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Identification of the Sialic Acids from the Egg Jelly Coat of the Sea Urchin *Pseudocentrotus depressus* (Okayama)

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Summary: The jelly coat substance of the sea urchin eggs of *Pseudocentrotus depressus* (Okayama) was found to contain 0.35% sialic acids. After isolation of the sialic acids from the purified sulfated sialoglycoprotein containing 11% of sialic acids, analysis by colorimetry, thin-layer chromatography and gas-liquid chromatography/mass spectrometry demonstrated the nature of the sialic acids to be *N*-glycoloylneuraminic acid and 9-*O*-acetyl-*N*-glycoloylneuraminic acid. The molar ratio between both sialic acids was found to be 1 : 1. Since it is known that *O*-acyl groups are partially released during the hydrolysis and isolation procedure, it can be assumed that in the native glycoprotein the percentage of *O*-acetylation is much higher than 50%. The sugar and sulfate composition of the glycoprotein is also described.

Identifizierung der Acylneuraminsäuren in der Eihülle des Seeigels Pseudocentrotus depressus (Okayama)

Zusammenfassung: Die gelartige Hüllensubstanz der Eier des im Japanischen Meer vorkommenden Seeigels *Pseudocentrotus depressus* (Okayama) enthält 0.35% Acylneuraminsäuren. Diese sind Bestandteil eines sulfatierten Sialoglycoproteins, das in gereinigter Form 11% Acylneuraminsäuren enthält. Die isolierte Sialinsäurefraktion ist aus *N*-Glycoloylneuraminsäure und 9-*O*-Acetyl-*N*-glycoloylneuraminsäure im molaren Verhältnis von 1 : 1 zusammengesetzt, wie durch Kolorimetrie, Dünnschichtchromatographie und Gas-Chromatographie/Massenspektrometrie ermittelt wurde. Der *O*-Acetylgehalt der Neuraminsäurereste dürfte im nativen Glycoprotein weit höher als 50% liegen, da *O*-Acetylgruppen während der Isolierungsprozedur der Acylneuraminsäuren teilweise abgelöst werden. Der Gehalt des Glycoproteins an anderen Zuckerresten und Sulfat wurde ebenfalls bestimmt.

Key words: Sialic acids, *O*-acetylated, gas-liquid chromatography/mass spectrometry, sea urchin, egg jelly coat.

Abbreviations:
Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycoloylneuraminic acid; Neu9Ac5Gc, 9-*O*-acetyl-*N*-glycoloylneuraminic acid.

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The jelly coat of sea urchin eggs has been found to play an important role in the fertilization process[1,2]. The coat consists of glycoproteins and polysaccharide-protein complexes of varying compositions[3]. Homogeneous sialoglycoproteins containing about 70% of sialic acid have been isolated from the egg jelly coat of the sea urchin species *Pseudocentrotus depressus* (Misaki), *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus*[3,4]. The main sialic acid in these glycoproteins was found to be *N*-glycoloylneuraminic acid. In addition, small amounts of *N*-acetylneuraminic acid were detected in the latter two species, whereas in *P. depressus* (Misaki) an unique sialic acid, *N*-acetoglycoloyl-4-methyl-4,9-dideoxyneuraminic acid has been reported to occur[3,5]. Mild acid hydrolysis of the jelly coat substance of *P. depressus* (Misaki) led also to the isolation of the disaccharide fucopyranosyl-(1 → 4)-*N*-glycoloylneuraminic acid[6].

Here the identification of the sialic acids from the egg jelly coat substance of the sea urchin species *P. depressus* (Okayama) is described.

Materials and Methods

Isolation of homogeneous sialoglycoprotein material

Sea urchin eggs of the species *Pseudocentrotus depressus* (Okayama) were collected from the Japan Sea. The isolation of the crude sulfated sialoglycoprotein from the powdered jelly of the eggs as well as the subsequent purification procedures were carried out as reported earlier for the other species[3,4,7].

Isolation of sialic acids from the sialoglycoprotein[8]

Sialic acids were released from 3.6 mg purified sialoglycoprotein by hydrolysis in 5 m*l* aqueous formic acid, pH 2, at 70 °C for 1 h, followed by dialysis in 50 m*l* distilled water at 2 °C for 12 h and by a second hydrolysis in 5 m*l* 0.1N HCl at 80 °C for 1 h. After dialysis, the sialic acids in the combined diffusates were purified by ion-exchange chromatography at 4 °C. The solution was passed through a column (5 m*l*) of Dowex 50 W X 8, $H^{\oplus}$-form (20–50 mesh). The resin was washed with 25 m*l* of distilled water and the total eluate was concentrated to 2 m*l* by rotary evaporation at 30 °C. Then the sialic acid mixture was adsorbed on a column (5 m*l*) of Dowex 2 X 8, $HCOO^{\ominus}$-form (200–400 mesh). The column was washed with 25 m*l* of distilled water and the sialic acids eluted with 25 m*l* of 1N formic acid. Finally, the acidic eluate was lyophilized.

Quantitative analysis procedures

Sialic acids were determined by the 4-dimethylaminobenzaldehyde/HCl (direct Ehrlich)[9], the orcinol/$Fe^{3\oplus}$/HCl (Bial)[10], and the periodic acid/thiobarbituric acid (Warren)[11] assays. Neu5Gc was used as a standard.

Fucose was analyzed via a periodate oxidation procedure; the formed acetaldehyde was measured using 4-hydroxybiphenyl[12]. Total hexose was measured by the phenol/H_2SO_4 method[13], after correction for methylpentose. Galactose was used as a standard. Hexosamines were analyzed on the amino acid analyzer after hydrolysis of the glycoprotein in 4N HCl (100 °C for 6 h).

The amount of glycoloylsialic acids was determined via the glycolic acid content[14]. The amount of *O*-acyl groups of sialic acids was measured with alkaline hydroxylamine and ferric chloride[15].

The sulfate content was calculated from the element analysis for sulfur. The values were corrected for methionine and cysteine. Protein was determined according to Lowry[16] with bovine serum albumin as standard.

Chromatographic methods and mass spectrometry[8]

Sialic acid mixtures were analyzed by one- and two-dimensional thin-layer chromatography on 0.2 mm cellulose plastic sheets (E. Merck, Darmstadt) using the solvent system 1-butanol/1-propanol/0.1N HCl 1:2:1 (v/v). For the two-dimensional chromatography an intermediate ammonia treatment was introduced, leading to a saponification of *O*-acyl groups, if present[17]. Before use the cellulose plates were prerun in 0.1N HCl, and dried under a current of air at room temperature for 30 min. Crystalline Neu5Ac and Neu5Gc were used as reference compounds. Sialic acid spots were visualized using the orcinol/$Fe^{3\oplus}$/HCl spray reagent[17].

Acylhydroxamates were analyzed by thin-layer chromatography on cellulose (see above) with the solvent system 1-propanol/10% ammonium carbonate/5M ammonium hydroxide 6:2:1 (v/v). The acylhydroxamate spots were stained by spraying with a 10% $FeCl_3$ solution in water[18]. Reference acetylhydroxamate was prepared from ethyl acetate[8].

Gas-liquid chromatography and combined gas-liquid chromatography/mass spectrometry of trimethylsilylated sialic acid methyl esters using 3.8% SE-30 as stationary phase were performed as described previously[18–20].

Results and Discussion

The sialic acid content of the powdered jelly coat of the sea urchin eggs of *P. depressus* (Okayama)

was found to be 0.35% (direct Ehrlich reaction). This is a much lower value than reported earlier for the jelly coat powders from the species *P. depressus* (Misaki) (3.6%), *A. crassispina* (4.1%) and *H. pulcherrimus* (2.3%)[7].

In Table 1 the composition of the purified sulfated sialoglycoprotein of *P. depressus* (Okayama) is presented; for comparison the details of *P. depressus* (Misaki) are included[3,4]. Both glycoproteins differ in several aspects.

Based on the direct Ehrlich and Bial reactions, for *P. depressus* (Okayama) 36 μmol sialic acid/100 mg of purified glycoprotein were found. This amount corresponds quite well with the glycolic acid content, being 33 μmol/100 mg, which suggests the presence of almost exclusively *N*-glycoloylneuraminic acids. However, using the thiobarbituric acid assay, after hydrolysis with dilute acid of the sialoglycoprotein and subsequent isolation of the sialic acids, a much lower value for sialic acid was measured (17 μmol/100 mg glycoprotein). This value of free sialic acids increased to 38 μmol/100 mg glycoprotein when using the orcinol assay. This observation points to a high degree of *O*-acylation in the glycerol side chain of the sialic acids[8]. This was also proven by the hydroxamate assay carried out on the isolated sialic acid fraction[15] and by thin-layer chromatography of the free sialic acids demonstrating that 50% of the isolated sialic acids was *O*-acylated.

Table 1. Composition of the sialoglycoproteins isolated from the jelly coat substances of the sea urchin eggs of *Pseudocentrotus depressus* (Okayama) and *Pseudocentrotus depressus* (Misaki).

Component	μmol/100 mg glycoprotein	
	(Okayama)	(Misaki)[3,4]
Sialic acid		
Direct Ehrlich assay	36	216
Orcinol assay	38	n.d.
Thiobarbituric acid assay	17	97
Fucose	142	56
Mannose, Galactose, Glucose (Hexoses)	48	21
Hexosamines	n.d.	13
Galactosamine	13	n.d.
Glucosamine	16	n.d.
Glycolic acid	33	216
Sulfate	159	79
Protein (Lowry) [%]	35	6

One-dimensional thin-layer chromatography of the isolated sialic acids gave rise to two distinct bands of similar intensity with R_F values of 0.54 and 0.75, corresponding to those of Neu5Gc and mono-*O*-acetyl-Neu5Gc, respectively. Two-dimensional thin-layer chromatography with intermediate ammonia treatment led to conversion of the substance with R_F 0.75 to the substance with R_F 0.54. Thin-layer chromatography of the acylhydroxamate obtained from the free sialic acid mixture revealed the presence of acetylhydroxamate, thus demonstrating the occurrence of *O*-acetyl groups in the Neu5Gc derivative.

Further characterization of the sialic acid mixture was carried out by gas-liquid chromatography and combined gas-liquid chromatography/mass spectrometry after esterification with diazomethane and trimethylsilylation[19,20]. Using this approach similar amounts (peak areas) of the trimethylsilylated methyl ester derivatives of Neu5Gc (R_{Neu5Ac} = 1.81) and 9-*O*-acetyl-*N*-glycoloylneuraminic acid (Neu9Ac5Gc) (R_{Neu5Ac} = 2.04) were detected. The retention times of both derivatives are given relative to the trimethylsilylated methyl ester derivative of Neu5Ac. The mass spectra (Fig. 1 and 2) were interpreted on the basis of a series of characteristic fragment ions, which indicate the molecular weight, the *N*-acyl substituent, and the number and positions of the *O*-acyl substituents, as reported earlier[19,20]. In Table 2 the most important mass spectrometric data are summarized.

In conclusion the isolated sialic acid mixture contains Neu5Gc and Neu9Ac5Gc in similar amounts. Since it is known that *O*-acetyl groups are partially released during the hydrolysis and isolation procedure of sialic acids from glycoconjugates, it can be assumed that the percentage of *O*-acetylation at position C-9 is much higher than 50% in the native sialoglycoprotein from *P. depressus* (Okayama). Furthermore, this study has shown that the species of *P. depressus* (Okayama), collected in the Japan Sea, and of *P. depressus* (Misaki), collected in the Pacific

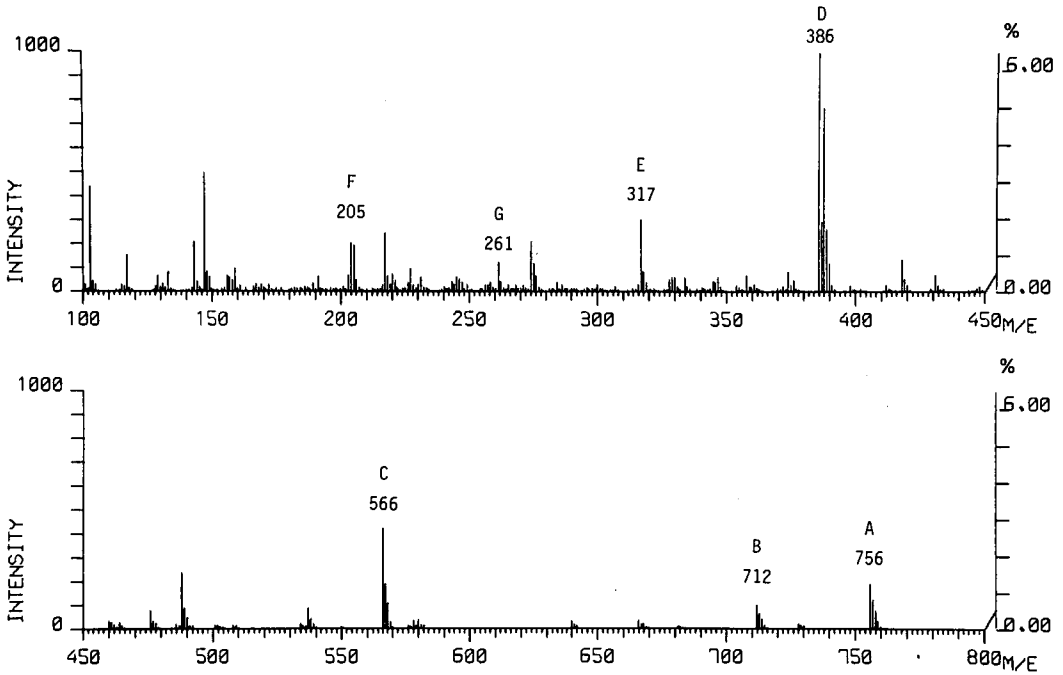


Fig. 1. 70 eV mass spectrum of the trimethylsilylated derivative of the methyl ester of *N*-glycoloylneuraminic acid.

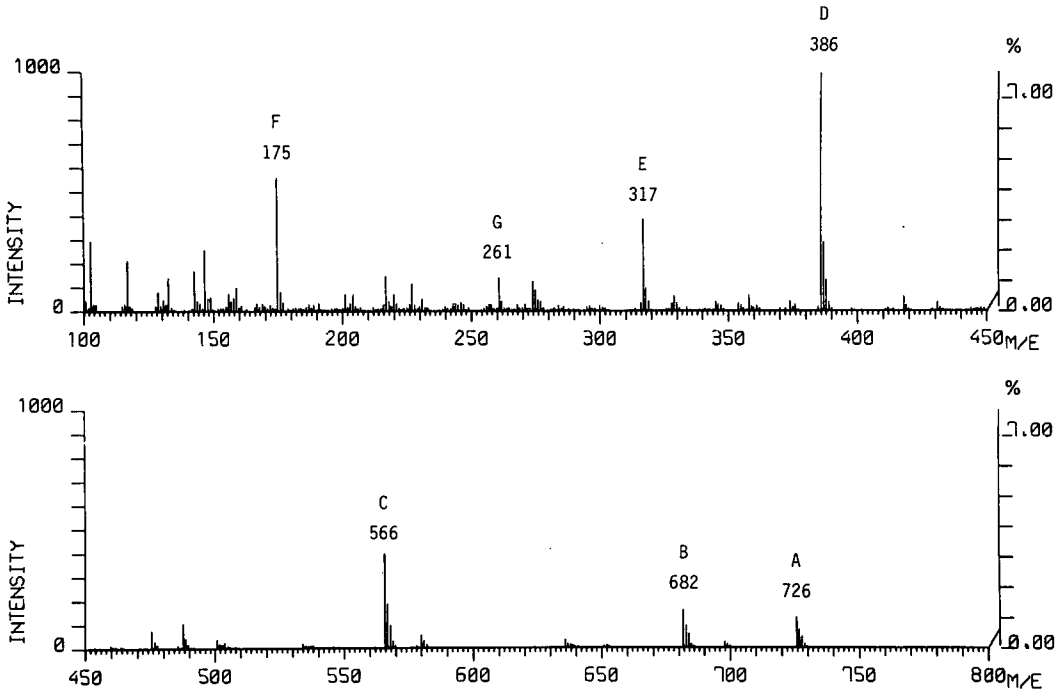
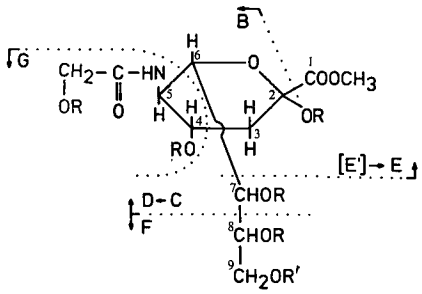


Fig. 2. 70 eV mass spectrum of the trimethylsilylated derivative of the methyl ester of 9-*O*-acetyl-*N*-glycoloylneuraminic acid.

Table 2. Specific fragment ions A to G in the mass spectra of the trimethylsilylated derivatives of the methyl esters of *N*-glycoloylneuraminic acid (Fig. 1) and 9-*O*-acetyl-*N*-glycoloylneuraminic acid (Fig. 2).
For a full discussion of the applied mass spectrometric method, see ref.[19] and the review in ref.[20]. The gas chromatographic retention times on 3.8% SE-30 at 215 °C are given relative to the trimethylsilylated derivative of the methyl ester of Neu5Ac[20] (R_{Neu5Ac}).

$(Me_3Si)_6$Neu5Gc (R_{Neu5Ac} 1.81) *m/e*	$(Me_3Si)_5$Neu9Ac5Gc (R_{Neu5Ac} 2.04) *m/e*	Explanation
756	726	A: $M^{\oplus\cdot} - {}^{\cdot}CH_3$ (from Me_3Si-)
712	682	B: $M^{\oplus\cdot} - {}^{\cdot}COOCH_3$
566	566	C: $M^{\oplus\cdot} - ROCH^{8}\text{-}CH_2^{9}OR'$
386	386	D: $M^{\oplus\cdot} - ROCH^{8}\text{-}CH_2^{9}OR' - ROH$ (from C-2) $- ROH$ (from C-4)
317	317	E: $M^{\oplus\cdot} - ROCH^{7}\text{-}CH(OR)^{8}\text{-}CH_2^{9}OR' - ROCH_2\text{-}CO\text{-}NH_2$ (from C-5)
205	175	F: $RO^{\oplus}{=}CH^{8}\text{-}CH_2^{9}OR'$
261	261	G: $ROCH_2\text{-}CO\text{-}NH^{\oplus}{=}CH^{5}\text{-}CH^{4}HOR$

R = $(CH_3)_3Si$-
R′ = $(CH_3)_3Si$- (in case of Neu5Gc)
R′ = CH_3CO- (in case of Neu9Ac5Gc)



Ocean, contain different sialoglycoproteins. Besides of large variations in the amounts of sialic acid, neutral monosaccharides, hexosamines, sulfate and protein (Table 1), the most marked difference has been found in the nature of the sialic acids which occur in addition to Neu5Gc: 9-*O*-acetyl-*N*-glycoloylneuraminic acid in the Okayama species as reported here and *N*-acetoglycoloyl-4-methyl-4,9-dideoxyneuraminic acid in the Misaki species as reported earlier[3,5].

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