled with 850 μl of MSS, 50 μl of brown shrimp agglutinin and 100 μl of glutaraldehyde-treated bacteria suspension. When inhibitors were used, 50 μl of each were added 15 min before the bacteria were introduced. The results are expressed as the percentage of the difference in optical density versus time.

RESULTS

Haemagglutinating activity of the plasma

The cell-free plasma (obtained using an anticoagulant solution) from brown shrimp (*Penaeus californiensis*) showed important haemagglutinating activity against erythrocytes from several vertebrate species (Table 1). As with other invertebrate agglutinins, the shrimp haemolymph showed different reactivity with the vertebrate erythrocytes tested, but was incapable of differentiating between human AB0 types. The major reactivity of the haemolymph was against mouse erythrocytes (16 times more than human RBC), while no activity against horse RBC was detected. For the other vertebrate RBCs, haemagglutinating activity was less than for human RBCs.

Purification of the agglutinin

Since the haemagglutinating activity of the brown shrimp haemolymph was inhibited partially by GalNAc and fetuin, a GalNAc–agarose column was used for its purification. Two fractions with haemagglutinating activity were detected (Fig. 1). The unretained fraction (Fraction A) contained the major part of the haemolymph proteins and haemagglutinating activity against the mouse, but not against the human erythrocytes. The EDTA-eluted fraction (Fraction B) showed haemagglutinating activity against both human and mouse RBC. This last fraction, designated as BSH-I, was applied to a Sepharose 6B column (1 × 56 cm) to separate minor contaminants. The results are summarized in Table 2, where the degree of purification is shown.

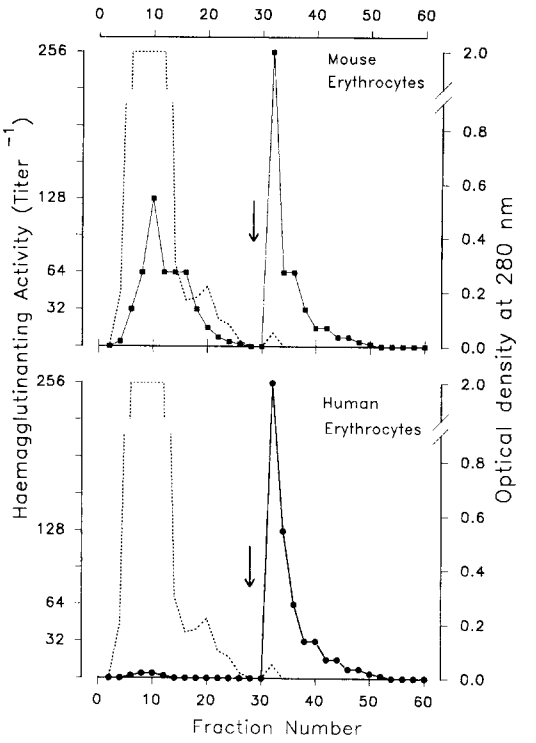

Fig. 1. Affinity chromatography of brown shrimp (*P. californiensis*) haemolymph on GalNCa–agarose column (5 ml). The column was washed with TBS–Ca until the absorbance became negligible, then the eluting buffer was changed to TBS–EDTA at the point indicated by the arrow. For each fraction, absorbance at 280 nm (dashed line) and haemagglutinating activity (solid line) was tested against both human and mouse erythrocytes.

Molecular weight of haemagglutinin

The apparent molecular weight of the purified native brown shrimp haemagglutinin (BSH-I) was 180 kDa, as estimated by gel-filtration (Fig. 2). A comparable result (170 kDa) was obtained by non-

Table 2. Purification of the brown shrimp (*Penaeus californiensis*) agglutinin

Fraction	Protein (mg/ml)	Volume (ml/	Mouse erythrocytes Titre	HAU	P.F.	Human erythrocytes Titre	HAU	P.F.
Haemolymph	42.75	8.0	1:2048	20.87	1.00	1:128	33.99	1.00
H. dialysed	30.06	8.0	1:2048	14.67	1.42	1:128	234.80	1.42
Fraction A	18.33	13.5	1:512	35.80	0.58	1:4	4582.50	0.10
Fraction B	0.06	15.0	1:128	0.44	47.40	1:64	0.89	375.26
Sepharose	0.10	6.0	1:256	0.39	53.50	1:128	0.78	428.19

HA unit = Haemagglutinating unit is defined as the amount of protein (μg) required to give one well of haemagglutination. P.F. = purification factor.

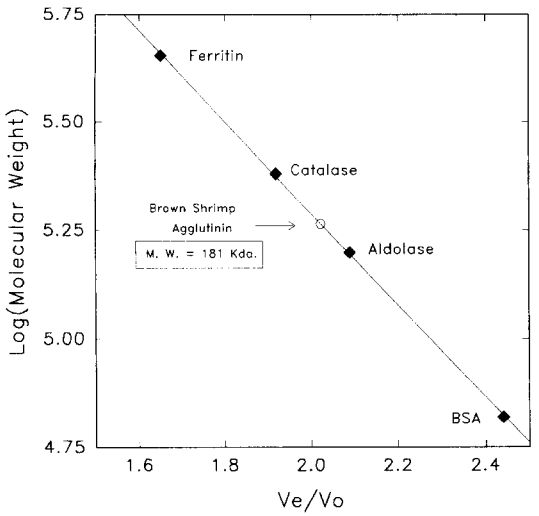

Fig. 2. Molecular weight of brown shrimp agglutinin was determined using a Sepharose column. Molecular marker (Pierce), samples and column were previously stabilized with TBS–Ca. The molecular markers were detected by absorbance at 280 nm, while the agglutinin was detected by haemagglutination.

denaturing PAGE (not shown). In addition, the apparent molecular weight of the dissociated agglutinin, estimated by SDS–PAGE under non-reducing conditions, yielded a single protein band of about 85 kDa (Fig. 3), but under reducing conditions, the only band observed showed an apparent molecular weight of 41 kDa. Thus, it is apparent that this agglutinin is built up by four subunits of similar molecular weight, but linked in two different ways to one another.

Effect of calcium

The BSH-I was dialysed against TBS–EDTA and TBS (EDTA and Ca^{2+}-free). When a Ca^{2+}-free buffer was used for the haemagglutination assay, no haemagglutinating activity was shown. However, when 10 mM $CaCl_2$ were added to the assay, the haemagglutinin regained its original activity.

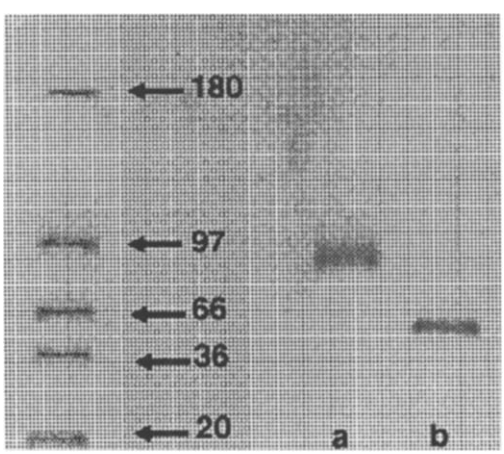

Fig. 3. Polyacrylamide gel electrophoresis of brown shrimp agglutinin isolated by affinity chromatography and gel-filtration under SDS non-reducing conditions (lane a) and SDS reducing conditions (lane b). The molecular markers are indicated.

Inhibition of haemagglutination

The effect of various sugars, glycoproteins and bacterial LPS on the haemagglutinating activity of the BSH-I is shown in Table 3. GalNAc, GluNAc, NANA, fetuin, submaxillary bovine mucin, and *E. coli* LPS were able to inhibit the agglutination of human erythrocytes. The reduced inhibitory effect of asialofetuin suggests that the sialic acid residues may contribute to the specificity of the BSH-I, although NANA had little inhibitory effect on haemagglutination. Thus, the binding site of the BSH-I appears complex.

Agglutination of bacteria

Since LPS is a component of the cell wall of Gram-negative bacteria and this compound showed an interesting inhibitory effect, it is possible to assume that BSH-I would bind to Gram-negative bacteria. This was demonstrated by an increase in the sedimentation rate of suspended bacteria in buffer solution. As shown in Figs 4 and 5, no difference was found between the different *Vibrio* strains tested. As observed in Fig. 5(b), the addition of LPS to the *V. parahaemolyticus*/BSH-I assay, produced a bacteria sedimentation rate similar to that when agglutinin was not added. Similarly, but to a lesser extent, the GalNAc showed an inhibitory effect. These results are in agreement with those obtained by the inhibition of haemagglutination test.

DISCUSSION

The haemolymph of many invertebrates is known to agglutinate foreign erythrocytes. The lectins responsible for haemagglutination have been isolated and characterized from haemolymph and haemocytes. Their sugar specificity, molecular structure and

Table 3. Effect of sugars, glycoproteins and bacterial LPS on the haemagglutinating activity of the brown shrimp agglutinin

Glycoproteins (mg/ml)	
Fetuin	0.62
Fetuin desialized	5.00
Porcine mucin	NI
Bovine submaxillary mucin	1.25
Orosomucoid	5.00
Human transferrin	5.00
Carbohydrates (mM)	
Fucose	NI
Galactose	NI
Glucose	NI
Mannose	NI
N-Acetyl galactosamine	25
N-Acetyl glucosamine	50
N-Acetyl neuraminic acid	50
Lipopolysaccharide (mg/ml)	0.012

NI: no inhibition at 10 mg/ml of glycoprotein or 100 mM of sugar.
The values are given as the minimal concentration required to inhibit four haemagglutinating units.

ionic requirements differ widely, and the exact functions of the invertebrate agglutinins remain to be clarified. There are apparent contradictions as to how agglutinins can participate in the elimination of foreign material. For example, haemolymph lectins from *Homarus americanus* (Hall and Rowlands, 1974b), *Mytilus edulis* (Renwrantz and Stahmer, 1983) and *Aplysia californica* (Pauley *et al.*, 1971) act as opsonins which enhance phagocytosis against foreign particles. In several animals such as *Mytilus edulis* (Renwrantz and Stahmer, 1983), *Crassostrea virginica* (Vasta *et al.*, 1984), *Lymnaea stagnalis* (van der Knaap *et al.*, 1981) and *Leucophaea maderae* (Amirante and Mazzalai, 1978), haemolymph agglutinins have been found in haemocyte membranes by using polyclonal antibodies as probes. This association with haemocyte plasma supports the idea that they act as membrane-bound receptors which can recognize "foreignness". On the other hand, there is evidence which indicates that haemolymph itself is not opsonic (Scott, 1971; Fuke and Sugai, 1972; Rowley and Ratcliffe, 1980; Abul-Salam and Michelson, 1980), and that haemocytes show no immunoreactivity against haemolymph lectin (Stein and Basch, 1979; Suzuki and Mori, 1990). Hence, it seems that the definitive physiological function of invertebrate haemolymph lectins has yet to be determined.

Because coagulation is a fast process in the shrimp and other crustaceans (Durliat and Vranckx, 1981), an anticoagulant solution is required to obtain a cell-free preparation. Although one anticoagulant solution has been used previously to obtain haemocytes from marine crustaceans (Smith and Söderhäll, 1983), this solution was not useful in extracting the brown shrimp haemolymph as protein precipitation was observed (data not shown). Based on an earlier study of sodium and potassium concentrations, pH and osmolality of shrimp haemolymph (Vargas-Albores and Ochoa, 1992), we designed an anti-

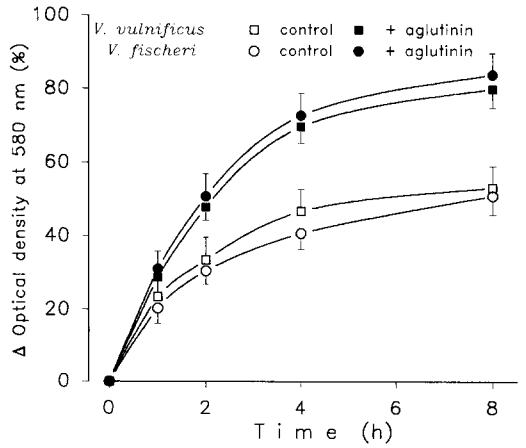

Fig. 4. The rate of sedimentation of *Vibrio vulnificus* and *V. fischeri* in the presence (filled) and absence (empty) of the BSH-I.

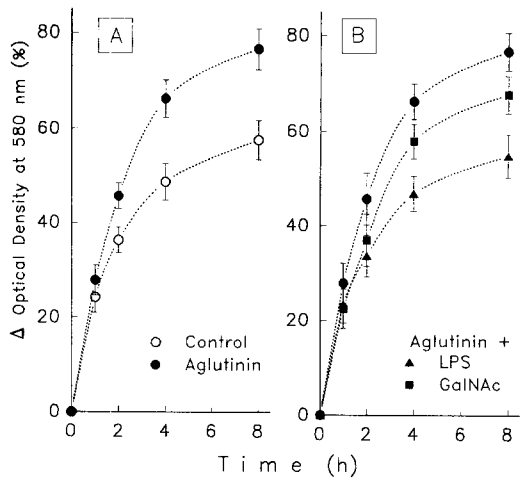

Fig. 5. Interaction of the BSH-I and *Vibrio parahaemolyticus*. The BSH-I increase in the rate of sedimentation (A), could be inhibited by GalNAc and LPS (B).

coagulant solution to obtain the brown shrimp haemolymph easily, without protein precipitation or coagulation. The integrity of the cells was verified by microscopic observation to ensure that no cell contamination was present in our plasma preparation. Because the osmolality of the shrimp haemolymph is high, and because of the presence of EDTA in the anticoagulant, it was necessary to dialyse the shrimp plasma before using it in haemagglutination tests. During the dialysis, some proteinaceous material, without haemagglutinating activity, was precipitated.

Similarly to other invertebrates, the brown shrimp haemolymph showed agglutinating activity against vertebrate RBCs. Instead of using haemoabsorption to detect different haemagglutinating activities as carried out in other studies on invertebrate agglutinins, we separated these different haemagglutinating activities by affinity chromatography. Our results showed that brown shrimp haemolymph has more than one haemagglutinating activity: the first was against mouse RBCs, and the second against mouse and human RBCs (Table 2 and Fig. 1). For the latter, a gel-filtration step was necessary to obtain a single protein band (BSH-I) in PAGE. Multiple serum agglutinins have been noted for other crustaceans such as *Callinectes sapidus* (Cassels *et al.*, 1986), *Homarus americanus* (Hall and Rowlands, 1974a,b), *Macrobrachium rosenbergii* (Vasta *et al.*, 1983) and *Birgus latro* (Vasta and Cohen, 1984).

Because of their variability, the molecular weight of invertebrate agglutinins and the number and size of their subunits make them difficult for comparative or phylogenetic studies. In general, the only characteristic that is common to all invertebrate agglutinins is their ability to interact with saccharide residues. Thus, many differences in molecular structure can be observed between related species. For example, in the genus *Penaeus*, the BSH-I is a tetrameric protein with a molecular weight of 175 ± 5 kDa; *P. monodon* lectin

is a 420 kDa protein, built up by subunits of 27 kDa (Ratanapo and Chulavatnatol, 1990), while the *P. stylirostris* agglutinin has an apparent molecular weight of 30 kDa (Vargas-Albores, unpublished data). On the other hand, the calcium-dependence in agglutination activity displayed by BSH-I seems to be a common feature for agglutinins from *P. monodon* (Ratanapo and Chulavatnatol, 1990), and *P. stylirostris* (Vargas-Albores *et al.*, 1992), as well as from other crustaceans such as the lobster, *Homarus americanus* (Hall and Rowlands, 1974a), the crab *Cancer antennarius* (Ravindranath et al., 1985), and the blue crab, *Callinectes sapidus* (Cassels *et al.*, 1986).

The specificity of the agglutinins from arthropods is diverse, although sialic acid-specific lectins have been found among several species such as *Homarus americanus* (Hall and Rowlands, 1974a), *Macrobrachium rosenbergii* (Vasta *et al.*, 1983), *Birgus latro* (Vasta and Cohen, 1984), *Cancer antennarius* (Ravindranath *et al.*, 1985) and *Penaeus monodon* (Ratanapo and Chulavatnatol, 1990). However, *H. americanus* also has a GalNAc-specific agglutinin (Hall and Rowlands, 1974a), and both *M. rosenbergii* (Vasta *et al.*, 1983) and *B. latro* (Vasta and Cohen, 1984) have a second agglutinin with undetermined specificity. Thus, the presence of multiple agglutinins is frequent in marine arthropods, and one of these agglutinins is apparently sialic acid-specific.

At least two agglutinins were also detected in brown shrimp haemolymph. The BSH-I showed a partial specificity for GalNAc and NANA. Additionally, the BSH-I bound glycoproteins, sugars and bacterial LPS (Table 3), indicating a considerable variation in specificity. The BSH-I binding ability against LPS (Table 3) suggests that BSH-I is capable of binding to Gram-negative bacteria. This was confirmed by our bacteria sedimentation experiments (Figs 2 and 3). The fact that the brown shrimp agglutinin can bind and agglutinate marine bacteria (including pathogens) is significant to the physiological role of haemagglutinin.

LPS-binding proteins which are involved in defence mechanisms have been reported in other animals. An LPS-binding glycoprotein (60 kDa) could be detected in rabbit plasma during the acute-phase of inflammation (Tobias *et al.*, 1986). This protein was able to bind to the surface of *Salmonella* or to LPS-coated erythrocytes, enhancing their attachment to macrophages (Wright *et al.*, 1989). Two LPS-binding proteins have also been isolated from the haemocytes of the horseshoe crab. One protein (11.6 kDa) is called "anti-LPS factor" (Aketagawa *et al.*, 1986), while the other, a zymogen of serine protease, is called "factor C" (Nakamura *et al.*, 1988). In another study, a haemagglutinin which was able to bind to LPS was detected in the haemocyte, but not in the plasma, of *Halocynthia roretzi* (Azumi *et al.*, 1991).

In summary, BSH-I is a calcium-dependent protein with a molecular weight of 175 ± 5 kDa which can be inhibited by sugars (GalNAc, NANA), glycoproteins (fetuin) and bacterial LPS. Furthermore, BSH-I is able to react with bacteria, which could help to explain the physiological role of this particular invertebrate serum agglutinin.

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