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Characterization of glycoconjugates in giant freshwater prawn embryos by lectin histochemistry

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Abstract

Lectin binding sites in early and late-stage embryos were studied to discover markers of primordial germ cells as tools to study innate immunity and the defensive mechanism of the giant freshwater prawn *Macrobrachium rosenbergii*. Changes in carbohydrate content of early and late embryos were examined using peroxidase-labeled lectins, *Helix pomatia* agglutinin (HPA), concanavalin A agglutinin (Con A), *Ricinus communis* agglutinin (RCA-I), *Ulex europaeus* agglutinin (UEA-I), and wheat germ agglutinin (WGA). Results were preincubated with 0.2 M specific inhibitory sugars, *N*-acetyl-D-galactosamine (NAG) for HPA, α-D-mannose for Con A, β-D-galactose for RCA-I, NAG for WGA and α-L-fucose for UEA-I. HPA binding sites were predominantly located at the inner embryonic envelope and primordial germ cells, and to a lesser extent, at the yolk and vegetative pole. Nuclei of primordial germ cells responded to HPA, whereas the vegetative pole or pole cells and yolk granules bound to RCA-I and UEA-I rather than to Con A, WGA or HPA. All lectins had detectable labelling on outer shell layers of prawn embryos. In late-stage embryos, the oligosaccharide moieties of cell surfaces on different cells were different; primordial germ cells (PGC) bound with HPA, muscle bound with UEA-I, and nerve tissue bound with WGA, indicating prevention of the action of antigenic agents such as antibacterial, antifungal, and antiviral peptide systems as innate immunity. Lectins were used to recognize changes in the location of glycoconjugates during the embryonic development of the giant freshwater prawn. Glycosylation of early and late-stage embryos can be used to predict innate immunity.

Keywords: Lectins, Primordial germ cells, Giant freshwater prawn, Cell differentiation, Cell glycoconjugates, Glycosylation, Embryos

1. Introduction

Lectins are proteins or glycoproteins, derived from both animal or plant material, that recognize and bind to specific sugar moieties. They are used as tools to analyze animal cell glycoconjugates because of their capacity to discriminate the numerous complex carbohydrate structures found on the surface of cells and attached to soluble glycoproteins. All cells have carbohydrates on their surfaces. Lectins are present in several plants such as *Ulex europaeus* (Gorse), *Triticum vulgaris* (Wheat germ), *Canavalia ensiformis* (Jack bean) and *Ricinus communis* (Castor bean). Binding of the lectin from the snail *Helix pomatia* agglutinin (HPA), indicates *N*-acetylgalactosamine (GalNAc), whereas concanavalin A agglutinin (Con A), wheat germ agglutinin (WGA), *Ricinus communis* agglutinin (RCA-I), *Ulex europaeus* agglutinin (UEA-I), indicate α-D-mannose, *N*-acetyl-D-glucosamine (NAG), β-D-galactose and α -L-fucose, respectively.

HPA is a binding lectin found in the albumen gland of the snail [1,2]. As a constituent of perivitelline fluid, HPA protects fertilized eggs from bacteria and is part of the innate immunity system of the snail [1]. Variations of sugar moieties on cell surfaces indicate glycosylation, which is an indicator of cell differentiation; for example, in rat microglial cells [3] and provides instructions that enable adaptive immune responses to enhance immunogenicity employing phylogenetically ancient defense mechanisms, also known as innate immunity [4-6]. This system is the first line of defense that helps to limit infection at an early stage and relies on germline-

encoded receptors that recognize conserved molecular patterns on micro-organisms to destroy antigens and protect and preserve themselves. Invertebrates secrete a rigid, wax-covered cuticle that serves as a mechanical barrier. They can also rapidly produce effective innate immune responses during infection. Invertebrates do not contain antigen-specific lymphocytes and do not produce immunoglobulins [6]. However, they contain several soluble molecules that bind to and lyse micro-organisms. Typically, such molecules are lectin-like proteins, which bind to carbohydrates present on microbial cell walls, and hence initiate various immune responses including agglutination of the invading micro-organisms [7-9].

In this study, lectins were used in histochemical studies to probe for localization for sugar residues, cell-to-cell interactions and variation of biological activity.

The freshwater giant prawn, *Macrobrachium rosenbergii* de Man (1879), is one of the most economically important aquatic species of several countries in Asia, such as Bangladesh, Vietnam, China, Taiwan and Thailand. Several projects have been undertaken to improve the management of heterogeneous prawn populations in nursery ponds and recognize the special requirements of harvesting and processing, to ensure that only high-quality products reach the market [10]. *M. rosenbergii* lives in tropical freshwater environments with turbid conditions caused by adjacent brackish areas of water. Because its larval development must occur in brackish water, gravid females migrate downstream to estuaries where the eggs hatch as free-swimming larvae. After hatching, the eggs develop into planktonic larvae that undergo several zoeal stages. After metamorphosis, postlarvae adopt a more benthic lifestyle and commence an upstream migration toward freshwater to become adults [11].

Primordial germ cells (PGCs) are the precursors of gametes. They enter the genital crest when the gonad forms [12]. In many insect species, the PGCs are formed before blastoderm formation. The polar granules are incorporated into a single cell that buds off at the posterior pole to form what is called a pole cell. The PGC emerges from the pole cell [12,13]. The PGCs of various organisms such as chickens *Gallus gallus*, *Xenopus*, marsupials and humans are large round cells compared to the surrounding somatic cells [12-14]. When examined by phase-contrast microscopy, the PGCs show as a cluster at the animal pole of the yolky egg of the giant freshwater prawn. They can be seen in the early embryonic stage and can be better distinguished with increasing of their development. They are large round cells compared to the surrounding cells. The nucleus is massive, with 2-3 prominent nucleoli, and the cytoplasm is granulated [15-16]. On day 6, migrating PGCs are discovered in the posterodorsal region of the brain. They migrate along the coelomic membrane, arrive at their destination on day 10 and become permanent residents on days 15-16 [12]. They show no distinguishing cytoplasm but have numerous condensed chromatins in the nucleus [12,14-16]. They divide at the anterior of the heart and then enter into the gonad [15-16].

The glycoconjugate pattern in two-stages of prawn embryos and the occurrence and changes of carbohydrate moieties during early and late development were investigated using peroxidase-labeled lectins of HPA, Con A, RCA-I, UEA-I and WGA. The new finding was embryonic glycosylation. We found that HPA was a marker for PGCs as the original sex cells. WGA was a marker for nerve tissue, while UEA-I was a marker for prawn embryo muscles.

2. Materials and methods

The embryos of giant freshwater prawn, *M. rosenbergii*, are shown as developed by day 0.5 (Figure 1A), 5.5 (Figure 1B), 8.5 (Figure 1C), 12.5 (Figure 1D) and 18.5 days (Figure 1E) until hatching (Figure 1F). Clusters of 5.5-day-old and 12.5-day-old embryos were collected from embryonic culture in 15% artificial seawater.

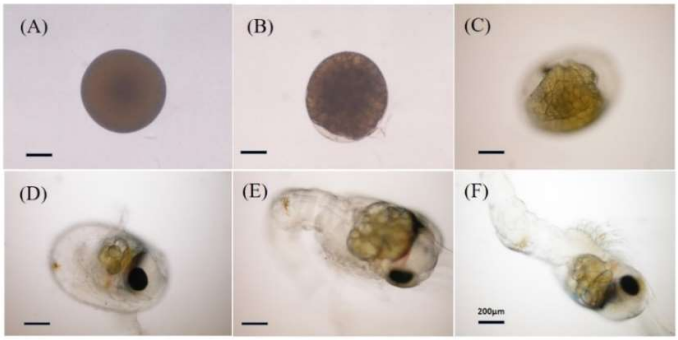

Figure 1 Embryonic stages collected from brooder before testing with conjugated plant lectins. (A) 0.5-day-old embryo, (B) 5.5-day embryo, (C) 10.5-day embryo, (D) 12.5-day embryo, (E) 16.5-day embryo and (F) 18.5-day hatching larva.

The collected specimens were fixed with Bouin's solution, dehydrated in an alcohol series, embedded in paraplast and sectioned to 5 µm thickness using a rotary microtome. Sections were incubated with five peroxidase-labeled lectins, 20 µg/mL HPA, 10 µg/mL Con A, 50 µg/mL RCA-I, 20 µg/mL UEA-I and 50 µg/mL WGA for 1 h at room temperature (Table 1). All lectins were purchased from Sigma Chemical Co. The sections were then washed with 0.05 M Tris buffer (pH 7.6) and incubated with 0.5 mg/mL 3',3' diaminobenzidine (DAB) containing 0.01% H_2O_2 for 15 min. After incubation, the sections were rinsed with deionized water, dehydrated and mounted with an adhesive mount (Permount, Sigma Chemical Co.). Control sections underwent a 30 min pre-incubation with the specific inhibitory sugars shown in Table 1. Sections were incubated with 0.5 mg/mL DAB combined with 0.01% H_2O_2 for 15 min and prepared as controls for endogenous peroxidase [17].

The sections were examined under a bright-field microscope (Olympus BX 51) and images were captured using an Olympus DP 50 digital camera. Primordial germ cells were identified according to [16,17] and the intensity of labeling was evaluated by Photoshop CS6 to analysis color intensity and was classified as either intense labeling represented by + + + + + = very intense; + + ++ = intense; + + + = moderate; ++= weak; + = very weak; – = none [17,18].

Table 1 Lectins used in this study: their acronyms, concentration used and their sugar-binding specificities.

Lectin source	Acronym	Concentration (µg/mL)	Specificity*	Inhibitory/binding sugar	References
H. pomatia	HPA	20	GalNAcα (or ß)-1,3Gal >D-Gal	GalNAc Lactose	[1,17,18]
R. communis	RCA-I	50	D-Gal > D-GalNAc	N-Acetylglucosamine	[17,19]
Triticum vulgaris	WGA	50	(GlcNAc ß-1,4) n >NeuNAc	α -D-methyl-mannose	[17,20]
Canavalia ensiformis	Con-A	10	Man α-1 >Glcα1>GlcNAcα-1	L-Fucose	[17,19]
Ulex europaeus	UEA-I	20	L-Fucα-1,2Gal		[17,20]

*Abbreviations: *N*-acetylgalactosamine (GalNAc); galactose (Gal); *N*-acetyl-β-D-glucosamine (GlcNAc); *N*-Acetylneuraminic acid (Neu5Ac); Mannose (Man); D-glucose (Glc); L-fucose (LFuc).

3. Results and discussion

All five lectins tested bound strongly or not at all to different parts of the early and late embryos (Table 2). Results showed that HPA conjugated cells were remarkable for PGCs in both early and late embryos (Figure 2, 3B and E). WGA conjugated strongly with nerve cells of late embryos (Figure 3C and F). UEA-I (Figure 4A and C) and RCA-I lectins (Figure 4B and D) bound strongly to yolk granules. Con A lectins bound to most of the various groups of cells except for yolk granules (Figure 3A and D). HPA intensely labeled the primordial germ cells of early embryos and primordial sex cells in the late embryonic stage, while HPA binding sites were found on ventral nerves, tail and leg muscles of late embryos (Figure 2).

In addition, HPA bound moderately to cell mass of the outer and inner envelope in both embryonic stages. Eggs of the giant freshwater prawn developed to embryos from a unicellular fertilized oocyte to cleavage, blastula and gastrula stages as 5.5-day-old embryos. PGCs, when binding to HPA, became clear and could be identified as the original fertilized egg. [12,15-16].

Moreover, HPA can identify glycosylation changes associated with PGC development to recognize aberrant O-linked α-*N*-acetylgalactosamine (GalNAcα) glycosylation, as shown by studies of cell proliferation associated with cancer detected by HPA [20-22]. HPA is an GalNAc binding lectin found in the albumen gland of the snail [23] similar to HPA bound strongly to PGCs and yolk of prawn embryos in this study.

Similar findings were reported from mouse placental GMG cells in which the binding site of HPA was at the cytoplasmic granules [24]. The granules distributed in the cells responded to HPA, possibly due to *N*-acetyl-α-D-galactosamine in the cytoplasm. By contrast, PGCs in *Xenopus* sp. was not specific to HPA [25] which bound to the outer envelope of late embryos in contrast to the inner envelope in early embryos. Several suggestions as to why this altered glycosylation is associated with the change in behavior of the PGCs have been put forward [26]. However, the HPA-binding glycoconjugates have not yet been identified, nor have any cellular functions related to the metastatic cascade been attributed to identify HPA-binding glycoconjugates to histochemically analyze the lectin-binding properties of human breast cancer [20] and pancreatic cancer [23].

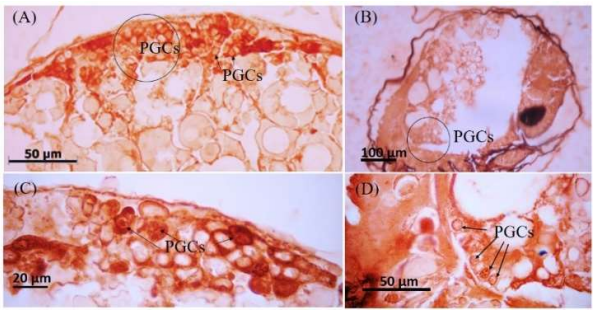

Figure 2 (A and C) HPA labeled to the primordial germ cells in early embryos and (B and D) HPA in late embryos bound on a group of primordial germ cells.

Table 2 Lectin binding patterns found in stages of embryonic giant freshwater prawn.

Stages	Location	HPA	WGA	RCA-I	UEA-I	Con-A
Early-stage embryo	Outer embryonic envelope	+ + +	+	+	+	+ +
Early-stage embryo	Inner embryonic envelope	+ + +	–	–	–	+ +
Early-stage embryo	Embryonic cells	+ + +	+ + + + +	–	–	+ +
Early-stage embryo	Yolk	+ +	–	+ + + +	+ + +	–
Early-stage embryo	PGCs	+ + + + +	–	–	–	+ +
Late-stage embryo	Outer embryonic envelope	+ + +	+	+ +	+ +	+ +
Late-stage embryo	Inner embryonic envelope	+ + +	–	–	–	+ +
Late-stage embryo	Muscle cells	–	–	–	+ + + + +	+ +
Late-stage embryo	Ventral nerve cord	–	+ + + + +	–	+	+ +
Late-stage embryo	Yolk	+	–	+ + + +	+ + +	+ +
Late-stage embryo	Primordial sex cells	+ + + +	–	–	–	+ +

Intensity of staining: + + + + + = very intense; + + ++ = intense; + + + = moderate; ++= weak; + = very weak; – = none.

Con A reactivity was found at the muscles, nerves, primordial germ cells and primitive gut. From the results, PGCs were stained with HPA, Con A and WGA. Con A lectins bound most of the different groups of cells except for yolk granules. Con A provided visualization of many structures and positively indicated terminal sucrose, D-glucosyl and NAG residues. Con A labeled primordial germ cells in the early embryos and also the inner envelope, embryonic cells, primordial germ cells, and yolk (Figure 3A and D). It showed a higher intensity of staining on the inner envelope than on the outer envelope. It also strongly bound to embryonic cells, including primordial germ cells, due to the intensity of the cytoplasmic portion of PGCs. The reactivity of Con A bound to the cytoplasm at high density, resulting in the ability to distinguish differentially labeled granules. No differences were found in the intensity of Con A reactivity on muscle, nerve, primordial germ cells, or primitive gut (Figure 3A and D). An intense response was seen on the outer envelope. The staining pattern of Con A in the yolk was uniform and moderately intense. Con A binding particles were found in the yolk cytoplasm. In addition, Con A stained weakly on the inner envelope but bound moderately well on the developing intestine and primordial germ cells. Con A indicates the presence of D-mannose. It showed a great affinity for embryonic cells, primordial germ cells, the inner envelope in early embryos and a similar response to muscles, envelope, nerves, yolk, intestine, tail and legs in late embryos. Therefore, labelling for primordial germ cells cannot be observed in early embryos labeled with Con A. Similarly, in mice, Con A showed uniform responses to germ cells [27], non-germ cells [28] and proliferative cells or cancer cells [28]. The binding sites of Con A on the envelope were also altered. Thus, the sugar residues found on the envelopes were different at each developmental stage.

WGA indicates the presence of GlcNAc or its oligomers. WGA labelling on the cytoplasm of embryonic cell mass was more intense than the nucleus when both embryonic and primordial germ cells were observed (Figure 3C). WGA staining appeared pale throughout the late embryo stage, except for it being very intensely detected in brain and ventral nerves (Figure 3F). Non-reactivity of WGA staining was detected in the inner integument, yolk, muscles and primordial germ cells. WGA intensely bound to several embryonic zones, but no reactive sites were found on the yolk part, indicating the absence of GlcNAc or its oligomers [28]. The two layers of the embryonic envelope bound to WGA. WGA labelling of the cytoplasm stained more strongly than the nucleus in every cell in early embryos. Surprisingly, WGA staining was generally pale on the entire late embryo, with the exception of a very intense staining detected on the brain and ventral nerve cells. Non-reactive WGA staining was found on the inner integument, yolk, muscles and intestine. WGA also reacted strongly on the outer integument and was distinguishable. WGA indicates the presence of GlcNAc or its oligomers [18]. WGA on the envelope indicates *N*-acetyl-β-D-glucosamine localization. The early embryos required a higher concentration of *N*-acetyl-β-D-glucosamine to form the cuticle than did the older specimens [29]. WGA strongly stained nerve cells of the late embryos and all cells in early embryos; therefore, WGA found in prawn embryos referred to

Neu5Ac residues found in many nervous systems [29]. WGA binds specifically to NAG and Neu5Ac (sialic acid) residues, which are both ubiquitous in neural membranes [30].

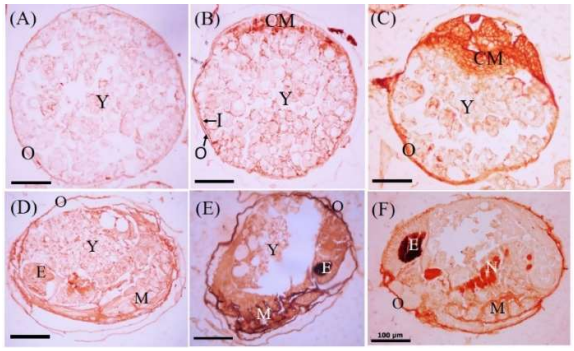

Figure 3 Comparison of 5.5-day-old embryo sections (A, B and C) and 12.5-day-old late embryo sections (D, E and F). The conjugate lectin showed more intensity in older embryos, shown as 3 types of lectin binding. Highest intensities were shown by WGA (C and F), HPA (B and E) and Con A, respectively (A and D), in both early and late-stage embryos. HPA bound to PGC and primordial sex cells intensely (B and E), whereas WGA bound to mesodermal cells and nerve cells intensely (C and F). E = eye; M = muscle; I = inner shell membrane; O = outer shell membrane; N = nerve cells; Y = yolk; CM = cell mass; scale bar =100 µm.

The RCA-I binding site was only perceived in the yolk of both stages. The outer embryonic envelope showed weak binding to RCA-I (Table 2). RCA-I staining results also showed non-reactivity in the entire late embryos, except for high localization on the yolk granules. RCA-I binding sites were detected only at the outer embryonic envelope (Figure 4A and C). Embryonic cells, primordial germ cells, and yolk were not reactive, so the entire embryo lacked binding sites for RCA-I. RCA-I indicated β-D-galactose [31]. The embryonic cells were not reactive to RCA-I. β-D-galactose has been reported to be involved in cell-cell and cell-matrix interactions [32]. It was spread on the envelope but was not found in embryonic cells of early and late embryos. It has also been found on the surface of sheep embryos [33]. It is possibly used for compaction. In general, the individual embryos of giant freshwater prawn interact in the brood chamber. Therefore, β-D-galactose may play an important role in embryonic cell interaction. Results of staining with RCA-I also showed non-reactivity on the entire late embryos except for a very pale localized staining on the outer integument. The results suggested that the components of the envelope changed during embryonic development. In *Xenopus*, the cell surface envelope of embryos was reported to change and was detected by lectins [33].

Action of UEA-I against glycosphingolipids was investigated. Muscle and nerve cells in late embryos were intensely stained with UEA-1 Figure 4D. Moreover, the yolk cells were also intensely stained with UEA-I. Yolk granules labeled with UEA-I below the embryonic envelope showed distinct responses in high density, higher than those distributed in the middle part of the early embryos (Figure 4B). The outer layer was weakly labeled with UEA-I (Figure 4B and D).

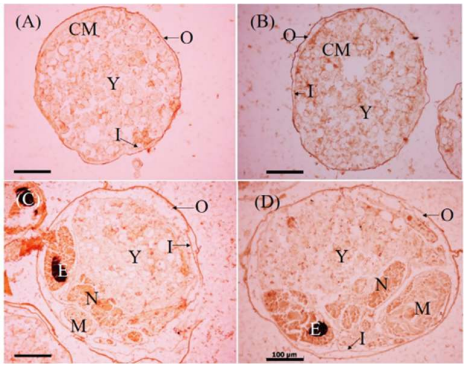

Figure 4 Early and late-stage embryos of shell membranes bound strongly with both RCA I (A and C) and UEA I (B and D), showing intensity present at muscle and nerve cells in late embryos (D).

4. Conclusion

Five lectins showed different binding of early and late embryos. We found HPA strongly at PGC. WGA bound with nerve. Con A bound with all types of cells. The binding of HPA, Con A, WGA and RCA-I showed

the presence of *N*-acetyl-α-D-galactosamine, α-D-mannose, *N*-acetyl-β-D-glucosamine and β-D-galactose, respectively. These new findings showed cell differentiation on early and late embryos by glycosylation and can predict innate immunity using the power of lectins to recognize changes and distribution of glycoconjugates during embryonic development of the giant freshwater prawn.

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