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Minireview

β-Glucan in invertebrates

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Abstract

β–Glucans, as biologically active polysaccharides, have been used for decades but only recently have become a focus of evolutionary studies. As β–glucans are shown to be active in all animal species studied - from earthworms to humans - we can safely conclude that β–glucan immunostimulation is one of the first defensive mechanisms active across the entire evolutionary spectrum. The recognition of β–glucans as major components of yeast, fungal, and bacterial cell walls belongs to the first defense mechanisms that evolved during phylogenetic processes.

Key words: glucan; invertebrates; immunity; PPO

Introduction

Polysaccharides and particularly glucans have a long history as immunomodulators. Interest in glucans has increased after experiments showing that zymosan stimulates the macrophages via the activation of complement system.

β-1,3 glucans are structurally complex homopolymers of glucose, usually isolated from yeast and fungi. The number of individual glucans is almost as great as the number of sources used for isolation. Different physicochemical parameters, such as solubility, primary structure, molecular weight, branching, and polymer charge, influence the biological activities of β-1,3 glucans. It is not surprising that β-glucans have been extensively studied for their immunological and pharmacological effects. More than 600 papers describing the biological activities of glucans exist. Up until now, strong immunostimulating effects of β-1,3 glucans have been demonstrated in all tested animal species including earthworms (Beschin *et al*., 1998), shrimps (Duvic and Söderhäll, 1990), fish (Anderson, 1992), mice, rats (Feletti *et al*., 1992), rabbits, guinea pigs (Ferencik *et al*., 1986), sheeps, pigs (Benkova *et al*., 1991), cattle (Buddle *et al.,* 1988) and humans.

The immunomodulating effects of β-glucan are well established during the development of immune reactions. At the same time, it is easy to understand why glucan, forming part of the bacterial and fungal cell walls, is an important molecule to be recognized by the defense system. However, the importance for the invertebrates, often less concerned with the sterility of the inner milieu, is not as clear.

In invertebrates, as in all metazoans, each phylum represents an appropriate fundamental morphological pattern according to the evolutionary history of the phylum and adaptation to the environmental conditions in which it has radiated. However, from the defense standpoint, three major principles, which may well be based on varying molecular and biochemical backgrounds, are common to all of them. The animals are capable of recognition, processing and elimination of non-self. Invertebrates have evolved a wide variety of active defense mechanisms enabling them to use their highly effective innate defense pathways to protect themselves against invading pathogens despite the absence of an adaptive immune system based on lymphocytes or antibodies. At the same time, microorganisms possess distinctive molecular patterns, including β-glucans. Pattern recognition proteins binding to β-glucan have been implicated in the activation of the innate defense reactions.

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Phenoloxidase

Invertebrates do not use molecules of the immunoglobulin superfamily as receptors for recognition of non-self structures. Regardless of this, they exhibit considerable selectivity in their defense reactions (Ratcliffe and Rowley, 1979; Vitvièka and Šíma, 1998). In general, arthropods, molluscs, and deuterostomian tunicates recognize non-self material, the microbial surface determinants that are conserved and ubiquitous among microorganisms but not present in the eukaryotic host. These structures - mainly lipopolysaccharide (LPS), peptidoglycan (PGN), mannan, and β-1,3 glucan - are recognized by means of a group of germ-line encoded receptors, usually termed pattern recognition receptors (PRR). Using highly selective recognition processes, signaling cascades are thus activated. These cascades regulate production of defense substances, agglutinins and poisonings (generally lectins), and non-agglutinin factors, inducible or constitutive antibiotic peptides and the components of the prophenoloxidase (PPO) complex in the host (Ratcliffe, 1991; Hoffmann *et al*., 1999; Andreu and Rivas, 1998; Hoffmann, 2004). The PPO of tyrosinase type, a Cu-containing enzyme, is widely distributed both in prokaryotes and eukaryotic organisms. It was hypothesized that the Cu-binding domains of the PPO evolved from similar regions of an ancestral hemocyanin molecule of arthropods (Fujimoto *et al*., 1995; Kawabata *et al*., 1995; van Holde *et al*., 2001).

Specific non-self recognition mechanisms of the PPO system, as a basic part of immune defense of invertebrates, are involved during a row of hierarchized processes like cell cooperation and communication in the course of phagocytosis (Smith and Söderhäll, 1983; Söderhäll *et al*., 1990), nodule and capsule formation (Kobayashi *et al*., 1990), melanin synthesis (melanization of foreign bodies) and sclerotization (cuticle tanning and hardening) (Lackie, 1988; Marmaras *et al*., 1996; Söderhäll and Cerenius, 1998), hemocyte locomotion (Takle and Lackie, 1986) and coagulation of blood (Durliat, 1991).

Participation of the PPO during melanization, a characteristic defense reaction of arthropods, was first documented in grasshopper eggs in 1941 (Bodine and Allen, 1941). The PPO catalyzes two key steps in the melanin synthesis (Mason, 1955, 1956). Both cellular and humoral immunity are commonly associated with melanin deposits (Rattcliffe, 1991) which formation could be very rapid: in *Galleria melonella* the entrapped microbes melanize during 5-60 min (Rattcliffe and Gagen, 1977).

Similarly, the PPO can control terminal differentiation of blood cells. In crayfish *Pacifastacus leniusculus* the administration of β-1,3 glucan induces a rapid decrease of hemocytes followed by an accelerated increase of maturation of new cells expressing PPO transcript and their release into circulation (Söderhäll *et al*., 2003).

Some activities of the PPO system resemble reactions of vertebrate lectin pathway of the complement system (C), e.g. cell lysis, opsonization, and substance release (Söderhäll, 1982; Cerenius and Söderhäll, 2004; Ma *et al*., 2004). Both the lectin pathways of C and the PPO are proteolytic and comprise PRR proteins, serine proteases activation and their inhibitors, generation of opsonins and Ca^{2+} presence (Ashida and Dohke, 1980; Aspán *et al*., 1990; Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004). The role of Ca^{2+} during the β-1,3 glucan-dependent PPO activation has been repeatedly documented but it is still unclear (Ashida *et al*., 1983; Lee *et al*., 2004).

In addition to the substances of microbial origin - mainly PPS, PGN, mannans, and β-1,3 glucans - various physical and chemical stimuli such as temperature, pH, detergents, denaturing agents or proteases activate the PPO cascade (Söderhäll and Unestam, 1979; Ashida and Yoshida, 1988; Dunphy, 1990; Brivio *et al*., 1996).

The PPO is discharged from cellular granules in an inactive form (pro-enzyme). After the conversion into its active form, the PPO cascade molecules are released from hemocytes into the hemolymph pool (Preston and Taylor, 1970; Ashida *et al*., 1983; Durlay and Lackie, 1985). When the PPO is activated at the surfaces of microbial cell, it generates highly reactive and toxic quinone intermediates (Johanson and Söderhäll, 1989). The reaction consists of active catalyzation of the oxygenation of monophenols to *o*-diphenols and their further oxidation to *o*-quinines (Aspán *et al*., 1995; Chase *et al*., 2000).

β-1,3 glucans are first recognized by PRR which activate serine proteases of PPO system (see below). The PPO system activating factors (similar to *Drosophila* easter-type serine proteases) cleave PPO to phenoloxidase (PO). By means of oxidation of phenols to melanin, the PO produce toxic antimicrobial substances, which process is accompanied by sclerotization of cuticle (Lee *et al*., 1998, 2004; Satoh *et al*., 1999). The smallest structure able to activate PPO cascade is a laminaripentose as has been demonstrated in crayfish PPO system (Söderhäll and Unestam, 1979). It is remarkable that the invertebrate peptidoglycan and β-1,3 glucan PRR triggering the PPO cascade are non-enzymatic homologues of bacteriophage T7 lysozyme and bacterial β-1,3 glucanase (Ochiai and Ashida, 1999; Zhang *et al*., 2003). Moreover, some PRR recognizing PGN can also function to recognize β-1,3 glucan and induce the PPO cascade (Lee *et al*., 2004). In a preliminary study, the β-1,3 glucans isolated from yeast cell walls exhibited a significant stimulation of the PPO system activity in hemocytes *in vitro* and in hemolymph *in vivo* of black tiger shrimp, *Penaeus monodon* (Suphantharika *et al*., 2003). The complex of PPO and IL-1 like molecule found in *Manduca sexta* (tobacco hornworm) could be regarded as an evolutionary novelty in invertebrate type defense (Beck *et al*., 1996).

Glucan-binding protein

Invertebrates are using innate immune mechanisms conserved throughout the animal kingdom. A heterologous group of hemolymph proteins, among others, serves as a surveillance mechanism by binding to the surface of invading microbes. One member of this group is so called β-glucan-binding protein (GBP).

Proteins binding to the β-glucan have been identified in numerous arthropod species. Their activity is usually to stimulate the PPO activation cascade. Subsequent purification and identification revealed similar properties: proteins containing carboxyl-terminal glucanase-like domain without enzymatic activity. Glucan is bound via less conserved amino-terminal domain. In *M. sexta,* two different GBPs are 57% identical in amino acid sequence (Kanost *et al*., 2004). Both these GBP differ with respect to their presence and up regulation upon the immune challenge. In both cases, they are involved in potentiation of PPO activation and in agglutination of bacteria and yeast. GBP, sometimes also named as glucan-receptor, was also isolated from plasma of a silkworm, *Bombyx mori* (Yoshida *et al*., 1986). Later studies showed that these molecules are 30 kDa lipoproteins (Ujita *et al*., 2002). For a detailed study of a GBP from *B. mori,* see (Ochiai and Ashida, 2000).

Glucan binding proteins are commonly found in crustaceans. They usually have a size app. 100 kDa and besides binding glucan, bacteria and hemocytes, they have strong ability to act as opsonins (Cerenius *et al*., 1994). Even as numerous proteins with similar properties (such as mannan-binding, LPS-binding or factor G) have been found (for review see Söderhäll and Cerenius, 1998), the GBPs are probably the most important. There are suggestions that these proteins might develop from a primitive glucanase and later evolved into glucan-binding molecules without any enzymatic activity. In addition, crayfish GBP is identical to the shrimp protein LP1, which is involved in lipid transport to the ovary. Both molecules react to the glucan binding by binding to the surface of the hemocytes, probably via an Arg-Gly-Asp motif (Holmblad *et al*., 1997). A different, high-density glucan-binding lipoprotein has been found in the white shrimp *Panaeus vannamei,* having only significant similarity to the GBP from the crayfish (Romo-Figueroa *et al*., 2004) and to ovarian vitellin (Garcia-Oroyco *et al*., 2002). Söderhäll's group isolated and characterized a GBP from the crayfish *Pacifastatus leniusculus* and found that this 40 kDa protein has a strong similarity to bacterial glucanases and to the GBPs from *Eisenia foetida.* This protein bound both linear and branched glucans as well (Lee *et al*., 2000).

A detailed study evaluated cDNA cloning, purification, properties and functions of a GBP from a moth, *Plodia interpunctella.* The report showed that the protein contains an open reading frame that encodes 488 amino acids of which the first 17 residues comprised the secretion signal peptide. Carboxyl-terminal domain was similar to other invertebrate GBPs as well as β-glucanases from bacteria and sea urchin. In addition, this GBP was constitutively expressed in all life stages and no microbial challenged changed its expression (Fabrick *et al*., 2003). Subsequent study showed that the amino-terminal domain consists primarily of an α-helix secondary structure with only minor β-structure. Functional data revealed that this GBP bound only to the 1,6-branched glucans (as it bound to laminarin, but not to curdlan). Hence, this GBP has two binding domains separated by a putative linker region, one for glucan and the second for the stimulation of the PPO cascade (Fabrick *et al*., 2004).

A surprising observation has been made by Bilej's group. These authors found that the cytolytic factor 1, present in the coelomic fluid of *E. foetida* earthworms, has significant homology with the catalytic region of β-1,3-glucanase and strongly binds β-1,3-glucan. In addition, this molecule also participates in activation of the PPO cascade (Beschin *et al*., 1998).

Other effects of β-glucan

Immunostimulating effects of glucans have also a significant commercial potential. β-glucans have successfully been used to increase the resistance of shrimp *Panaeus japonicus* against vibriosis (Itami *et al*., 1994), further studies using *P. monodon* showed protection against vibriosis, white spot syndrome virus and *Vibrio damsela* (Su *et al*., 1995; Song *et al*., 1997) and also enhancement of survival and immunity during brood-stock rearing (Chang *et al*., 2000). All these effects were caused by direct impact on haemocytes via stimulation of phagocytosis, cell adhesion and superoxide anion (Chang *et al*., 2000) and superoxide dismutase production (Chang *et al*., 2003). Surprisingly, the glucan-induced resistance was maternally transmitted (Huang and Song, 1999). In addition, glucan injection reduced expression of peroxinectin (Sritunyalucksana *et al*., 2001).

Surprising data was obtained in the freshwater crayfish, *P. lenieusculus*. The injection of glucan caused a short-term severe loss of hemocytes, followed by a rapid recovery due to the accelerated release of cells from the hematopoietic organs (Söderhäll *et al*., 2003). When compared to other effects of glucan in invertebrate, this function on stem cells is the only one, which is completely comparable to vertebrates including humans (Patchen and MacVittie, 1983).

Another potentiation of defense reaction by glucan was documented in earthworms. A study of *E. foetida* showed that earthworms responded to the glucan challenge by increase in coelomic cytolytic factor and lysozyme-like activity and that these effects were caused by direct binding of glucan to hemocytes (Kohlerova *et al*., 2004).

In mosquito *Anopheles gambiae,* injection of glucan leads to the induction of hemolymph proteins. When compared with the effects of LPS or *Escherichia coli,* the study showed that each stimulus induced different proteins, suggesting that mosquitoes have the ability to discriminate between elicitors (Han *et al*., 1999). Previous experiments using a *Drosophila* cell lines showed strong *in vitro* induction of cecropin genes by addition of algal glucan (Samakovlis *et al*., 1992) suggesting the role of hemocytes in glucan-induced effects.

The means of how invertebrates recognize glucan are still not completely understood. A novel approach was used during studies of an insect apolipoprotein. It has been shown that insect apolipoprotein, homologous to mammalian apoE, strongly binds β-glucan and probably acts as a pattern recognition molecule (Whitten *et al*., 2004).

The last species from the invertebrate kingdom used in glucan-research was a pulmonate snail, *Biomphalaria glabrata.* However, rather than a detailed study of the glucan effects, the experiments

were focused on use of glucan in evaluation of lectin-phagocytosis in molluscs. Sufficient proof exists, however, to support the theory of the existence of glucan receptors on mollusc hemocytes (Bayne and Fryer, 1994).

Conclusions

Two different reasons for the studies of β-glucan in invertebrates exist: one is the general progress of our knowledge of the fundamental defense reactions in invertebrates, including phagocytosis, lectins or phenoloxidase systems; the other being the ever increasing need to find more natural treatment for invertebrates stressed by extensive farming. Many species of invertebrates, particularly the arthropods, also contribute to the massive spread of the most devastating infectious diseases throughout the world.

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