Carbohydrate Components of Lipovitellin of the Sand Crab *Emerita asiatica*

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ABSTRACT Lipovitellin II (Lv II), the major yolk protein of the anomuran crab *Emerita asiatica*, was purified using heparin–sepharose affinity column chromatography. The purified Lv II was a glycoprotein as it was stainable with periodic acid–Schiff's reagent. Quantitative analysis of sugars showed the presence of fucose, mannose, galactosamine, *N*-linked oligosaccharides, as well as *O*-linked oligosaccharides containing *N*-acetyl hexosamine as the terminal residue. The amount of *N*-linked oligosaccharides is higher than that of the *O*-linked oligosaccharides. Biogel P-4 column chromatographic separation of the radiolabeled oligosaccharides of Lv II showed the presence of five different *O*-linked oligosaccharides and four different *N*-linked oligosaccharide species. HPTLC separation of the neoglycolipids prepared from the *O*-linked oligosaccharides also showed the presence of five different *O*-linked oligosaccharide species. *N*-linked oligosaccharides contain significant quantities of mannose. Unisil column chromatographic purification in conjunction with HPTLC separation revealed three neutral glycolipid species such as monoglycosylceramide, diglycosylceramide, and triglycosylceramide in the Lv II. The functional significance of these carbohydrate components of the major yolk protein during embryogenesis of the sand crab is discussed.

Key Words: vitellogenin; neoglycolipids; *N*-linked oligosaccharides; *O*-linked oligosaccharides; neutral glycolipids

INTRODUCTION

Oocytes in crustaceans and many other oviparous animals contain large stores of protein, lipid, and carbohydrates which give nutritional support to the developing embryo (Wall and Patel, 1987). Hence, vitellogenesis or yolk formation is one of the crucial events of oogenesis in decapod crustaceans. Yolk formation in most crustaceans occurs in a biphasic manner; an initial protractive event of autotransynthesis (synthesis within the oocyte) followed by a quick period of heterosynthesis (sequestration of the presynthesized yolk precursor protein, vitellogenin from outside) (Meusy, 1980; Adiyodi and Subramoniam, 1983; Meusy and Payen, 1988). In most insect species, the vitello-

genin is synthesized by the fat body (see Engelmann, 1985). In vertebrates, the yolk precursor protein is produced by the liver, released into the circulation and selectively sequestered in developing oocytes via receptor-mediated endocytosis (Stifani et al., 1990). However, in most crustaceans, the yolk precursor protein has been reported to be synthesized by either one of the three extra-ovarian sites such as hepatopancreas (Paulus and Laufer, 1987), subepidermal adipose tissue (Vasquez-Boucard, 1985; Charniaux-Cotton and Payen, 1988), and hemocytes (Kerr, 1968). Upon arrival in the oocyte, the vitellogenin is stored as such or cleaved into two or more major yolk proteins, the lipovitellins, by the specific action of trypsin-like enzymes (Meusy, 1980). As early as 1967, Wallace and his co-workers characterized the crustacean yolk protein as a high-density glycolipoprotein with high percentage of lipids invariably conjugated to carotenoid pigment. Despite much work that has been done on the lipid and protein components of invertebrate and vertebrate lipovitellin (Fyffe and O'Connor, 1974; Ohlendorf et al., 1977; de Chaffoy de Courcelles and Kondo, 1980; Raikhel and Dhadialla, 1992; Tirumalai and Subramoniam, 1992; Subramoniam et al., 1999), only few reports are available on the carbohydrate components of the major yolk protein. Work on insect lipovitellin revealed the presence of high-mannose oligosaccharides which have been reported to be necessary for binding to its receptors on the oocyte membrane (Raikhel and Dhadialla, 1992). In vertebrates, report on the oligosaccharide component is available only for the phosphovitellogenin (Shainkin and Perlmann, 1971a). To our knowledge, no report is available on the *O*-linked oligosaccharides of the vitellogenin/vitelin of oviparous animals. Our present work on *Emerita* lipovitellin showed the presence of five different *O*-linked oligosaccharides and four different *N*-linked oligosaccharides, as well as three neutral glycolipid species. The functional significance of oligosaccharides and neutral glycolipid species of the lipovitellin during vitellogenesis and embryogenesis is discussed.

MATERIALS AND METHODS

Experimental Animal

The sand crab *Emerita asiatica* was collected from the intertidal region of Marina beach and Elliot's beach, Chennai, and brought to the laboratory. The female crabs were identified based on the occurrence of three pairs of pleopodal hairs (Subramoniam, 1979).

Sample Preparation

Freshly laid egg was removed from the pleopodal hairs using a fine forceps. The egg was homogenized (1 g/10 ml) with Tris-Cl buffer (20 mM, 5 mM EDTA, pH 7.2) and centrifuged at 6000 *g* for 15 min at 4°C in a microcentrifuge (Kobota, Japan). The clear supernatant was collected and kept in the freezer until use.

Heparin–Sephacrose Affinity Column Chromatographic Purification of Lipovitellin

Five hundred microliters of the clear egg supernatant was loaded to heparin–sephacrose affinity column (15 × 1.1 cm) which was pre-equilibrated with Tris-Cl buffer (pH 7.2). The unbound proteins were washed with Tris-Cl buffer and the bound proteins were eluted with increasing concentrations (0.1–1.0 M) of sodium chloride in Tris-Cl buffer as stepwise gradient. Two milliliter fractions were collected using an automatic fraction collector (LKB, Sweden) and the absorbance was read at 280 nm in a spectrophotometer (Hitachi, Japan). Purity of the Lv II was tested using native polyacrylamide gel electrophoresis.

Native Polyacrylamide Gel Electrophoresis

Native polyacrylamide gel electrophoresis (PAGE; Davis, 1964) was used to test the purity of Lv II. The glycoprotein nature of the affinity column chromatographically purified Lv II was tested by staining the electrophoretically separated protein with periodic acid–Schiff's (Smith, 1968) reagent. After electrophoresis, the gel was fixed in 7% acetic acid for 30 min and then rinsed in distilled water for 30 min. After incubating and rinsing the gel with 1% periodic acid and distilled water for 30 min each, the gel was stained with periodic acid–Schiff's reagent in dark at 13°C for 12–14 hr, destained, and stored in 1% sodium metabisulfite.

Quantification of Protein-Bound Carbohydrates

To quantify the protein-bound sugars of Lv II, the purified major yolk protein was treated with chloroform:methanol (2:1, v/v) to remove the lipids (Folch et al., 1957) and then the individual sugars were quantified spectrophotometrically. Hexoses were quantified following the procedure of Niebes (1972). Hexosamine and galactosamine were quantified by the method of Wagner (1979). Mannose was quantified following the procedure of Ashwell (1957). Fucose was quantified by the method of Disch and Shettles (1948). *N*-acetyl hexosamine terminating *O*-linked oligosaccharide was quantified by the method of Bhavanandan

et al. (1990). Sialic acid was estimated by the method of Jourdan et al. (1971). Glucose was quantified using glucose oxidase and peroxidase (Hugget and Nixon, 1957) method.

Characterization of *O*-Linked Oligosaccharides

Radiolabeling of *O*-linked oligosaccharides. To release the *O*-linked oligosaccharides from the purified Lv II, 50 mg of the delipidated Lv II (Folch et al., 1957) was treated with 0.1 M sodium hydroxide (Montreuil et al., 1986) and incubated for 16 hr at 45°C. The mixture was centrifuged at 6000 *g* for 10 min. The clear supernatant containing *O*-linked oligosaccharides was evaporated to dryness and the residue was freed from alkali by evaporation with 3 ml of double distilled water five times. The released *O*-linked oligosaccharides were dissolved in 30 µl of double distilled water and mixed with 300 µl of alkaline [³H]-sodium borohydride solution (dimethylformamide solution of NaB³H₄). The reaction mixture was incubated at 30°C for 4 hr and reaction was stopped by adding 1.5 ml of glacial acetic acid. The mixture was then evaporated to dryness and the residue was dissolved in 0.5 ml of 1 M acetic acid and again evaporated to dryness. Evaporation with 1 M acetic acid was repeated five times to ensure the removal of any exchangeable tritium as [³H]-H₂O (Takasaki and Kobata, 1974; Nishigaki et al., 1978). The dried oligosaccharide was dissolved in water and dialyzed against distilled water overnight at 4°C and then lyophilized.

Biogel P-4 column chromatographic separation of *O*-linked oligosaccharides. For the separation of different species of *O*-linked oligosaccharides, the labeled *O*-linked oligosaccharide powder was dissolved in double distilled water and loaded onto the Biogel P-4 column (62 × 0.8 cm). The column was eluted with double distilled water (Natowicz and Baenziger, 1980). Eluents were collected (5 drops/test tube) and the radioactivity was measured using a Rackbeta scintillation counter (Pharmacia, Sweden).

HPTLC separation of *O*-linked oligosaccharides. For the separation of *O*-linked oligosaccharides through high performance thin layer chromatographic plate (HPTLC), approximately 100 mg of delipidated Lv II was treated with 1 M sodium borohydride in 0.1 M sodium hydroxide (Montreuil et al., 1986) and incubated for 16 hr at 45°C (β-elimination). The mixture was centrifuged at 6000 *g* for 10 min. The pH of the clear supernatant was adjusted to 8.0 with 4 M acetic acid. Boric acid was removed by the repeated addition and evaporation of methanol. Solution containing the oligosaccharide was lyophilized and kept in the freezer. The total hexose content of the released *O*-linked oligosaccharide was quantified using the phenol–sulphuric acid reagent (Dubois et al., 1956).

To convert the released *O*-linked oligosaccharide alditols to aldehydes, 1 mg of *O*-linked oligosaccharide was treated with 4 mg of sodium metaperiodate and mixed vigorously using a vortex mixture. After incubating the mixture on ice for 5 min, glycerol was added

to stop the reaction. To link the *O*-linked oligosaccharide aldehydes to the carrier molecule phosphatidyl ethanolamine (PE), the PE and the oligosaccharide were mixed in 6:1 ratio and the pH of the solution was adjusted to 8.0 with 1 N NaOH (Stoll et al., 1990). The PE linked oligosaccharides (neoglycolipids) were reduced by incubating with 1 M sodium borohydride in 0.05 N NaOH at 80°C for 10 min. To separate the neoglycolipids from the unreacted PE, the mixture was treated with 5 volumes of chloroform:methanol (2:1, v/v) and one volume of distilled water. After vigorous shaking, the lower phase containing the neoglycolipid was collected and dried in a pen evaporator. To separate the different neoglycolipid species, 10 µg carbohydrate equivalent of neoglycolipids was spotted on HPTLC plate and developed in chloroform:methanol:water (60:35:8, by volume). The neoglycolipids were detected by moderately spraying with orcinol reagent and heating the HPTLC plate at 100°C for 5–10 min.

Characterization of *N*-Linked Oligosaccharides

Preparation of *N*-linked oligosaccharides. First, the *O*-linked oligosaccharides were released from the delipidated Lv II by incubating the protein in 0.1 M NaOH solution for 16 hr at 45°C. The mixture was then centrifuged for 10 min at 6000 *g*. Protein precipitate (sediment) collected after centrifugation was incubated in 1 M NaOH solution at 100°C for 6 hr to release the *N*-linked oligosaccharides (Montreuil et al., 1986). The solution was centrifuged for 10 min at 6000 *g*. The clear supernatant containing *N*-linked oligosaccharide was evaporated to dryness and the residue was freed from alkali by evaporation with double distilled water five times. The *N*-linked oligosaccharide powder was dissolved in distilled water and labeled with NaB³H₄, using the procedure described above for labeling the *O*-linked oligosaccharides.

Biogel P-4 gel filtration column chromatographic purification of *N*-linked oligosaccharides. The radiolabeled *N*-linked oligosaccharide was dissolved in distilled water and loaded onto Biogel P-4 column (62 × 0.8 cm). The *N*-linked oligosaccharides were eluted with warm (50°C) distilled water (Natowicz and Baenziger, 1980). The eluents (3 drops/test tube) were collected and the radioactivity was measured using a Rackbeta scintillation counter (Pharmacia, Sweden).

Purification and Characterization of Neutral Glycolipids

To purify and characterize the neutral glycolipids of Lv II, the lyophilized powder of the purified major yolk protein was treated with 57 ml of chloroform:methanol (2:1, v/v) to extract the total lipids (Folch et al., 1957). After filtration, the residue was again treated with 10 volumes of chloroform:methanol with the reverse ratio (1:2, v/v) and with the addition of 5% water (Suzuki, 1965). Both filtrates were combined and an appropriate amount of chloroform was added to yield a final concentration of chloroform:methanol of 2:1 (v/v). To

this, 0.2 volume of 0.88% KCl was added, shaken vigorously, and kept for 2 hr. The lower phase containing the neutral glycolipids was drained and dried under nitrogen atmosphere. Amount of neutral lipid (Barnes and Blackstock, 1973) and total glycolipids (Roughan and Batt, 1968) in the lower phase fraction was quantified spectrophotometrically. Total lipid-bound carbohydrate was quantified by treating with anthrone reagent (Carroll et al., 1956). The neutral glycolipids were purified by the procedure described by Yogeewaran and his co-workers (1970). Chloroform–methanol containing 30 mg of the lower phase lipid was loaded onto an activated silicic acid (Unisil) column (10 × 0.8 cm) which was packed and equilibrated with chloroform. The column was washed with 30 ml of chloroform to elute the neutral lipids and 120 ml of acetone followed by 110 ml of acetone–methanol (9:1, v/v) to elute the neutral glycolipids and 40 ml of methanol to elute the phospholipids. All the three fractions were dried under nitrogen atmosphere and the amount of carbohydrate in all the three fractions were quantified (Carroll et al., 1956). The neutral glycolipid fraction containing 10 µg carbohydrate equivalent was spotted on HPTLC plate, and developed in chloroform:methanol:water (65:25:4, by volume). The plate was dried, sprayed moderately with resorcinol reagent, and heated at 100°C for 3–5 min. The glycolipids appeared as pink spots. The different neutral glycolipid fractions were identified by comparing their *R_f* values with the *R_f* values of authentic standards run under identical condition.

RESULTS AND DISCUSSION

Figure 1 shows the heparin–sepharose affinity column chromatographic purification of Lv II from the egg of the sand crab *E. asiatica*. The Lv II was detected in the eluents collected from the major bound protein peak (Fig. 1, peak A). The Lv II was identified based on its relative mobility in native PAGE, its glycolipoprotein nature, and the molecular weight (Tirumalai and Subramoniam, 1992). Native PAGE of the eluents from the fractions 59–65 revealed the presence of a single protein fraction with Coomassie blue R-250 (Fig. 1a), suggesting its purity. The purified protein fraction also gave positive reaction with periodic acid–Schiff's reagent (Fig. 1b), suggesting that the major yolk protein is a glycoprotein.

Quantitative analysis of the protein-bound carbohydrates of Lv II (Table 1) showed that the hexosamines are the major sugars followed by hexoses. Spectrophotometric analysis also showed the presence of higher amount of mannose in the delipidated major yolk protein as well as in the *N*-linked oligosaccharides of Lv II. Fucose and *O*-linked oligosaccharides with *N*-acetyl hexosamines as terminal residues constituted only 2.5% of the total carbohydrate content of the delipidated Lv II. However, reaction with the resorcinol reagent revealed the absence of sialic acids in the purified Lv II, suggesting the asialoglycoprotein nature of the major yolk protein. Reaction with the glucose

Fig. 1. Heparin-sepharose affinity column chromatographic purification of Lv II of *E. asiatica*. The unbound proteins were eluted with Tris-Cl buffer (peak A). The bound proteins (peak B) were eluted with Tris-Cl buffer containing different molar concentrations of sodium chloride as a step wise gradient. Purity of the Lv II was tested by PAGE (7% gel) stained with Coomassie blue R-250 (a). Glycoprotein nature of the major yolk protein was tested by staining the native PAGE separated protein with periodic acid-Schiff's reagent (b).

oxidase and peroxidase revealed the absence of glucose in the delipidated major yolk protein. Our previous work on the Lv II of *E. asiatica* showed the presence of both protein and lipid-bound carbohydrate (Tirumalai and Subramoniam, 1992). The protein-bound carbohydrate was higher than the lipid-bound carbohydrate. One of the characteristic features of invertebrate lipovitellin is the absence of sialic acids. Higher amounts of mannose and absence of sialic acids in *N*-linked oligosaccharide fraction of lipovitellin suggest that the *N*-linked oligosaccharides may be simple/high mannose type.

In vertebrates, it is now known that many secreted molecules and virtually all cell surface molecules, are to

some extent glycosylated (Berman and Lasky, 1985). Glycosylation serves different functions in different proteins. Glycosylation increases the solubility of the proteins, important to the folding and transport processes and to protect the protein from intracellular proteases (Gibson et al., 1979; Berman and Lasky, 1985). It can increase the antigenicity of the proteins (Alexander and Elder, 1984), and helps in the recognition of receptors on the membranes (Raikhel and Dhadialla, 1992). Interestingly, the yolk-precursor protein (vitellogenin) and the major yolk protein (lipovitellin) contain carbohydrates apart from the lipids. The total carbohydrate content of the crustacean (de Chaffoy de Courcelles and Kondo, 1980; Tirumalai and Subramoniam, 1992) and the amphibian vitellogenin/vitellin (Wallace, 1985) was found to be constant. However, the carbohydrate content of the insect vitellogenin/vitellin varies (Table 2) from species to species (Engelmann, 1985). Among the insects, *Nau-phoeta cinerea* contains the highest amount of carbohydrate reported so far (Imboden et al., 1987). The functional significance of such striking difference in the carbohydrate content of the yolk/yolk-precursor protein is not known. The insect vitellogenin/vitellin contains higher amount of mannose and glucosamine (Masuda and Oliveira, 1985). However, the galactosamine has been reported only in the crustacean lipovitellin and constituted as much as 80% of the total hexosamines of the crustacean major yolk protein (de Chaffoy de Courcelles and Kondo, 1980). The presence of higher amount of galactosamine in crustacean lipovitellin suggests the possible presence of *O*-linked oligosaccharides in crustacean major yolk protein, which has not been reported so far in the vitellogenin/vitellin of oviparous animals.

Crustacean yolk proteins are characterized by the presence of one or more lipovitellins with relatively higher percentage of lipids and carbohydrates in comparison with those of vertebrate eggs. Decapod lipovitellins are high density lipoproteins with carbohydrate as the major covalently linked prosthetic group. In recent years, several investigations have been made to determine the biochemical constituents of eggs as well as the alterations that they undergo during embryonic development in the anomuran crab *E. asiatica*. The two lipovitellins (Lv I and Lv II) of this crab constitute as much as 90% of the total yolk proteins (Tirumalai, 1996). The lipids that form up to 30% include neutral lipids, glycolipids, and phospholipids. The carbohydrates of *Emerita* yolk exists in three forms namely free carbohydrate, protein-bound carbohydrate, and lipid-bound carbohydrate. A recent investigation on *Emerita* embryogenesis reported the biochemical alterations in the major organic substances as well as the activity of nonspecific esterase during egg development (Subramoniam, 1991). As for the carbohydrates, this study showed an increase in the concentration of free carbohydrates and glycogen with concomitant decline in the protein-bound carbohydrates during embryogenesis. Interestingly, the exis-

TABLE 1. Sugar Composition of Delipidated Lv II of *E. asiatica*

Carbohydrates	mg/100 mg delipidated Lv II
Hexose	1.375 ± 0.32
Hexosamine	1.460 ± 0.14
Galactosamine	1.020 ± 0.09
Mannose	0.730 ± 0.11
Fucose	0.120 ± 0.07
Glucose	ND
Sialic acid	ND
<i>N</i> -linked oligosaccharides	1.690 ± 0.11
Mannose ^a	0.680 ± 0.04
<i>O</i> -linked oligosaccharides	1.045 ± 0.16
<i>O</i> -linked oligosaccharides with <i>N</i> -acetyl hexosamine as the terminal residue	0.192 ± 0.03

^aIndicates the amount of mannose in the *N*-linked oligosaccharides.

ND, not detectable level.

TABLE 2. Carbohydrate Components of Vitellogenin and Vitellin

Animals name	Carbohydrates (%)		References
Crustacea			
<i>Artemia salina</i>			
Vitellin	Total	3.6	de Chaffoy de Courcelles and Kondo (1980)
	Hexose	2.2	
	Mannose	1.8	
	Galactose	0.4	
	Hexosamine	1.4	
	Galactosamine	1.2	
	Glucosamine	0.2	
<i>Emerita asiatica</i>			
Lipovitellin II	Total	2.8	Tirumalai and Subramoniam (1992)
	Glycolipid	0.5	
Myriapoda			
<i>Spirostreptus asthenes</i>			
Vitellogenin	Total	2.2	Prasad and Subramoniam (1991)
Vitellin	Total	2.3	
Insecta			
<i>Hyalopora cercopia</i>			
Vitellogenin	Total	1.0	Kunkel and Pan (1976)
<i>Phylosamia cynthia</i>			
Vitellogenin	Total	2.5	Chino et al. (1976)
<i>Manduca sexta</i>			
Vitellogenin	Total	3.0	Mundall and Law (1979)
<i>Blatella germanica</i>			
Vitellogenin	Total	4.5	Kunkel and Pan (1976)
<i>Leucophaea maderae</i>			
Vitellin	Total	8.3	Dejmal and Brookes (1972)
<i>Locusta migratoria</i>			
Vitellin	Total	14.0	Chen et al. (1976)
Vitellin	Mannose	12.3	Chen et al. (1978)
	Glucosamine	1.3	
<i>Aedes aegypti</i>			
Vitellin	Glucosamine	0.9	Dov and Whitney (1987)
<i>Apis mellifera</i>			
Vitellogenin	Total	2.0	Wheeler and Kawooya (1990)
Pisces			
<i>Salmo gairdneri</i>			
Vitellin	Hexosamines	0.2	Riazi et al. (1987)
	Hexoses	0.26	
Phosvitin	Hexosamines	5.5	
	Hexoses	3.5	
Amphibians			
<i>Xenopus laevis</i>			
	Hexose	0.6	Ansari et al. (1971)
	Hexosamine	0.8	
	Sialic acids	0.23	
Aves			
<i>Gallus gallus</i>			
Phosvitin	Total	6.5	Shainkin and Perlmann (1971b)
	Hexose	2.7	
	Hexosamine	2.2	
	Sialic acid	1.7	

tence of two forms of α -galactosidases and three forms of β -galactosidases as well as α - and β -glucosidases have been investigated in the developing embryos of *E. asiatica* (Gunamalai, 1993). The present study also showed the occurrence of both glucose- and galactose-containing glycolipids in the lipovitellin of *E. asiatica*. However, further work is needed to investigate the exact role of these carbohydrases in the hydrolysis

of the carbohydrate component of the major yolk protein.

Spectrophotometric analysis showed the presence of higher amount of *N*-linked oligosaccharides when compared to *O*-linked oligosaccharides (Table 1). *O*-linked and *N*-linked oligosaccharides of the Lv II of *E. asiatica* were released by treating the delipidated protein with mild and strong alkali reagent. The

Fig. 2. Biogel P-4 gel filtration column chromatographic separation of *O*-linked oligosaccharides of Lv II of *E. asiatica*. *O*-linked oligosaccharides obtained from Lv II by treating with mild alkali reagent were labeled with NaB^3H_4 and then loaded on to the gel filtration column. The eluents were collected (5 drops) and the radioactivity was determined with a liquid scintillation counter.

present study reveals higher amounts of galactosamine as well as *O*-linked oligosaccharides containing *N*-acetyl hexosamine as the terminal residue in the *Emerita* lipovitellin. To separate the different species of *O*-linked and *N*-linked oligosaccharides, the released oligosaccharides were radiolabeled with tritiated sodium borohydride and passed through Biogel P-4 gel filtration column. Analysis of the oligosaccharides using Biogel P-4 column showed the presence of five different *O*-linked oligosaccharides (Fig. 2) and four different *N*-linked oligosaccharide (Fig. 4) species in the Lv II.

Figure 3 shows the HPTLC separation of the different *O*-linked oligosaccharide species of the major yolk protein. Since conventional thin layer chromatographic separation of the oligosaccharides is difficult due to their microheterogeneity, the released *O*-linked oligosaccharides from the Lv II were linked to the carrier molecule phosphatidyl ethanolamine and then separated on thin layer chromatographic plate. HPTLC analysis also showed the presence of five different *O*-linked oligosaccharides in the Lv II of *E. asiatica*, confirming the gel filtration chromatographic result.

Oligosaccharides of the glycoproteins have been reported to act as specific markers for targeting the proteins either intra- or extra-cellularly. The targeting activity is mediated by the biantennary or triantennary oligosaccharides containing galactose or *N*-acetyl galactosamine residues (Chiu et al., 1994). Work on insect vitellin revealed the presence of high-mannose oligosaccharides (Raikhel and Dhadialla, 1992). A few authors (Osir et al., 1986; Konig et al., 1988; Raikhel and Bose, 1988) have reported the role of carbohydrate moieties in the vitellin polypeptides with the receptors on the follicle membrane. Analysis of the oligosaccharides from the vitellin of the insects *Simpoce capitata* and *Blatella germanica* revealed the difference in the total carbohydrate content of the individual oligosac-

Fig. 3. HPTLC separation of *O*-linked oligosaccharides (neoglycolipids) of Lv II of *E. asiatica*. The *O*-linked oligosaccharides released from Lv II were linked to the carrier molecule, phosphatidyl ethanolamine and spotted on HPLC plate. The chromatogram was developed in chloroform:methanol:water (60:35:8, by vol). The neoglycolipids were detected by spraying the chromatogram with orcinol reagent. o, origin; s, solvent front.

charide species (Konig et al., 1988). However, these differences do not correlate with the ability of vitellin to compete for binding. These authors reported that high-mannose oligosaccharides of vitellin are necessary but not sufficient for binding to the vitellogenin receptors. However, different results were obtained for the binding of *Manduca sexta* vitellogenin to its receptors on the follicle membrane (Osir and Law, 1986). Vitellogenin deglycosylated by treatment with endo-

Fig. 4. Biogel P-4 gel filtration column chromatographic separation of *N*-linked oligosaccharides of Lv II of *E. asiatica*. *N*-linked oligosaccharides obtained from Lv II by treating with strong alkali reagent were labeled with $1\text{ M NaB}^3\text{H}_4$ and then loaded on to the gel filtration column. The column was eluted with warm (50°C) distilled water and the radioactivity in the eluents (3 drops) was determined using a liquid scintillation counter.

glycosidase-H competed effectively with the native vitellogenin for binding to follicle membrane and was taken by the follicle. In vertebrates, reports on the oligosaccharide components are available only for the phosvitin of vitellogenin. In contrast to the invertebrate vitellogenin, vertebrate vitellogenin (phosvitin) is a sialoglycoprotein containing a single high-mannose *N*-linked oligosaccharide species (Shainkin and Perlmann, 1971a). Competition studies indicate that the phosvitin portion of vitellogenin molecule mediates the binding and uptake (Woods and Roth, 1984). However, no report is available on the oligosaccharide component of the crustacean vitellogenin/vitelin. In addition, we do not have adequate knowledge of the functional significance of the oligosaccharide components of these proteins. It is not known whether the oligosaccharides of these proteins play a role in the secretion, transport, yolk subunit assembly, and stabilization of the major yolk proteins against proteolytic cleavage during yolk degradation. The present work on *Emerita* lipovitellin revealed the presence of *O*-linked oligosaccharides containing terminal *N*-acetyl hexosamine residues. It remains to be seen whether the *O*-linked oligosaccharides of crustacean lipovitellin play any role in secretion of the yolk precursor protein during yolk synthesis and recognition of its receptors on the oocyte membrane during yolk accumulation. It is also not clear whether the *O*-glycosylation renders the major protein resistant to proteolytic cleavage (Berman and Lasky, 1985) during yolk degradation. *Emerita* lipovitellin also contains fucose. Fucosylation may reduce the metabolic rate (degradation) of the major yolk protein during embryogenesis (Chiu et al., 1994). However, further work is needed to elucidate the structure and function of the *O*-linked and *N*-linked oligosaccharides of the crustacean lipovitellin.

The neutral glycolipid fraction of the major yolk protein was purified using Unisil column chromatography. The amount of carbohydrate in the lipid fraction of Lv II was quantified spectrophotometrically (Table 3). After subjecting the lower phase lipid to the column, the carbohydrate content in the individual lipid (neutral lipid, glycolipid, and phospholipid) fractions was determined in order to assess the elution

TABLE 3. Distribution of Lipid-Bound Carbohydrates in the Florosil Column Chromatographically Purified Neutral Lipid, Glycolipid, and Phospholipid Fraction of Lv II of *E. asiatica*

	Amount of LBC (μ g)
Total LBC in 30 mg of LP	267.760 $\pm$ 1.02
After florosil column chromatography	
LBC in NL fraction	15.200 $\pm$ 1.02
LBC in GL fraction	223.400 $\pm$ 0.87
LBC in PL fraction	23.300 $\pm$ 0.90

LBC, lipid-bound carbohydrate; LP, lower phase lipid; NL, neutral lipid fraction; GL, glycolipid fraction; PL, phospholipid fraction.

Fig. 5. HPTLC separation of the neutral glycolipids of the Lv II of *E. asiatica*. Unisil column chromatographically purified neutral glycolipids of the Lv II was spotted on HPTLC plate and the chromatogram was developed in chloroform:methanol:water (65:25:4, by vol). The neutral glycolipids were identified by spraying with orcinol reagent. a, standard glycolipids; b, neutral glycolipids of Lv II; c, tetraglycosylceramide; d, triglycosylceramide; e, diglycosylceramide; f, monoglycosylceramide; g, solvent front; *, unidentified glycolipid fraction.

pattern of the glycolipids. Column chromatographic purification revealed 82% recovery of the glycolipid fractions and distribution of minor quantities of glycolipids in both neutral lipid and phospholipid fractions. The meager amount of carbohydrate in the phospholipid fraction could be due to the presence of the carbohydrate-containing glycerophospholipid, phosphatidyl inositol (Tirumalai and Subramoniam, 1992). Thin layer chromatographic separation of purified glycolipids showed the presence of three neutral glycolipid species such as monoglycosylceramide, diglycosylceramide, and triglycosylceramide (Fig. 5). The glycolipids of the major yolk protein have been reported for the first time in *Emerita* lipovitellin (Tirumalai and Subramoniam, 1992). In *E. asiatica*, glycolipid formed the minor lipid species and constituted 2% of the total lipid fraction of the Lv II. Present work on *Emerita* lipovitellin revealed the presence of both glucose (monoglycosylceramide) and galactose (diglycosylceramide) containing glycolipids. The galactolipids of yolk/yolk precursor protein may be involved in the recognition of its receptors on the oocyte membrane (van Berkel et al., 1985). However, it is not known whether the lipovitellin glycolipids are utilized for the formation

of stage-specific embryonic antigens during embryogenesis (Gooi et al., 1981).

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