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Lectins, as non-self-recognition factors, in crustaceans

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Abstract

Invertebrate immune system must rely on non-self-recognition molecules to ensure efficient defence responses against infectious pathogens that continuously threaten their survival. Lectins from the hemolymph of invertebrates, including crustaceans, have been regarded as potential molecules involved in immune recognition and microorganism phagocytosis through opsonization. This report presents an overview on the molecular characterisation, physiological role, synthesis and induction upon infection of arthropod lectins, with special emphasis on crustaceans. Although the participation of arthropod lectins in immune surveillance is not fully demonstrated, some recent reports in insects and horseshoe crabs appear to support this concept more convincingly. Unfortunately, such unambiguous evidences have not been thus far demonstrated in crustaceans. The results obtained in related arthropod groups could, however, be predictable for crustaceans. The precise mechanisms underlying non-self-recognition represent the basis to prevent and control infections as well as to stimulate animal resistance. This is particularly relevant for cultivated aquatic species, especially penaeid shrimps, which are frequently constrained by recurrent diseases that often provoke great economic losses.

Keywords: Lectin; Non-self-recognition; Immune system; Arthropods; Crustaceans; Shrimps; Penaeids

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1. Introduction

Agglutinin/lectins are proteins or glycoproteins usually without catalytic activity that have the ability to bind to specific carbohydrates expressed on different cell surfaces. Due to the fact that they are, in general, at least bivalent (i.e. the molecule has at least two specific binding sites), they can bind cells and an agglutination reaction occurs. The term agglutinin is derived from this functional capacity and may be distinguished from the most restricted denomination lectin, which corresponds to agglutinins whose capacity to selectively react to a specific sugar group has been unequivocally demonstrated.

Lectins exist in almost all living organisms. They were first identified before the turn of this century as plant proteins capable of agglutinating red blood cells, but their ubiquity is now well established. Agglutinin molecules may occur at the surface of viruses, bacteria, yeast, protozoan and throughout all animal and plant kingdoms (Sharon and Lis, 1989). Their specificity is always determined by the type of carbohydrate to which they bind. Hemagglutination inhibition assays in which the agglutinin is incubated with various carbohydrates or glycoconjugates before the addition of the target red blood cell are the mean to establish this specificity.

In spite of their apparent ubiquity, and the remarkable number of recent publications on their occurrence, structure, and specificity, the natural functions of lectins are still not fully understood. Interaction between lectins and carbohydrates have been shown to be involved in various activities. Therefore, different biological roles have been proposed for these molecules, including the cellular and tissue transport of carbohydrates, glycoproteins and calcium (Goldstein et al., 1980; Ravindranath and Cooper, 1984; Vasta, 1992), correlation to insect development (Komano et al., 1983; Kubo et al., 1990, 1993; Natori, 1990), cytolytic and cytotoxic factors (Komano and Natori, 1985; Hatakeyama et al., 1995; Armstrong et al., 1996), cell adhesion, migration and apoptosis (Peters et al., 1983; Perillo et al., 1995; Kasai and Hirabayashi, 1996; Ni and Tizard, 1996; Vasta et al., 1999).

Most current research has emphasised the possible role of lectins as non-self-recognition molecules in vertebrate and invertebrate immunity (Renwrantz, 1986; Arason, 1996; Matsushita, 1996; Vasta et al., 1999; Wilson et al., 1999). Invertebrates do not possess an adaptative immune system based on a multitude of highly specific antibodies and antigen receptors equivalent to that of vertebrates. However, the great success of those more primitive organisms, particularly arthropods, is definitely attested by their enormous numbers on earth and the diversity of environments they can colonise, which often abound in opportunistic microbes. Their success must certainly rely on efficient immune defences capable of protecting these animals against the multiple invading microorganisms, which continuously threaten their survival.

Due to the fact that lectins have the ability to bind carbohydrate and promote the agglutination of different cells, such as bacteria and other invading pathogens, it is reasonable to assume that these molecules may be regarded as having a potential role in invertebrate non-self-recognition reactions. As with vertebrate immunoglobulins, they can agglutinate microorganisms and enhance their phagocytosis by mediating binding between the hemocyte surface and a foreign body (opsonic role), and are apparently

synthesised by invertebrate immune cells (hemocytes). However, in contrast to immunoglobulins, the specificity of invertebrate agglutinins is restricted only to sugar residues.

Among arthropods, crustaceans assume a particular economic importance as a food source and their use in aquaculture, especially penaeid shrimps, is currently expanding. The success of shrimp cultivation is, however, seriously endangered by diseases that are the most limiting factors capable of producing great economic losses (Roch, 1999). In this context, studies on crustacean and shrimp immunity should be deeply stimulated, in order to generate the basis to prevent and control infections as well as increase animal resistance.

One of the first points to be addressed on crustacean and shrimp immunity should concern non-self-recognition mechanisms; here, the hemolymphatic lectins may gain their important role. However, compared to other arthropod groups, such as insects and horseshoe crabs, the current knowledge on lectin involvement in crustacean non-self-recognition is still much less well established. In contrast, studies on the prophenoloxidase (proPO)-activating system and related molecules (Söderhäll and Cerenius, 1992; Söderhäll et al., 1996) and, more recently, on clotting proteins (Kopacek et al., 1993a; Komatsu and Ando, 1998; Hall et al., 1999 in crustaceans have progressed well.

This review will mainly focus on the current status of occurrence, specificity and biological role of crustacean lectins, primarily those of shrimps. To support some views, especially concerning the biological role and inducibility of lectins, we will provide some examples from insects and horseshoe crabs, to which crustaceans are phylogenetically related, and in which the evidences are particularly convincing. Similar roles may be predictable for crustaceans, even though not yet demonstrated.

2. Structural aspects and carbohydrate specificity

Animal lectins are a heterogeneous class of molecules, which exhibit a high structural diversity. They have been initially classified in two different groups: C- and S-type lectins, based on amino-acid sequence similarities, particularly in the carbohydrate-recognition domain (CRD), along with overall domain organisation and physico-chemical properties, such as divalent cation dependence and free thiol requirement (Drickamer, 1988). Calcium dependence was the main property associated with C-type lectins, whereas thiol dependence was the S-type group's characteristic. Recently, as a consequence of three-dimensional structure studies and the availability of additional primary sequence data, a more complete classification has emerged. Therefore, C-type lectins have been further characterised into distinct subgroups, and S-type lectins reclassified as galectins; but the existence of new categories was also established (Barondes et al., 1994; Drickamer, 1995; Vasta et al., 1996; Vasta et al., 1999).

In arthropods, the majority of lectins thus far purified cannot be clearly placed in the preestablished lectin families. Nevertheless, some examples of C-type-like lectins have been reported in arthropods, as in the flesh fly *Sarcophaga peregrina* and the tobacco hornworm *Manduca sexta*, (Komano et al., 1980; Yu et al., 1999). Interestingly, in the latter, the lectin primary structure indicated the presence of two CRD, a characteristic

that is not frequently found within the soluble lectins belonging to the C-type family. The occurrence of C-type lectins has also been reported in crustaceans, as for two species of acorn barnacles, *Megabalanus rosa* and *Balanus rostratus* (Muramoto and Kamiya, 1989; Toda et al., 1998). It is relevant to point out that among C-type lectins, little or no homology may be present in domains other than that involved in CRD (Vasta et al., 1996).

Also in arthropods, the occurrence of lectins sharing homology with vertebrate acute-phase C-reactive (CRP) and serum amyloid P component (SAP) proteins was equally demonstrated. The latter are lectins that participate in the vertebrate immune system (see next section) and belong to the family of pentraxins. The term pentraxin refers to the pentameric structure of these molecules, which are composed of identical subunits of 20 to 30 kDa, usually noncovalently bound (Vasta et al., 1999). Several lectins purified from different species of horseshoe crabs and from cockroaches (see below) have been demonstrated to share homology to this lectin family (Bishayee and Dorai, 1980; Robey and Liu, 1981; Shishikura and Sekiguchi, 1983; Dodds and Day, 1996; Wilson et al., 1999).

Concerning carbohydrate specificity, invertebrate lectins can vary from specific molecules to others that exhibit a broader spectrum of recognition. The specificity of a lectin is related to the carbohydrate for which it shows the highest affinity, but most lectins that are considered specific for one monosaccharide may also bind, although with lower affinity, to other carbohydrates, which are structurally related. The agglutination of many different types of cells/glycoconjugates may actually reflect the ubiquity of the ligand. A lectin from the horseshoe crab *Limulus polyphemus* that binds sialic acid can illustrate this statement and be considered as a lectin of a broad spectrum of recognition, since the fact that it agglutinates a variety of cells can be associated to the wide distribution of this monosaccharide among cell surfaces (Cohen et al.,1983; Vasta, 1992).

In spite of the enormous diversity of lectins, one common aspect of their molecular organisation has emerged recently. In most lectin molecules, including those of invertebrates, a globular region, comprising less than 200 amino acids, can be ascribed to the sugar-binding activity. This region is that referred above as CRD (Weis and Drickamer, 1996). As a consequence, mechanisms of sugar recognition by these molecules share some key aspects in diverse protein structural frameworks. Therefore, the structural basis for saccharide–lectin interaction and recognition implies different interactions, such as hydrogen bonding to different sugar OH groups, and explains how high affinity and highly selective binding are achieved for complex saccharides (Weis and Drickamer, 1996). Some agglutination inhibition studies using derivatives of sialic acid (*N*-acetylneuraminic acid; NeuAc), for example, have pointed out the importance of hydrophobic bonding and negative charge, such as those of carboxylic acid, for the agglutination reaction (Cohen et al., 1983). Studies on the binding of the mannose-binding protein (MBP), a mammalian acute-phase reactant that plays an important role in the innate immune system (see next section), have provided a good understanding of the concept of ligand recognition based on molecular micro- and macropatterns. This protein forms oligomers composed of six trimers and selectively recognises the carbohydrate patterns on the surface of different microorganisms but not the sugars that are present in

self-glycoproteins (Thiel and Reid, 1989). This observation is related to the equatorial orientation of the C3–OH and C4–OH groups of the sugar moiety (Weis and Drickamer, 1996), a configuration represented in the hexoses *N*-acetylglucosamine (GlcNAc), glucose and fucose as well as mannose. Combinations of these sugars are found on the cell wall structures of different microorganisms. On the other hand, the configurations of OH groups in two other carbohydrates, galactose and sialic acid, found in the penultimate and ultimate position of mammalian glycoproteins, respectively, do not fit in this pattern of recognition (Weis and Drickamer, 1996). Based on three-dimensional studies, an additional feature of the molecular pattern recognised by MBP oligomers involves the distances between CRD within a trimer and between CRD of different trimers in order to achieve high-affinity binding. Therefore, the highly repetitive structure of the ligands found in microbial cell wall would favour this recognition in opposite to the glycoproteins of higher organisms, which are not displayed in a repetitive manner in the cell membrane (Hoffmann et al.,1999). For some lectins, another important feature in carbohydrate binding is the presence of calcium binding sites within the CRD. Some amino acid residues in these sites may also be involved in ligand binding specificity (Weis et al., 1992). The presence of glutamic acid and asparagine are usually associated with a binding specificity towards mannose or glucose, or sugars with the same configuration in the C3–OH and C4–OH as in mannose. On the other hand, galactose-specific CRD exhibits glutamine and aspartic acid at these positions instead. Although in arthropods few examples are available to draw comparisons to vertebrate lectins, in the tobacco hornworm *M. sexta* lectin, where two CDR domains are present, conservation of these residues in one of them is found and is related to the binding of mannose or glucose, whereas in the other domain, the putative residues required for calcium binding are not well conserved (Yu et al., 1999).

In decapods, the specificity of lectins towards carbohydrates is mainly related to *N*-acetylated carbohydrates, such as NeuAc, GlcNAc, and *N*-acetyl-D-galactosamine (GalNAc), as can be depicted by the data partially summarised in Tables 1 and 2.

Although the recognition of NeuAc is a common feature among crustacean lectins (Tables 1 and 2) some of them exhibit a particular pattern of specificity towards *O*-acetylated sialic-acid derivatives (Hall and Rowlands, 1974a; Cassels et al., 1986). The report of Ravindranath et al. (1985) has shown the specificity of the marine crab *Cancer antennarius* hemolymph lectin to 9-*O*/4-*O*-acetyl sialic acid, whereas the hemolymph lectins of the freshwater prawn *Macrobrachium rosenbergii* and the marine crab *Liocarcinus depurator* have been shown to specifically recognise 9-*O*-acetyl sialic acid (Vàzquez et al., 1993; Fragkiadakis and Stratakis, 1997a). The agglutination of the bacterium *Bacillus cereus* by *M. rosenbergii* hemolymph lectin can be related to the recognition of these *O*-acetylated sugars on the bacteria cell surface (Vàzquez et al., 1996).

It is noteworthy that although crustacean lectins apparently share no common structural relation, the specificity of recognition seems to have been well conserved within this group of invertebrates (Tables 1 and 2). On the other hand, a sialic acid-binding lectin with specificity for *N*-glycolylneuraminic acid (NeuGc) was purified from the hemolymph of the marine crab *Scylla serrata* (Mercy and Ravindranath, 1993).The unique binding specificity of this lectin distinguishes it from other known

Table 1
Some lectins characterised among crustaceans

Crustaceans	Divalent cation	MW (kDa)	Subunits (kDa)	Carbohydrate/glycoprotein/LPS inhibition	Biological activity	References
Freshwater prawn						
M. rosenbergii	CD	19	9.6	NeuAc; GalNAc; GlcNAc; OSM; Fetuin	Agglutination of bacteria (*B. cereus*; *Aeromona* sp., *P. haemolytica*; *P. multocida*, *Sta. aureus*)	Vasta et al., 1983; Vàzquez et al., 1993; 1996; 1997
Crabs						
S. serrata	CD	55, 70	25–30	NeuGc; Colomic acid; Thyroglobulin; BSM; Fetuin	Opsonisation	Mercy and Ravindranath, 1993, 1994. Kongtawelert, 1998
Liocarcinus depurator	CD	700	38	NeuAc; BSM; LPS	Agglutination of bacteria (*E. coli* K12; *E. aerogenes*; *P. aeruginosa*); Mitogenic activity (human lymphocytes)	Fragkiadakis and Stratakis, 1997a
C. japonicus	CD	300	19	α-galactosyl; α-glucosyl	n.d.	Umetsu et al., 1991
C. antennarius	CD	140	36	NeuAc; BSM; ESM	n.d.	Ravindranath et al., 1985
Lobsters						
H. americanus LAg-1; LAg-2	CD	19S; 11S	55; 55	NeuAc; GalNAc	n.d.	Hall and Rowlands, 1974a,b
Jasus novaehollandiae JN-2[#]	CD	400	85, 81, 63	PSM; Asialo-PSM; Fetuin; D-ribose; D-arabinose; D-galactose; D-mannose; D-glucosamine	Agglutination of bacteria (*P. aeruginosa*)	Imai et al., 1994
J. verreauxi	CI	n.d.	n.d.	BSM; Fetuin; NeuAc; GlcNAc	n.d.	Imai et al., 1994

Crayfishes						
P. leniusculus	CD	420	65–80	PSM; BSM; Fetuin; LPS; GlcNAc; α-L-rhamnose	n.d.	Kopacek et al., 1993b; Middleton et al., 1996
Austropotamobius pallipes	CPD	n.d.	n.d.	Fetuin	n.d.	Middleton et al., 1996
Astacus astacus	CD	n.d.	n.d.	Fetuin; D-xylose	n.d.	
Astacus leptodactylus	CD	n.d.	n.d.	Fetuin	n.d.	
Procambarus clarkii	CI	n.d.	n.d.	Fetuin; GalNAc; GlcNAc; Maltose	n.d.	
Acorn barnacles						
Megabalanus rosa BRA-1	CD	330	22	Gal	Biomin. (+)	Muramoto et al., 1985; Muramoto and Kamiya, 1986; Muramoto and Kamiya, 1989; Muramoto et al., 1994
BRA-2	CD	140	22	Gal		
BRA-3	CD	64	16	Gal		
Balanus rostratus	CD	120	25	Gal; lactose; melibiose	Biomin. (−)	Toda et al., 1998
Mantis shrimp						
Squilla mantis, lect anti-H	CD	192	–	L-Fucose	Capsule formation	Amirante and Basso,1984; Battistella et al., 1996
lect anti-A		192		GalNAc		

Abbreviations: CD: cation dependent; CI: cation independent; CPD: cation partially dependent; BSM: bovine mucin; OSM: ovine mucin; ESM: equine mucine; PSM: porcine mucine; GlcNAc: *N*-acetylglucosamine; GalNAc: *N*-acetylgalactosamine; ManNAc: *N*-acetylmannosamine; NeuAc: *N*-acetylneuraminic acid; NeuGc: *N*-glycolylneuraminic acid; LPS: lipopolysaccharide (the type/source is not specified). Bacteria: *B. cereus*; *E. coli*, *E. aerogenes*, *Pasteurella haemolytica*; *P. multocida*; *P. aeruginosa*; *Staphylococcus aureus.* n.d. — not determined; Biomin.: biomineralization; (+) positive; (−) negative; S: Svedberg units.

[#] The same authors reported that another species, *J. edwardsii*, was found to posses lectins similar to those of *J. novaehollandiae.*

Table 2
Lectins characterised in different species of penaeids

Species	Divalent cation	MW (kDa)	Subunits (kDa)	Carbohydrate/glycoprotein/ LPS inhibition	Biological activity	References
P. japonicus	CD	330	33	GlcNAc; GalNAc NeuAc; Ribose BSM; PSM; Fetuin	Opsonic activity (serum)	Kondo et al., 1992; 1998
	CI	n.d.	n.d.	GlcNAc; GalNAc NeuAc; BSM PSM; Fetuin	n.d.	
P. indicus	CI	n.d.	n.d.	GlcNAc; GalNAc ManNAc; NeuAc BSM; Fetuin; LPS	n.d.	Maheswari et.al., 1997
P. californiensis	CD	175	41	GlcNAc; GalNAc NeuAc; BSM Fetuin; LPS	Agglutination of bacteria (*V. fischeri*; *parahaemolyticus*; *vulnificus*); Opsonic activity	Vargas-Albores et al., 1993; Vargas-Albores, 1995
P. stylirostris	CD	30		n.d.	n.d.	Vargas-Albores et al., 1993
P. monodon	CD	420	27	GlcNAc; GalNAc ManNAc; NeuAc BSM; Fetuin	Agglutination of bacteria (*V. vulnificus*) (*V. parahaemolyticus*; *A. hydrophila*; *P. shigelloides*; *P. aeruginosa*; *E. coli*; *B. subtilis*)	Ratanapo and Chulavatnatol l990; 1992 Sritunyalucksana et al., 1999
P. paulensis	CI	153	31	NeuAc; GalNAc GlcNAc; Fetuin LPS	Opsonic activity (serum) (*V. harveyi*; *B. cereus*)	Our results (unpublished data)
P. schmitti	CPD	153	31, 34	NeuAc; GalNAc GlcNAc; Fetuin; LPS	n.d.	Our results (unpublished data)
P. longirostris	CD	440$^{(1)}$, 210 $^{(2)}$	27$^{(1)}$, 36$^{(2)}$	NeuAc$^{(1)}$; Gal$^{(1)}$; GalNAc$^{(2)}$; BSM; Fetuin	Agglutination of bacteria (*P. aeruginosa*; *E. coli*)	Fragkiadakis and Stratakis, 1995

Abbreviations: CD: cation dependent; CI: cation independent; CPD: cation partially dependent; BSM: bovine mucin; OSM: ovine mucin; ESM: equine mucine; PSM: porcine mucine; GlcNAc: *N*-acetylglucosamine; GalNAc: *N*-acetylgalactosamine; ManNAc: *N*-acetylmannosamine; NeuAc: *N*-acetylneuraminic acid; LPS – lipopolysaccharide (the type/source is not specified). Bacteria: *Aeromonas hydrophila*; *B. cereus, B. subtilis, E. coli*; *Plesiomonas shigelloides*; *P. aeruginosa*; *V. fischeri*; *V. harveyi*; *V. parahaemolyticus*; *V. vulnificus*. n.d. — not determined.
(1) and (2) correlates the corresponding MW, subunits and particular inhibition pattern of each lectin.

sialic acid-specific lectins (Tables 1 and 2), particularly from those of a broader range of specificity towards this ligand, as seen, for example, in the American horseshoe crab *L. polyphemus* (Mercy and Ravindranath, 1993). Interestingly, the specificity towards NeuGc has also been reported in different species of molluscs (Swarnakar et al., 1991; Dam et al., 1993).

In spite of the *N*-acetylated carbohydrate recognition mentioned above, different sugar specificity has also been observed among crustaceans. Three galactose-binding lectins have been described in the hemolymph of the acorn barnacle *M. rosa* (Table 1), and the recognition towards the same monosaccharide has been assigned to the lectin present in the hemolymph of a different species of barnacle, *Balanus rostratus* (Toda et al., 1998). However, specificity to galacturonic and glucuronic acids and also to NeuAc has been displayed by a lectin occurring in a different species of barnacle, *Balanus balanoids* (Ogata et al., 1983). On the other hand, galactose binding is not restricted to barnacles, since Fragkiadakis and Stratakis (1997b) have also shown the presence of a galactose-recognising lectin in the hemolymph of the crustacean *Potamon potamios.* Additional carbohydrate specificity might be exhibited by crustacean lectins, as seen for the serum lectin of the freshwater crab *Parathelphusa hydrodromus*, which appears to be unique among all the known crustacean agglutinins in being specific for nonreducing terminal glucose with $\alpha\ 1 \rightarrow 2$ glycosidic linkage (Nalini et al., 1994).

The range of specificity seems to be more restricted among penaeids. As seen in Table 2, NeuAc, a sialic acid with an acetyl group on C-5, is an effective inhibitor of the agglutination activity, as seen for some other crustacean species (Table 1). However, this sugar specificity is not absolute but shared by other *N*-acetylated amino-sugars, mainly GalNAc and GlcNAc, which contain the acetyl group on C-2. Although an acetyl group seems essential for lectin–ligand interaction, the latter two monosaccharides are usually less efficient in their hemagglutination inhibitory capacity, as seen, for example, in *Penaeus monodon* (Ratanapo and Chulavatnatol, 1990), *P. schmitti* and *P. paulensis* (our results). This could indicate the importance of the position of this group as well as its equatorial arrangement. One exception to this behaviour is the lectin of *P. indicus*, for which GalNAc, GlcNAc, ManNac were as effective as NeuAc in inhibiting the serum agglutination activity (Maheswari et al.,1997). Sialoglycoproteins, known to contain predominantly terminal NeuAc, are also characterised as effective inhibitory moieties of penaeid hemagglutination activity (Table 2). Bovine mucin (BSM) usually shows a greater inhibitory potency when compared to fetuin, as reported, for example, in *P. indicus* (Maheswari et al.,1997) and seen also in *P. schmitti* and *P. paulensis* (our results). This observation could reflect the difference in the arrangement of the terminal NeuAc in the oligosaccharide of these two proteins, which can be $\alpha\ 2 \rightarrow 6$ linked to GalNAc in BSM (Tsuji and Osawat, 1986) or $\alpha\ 2 \rightarrow 3$ linked to galactose in fetuin (Nilsson et al., 1979). The presence and the position of *O*-acetyl groups in derivatives of sialic acid may also be important for the binding of at least some penaeid lectins as suggested by the high titres of agglutination observed towards rat and rabbit erythrocytes, as seen, for example, in *P. schmitti* and *P. paulensis* (our results). These blood cell types posses great amounts of 9-*O*-acetylsialic-acid residues on their surface, derivatives also seen in BSM (Schauer, 1982). On the contrary, different red blood cells

may predominantly exhibit other sialic-acid derivatives, such as 4-*O*-acetylated residues, which can be associated with lower agglutination titres in some cases (Vàzquez et al., 1993).

Most of the lectins reported in decapods are known to be dependent upon divalent cations, usually calcium, and they are sensitive to divalent chelators in a reversible or irreversible manner (Ratanapo and Chulavatnatol, 1990; Nalini et al., 1994). It should be also emphasised that some degree of variability in divalent cation dependency among crustaceans can be seen, even in related species (Tables 1 and 2). The serum lectins of *P. indicus* (Maheswari et al., 1997) and that of *P. paulensis* (our results) do not require cations (Ca^{2+} and Mg^{2+}) for their activity, a characteristic quite uncommon among crustacean lectins. Moreover, in some cases, the molecule behaviour in the presence of cation chelators can suggest a partial dependency, as observed in some species of crayfishes (Middleton et al., 1996) and in the penaeid *P. schmitti* (our results).

Concerning their molecular structure, crustacean lectins exhibit differences related to molecular mass, number and size of subunits, as revealed by the data partially summarised in Tables 1 and 2. Structural diversity can be observed even between related species as seen for penaeids. A lectin purified from *P. californiensis* hemolymph, BSH-1, was shown to be a 175 kDa oligomer, made up of subunits of 41 kDa, whereas in *P. monodon*, a 420 kDa hemolymph lectin, called monodin, comprises subunits of 27 kDa (Ratanapo and Chulavatnatol, 1990; Vargas-Albores et al., 1993). In *P. paulensis*, using affinity-chromatography on fetuin-agarose, followed by gel filtration on Superose 12, we have isolated a lectin from the hemolymph of apparent molecular mass of 153 kDa. Upon reduction, this lectin displayed subunits of 31 kDa on SDS-PAGE (unpublished data).

In spite of the apparent structural diversity of naturally occurring lectins of prawns, the lectin of *Parapenaeus longirostris* has the same native and subunit molecular mass as monodin (Ratanapo and Chulavatnatol, 1990), in addition to the fact of sharing the same NeuAc specificity. This finding might represent a common feature among some penaeid prawns, as suggested by Fragkiadakis and Stratakis (1995). Furthermore, monodin was immunologically detected in two species of prawns, *P. merguiensis* and *Metapenaeus monceros* (Ratanapo and Chulavatnatol, 1990). Correspondingly, using a monoclonal antibody to *P. japonicus* lectin (Bachère et al., 1995), we have observed cross-reactivity against the partially purified lectin of *P. schmitti*, obtained by affinity chromatography on a fetuin-agarose column. The apparent molecular weight of this lectin was shown to be 153 kDa by gel filtration, as determined for *P. paulensis*, although two distinct subunits (31 and 34 kDa) were seen on SDS-PAGE under reducing conditions (unpublished data).

Due to the limited data available, more information concerning primary structure as well as nucleotide sequence and gene organisation of crustacean lectins, including penaeids, is undoubtedly required. These additional data might contribute to the establishment of a clearer and more complete view of structural homology relations, a knowledge that could be valuable to the understanding of the effector functions of these molecules and the mechanisms associated to invertebrate defence strategies, as well as their potential use and applications.

3. Biological role

Numerous reviews reporting agglutinin involvement in arthropod defence and recognition mechanisms have been reported (Amirante, 1986; Olafsen, 1986, 1988; Vasta, 1992; Millar and Ratcliffe, 1994, Vargas-Albores, 1995; Vasta et al., 1996; Natori et al., 1999). Nevertheless, the major problem regarding the role of agglutinins as non-self-recognition molecules relies on the molecular basis for the generation of binding diversity and specificity to account for an efficient immunorecognition, as that provided by the immunoglobulins of the vertebrates. In this context, two recent examples in arthropods appear to support the view that invertebrate lectins may exhibit the required binding diversity to efficiently discriminate non-self-particles. The first, concerns the cockroach *Blaberus discoidalis*, which contains multiple plasma lectins (namely, BDL1, BDL2, BDL3, and GSL) each with different carbohydrate-binding specificities (Chen et al., 1993; Wilson et al., 1999) and, consequently, able to potentially recognise different invading pathogens. It was demonstrated that each of these purified molecules was capable to induce a specific and enhanced phagocytic response towards different microorganisms, such as yeast (*Saccharomyces cerevisiae*) and bacteria (*Escherichia coli* and *B. cereus*). This response was related to the carbohydrate exposed on the microorganism surface and to the sugar specificity of each lectin (Wilson et al., 1999). The cockroach BDL1 and GSL, a mannose-specific and a β-1,3-glucan-specific lectin (GSL), respectively, both increased the phagocytosis of the yeast that expose these carbohydrates in their cell surface. On the other hand, the cockroach BDL2, a GlcNAc/GalNAc-specific lectin, strongly induced the phagocytosis of the Gram-positive *Bac. cereus*, which contains GlcNAc on its peptidoglycan cell wall. Finally, the cockroach BDL3, a GalNAc-specific lectin, enhanced the phagocytosis of *E. coli* (K12), which exhibits galactose residues on its LPS portion.

It should be mentioned, however, that to be true ospsonic factors, arthropod lectins, must be able not only to specifically bind to non-self-particles surface but also to adhere to virtually specific receptors on the phagocyte (hemocytes) surface, thus facilitating phagocytosis in an analogous manner to the Fc portion of mammal immunoglobulins or the C3b fragments of the vertebrate complement system. In *Bla. discoidalis*, Wilson et al (1999) showed that the binding of endogenous lectin to cockroach hemocyte receptors was apparently independent of carbohydrate recognition. This was determined by using competitive exogenous lectins with similar sugar specificity than cockroach endogenous lectins. The authors demonstrated that the intensity of binding of endogenous lectin-opsonized microorganisms to the hemocyte surface was not reduced by exogenous lectins. Nevertheless, the mechanism of lectin binding on putative hemocyte receptors, that would confirm the opsonic properties of these molecules was not investigated. The specific binding of lectins onto phagocyte surface after reacting with non-self-particles is still largely unknown in arthropods. One exception is the report of Nagasawa et al. (1993) in *S. peregrina*, where a specific receptor for the flesh fly lectin, purified from an embryonic cell line, was immunodetected on the larval hemocyte surface.

Another interesting example of multiple lectins with different sugar specificities and playing distinct functional roles in non-self-recognition was also provided by horseshoe crabs. In the Japanese horseshoe crab *Tachypleus tridentatus*, several lectins, named

tachylectins, were purified from hemolymph. Tachylectin 1 had a broad specificity to S and R types of lipopolysaccharides (LPS) from Gram-negative bacteria cell walls and was also capable to inhibit the growth of Gram-negative bacteria (Saito et al., 1995). Tachylectin 2 had a binding specificity for GlcNAc and GalNAc and promoted the agglutination of certain strain of Gram-positive *Staphylococcus* and recognised several kinds of LPS (Okino et al., 1995; Kawabata and Iwanaga, 1999). Tachylectin 4 had a binding specificity for fucose and specifically recognised S-type of LPS from several Gram-negative bacteria through *O*-specific polysaccharides (*O*-antigen) (Saito et al., 1997). More recently, two new lectins, named tachylectin 3 and 5, were also purified in the same horseshoe crab and proved to be structurally and functionally distinct from the others (Inamori et al., 1999; Kawabata and Iwanaga, 1999). Tachylectin 3 was specific to the human blood group A antigen and recognised with highly specificity (more than tachylectin 4) the sugar moiety *O*-antigen from LPS of several Gram-negative bacteria (Inamori et al., 1999). In contrast to the others, tachylectin 5 was identified in the horseshoe crab plasma (and not in hemocyte extracts) and might probably be the primary lectin to recognise microbes. This lectin type had a broad specificity to *N*-acetetylated substances and promoted the strongest agglutinating activity out of the 5 tachylectin types, against both Gram-positive and Gram-negative bacteria (Kawabata and Iwanaga, 1999).

It is particularly interesting that the tachylectins thus far purified from *T. tridentatus* exhibit different degrees of sugar specificity (from broad to highly specific) and tachylectins 1–4, which are predominantly expressed in the horseshoe crab blood cells, are released from the hemocytes in response to external stimuli. In view of these observations, Inamori et al. (1999) and Kawabata and Iwanaga (1999) proposed that the innate immune system of the horseshoe crab may recognise invading pathogens through a combinatorial method by using broad and highly specific lectins against molecules exposed on pathogen surface and function synergistically to ensure an effective host defence.

Apart from these two well-characterised examples, multiple lectins have also been purified in other insects, such as the silkworm *Bombyx mori* (Amanai et al., 1990; Yoshida et al., 1996) and the American cockroach *Periplaneta americana* (Kubo and Natori, 1987; Jomori et al., 1990; Kubo et al., 1990, 1993; Kawasaki et al., 1996). Likewise, in the American horseshoe crab *L. polyphemus*, multiple lectins have also been isolated (Marchalonis and Edelman, 1968; Oppenheim et al., 1974; Roche and Monsigny, 1974; Robey and Liu, 1981; Brandin and Pistole, 1983; Fujii et al., 1992; Tsuboi et al., 1993).

The two detailed examples on insect and horseshoe crab described above apparently suggest a certain degree of functional analogy between invertebrate lectins and vertebrate antibodies. However, it is important to point out that these two classes of molecules do not really share any structural and biochemical homology and, thus, do not seem to be phylogenetically related. Moreover, the presence of lectins participating on the vertebrate innate immune system is also well evidenced by now. One of the most studied examples concerns the mannan-binding protein (MBP), which binds to mannans exposed on the cell walls of yeast and other microorganisms (Kawasaki et al., 1983). MBP not only acts as an opsonin towards microorganisms but also has the ability to

activate the complement system, in a pathway which is independent of immunocomplexes (Matsushita, 1996). The CRP and SAP proteins are other examples of vertebrate lectins (Matsushita, 1996), which can also activate the complement system after reacting with microorganism surface components (Ni and Tizard, 1996). These types of carbohydrate-binding proteins, capable of recognising surface structures common for different pathogens and participating in the innate immune system of both vertebrates and invertebrates, could thus be regarded as phylogenetically related. In effect, it was found that BDL1 of *B. discoidalis* shared similarities to the vertebrate MBP in terms of structure and specificity (Wilson et al., 1999). Also in the flesh fly *S. peregrina*, a purified lectin was shown to have a certain degree of homology with the mammal MBP (Takahashi et al., 1985). Also in a number of horseshoe crab species, as *T. tridentatus* (Shishikura and Sekiguchi, 1983), *Carcinoscorpius rotundacauda* (Bishayee and Dorai, 1980) and *L. polyphemus* (Robey and Liu, 1981), the occurrence of CRP was referred. The CRP of *L. polyphemus* shares 35% of homology with the human CRP and SAP (Dodds and Day, 1996). These findings apparently suggest that non-self-recognition molecules, such as lectins from vertebrate innate immune system, might have had their evolutionary origin from invertebrates. On the other hand, the mammal adaptative immune system, consisting of specific antibodies and memory, might have been a later acquisition.

Besides lectins, the immune system of arthropods includes also other defence mechanisms involving microorganism-carbohydrate recognition. A complex proteolytic cascade known as proPO system is triggered by components of microbial cell walls, (Söderhäll and Cerenius, 1992; Söderhäll et al., 1996; Ashida and Brey, 1997). Recently, an interesting connection between lectins and the proPO activating system of arthropods was reported in insects. Chen et al. (1995) demonstrated that the GSL from the cockroach *B. discoidalis* significantly enhanced the proPO activation by laminarin (a β-1,3-glucan). These observations were also confirmed for three other cockroach species (Chen et al., 1998). Still in *B. discoidalis*, Wilson et al. (1999) obtained evidences that the level of phagocytosis of the distinct microorganisms mentioned above could be increased by the several lectins, in a proPO-dependent and/or proPO-independent mechanism. Therefore, the authors suggested that the insect innate immunity based on multiple lectins could potentiate the uptake of a range of microorganisms through their direct recognition and opsonization and through the activation of the proPO cascade. These immune responses in combination with the intracellular killing of the pathogen through degrading enzymes and the generation of toxic molecules, such as reactive oxygen intermediates, would be sufficient to protect the invertebrate host against a variety of microorganisms despite of the lack of an Ab-based recognition system.

To our knowledge, a link between hemolymphatic lectins and the proPO activation system has so far been reported in the cockroaches as described above and also in the tobacco hornworm *M. sexta* (Yu et al., 1999). In the latter, a lectin named immulectin, synthesised in response to microbe infections (see below), was also shown to trigger the proPO system. Immulectin has a binding site for LPS and is capable to activate the proPO system when added to the insect plasma, especially when LPS are present. Also in the silkworm *B. mori*, a peptidoglycan recognition protein (PGRP) purified from the insect hemolymph was demonstrated to trigger the proPO cascade after reacting with

peptydoglycans (Yoshida et al., 1996; Ochiai and Ashida, 1999). However, the authors could not effectively confirm the lectin nature of PGRP based on sugar inhibition tests. It shall be noted that the potential link between lectins and the proPO system is just emerging and only few insect species have been examined. It would be of great interest to investigate whether the proPO system and the natural lectins from other arthropods, including crustaceans, are also functionally connected. Future research, using a number of different arthropod species, could confirm the direct involvement of lectin in the proPO activating system. This would give support to the concept of a possible integration of several immune reactions in arthropod defence systems. In this context, it could be said that lectins, as recognition factors, are common to invertebrate and vertebrate innate immune systems, where they can trigger proteinase cascades such as the proPO and the complement systems, respectively.

As far as crustaceans and shrimps are concerned, multiple lectins with diverse sugar-binding specificities related to different functional activities are still very limited compared to insects and horseshoe crabs. The first substantial evidence of multiple lectins in crustaceans was reported more than two decades ago in the lobster *Homarus americanus* by Hall and Rowlands (1974b). The authors purified two different plasma agglutinins, namely LAg-1 and LAg-2, but they did not exclude the occurrence of some others. These agglutinins, which were encountered either in the hemocyte extracts either in the plasma, reacted with human (LAg-1) and mouse erythrocytes (LAg-2). In a further report (Hall and Rowlands 1974a), they characterised the sugar specificity of both purified lobster lectins (Table 1). By the early 70s, the authors already emphasised the fact that multiple lectins with different sugar specificity could constitute an important evidence of agglutinin heterogeneity and, thus, support the hypothesis of the role of agglutinins as non-self-recognition molecules in crustacean defence. Unfortunately, due to technical difficulty, the authors could not precisely demonstrate the role of the purified lobster lectins as opsonic factors (Hall and Rowlands, 1974a). Still, they showed that the lobster hemocytes were not capable to uptake erythrocytes in the absence of the purified agglutinins, whereas in their presence, phagocytosis was occasionally enhanced.

Unfortunately, since then, very few other examples of multiple lectins in crustaceans and shrimps have been reported to effectively validate the view that the multiplicity of non-self-materials could be recognised by functional heterogeneous lectins. Among the existing few examples, we can point out the purification of two distinct carbohydrate-specific lectins in the hemolymph of the stomatopod *Squilla mantis*, and three isolated lectins in the barnacle *M. rosa*, whose binding specificity was, however, towards the same sugar group (see Table 1).

Most of the literature dealing with crustacean lectins concerns the purification and characterisation of a unique lectin from the hemolymph and very rarely is its biological activity determined (see Tables 1 and 2). This is quite intriguing, in view of the great economic interest of crustaceans, especially penaeid shrimps. We will consider below some examples of crustacean lectins, mainly from shrimps, functioning as potential non-self-recognition factors.

In the penaeid *P. monodon*, Ratanapo and Chulavatnatol (1992) reported the agglutination of the highly pathogenic bacteria *Vibrio vulnificus* by a purified lectin called monodin. In the other penaeid, *Pen. californiensis*, Vargas-Albores et al. (1993)

investigated the ability of the purified lectin to react with different marine species of *Vibrio*. They demonstrated that the agglutinin of this penaeid was able to react to at least three different *Vibrio* species, *V. vulnificus*, *V. fischeri and V. parahaemolyticus*. This reaction was specific and the agglutination of *V. parahaemolyticus* could be inhibited by GalNAc and LPS. The inhibition by LPS suggested that this natural ligand of the penaeid lectin could be one effective sign that triggered the shrimp immune system (Vargas-Albores et al., 1993). In the prawn *P. longirostris*, Fragkiadakis and Stratakis (1995) also referred that purified lectins from the hemolymph that recognised *N*-acylaminosugars strongly agglutinated formalin-fixed bacteria, *Pseudomonas aeruginosa* (strain ATCC 9027) and *E. coli* (strain XL-1).

The observations of Vàzquez et al. (1993, 1996, 1997) on the lectins of the hemolymph of the freshwater prawn *Mac. rosenbergii* are of particular interest. The authors (1993) purified and characterised a lectin from the prawn hemolymph and showed (1996) that it had the ability to agglutinate several bacteria species by recognising *O*-keto and *O*-methyl containing sugars and *N*-acetyl-sugar residues from the bacterial polysaccharide cell wall. In a later report (Vàzquez et al., 1997), they incubated the hemocytes (granulocytes) with different vertebrate erythrocytes pretreated with the shrimp serum but also with the purified lectin and an opsonization reaction was not encountered. Morevover, the authors also demonstrated that the granulocytes of *Mac. rosenbergii*, in spite of expressing a surface receptor, which seemed to correspond to the humoral purified lectin (see next section), had the ability to recognise foreign cells in an apparently nonmediated sugar recognition basis.

In our results with the penaeid *P. paulensis*, the uptake of Gram-negative (*V. harveyi*) and Gram-positive (*B. cereus*) bacteria by isolated granulocyte populations increased approximately three fold when these microorganisms were preincubated with the shrimp serum (unpublished data). However, a relation between the opsonic factor(s) and lectins has not yet been established.

It is important to note that apart from the potential involvement of arthropod lectins in non-self-recognition and opsonization, recent findings are emerging on the role of these molecules as immune effectors in microorganism neutralization. In effect, as previously mentioned tachylectin 1 from the horseshoe crab *T. tridentatus* has the ability to inhibit the growth of Gram-negative bacteria (Saito et al., 1995). Also in crustaceans, Majumder et al. (1997) reported that a lectin purified from the edible crab *Scy. serrata* (Table 1) exhibited an antimicrobial activity against *Bac. cereus* and *E. coli* by inhibiting endogenous respiration.

4. Cell-bound lectins

Little data exist on the occurrence of membrane-associated lectins in arthropods, compared to the soluble lectins found in the body fluids. Amirante (1976) was the first to establish that two agglutinins isolated from *Leucophaea maderae* hemolymph were encountered on the cell membrane and in the cytoplasm of numerous hemocytes of this cockroach. Later, Amirante and Basso (1984) demonstrated by specific immunolabelling assays that lectins specific to fucose and GalNAc were present also on the membrane of the granulocytes of the stomatopod *S. mantis*.

The most convincing and unambiguous example of hemocyte-bound lectin in crustaceans and functioning in non-self-recognition and phagocytosis was recently reported by Vàzquez et al. (1997) in the prawn *M. rosenbergii* as mentioned above. The authors showed that the *M. rosenbergii* granulocytes had a surface receptor, which recognised *N*-acetylated and *O*-acetylsialic-acid residues on different erythrocyte surfaces. The specificity of this receptor was very similar to the shrimp humoral lectin. Furthermore, the phagocytic activity of the shrimp granulocytes decreased 50% when antibodies raised against the purified lectin from the shrimp serum were used. These findings indicated the presence of common epitopes between this receptor and the soluble lectin of the serum, thus suggesting that both recognition factors could be the same molecule.

5. Lectin synthesis and induction

There are evidences that arthropod lectins might be synthesised by hemocytes. As already suggested by Hall and Rowlands (1974b), the agglutinating activity in the lobster *H. americanus* was strongly associated to hemocyte extracts and the authors suggested that they should be the main agglutinin source. In the horseshoe crab *T. tridentatus*, also referred to above, four of the five purified lectins came from hemocytes and were released from the granules upon LPS stimulation. Conversely, Fragkiadakis and Stratakis (1997a) demonstrated through in vitro labelling studies, that the lectin of the crab *L. depurator* was synthesised in the hepatopancreas, in spite of its presence within the crab granulocytes.

It is not yet determined whether lectins may be induced and thus increase their concentration in serum upon stimulation by non-self-particles. In the majority of arthropods, attempts to stimulate their production have met only limited success. The potential inducibility of lectins, especially in species of economic interest, such as shrimps, could be of particular relevance since if these molecules are really involved in immune defence reactions, the increase in their concentration could virtually confer a better protection to the host against invading pathogens. We could even speculate on the possibility of stimulating lectin increase by some potentially innocuous immunostimulant substances.

The most well-determined and convincing examples of lectin production and induction in arthropods concern, once more, the insects. The first detailed report was described to in the flesh fly *S. peregrina* larvae where a galactose binding C-type lectin was demonstrated to have dual functions, in defence and development (Natori, 1990). This lectin was found in the hemolymph of the flesh fly after immunisation with bacteria or red blood cells and also after body injury or during pupation (Komano et al., 1980, 1983; Takahashi et al., 1984; Komano and Natori, 1985). Thus, the *S. peregrina* lectin could be induced not only by foreign particles or body injury but also by the fly development. The fly lectin is synthesised in the fat body and secreted into the hemolymph upon microbial stimulation or during pupation. Once in the plasma, it is apparently internalised by the hemocytes. Very recently, a new *S. peregrina* lectin, denominated granulocytin, was also purified (Natori et al., 1999). Different from the first lectin, granulocytin is constitutively synthesised, stored in the granular hemocytes,

and its biological role is still not known. Similarly to the flesh fly, a lectin purified from the silk moth *B. mori*, named hemocytin (Suzuki and Natori, 1983), was also demonstrated to have dual functions (Yamakawa and Tanaka, 1999). The authors reported that the hemocytin gene of the silk moth was transcribed in the hemocytes after injection of *E. coli* and LPS and/or at the larval–pupal metamorphosis.

A very recent and interesting example of lectin induction upon microorganism challenge was provided by Yu et al. (1999) in the tobacco hornworm *M. sexta*. The authors described a C-type lectin, named immulectin, which was synthesised by the fat body and secreted to the hemolymph when the insect larvae were injected with Gram-positive, Gram-negative bacteria or yeast. Immulectin was capable of agglutinating bacteria and yeast in a calcium-dependent manner and was not present in naive insects. Its agglutination activity towards Gram-positive and Gram-negative bacteria was inhibited by LPS but not by laminarin (β-1,3-glucans). As previously described, in addition to its inducibility and microbe agglutination, immulectin, is also capable of triggering the proPO system.

To our knowledge, such conclusive examples of lectin synthesis and induction are far from being clearly demonstrated in crustaceans. Among the very few examples, we can mention the results of Battistela et al. (1996) in the mantis shrimp *S. mantis*. The authors reported the ex novo synthesis of an agglutinin by the hemocytes after stimulation with foreign particles. This production was induced in vitro by the incubation of the mantis shrimp hemocytes with cotton fibres. The authors suggested that this lectin, which promoted the agglutination of human B erythrocytes and that did not naturally occur in the hemolymph was newly produced and secreted in vitro by the hemocytes in order to promote the encapsulation of the foreign particles.

In the black tiger prawn *P. monodon*, Ratanapo and Chulavatnatol (1992) reported an elevation of the lectin (monodin) level in most of the prawns suffering from bacterial (*V. vulnificus*) infection. However, this finding could not be clearly associated to a possible inducible mechanism, since this increased lectin concentration was not observed in all infected shrimps. On the other hand, in the same shrimp species, Sritunyalucksana et al. (1999) failed to induce an increase of lectin concentration in vivo and in vitro by using components of microorganism cells wall, such as LPS, beta-glucans, peptidoglycan and also commercial stimulants.

6. Conclusion

The cultivation of aquatic species, such as crustaceans, molluscs and fish, has been expanding over the last two decades, contributing to the economic and social development of different Asian and Latin-American developing countries. Among crustaceans, shrimp aquaculture represents more than 75% in quantity and penaeids account for the greatest part of the production of this sector.

The major constraint for shrimp culture has been attributed to bacterial and viral diseases, which are frequently accompanied by elevated shrimp mortality and great economic loss. The present methods to diagnose and control diseases are still costly and of limited efficiency. In this context, a better understanding of the crustacean immune system is crucial in order to establish the basis of susceptibility and resistance of

shrimps to different pathogens. Moreover, the potential manipulation of shrimp defence responses in order to increase protection and resistance to infections could be fundamental for shrimp health management. Although the immune reactions are not yet fully elucidated among invertebrates, evidence points towards a role for lectins in non-self-recognition. Therefore, a better understanding of their molecular structure and specificity, as well as their induction, would provide the necessary support upon which the attempts to use these molecules as tools to protect and stimulate shrimp resistance could rely on.

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