Food Chemistry 166 (2015) 473–478

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Molecular species analysis of monosialogangliosides from sea urchin *Strongylocentrotus nudus* by RPLC-ESI-MS/MS

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ARTICLE INFO

Article history:
Received 19 June 2013
Received in revised form 23 October 2013
Accepted 8 June 2014
Available online 14 June 2014

Keywords:
Ganglioside
PRLC-MS/MS
Fragmentation
Ceramide moiety
Sialic acid
Molecular species

ABSTRACT

Sea urchin gangliosides have been proved to contain neuritogenic activities, which related to their molecular compositions. This study reports a method utilizing reversed-phase chromatography coupled to mass spectrometry for structure investigation and molecular species determination of the monosialogangliosides from sea urchin *Strongylocentrotus nudus*. Two types of sulfated and nonsulfated monosialogangliosides were isolated from the sea urchin ovary. In MS^2 spectra of both nonsulfated monosialoganglioside and sulfated monosialoganglioside, 2-6 linked sialic acids were identified by the characteristic fragments of $^{0,4}A_2$-CO_2 and $^{0,2}A_1$. Fragment ions at *m/z* 139.1 and *m/z* 169.1 of nonsulfated monosialoganglioside might be characteristic for 8-sulfated sialic acid residue. Retention time of the molecules was effectively used in the characterization of unknown molecules, and molecules that differ in mass by only 0.04 Da were easily differentiated.

1. Introduction

Gangliosides are a family of sialic acid (Sia) containing glycosphingolipids. The glycosphingolipids are involved in a variety of biological processes, such as cell to cell recognition and cell signalling. Disruptions in the expression and metabolism of glycosphingolipids, particularly gangliosides, could affect the brain functions, which cause various diseases (Yu, Nakatani, & Yanagisawa, 2009). When administered exogenously to neurons *in vitro* and *in vivo*, gangliosides exert two principal effects, namely neuronotrophic and neuritogenic activities. Moreover, gangliosides influence neuronal plasticity during development, adulthood and aging (Ledeen, 1984; Mocchetti, 2005).

Interestingly, apart from vertebrates, gangliosides have also been found in sea urchins (Ijuin et al., 1996; Kubo, Irie, Inagaki, & Hoshi, 1990; Yamada et al., 2008) and proved to possess neuritogenic activity. As compared with mammalian gangliosides, the basic sugar moiety of the sea urchin gangliosides is the simple disaccharide, Sia2-6Glc (Higuchi, Inagaki, Yamada, & Miyamoto, 2007). Furthermore to be hydroxylated at C-11 position, the sialic acid residues of sea urchin gangliosides were possibly methylated (Yamada et al., 2008) and sulfated at C8 position (Ijuin et al., 1996; Kubo et al., 1990). The ceramide moieties of the sea urchin gangliosides are typically comprised of both non-hydroxy and α-hydroxy fatty acids (FA), and phytosphingosine-type long-chain bases (LCB). The FA chain lengths vary a lot and many of them are containing more than 20 carbons. The variations in ceramide moiety result in a complex molecular composition of sea urchin gangliosides therefore may affect their bioactivities, which were revealed in the experiments toward rat pheochromocytoma cells (PC12 cells) (Kaneko, Yamada, Miyamoto, Inagaki, & Higuchi, 2007) of sea cucumber gangliosides SJG-1and HLG-1 (Kaneko, Kisa, Yamada, Miyamoto, & Higuchi, 1999; Yamada, Matsubara, Kaneko, Miyamoto, & Higuchi, 2001).

Electrospray ionization (ESI) mass spectrometric using collision-induced dissociation has provided valuable tools for investigating the linkages of complex sialylated gangliosides (Meisen, Peter-Katalinic, & Muthing, 2003) and the ceramide moieties of glycosphingolipids (Colsch et al., 2004). High heterogeneity of ganglioside extracts result in the difficulty in identifying each of the

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http://dx.doi.org/10.1016/j.foodchem.2014.06.028
0308-8146/

molecules by direct infusion ESI-MS methods. Coupling of liquid chromatography (LC) with MS via ESI provides an improved sensitivity, selectivity and the possibility for high-throughput experiments (Sisu, Flangea, Serb, Rizzi, & Zamfir, 2011). In general, sphingolipids in biological samples are usually quantified by running a triple quadrupole mass spectrometer in multiple reactions monitoring (MRM) mode (Shaner et al., 2009). However, MRM requires prior knowledge about which compounds are present in the given sample. Besides, as a given mass can often be assigned to two or more biologically relevant structures, assigning a molecular structure based only on precursor and product masses is not always feasible. In theory, the retention time of an LC column provides biochemical information of given molecule. Ikeda and coworkers reported a method that using reversed-phase (RP) column to separate mouse brain ganglioside molecular species in same class (Ikeda, Shimizu, & Taguchi, 2008). On the RP column, either the sphingoid chain or the fatty-acyl chains influence the elution sequence of the gangliosides, and therefore, molecular species in a ganglioside class will likely separated from each other. The aim of this research project was to develop a RPLC-MS based profiling approach for the determination of sea urchin gangliosides, in which the gangliosides are separated and identified on the basis of hydrophobicity and mass spectrometry.

2. Materials and methods

2.1. Materials

The sea urchin *Strongylocentrotus nudus* were purchased from a local market. The ovaries of the sea urchins were separated from the guts and shells then lyophilized. Chromatographic grade acetonitrile was purchased from Mallinckrodt Baker (Phillipsburg, NJ). Acetic acid and ammonium acetate were purchased from Sigma–Aldrich (St. Louis, MO). Pure water was obtained from a Milli-Q water system (Millipore, Millipore, MA). Other chemical reagents used for extraction and isolation were from local commercial sources.

2.2. Extraction and isolation of sea urchin gangliosides

The sea urchin gangliosides were extracted according to a method reported by Svennerholm and Fredman (1980). Briefly, the lyophilized powder of the sea urchin ovaries was extracted twice with three volumes of chloroform–methanol–water (4:8:3, v/v/v). The mixture was then stirred for 30 min at room temperature and then centrifuged at 2000*g* for 30 min. The supernatant was collected, filtered and pooled. Water was added to the supernatant to give a final chloroform–methanol–water ratio of 4:8:5.6 (v/v/v). After vortex mixing and centrifugation, the resulted upper phases were directly passed through a Daisogel-SP120-C8 column (3.5 × 20 cm, 40–60 μm, Daiso Chemical, Osaka, Japan) to clean and desalt. Recovered gangliosides were redissolved in chloroform–methanol–water (30:60:8, v/v/v) and applied to a DEAE-Sephadex A25 column (1.7 × 45 cm, 40–120 μm, GE Healthcare Bio-sciences AB, Sweden). After washing the column with 350 mL solvent A (chloroform–methanol–water 30:60:8, v/v/v) to clean up neutral lipids, a linear gradient from solvent A to solvent B (200 mM ammonium acetate dissolved in solvent A) was proceeded. Total elution volume was 1000 mL and 10 mL of each fraction was collected.

2.3. Mass spectrometry for investigation of cleavage pathways

Mass spectrometry experiments for investigation of cleavage pathway of sea urchin gangliosides were performed on an Agilent G6410B triple quadrupole instrument (Agilent Technologies, Santa Clara). Both positive and negative modes were employed. Conditions for ESI-MS were as follows: ESI voltage, 3750 V in negative ionization mode and 4000 V in positive ionization mode; Vaporizing gas flow, 6 L/min, at a temperature of 300 °C; Nebulizer pressure, 25 psi; MS^2 spectra were obtained from collision induced dissociation (CID) using nitrogen as the collision gas at a pressure of 0.2 MPa. The collision energy was adjusted from 30 eV to 60 eV. Supplementary MS^3 experiments were performed on an LTQ-Orbitrap XL (Thermo Electron Corporation, Waltham, MA) that was coupled with a syringe pump (Harvard Apparatus, Holliston, MA).

2.4. Reversed phase liquid chromatography

The liquid chromatographic system was an Agilent 1260 series system consisting of equipped with a binary pump. Chromatographic separations were conducted on an YMC-Pack Pro C8 column (2.0 × 100 mm, 3 μm, YMC Corporation, Tokyo, Japan) at 25 °C. Sea urchin gangliosides with different ceramides were eluted with a mobile phase prepared by mixing acetonitrile and 20 mM ammonium acetate at a ratio of 75:25 (v/v). Sample solutions were prepared by dissolving 2 mg purified sea urchin gangliosides into 1 mL of pure water. Typically, the inject volume was 5 μL and the column was eluted with mobile phase at a flow rate of 0.2 mL/min. Each run was completed in 50 min.

2.5. MRM analysis

To profile the ceramide structure and the relative content of each molecule in sea urchin gangliosides, MRM analyses were performed by monitoring sialic acid ions in negative ion modes and LCB ions in positive ion mode. Nitrogen severed as vaporizing gas at a flow rate of 8 L/min, the nebulizer pressure was 30 psi, and dwell time for each transition was 50 ms. Fragmentor values were 160 V for NMG and 260 V for SMG in negative ionization mode, and 280 V for NMG and 290 V for SMG in positive ionization mode respectively. The precursor ions to sialic acid ions in negative ionization mode and precursor ions to LCB ions in positive ionization mode were monitored during the MRM analyses.

3. Results

3.1. Mass spectrometry

Two types of gangliosides with disaccharide, Sia2-6Glc, from the sea urchins *S. nudus* were isolated and simply classified as nonsulfated-monosialogangliosides (NMGs, eluted at 0.09–0.14 M ammonium acetate) and sulfated-monosialoganglioside (SMGs, eluted at 0.16–0.19 M ammonium acetate). Molecular weights of NGMs and SMGs were inferred from their deprotonated molecular ions. In negative ion mode, NMG was detected as the singly charged state ion ($[M-H]^-$), while SMG was detected as both singly and doubly charged state ions ($[M-H]^-$ and $[M-2H]^{2-}$) (Fig. 1). SMGs transformed from mainly doubly charged ions to singly charged ions as the fragmentor value was increased to 260 V. The considerable heterogeneity observed in the spectrum was supposed mainly attribute to the variations in the ceramides and sialic acids.

MS^2 analyses performed on the $[M-H]^-$ ions of NMGs showed that they were possessing terminal sialic acid residue (either Neu5Gc: N-hydroxyacetylneuraminic acid or Neu5Ac: N-acetylneuraminic acid). In MS^2 spectra of precursor ion at *m*/*z* 1121.7, characteristic fragment ions (Meisen et al., 2003; Wheeler & Harvey, 2000) of 6-sialyated hexose as $^{0,4}A_2$-CO_2 ions (*m*/*z* 322.1), $^{0,2}A_1$-H_2O of the Neu5Gc (*m*/*z* 218.1) and $^{0,2}X_1$ ions were identified (Fig. 2). Consistent with the disaccharide core (Sia2-6Glc), a C_2 fragment ion (*m*/*z* 486.1) was identified. Negative CID-MS^2