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The Vitelline Envelope of Eggs from the Giant Keyhole Limpet *Megathura crenulata.* I: Chemical Composition and Structural Studies†

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ABSTRACT: The egg vitelline envelope of the marine invertebrate *Megathura crenulata* is a glycoprotein composed of 37.3 mol % protein and 62.7 mol % carbohydrate. Of the total amino acid content, 61 mol % consists of a single amino acid, threonine. The carbohydrate content includes galactosamine, galactose, and fucose. The molar ratio of threonine to galactosamine is about 1:1. Most of the threonine residues are linked to galactosamine residues via O-glycosidic bonds. A single peptide that was purified following alkaline borohydride treatment of the vitelline envelope had the structure: Abu-Pro-Abu-(Abu_6, Pro_1, Thr_1), where Abu is 2-aminobutyric acid. Several sugar residues have been isolated following the alkaline hydrolysis of the vitelline envelope that include an octasaccharide Gal_4Fu_4, an hexasaccharide Gal_3Fu_3, a trisaccharide Gal_3, fucose, and galactose. It is proposed that the vitelline envelope of *Megathura crenulata* eggs is composed of polypeptide chains built to a large extent of closely spaced threonine residues. Almost every threonine residue is linked to a saccharide moiety.

The egg of the marine invertebrate *Megathura crenulata* is surrounded by a thick jelly coat underneath of which lies the vitelline envelope. Both of these structures have to be penetrated by the spermatozoon to start the process of fertilization. Similar structures are present in eggs of other animals such as sea urchins (Runnstrom, 1966; Austin, 1968), amphibians (Wyrick et al., 1974), and mammals (Piko, 1969). The vitelline envelope and the mammalian zona pellucida contain sperm binding sites essential for fertilization since treatment of the envelopes with lectins or antibodies prevents sperm binding and blocks fertilization (Lallier, 1972; Aketa and Onitake, 1969; Oikawa et al., 1974; Shivers et al., 1972). Further, the vitelline envelope and the zona pellucida have been implicated in the block of polyspermy (Vacquier et al., 1973; Barros and Yanagimachi, 1971).

Although there is a wealth of information concerning the morphology of the vitelline envelope of various animals (Austin, 1968), little is known about the chemical composition and structure of this entity. Chemical studies have been conducted on the vitelline envelope of hen's egg (Bellairs et al., 1963) and the marine gastropod *Tegula pfeifferi* (Haino and Kigawa, 1966), both of which have been found to contain glycoproteins. The mammalian zona pellucida has also been characterized as glycoprotein (Lowenstein and Cohen, 1964), although the low abundance of mammalian eggs renders a detailed chemical study difficult.

This paper describes studies of the chemical composition of the vitelline envelope of the *Megathura crenulata* egg, its protein and carbohydrate composition, and the nature of the chemical link between these two components.

Experimental Section

Materials. Giant keyhole limpets (*Megathura crenulata*) were purchased from Pacific Bio-Marine, Venice, Calif., Dowex 50-X2 and Bio-Gel P-2 were from Bio-Rad Lab., Richmond, Calif., Sephadex G-10 was from Pharmacia, Piscataway, N.J., tetrazolium blue was from Sigma Chemical Co., St. Louis, Mo. *N*-Allyl-*N,N*-dimethylamine, 1-propanol, and trifluoroacetic acid used in the Edman degradation were sequanal grade from Pierce Chemical Co., Rockford, Ill. Phenyl isothiocyanate and benzene (Spectrar)

† Contribution No. 5128 from the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received July 28, 1975.* This research was supported by U.S. Public Health Service Grants No. NS 10294, GM 16424, and NS 12018 and a National Institutes of Health Career Development Award (M.A.R.).

were from Mallinckrodt Chemical Works, St. Louis, Mo. Chitohexaose (NAG_6[1]) and chitotetraose (NAG_4) were prepared according to Raftery et al. (1969). All other chemicals were of reagent grade.

Methods. Isolation of Vitelline Envelopes. Isolation of the vitelline envelopes (egg membrane) of *Megathura crenulata* has been described (Heller and Raftery, 1973).

Amino Acid Analysis. Samples (approximately 0.5 mg) were hydrolyzed under reduced pressure at 110 °C for 24 and 72 h in 6 N HCl. After removal of HCl under reduced pressure, the hydrolysate was analyzed according to Spackman et al. (1958) on a Beckman Model 120 C amino acid analyzer. Cysteine and methionine were determined after performic acid oxidation. Amino sugars were determined on the amino acid analyzer after hydrolysis under reduced pressure at 100 °C for 4 and 12 h in 4 N HCl.

Neutral Sugar Determination. Samples were hydrolyzed under reduced pressure at 100 °C for 1 and 4 h in 1 N HCl. The HCl was removed under reduced pressure and the samples were dissolved in 75% (wt/wt) ethanol in water. Samples (100–200 μl) were applied to a 0.5 × 100 cm column containing aminex A5 resin (Bio-Rad Lab.) in the trimethylammonium form (Hobbs and Lawrence, 1972) that was equilibrated with 75% (wt/wt) ethanol in water. The column was kept at 65 °C using a water mantle and attached to a Beckman Model 120 B amino acid analyzer. The column was developed with 75% (w/w) ethanol in water pumped at a rate of 15.7 ml/h. Detection of the emerging sugars was achieved by using a tetrazolium blue reagent (2 g. in 1 l. of 0.2 M NaOH (Mopper and Degens, 1972) pumped at a rate of 2.8 ml/h. The reaction coil was kept at 80 °C and the developed color was analyzed at 570 nm. Complete separation of sugars was achieved with mixtures containing ribose, fucose, xylose, mannose, glucose, and galactose (in their order of emergence from the column). Fucose emerged after about 2 h and galactose after about 3 h. As little as 5 nm of an individual sugar could be detected. As fucose and galactose were the only neutral sugars found in the present study, known amounts of xylose were added to samples prior to hydrolysis. Recoveries were between 80 and 85%. The column was used repeatedly without regeneration and with very reproducible results. Ninhydrin assay of amino groups was performed according to Duggan (1957). Phenol–sulfuric acid assay of neutral sugars was performed according to Ashwell (1966). The Dische–Shettles cysteine–sulfuric acid assay of neutral sugars was performed according to Spiro (1966). Phosphate was determined according to McClare (1971) and sulfate was determined according to Terho and Hartiala (1971). The thiobarbituric acid assay of sialic acid was performed according to Spiro (1966) following hydrolysis with 0.1 N H_2SO_4 at 80 °C for 1 h.

Alkaline Hydrolysis of the Vitelline Envelope and Isolation of Peptides. Packed vitelline envelopes (2.0 ml) were washed with 10 ml of H_2O and centrifuged in a clinical centrifuge. This procedure was repeated three times. The vitelline envelopes (2.0 ml) were then solubilized by incubation with 3 mg of dithiothreitol at room temperature for 1 h. A solution containing 83 mg of $NaBH_4$ in 0.2 ml of 5 N NaOH was added and the mixture was flushed with nitrogen and incubated in a capped glass stoppered tube at 37 °C for a total of 144 h. An additional 83 mg of $NaBH_4$ was added after 72 h. Aliquots were taken at time intervals during the incubation, hydrolyzed with 6 N HCl, and run on the amino acid analyzer for analysis of threonine and 2-aminobutyric acid. At the end of the incubation, the mixture was neutralized with HCl and made 25% in acetic acid in a total volume of 20 ml. The reaction mixture was then centrifuged at 40 000*g* for 1 h to remove insoluble material.

Dowex 50-X2 Chromatography. The neutralized alkaline hydrolysate was applied to a Dowex 50-X2 column (1 × 50 cm) equilibrated with 0.2 M pyridine–acetic acid, pH 3.1 at 55 °C. The column was washed with starting buffer and, after collecting about 120 ml, a linear gradient-starting buffer to 2 M pyridine–acetic acid, pH 5.0 in a total volume of 500 ml, was started. The emerging fractions were assayed for neutral sugars and amino groups following alkaline hydrolysis.

Sephadex G-10 Chromatography. Pooled fractions from the Dowex 50-X2 chromatography were applied to a G-10 column (1.5 × 90 cm) equilibrated with 5% acetic acid. Elution was performed with 5% acetic acid.

Paper Chromatography of Isolated Peptides. Samples from the G-10 chromatography were applied to a 57 × 45 cm Whatman 3 MM chromatography paper and developed with a mixture of butyl alcohol–pyridine–acetic acid–water (30:20:6:24) (v/v) for 20 h. The paper was dried and sprayed with ninhydrin reagent (0.1% ninhydrin in 95% ethanol containing 4 ml of collidine and 30 ml of acetic acid in a total volume of 100 ml).

Edman Degradation of Isolated Peptides. Subtractive Edman degradation was carried out according to the procedure described by Peterson et al. (1972).

Isolation of Carbohydrate Oligomers. Packed vitelline envelopes (2.0 ml) were washed with 10 ml of H_2O and centrifuged in a clinical centrifuge. The procedure was repeated three times. The vitelline envelopes were then suspended in 4 ml of H_2O and solubilized with 3 mg of dithiothreitol and the solution was made 0.5 M in NaOH and 1 M $NaBH_4$. The mixture was flushed with nitrogen and incubated for 92 h at 42 °C. The mixture was then neutralized with acetic acid at 4 °C, brought to pH 3.1 with HCl, and passed through a Dowex 50-X2 column (1 × 10 cm) which was equilibrated with 0.02 M pyridine–acetic acid at pH 3.1. The resin was washed with the same buffer and the combined effluent and wash were concentrated by removing the buffer under reduced pressure. Methanol was added and the boric acid removed by distillation as methyl borate.

Bio-Gel P-2 Column Chromatography. The neutralized hydrolysate was taken in 1 ml of 0.02 M pyridine–acetic acid, pH 5.0, and applied to a Bio-Gel P-2 column (1.5 × 94 cm) equilibrated with the same buffer and calibrated with chitohexaose, chitotetraose, raffinose, lactose, galactose, and fucose. The emerging fractions were assayed for neutral sugar by the Dische–Shettles cysteine–sulfuric acid reaction (Spiro, 1966). Samples from each fraction were run on TLC and analyzed for individual sugars on the sugar analyzer after hydrolysis with 1 N HCl and on the amino acid analyzer after hydrolysis with 4 N and 6 N HCl.

Thin Layer Chromatography. TLC was performed on Eastman Chromagram silica gel sheets 6060. The plate was developed with pyridine–ethyl acetate–acetic acid–water (5:5:1:3) and sprayed with naphthoresorcinol (0.5 g in 90 ml of methanol containing 10 ml of 85% phosphoric acid). Color development was effected by heating the plate at 120 °C for 10 min.

[1] Abbreviations used are: Abu, 2-aminobutyric acid; Fu, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; NAG_6, chitohexaose; NAG_4, chitotetraose.

Table I: Amino Acid Composition of the Egg Vitelline Envelope of *Megathura crenulata*.

Amino Acid[a]	Mol %
Lysine	1.2
Histidine	1.5
Arginine	0.5
Aspartic acid	1.4
Threonine	61.0
Serine	1.4
Glutamic acid	1.1
Proline	16.6
Hydroxyproline	4.6
Glycine	0.6
Alanine	2.6
Half-cystine[b]	1.3
Valine	0.5
Methionine	0.2
Isoleucine	1.4
Leucine	3.7
Tyrosine	0.2
Phenylalanine	0.2

[a] Amino acid values were computed from the 24 and 72 h hydrolyses. [b] Determined as cysteic acid and methionine sulfone, following oxidation with performic acid.

Table II: Relative Molar Proportions of the Egg Vitelline Envelope Components.

	Mol %
Total amino acids	37.3
Threonine	22.7
Galactosamine	22.1
Total neutral sugar	40.6
Fucose	15.9
Galactose	24.7

Table III: Conversion of Threonine to 2-Aminobutyric Acid following the Alkaline Sodium Borohydride Treatment.

	Hydrolysis Time (h)				
Amino Acid	0 h	24 h	48 h	96 h	144 h
Threonine[a] (%)	100	72	62	9.2	8.3
2-Aminobutyric acid[a] (%)	0	7	11	61	73

[a] Amount of threonine at zero time taken as 100%. Amount of 2-aminobutyric acid is represented as percent of this value.

Results

Composition. A striking feature of the amino acid composition of the egg vitelline envelope (Table I) was the high content (61 mol %) of the single amino acid threonine. Two amino acids, proline and hydroxyproline, accounted for about 17 and 5 mol % of the total, while all other amino acids were found in relatively low concentrations. The only amino sugar found was galactosamine, present in an amount about equal (on a molar basis) to the content of threonine residues (Table II). Fucose and galactose were also present. No sialic acid could be detected in vitelline envelope samples that contained as much as 400 μg of neutral sugar. The thiobarbituric acid assay for sialic acid has been performed following hydrolysis with 0.1 N H_2SO_4 at 80 °C for 1 h. Sialic acid added to vitelline envelope samples reacted normally in the thiobarbituric assay. No phosphate or sulfate were detected in the vitelline envelope.

Linkage between Carbohydrate Moiety and Protein. The composition of the vitelline envelope (Table II), showing an equality in molar ratio between threonine and galactosamine, was suggestive that the linkage between the protein and the carbohydrate moiety might be through an O-glycosidic bond as has been shown for several glycoproteins (Spiro, 1970; Marshall, 1972; Spiro, 1973). This type of linkage between threonine and galactosamine is known to be susceptible to β-elimination under alkaline conditions, resulting in the conversion of threonine to 2-aminocrotonic acid which can be reduced to 2-aminobutyric acid.

Incubation of vitelline envelopes in 0.5 N NaOH at 37 °C caused gradual loss of threonine (Table III). The conversion of threonine to 2-aminocrotonic acid was further confirmed by reduction of the latter with $NaBH_4$ to 2-aminobutyric acid (Table III). After 144 hr of incubation, about 8% of the original threonine content remained and 73% of the original threonine content was accounted for as 2-aminobutyric acid. These results indicate that at least 73% of the threonine residues were susceptible to β-elimination or, in other words, were linked to the amino sugar.

Peptide Studies. In order to confirm and extend these results, the egg vitelline envelope material which was subjected to alkaline hydrolysis in the presence of $NaBH_4$ was subjected to ion exchange chromatography on Dowex 50-X2 (Figure 1). Of the three main peaks that emerged, fraction I contained ninhydrin positive material and most of the neutral sugar, fraction II contained small amounts of neutral sugar and ninhydrin positive material, while fraction III contained only 2-aminobutyric acid and traces of other amino acids. Thus it was clear that the 2-aminobutyr-

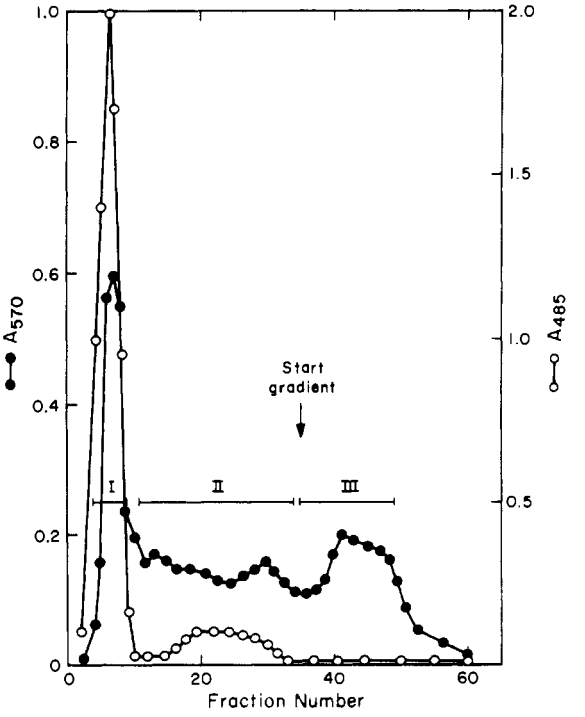

FIGURE 1: Dowex 50-X2 chromatography of vitelline envelope after treatment with alkaline sodium borohydride. The column (1 × 50 cm) was equilibrated with 0.2 M pyridine–acetic acid buffer, pH 3.1 at 55 °C. The fraction volume was 6 ml. Fractions were assayed for amino groups by the ninhydrin method (●—●) and for neutral sugar by the phenol–sulfuric acid method (○—○). Solid bars denote pooled fractions.

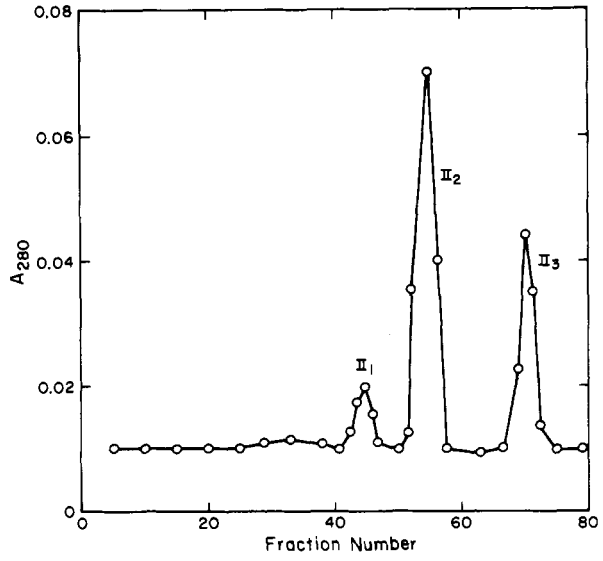

FIGURE 2: Sephadex G-10 chromatography of fraction II from the Dowex 50-X2 chromatography (Figure 1). The column (1.5 × 90 cm) was equilibrated with 5% acetic acid. Fraction volume was 1.5 ml.

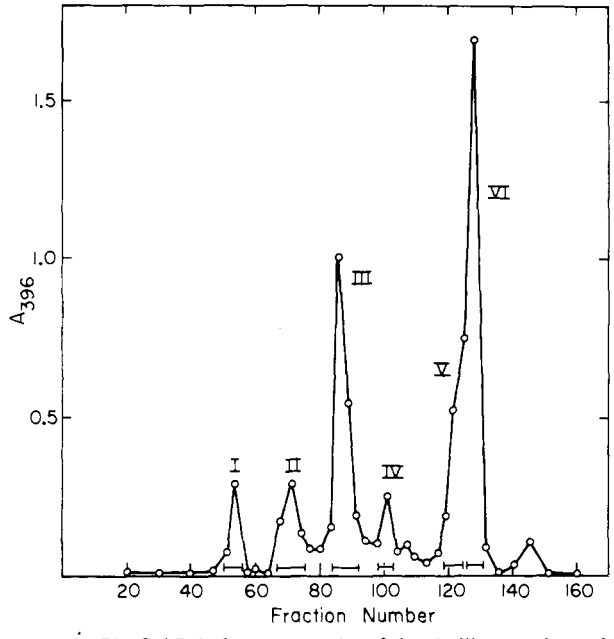

FIGURE 3: Bio-Gel P-2 chromatography of the vitelline envelope after treatment with alkaline sodium borohydride. The column (1.5 × 94 cm) was equilibrated with 0.02 M pyridine–acetic acid buffer, pH 5.0. The fraction volume was 1 ml. Fractions were assayed for neutral sugar by the cysteine–sulfuric acid method. Solid bars denote pooled fractions. The column was calibrated with chitohexaose, chitotetraose, raffinose, lactose, galactose, and fucose.

ic acid (which was derived from threonine linked to galactosamine) was cleaved from the amino and neutral sugars by the alkaline treatment. Fraction II was chosen for further study since it contained less carbohydrate than fraction I and was therefore more likely to yield peptide fractions which could readily be dealt with.

Fraction II was further fractionated on Sephadex G-10 to yield three new fractions (Figure 2). Fraction II_1 contained a single peptide as shown by paper chromatography and had the composition: Abu_8, Pro_2, Thr_1. Subtractive Edman degradation established the following sequence: Abu-Pro-Abu(Abu_6, Pro_1, Thr_1). The sequence determination could not be extended unequivocally beyond three steps. Fractions II_2 and II_3 (Figure 2) contained several peptides as judged by paper chromatography and were not further purified.

Oligosaccharide Studies. In order to study the nature of the carbohydrate moiety attached to the polypeptide chain, the vitelline envelope was hydrolyzed with 0.5 M NaOH containing 1 M $NaBH_4$ at 42 °C for 92 h. The neutralized mixture was then passed through a Dowex 50-X2 column equilibrated with 0.02 M pyridine–acetic acid buffer at pH 3.1 that retained over 95% of the peptide material. The carbohydrate containing material that came through was concentrated and fractionated on Bio-Gel P-2 (Figure 3). Each fraction was then analyzed on the sugar analyzer following hydrolysis with 1 N HCl and on the amino acid analyzer following hydrolysis with 4 N and 6 N HCl. Fraction I (51–57) did not contain any neutral sugar. Fraction II (68–75) contained equal amounts of fucose and galactose as did fraction III (82–91). Fraction IV (98–104) contained only galactose. Fraction V (119–124) contained free fucose and fraction VI (125–131) contained free galactose and other degradation products that were not characterized. Only trace amounts of galactosaminitol and aminobutyric acid were found in fractions II, III, and IV. Thin layer chromatography of fractions II, III, and IV revealed single spots after the plates were sprayed with naphthoresorcinol.

The ratio of galactose to fucose in fractions II and III, and their elution volumes on the calibrated Bio-Gel P-2 column, suggests that fraction II corresponds to an octasaccharide Gal_4Fu_4 and fraction III is an hexasaccharide Gal_3Fu_3. Fraction IV would then correspond to a trisaccharide Gal_3. The ratio of fractions II–III–IV was about 1:4:1 as determined by sugar analysis.

Discussion

The egg vitelline envelope of the marine invertebrate *Megathura crenulata* was found to be a glycoprotein with a rather unique composition, with over 61 mol % of the total amino acids being due to a single amino acid residue, namely threonine. The content of proline and hydroxyproline was also relatively high. The amino acids were linked to each other via peptide bonds as was shown by the isolation of several peptides with one of them being at least 11 residues long. From the partial sequence of one of these peptides, it was established that proline residues are interspaced between two threonine residues. If one assumes that threonine, proline, and hydroxyproline are interspaced and are found in accordance with their relative abundance, then some hypothetical sequences might be as follows:

$$\text{-Thr}_3\text{-Pro-Thr}_2\text{-Pro-Thr}_3\text{-Hyp-Thr}_2\text{-Pro-Thr}_3\text{-}$$

Other amino acids may of course be found interspersed to the extent of 17 mol %. The proximity of proline to Thr-GalNAc has been reported for other glycoproteins such as IgG H chain (Smyth and Utsumi, 1967) and bovine κ casein (Fiat et al., 1972).

The composition of the egg vitelline envelope of *Megathura crenulata* described in this paper is similar to that of *Tegula pfeifferi* (Haino and Kigawa, 1966) notably in the high percentage of threonine, galactosamine, fucose, and galactose. A significant difference is the absence of sulfate in the vitelline envelope of *Megathura crenulata*. Since no phosphate could be detected either, this suggested the absence of phospholipids. At least 73% of the threonine residues are linked to carbohydrate (most probably *N*-acetylgalactosamine) via O-glycosidic linkages, as was shown by

the β-elimination reaction. A similar linkage occurs in compounds like the ovine and porcine submaxillary glycoproteins and the M and N blood group substances (Spiro, 1970; Marshall, 1972; Spiro, 1973).

A recent analysis of the egg jelly coat of the toad *Bufo vulgaris* shows that threonine and serine accounted for approximately 70% of the total amino acids (Kawai and Anno, 1975). Large amounts of galactosamine, threonine, and serine were destroyed by the treatment of the jelly coat with alkali, indicating an O-glycosidic bond between *N*-acetylgalactosamine and the hydroxy groups of threonine and serine (Kawai and Anno, 1975). The destruction of the peptide-linked *N*-acetylgalactosamine moiety under the strongly alkaline conditions could explain the absence of the amino sugar from oligosaccharides isolated in the present study.

The data presented in this paper seem to suggest that the vitelline envelope of *Megathura crenulata* consists of polypeptide chains built to a large extent of closely spaced threonine residues. Most of the threonine residues are linked to *N*-acetylgalactosamine residues via O-glycosidic bonds. The amino sugar in turn is linked to a neutral sugar moiety which varies from an octasaccharide Gal_4Fu_4, an hexasaccharide Gal_3Fu_3, a trisaccharide Gal_3, galactose, and fucose. The polypeptide chains are linked to each other by disulfide bonds (Heller and Raftery, following article). Further studies on the structure of the oligosaccharides and their sequence along the polypeptide chain could shed light on their possible role in sperm recognition and early development.

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