Activation of shrimp cellular defence functions by microbial products

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ABSTRACT: Despite their relatively short life and assumed lesser complexity, crustaceans have mechanisms to detect foreign matter. For example, they recognize characteristics such as lipopolysaccharides (LPS) and β-glucans present in bacterial and fungal surfaces. In shrimp, these microbial components can activate cellular defence functions such as phagocytosis, melanization, encapsulation and coagulation. In spite of a direct stimulation, two kinds of recognition proteins have been detected in shrimp plasma. The LPS-binding agglutinin acts like an opsonin to increase the phagocytic rate. After reaction with β-glucans, the beta glucan binding protein (BGBP) induces degranulation and the activation of pro-phenoloxidase (proPO). The activation of proPO triggers cellular functions that inactivate, immobilise or destroy invaders. These plasma proteins stimulate cellular function only after reaction with LPS or β-glucans, in a manner resembling the secondary activities of vertebrate antibodies.

KEY WORDS: shrimp, cellular defence, proPO, BGBP

INTRODUCTION

Similar to other crustaceans, penaeid shrimp have molecules, cells and systems involved in defensive mechanisms to prevent invasions by microorganisms. As with vertebrates, an efficient defence system is expected to include recognition, effector and regulator components. Depending on the responding immunological defence mechanisms have been divided into delayed and immediate responses. As with most other invertebrates, it has been very difficult to demonstrate that a delayed response can be established in shrimp. However, some of their immediate response components have been studied and most of them have similar characteristics to those found in vertebrates and in other invertebrates.

Another interesting question concerning invertebrates, including shrimp, is the specificity of the defence system. While vertebrates apparently recognise and establish an immune response against an unlimited number of chemical structures, the invertebrates have a poor, if any, spectrum. There are few examples of invertebrates responding to challenge with proteins and other substances.

Besides other functions, the defence system in invertebrates is strongly compromised in pathogen elimination. Thus, the most important foreign signals that they have to recognise are those localized on the pathogen surface. In shrimp, two microbial compounds have been shown to be involved in the stimulation of cellular functions, i.e., lipopolysaccharides (LPS) and β–glucans. Although the primary interaction in both cases could be similar (i.e., directly or through a plasma protein), the cell targets and the activated systems are different. Microbial compounds are able to activate cellular functions directly, but the participation of plasma proteins improves the efficiency of the immune system response. These proteins, defined as recognition proteins, react with microbial compounds and either prevent unnecessary cellular activation or, if necessary, increase the response of effector cells.

RECOGNITION PROTEINS

In shrimp, two kinds of proteins are involved in the recognition of microbial products, and their activation of cellular functions has been described (Vargas-Albores 1995). The first group is constitutes multivalent sugar-binding agglutinins, also named hemagglutinins or lectins. The second group constitutes molecules that are apparently monovalent and do not induce agglutination, even though they are able to bind sugar residues. Agglutinating activities have been detected in plasma of *Penaeus monodon* (Ratanapo & Chulavatnatol 1990), *Penaeus stylirostris* (Vargas-Albores et al. 1992), *Penaeus californiensis* (Vargas-Albores et al. 1993a), *P. japonicus* (Bacheré et al. 1995) and *Penaeus indicus* (Maheswari et al. 1997). However, only the agglutinins from *P. monodon* and *P. californiensis* have been purified and their main properties studied. From *P. monodon* plasma, a 420-kDa glycoprotein formed by identical 27-kDa subunits was isolated by affinity chromatography using a fetuin-agarose column (Ratanapo & Chulavatnatol 1990). This lectin, named monodin, can react with NANA (N-acetyl neuraminic acid) and other N-acetyl amino sugars (GalNAc, GlcNAc and ManNAc) as determined by inhibition studies. Monodin induces the agglutination of *Vibrio vulnificus*, a major infective bacterium for prawns, and this agglutination

Vargas-Albores F, Jorge Hernández-López J, Gollas-Galván T, Montaño-Pérez K, Jiménez-Vega F, Yepiz-Plascencia G (1998) Activation of shrimp cellular defence functions by microbial products. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

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can be specifically inhibited by NANA (Ratanapo & Chulavatnatol 1992).

Similarly, the agglutinin isolated from *P. californiensis* plasma by affinity chromatography can be inhibited by monosaccharides (GalNAc, GlcNAc, NANA) and glycoproteins (fetuin, submaxillary bovine mucin). Although specificity has not been completely defined, this agglutinin can react with bacterial LPS and is also capable of agglutinating several Vibrios, including *V. parahaemolyticus*. Furthermore, the reaction can be inhibited by LPS (Vargas-Albores et al. 1993a). This shrimp LPS-binding agglutinin (LPS-BA) has an apparent molecular weight of 180-170 kDa and is formed of four 41-kDa subunits.

The second recognizing protein detected in shrimp plasma has the capability to react with beta glucan, and therefore it is named beta glucan binding protein or BGBP. The first crustacean BGBP was reported in crayfish (*Pacifastacus leniusculus*) by Söderhäll and colleagues (Duvic & Söderhäll 1990) who purified and characterized it exhaustively (Barracco et al. 1991, Duvic & Söderhäll 1992, Duvic & Söderhäll 1993, Thörnqvist et al. 1994). Antibodies against crayfish BGBP have been used to detect this protein in other crustaceans, including shrimp (Duvic & Söderhäll 1993, Thörnqvist et al. 1994, Vargas-Albores et al. 1996).

BGBP has been purified from *P. californiensis*, *P. vannamei* and *P. stylirostris* plasma as a 100-kDa monomeric protein. As shown in Table 1, the amino acid content of these shrimp BGBPs is nearly identical to the homologues from the freshwater crayfish, *P. leniusculus*. Major differences could be noted when comparing BGBP from crustaceans and insects, including differences in molecular masses and amino acid composition. *Bombyx mori* BGBP has a molecular mass of 62 kDa (Ochiai & Ashida 1988), significantly smaller than crustacean BGBP. In *Blaberus craniifer*, BGBP was reported as a 91 kDa protein that under reducing conditions split into subunits of 63 and 52 kDa (Söderhäll & Hall 1988), but such dimeric structure has not been reported in crustaceans.

When the known N-terminal amino acid sequence of crustacean BGBPs (*P. californiensis*, *P. vannamei* and *P. leniusculus*) are compared, high homology is observed. Crayfish BGBP was recently cloned and sequenced (Cerenius et al. 1994), but until now no other crustacean or insect BGBP complete sequence is available. Even though amino acid sequence comparison among BGBP can only be done with the N-terminus of the protein, the sequence appears to be highly conserved between shrimp and crayfish (Fig. 1). There are only 3 different residues between white and yellowlegs shrimp BGBPs and notably, all are substitutions by conserved amino acids. Whether this region corresponds to a specific domain important for protein function or is representative of the entire protein conservation, remains to be analysed once the complete sequence of more BGBPs are obtained. Although these kinds of proteins have also been found in insects (Ochiai & Ashida 1988, Söderhäll & Hall 1988), molecular masses and amino acid compositions are different to shrimp BGBP (Vargas-Albores et al. 1996). Unfortunately, studies on other beta glucan binding proteins that stimulate the proPO system are not yet available.

Table 1. Amino acid composition (mol %) of β-1,3-glucan binding proteins from the white shrimp (Vargas-Albores et al. 1997), brown shrimp (Vargas-Albores et al. 1996) and crayfish (Duvic & Söderhäll 1990).

Amino acid	White Shrimp	Brown Shrimp	Blue Shrimp	Freshwater Crayfish
Ala	6.53	7.14	6.98	5.74
Arg	4.83	5.91	5.70	5.00
Asx	12.36	12.87	15.34	12.22
Cys	n.d.	n.d.	n.d.	0.54
Glx	10.95	10.19	13.06	11.41
Gly	8.92	10.22	9.30	7.52
His	1.79	1.50	2.25	3.17
Ile	6.08	5.90	5.60	6.03
Leu	8.86	8.04	7.09	7.51
Lys	6.37	6.66	7.35	6.84
Met	0.95	0.79	0.58	1.18
Phe	5.34	5.21	4.28	5.55
Pro	3.56	3.14	1.92	3.20
Ser	8.44	9.01	7.47	7.70
Thr	6.31	5.91	5.02	6.23
Tyr	2.98	2.79	3.32	3.37
Val	5.62	4.55	4.74	6.78

n.d. not determined

THE SHRIMP PROPHENOLOXIDASE (PROPO) SYSTEM

In shrimp, like in all crustaceans, melanization is due to the action of phenoloxidase (PO) which promotes hydroxylation of phenols and oxidation of o-phenols to quinones, in response to foreign matter invading the hemocoele and during wound healing (for reviews see (Ashida & Yamazaki 1990, Johansson & Söderhäll 1989, Söderhäll 1992, Söderhäll et al. 1994). These quinones are subsequently transformed to melanin by non-enzymatic reactions. Although a direct antimicrobial activity has been described for melanin, the production of reactive oxygen

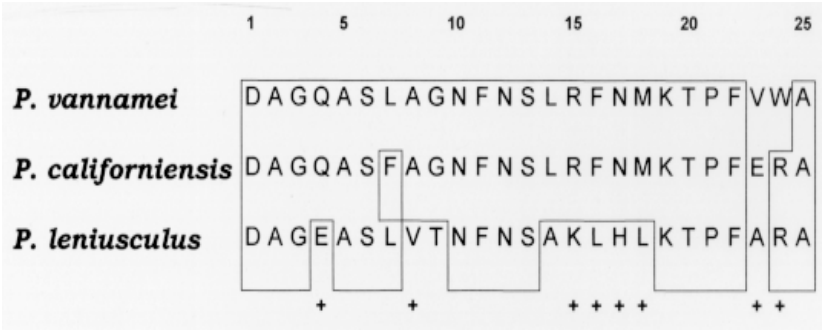

Figure 1. Alignment of *P. vannamei* (Vargas-Albores et al. 1997), *P. californiensis* (Vargas-Albores et al. 1996) and *P. leniusculus* (Duvic & Söderhäll 1990) BGBP N-terminal sequences. Identical sequences are boxed. Plus signs indicate conservative replacement between white shrimp and crayfish.

species such as superoxide anions and hydroxyl radicals during the generation of quinoids (Nappi et al. 1995, Song & Hsieh 1994) has also an important antimicrobial role. In addition, biological reactions like phagocytosis, encapsulation and nodulation are also activated.

Crustacean PO is located inside hemocytic granules as an inactive pro-enzyme called prophenoloxidase (proPO) and its transformation from proPO to PO involves several reactions known as the proPO activating system. As in other crustaceans (Ashida et al. 1983, Smith & Söderhäll 1983, Söderhäll & Hall 1984, Söderhäll & Unestam 1979), the shrimp proPO system is specifically activated by β-1,3-glucans (Vargas-Albores 1995, Vargas-Albores et al. 1996, Vargas-Albores et al. 1997) and LPS (Gollas-Galván et al. 1997, Hernández-López et al. 1996). Therefore, the crustacean proPO system has been considered as a recognition system (Ashida et al. 1983, Ashida & Yamazaki 1990, Johansson & Söderhäll 1989, Johansson & Söderhäll 1992, Lanz et al. 1993, Ratcliffe 1985, Ratcliffe et al. 1991, Ratcliffe et al. 1985, Söderhäll 1992, Söderhäll et al. 1990, Söderhäll et al. 1994). In addition, the proPO system has been proposed as the invertebrate counterpart of the vertebrate complement system since it can be activated by β-1,3-glucans (Ashida 1981, Leonard et al. 1985, Smith & Söderhäll 1983, Vargas-Albores et al. 1993b), has a cascade reaction, and involves proteinases (Aspán et al. 1990a, Söderhäll 1992, Söderhäll et al. 1994). However, other than these similarities, a direct lytic activity by the proPO system has not been detected.

Shrimp proPO can be obtained from hemocyte lysate supernatant (HLS) or by recovering the granular content by centrifuging the cells at 10000 g. However, neither HLS nor granular content could be directly activated by LPSs, including those from *Escherichia coli, Vibrio cholerae, Pseudomonas aeruginosa, Shigella flexneri, Klebsiella pneumoniae, Serratia marcencens* and *Salmonella thypi* (Hernández-López et al. 1996) if Ca^{2+} was absent. On the other hand, by adding Ca^{2+} (5 mM or more), activation of proPO is observed, but this activation occurs even in the absence of LPS. Moreover, during incubation of whole shrimp hemocytes with bacteria or laminarin, the activation of proPO can be detected only if Ca^{2+} (5 mM or more) is present. In absence of Ca^{2+}, proPO (but not PO) could be recovered from the supernatant, indicating that laminarin or LPS induced hemocyte degranulation only, but did not activate proPO.

The proposed proPO activation model for crustaceans (Johansson & Söderhäll 1989, Söderhäll 1992, Söderhäll et al. 1994) involves a proteolytic cleavage mediated by a serine-proteinase (Aspán et al. 1990a, Aspán et al. 1990b), namely proPO activating enzyme (PPAE). Although in shrimp, PPAE has been not purified, its presence and participation on proPO activation has been detected. Shrimp PPAE is contained as a zymogen inside the hemocyte granules, together with proPO and is also released during microbial stimulus, centrifugation or cellular lysis. The activation of PPAE is Ca^{2+}-dependent and the active enzyme can be inhibited by either melittin or soybean trypsin inhibitor (STI).

In shrimp, the activation of proPO involves in two steps. The first one is the degranulation that occurs when hemocytes are stimulated by bacteria, LPS or ß-glucans, and inactive forms of both proPO and PPAE are released. The second one requires the participation of Ca^{2+} for the conversion of inactive PPAE to an active proteinase that, in turn, transforms proPO to active PO (Gollas-Galván et al. 1997). Thus, under *in vivo* conditions, PPAE is activated by plasmatic Ca^{2+} after hemocyte degranulation, which is induced by external stimuli (Gollas-Galván et al. 1997), like LPS and ß-glucans.

THE COAGULATION SYSTEM

Another system activated by microbial products is coagulation. This is an essential defence response for crustaceans because it prevents both loss of hemolymph through breaks in the exoskeleton and the dissemination of bacteria throughout the body (Martin et al. 1991). According to Tait (1911), shrimp posses type C coagulation (i.e., there is a requirement for plasma proteins and cellular components). The key plasma protein which constitutes the clot has been named clotting protein or CP. It appears to be present in relatively high concentrations in hemolymph.

CP has been purified and characterized from other crustaceans, including the spiny lobster, *Panulirus interruptus* (Fuller & Doolittle 1971a), crayfish, *P. leniusculus* (Kopácek et al. 1993) and in the white shrimp, *P. vannamei* (Montaño-Pérez et al. 1998). In all cases, CP was reported as a lipoglycoprotein with a molecular mass about 420 kDa, and with two identical subunits linked by disulfide bridges. In addition, the amino acid composition of the white shrimp CP (Table 2) is similar to its homologues from crayfish (Kopácek et al. 1993), lobster (Fuller& Doolittle 1971a) and sand crab (Madaras et al. 1981), even though the latter species has a different type of coagulation (Ghidalia et al. 1981).

Table 2. Amino acid composition (mol %) of clotting protein from white shrimp (Montaño-Pérez et al. 1998), crayfish (Kop?cek et al. 1993), lobster (Fuller& Doolittle 1971a) and sand crab (Madaras et al. 1981).

Amino acid	*Penaeus vannamei*	*Pacifastacus leniusculus*	*Panulirus interruptus*	*Ovalipes bipustulatus*
Asx	10.39	9.05	9.90	11.94
Thr	7.68	8.72	7.12	6.66
Ser	7.92	7.65	8.19	6.75
Gxl	11.61	12.30	10.91	12.22
Pro	5.64	4.99	5.25	5.74
Gly	7.17	5.84	6.17	7.40
Ala	5.44	5.72	5.46	6.01
Cys	n.d.	1.53	1.31	1.27
Val	6.97	6.48	6.90	6.66
Met	1.38	1.49	1.69	1.75
Ile	5.69	6.31	5.12	5.09
Leu	8.79	8.32	9.60	8.14
Tyr	3.28	3.13	3.18	2.31
Phe	4.65	5.19	4.10	3.98
His	3.77	3.18	4.16	3.61
Lys	5.02	6.28	4.18	5.64
Arg	4.56	3.84	4.75	4.81

n.d. not determined

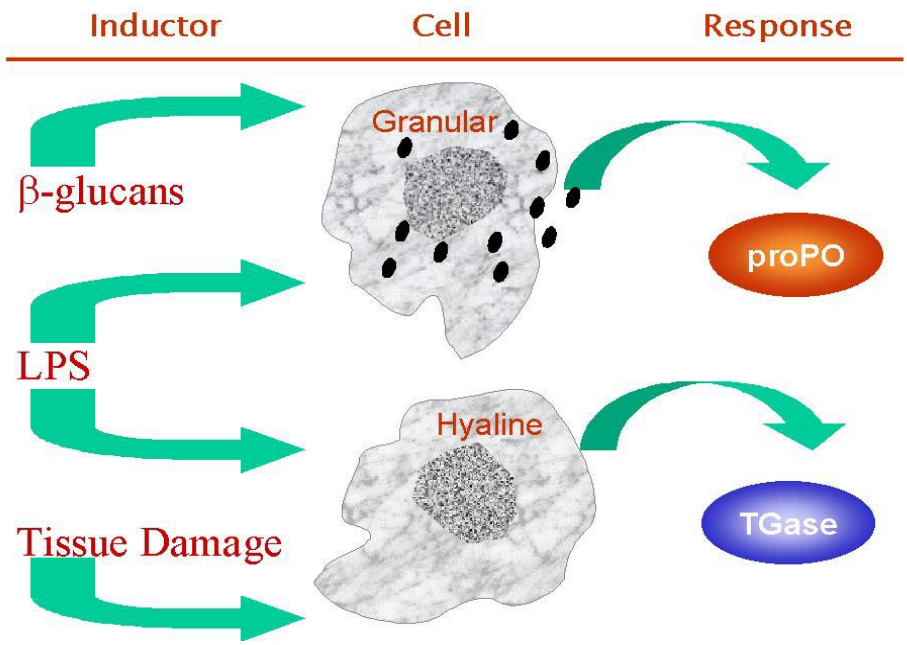

Figure 2. b-glucans and LPS stimulate shrimp hemocytes. Granular cells are stimulated by both microbial compounds producing degranulation, whereas hyaline cells are stimulated by LPS (and tissue damage) releasing TGase.

Apparently, cleavage of the N-terminus is not involved in the crustacean event, since the same N-terminal sequence is present before and after gelation (Fuller& Doolittle 1971b). However, the basis of coagulation is a cellular transglutaminase (TGase) supplied by hemocytes (probably hyaline cells) under external stimulus. The TGase-catalyzed reaction results in the formation of intermolecular ε–(γ-glutamyl)-lysine cross-links between the side chain of a glutamine residue on one polypeptide and the side chain of a lysine residue on another polypeptide (Lorand & Conrad 1984).

In shrimp, as well as in crayfish, polymerization of purified shrimp CP occurs by adding guinea pig liver TGase in the presence of Ca^{2+}. Polymerization of *P. vannamei* CP was also possible with either *P. californiensis* or *P. stylirostris* hemocyte lysate, in the presence of Ca^{2+}. Thus, considering that the reaction was also observed using guinea pig TGase, polymerization of CP is not species-specific. In addition, since polymerization of shrimp CP can be catalyzed by guinea pig liver TGase, it is possible that other cellular factors are not required for clot formation. Therefore, in spite of structural or probable differences in origin, vertebrate and invertebrate coagulation systems have the same mechanisms, although different strategies. In both cases, the clot is formed by the action of a Ca^{2+}-dependent TGase on a clottable plasma protein, fibrinogen or clotting protein, respectively. However, in vertebrates, a previous proteolytic processing reaction is required (fibrinogen to fibrin) to obtain a substrate for the TGase which is found in plasma. By contrast, shrimp TGase is compartmentalised in hemocytes (as in some other invertebrates) and acts immediately after release. Considering that prior proteolytic cleavage is not required, stimulation of hyaline hemocyte with bacterial LPS appears to be enough to induce the release of TGase and lead to subsequent plasma coagulation.

CELLULAR ACTIVATION

Both LPS and ß-glucans are capable of stimulating shrimp hemocytes to release cellular components. ß-glucans stimulate the proPO activating system, whereas LPS can stimulate both the proPO system and the coagulation system by activating different hemocyte populations. In addition to direct activation, the participation of recognition proteins (LPS-BA and BGBP) is very important in shrimp both for preventing undesirable activation and for stimulating cell activation. In despite of some differences, shrimp recognition proteins share a characteristic with vertebrates antibodies: the activation of cellular mechanisms after reaction with antigens (Fig. 2).

When mice erythrocytes (used as target cells) were incubated with shrimp hemocytes and LPS-BA, a stimulation in phagocytosis was observed. In addition, when the erythrocytes were previously incubated with sub-agglutinating doses of LPS-BA, washed and followed by incubation with shrimp hemocytes, similar stimulation of phagocytosis was recorded. Thus, LPS-BA acts as an opsonin rather than a direct activator of shrimp hemocytes. These results help to explain why *in vivo* spontaneous hemocyte agglutination does not occur, even though the agglutinin seems to bind to hemocyte membranes. Similarly, BGBP potentiates laminarin stimulation of the proPO system. However, the real stimulator is the complex BGBP-laminarin, as demonstrated in crayfish where a corresponding receptor on the hemocyte surface has been

reported. These results indicate that recognition proteins require a prior reaction with ß-glucans before they can stimulate cellular functions.

On the other hand, direct hyaline cell stimulation by LPS induces the release of a polymerizing TGase-type enzyme that in turn causes clot formation. Thus, in shrimp several defensive responses are activated by microbial products (LPS and b-glucans). Even though these products can directly activate proPO and the coagulation system, plasma recognition proteins that enhance the cellular defence mechanism also play key roles. Paradoxically the signal to activate the defence mechanisms is given by pathogens themselves through their surface components like LPS or glucans.

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