Isolation of *N*-acetylneuraminic Acid and *N*-glycolylneuraminic Acid from *Pleurocybella porrigens*

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Pleurocybella porrigens (*P. porrigens*) is a traditional food consumed in Japan. Toxicity was first reported in 2004, following which a series of poisonings were reported in 2007. More than 59 people who consumed *P. porrigens* suffered from similar severe cryptogenic encephalitis, with an overall death rate of approximately 29%. *P. porrigens* is believed to be a major etiological agent of this disease, but the mechanism of pathogenesis is not clear. To elucidate the toxic properties of *P. porrigens* in the 2004 and 2007 poisonings, we compared the oligosaccharide constituents of mushroom samples collected in these years with those collected in other years. Water extracts (90°C and 4°C) of *P. porrigens* were dialyzed, and the oligosaccharides obtained from the high-molecular-weight fraction (> 7.8 kDa) were subjected to acid hydrolysis for modification and labeling. Resultant saccharides were analyzed by high performance liquid chromatography on an octadecyl silane (ODS) column. Our analysis revealed that the concentration of *N*-acetylneuraminic acid (NeuAc) was abundant in all samples, however, *N*-glycolylneuraminic acid (NeuGc) was present only in significant amounts in the *P. porrigens* samples collected in 2004 and 2007.

Key words —— *Pleurocybella porrigens*, encephalopathy, sialic acid, *N*-glycolylneuraminic acid, *N*-acetylneuraminic acid

INTRODUCTION

The fungus *Pleurocybella porrigens* (*P. porrigens*; "*sugihiratake*" in Japanese), which is a basidiomycete of the Tricholomataceae family, is a lignicolous agaric mushroom that produces pure white, relatively small pleurotoid fruiting bodies on rotting conifers, often in abundance.[1] This edible mushroom grows from late summer to autumn and is native to Japan, and it is particularly common in the Tohoku and Hokuriku districts. *P. porrigens* was suspected to have caused a characteristic acute encephalopathy, including coma and spasm, in the Tohoku and Hokuriku districts in 2004.[2–4] A similar "*P. porrigens*-related encephalopathy" was reported in Niigata Prefecture (part of Hokuriku district) in 2007. We recently reported that oral administration of even 5.0 g/kg of hot water extract of *P. porrigens* could not cause acute toxic in normal mice.[5] In contrast, when only 1.0 g/kg of hot water extracts were treated to the model mice with renal failure on oral administration, the acute severe toxicity was observed.[6] Nevertheless, the toxic constituents responsible for the critical encephalopathy and nephrosis seen in affected patients have not been fully evaluated.

Therefore we isolated some of the toxic constituents from *P. porrigens* and elucidated the structure and cytotoxic activity of a new ketonic fatty acid conjugated with "porrigenic acid".[7]

However, the relationship between porrigenic acid and encephalopathy has not yet been confirmed. On the other hand, cyanide and thiocyanate were detected from the samples of *P. porrigens* col-

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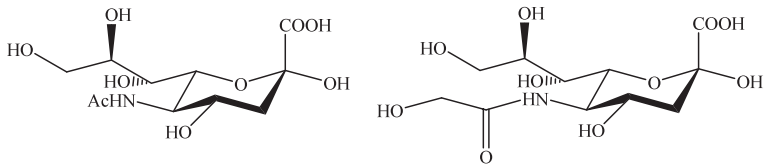

Fig. 1. NeuAc and NeuGc

Table 1. Saccharide Contents of the First-peak-fractions and Second-peak-fractions of Water Extracts

Extract	Year[a)]	Temp[a)] (°C)	Yield of fraction[b)] (g)	Fraction[c)]	Weight (mg)	Saccharide content[d)] (%)
1	2004	90	1.80	F	880	90
				S	720	42
2	2005	90	1.08	F	120	88
				S	720	36.8
3	2006	90	1.20	F	260	88.4
				S	910	66.2
4	2007	90	1.20	F	400	92.6
				S	1100	45.2
5	2004	4	1.28	F	320	60
				S	410	41
6	2005	4	1.60	F	180	52.9
				S	840	52.7

a) The fruiting bodies of *P. porrigens* were extracted with Milli-Q water at 90°C for 30 min or at 4°C for 24 hr. *b*) Water extracts 1–6 were dialyzed against Milli-Q water for 1 day and freeze dried to yield high-molecular-weight fractions (Fr.1–6). *c*) Fr. 1–6 (1.0 g) were separated by Sephadex G-75 column chromatography (2.6 cm $\phi \times 30$ cm) and fractions were eluted with Milli-Q water. Fractions were then collected from absorbance at 280 nm to obtain first-peak-fractions (F) and second-peak fractions (S). *d*) The saccharide content was estimated by the phosphophenol method.

lected in 2005 and 2006.[8)] However there is no report that there were cyanide and thiocyanate in *P. porrigens* collected in 2007.

In this study, we analyzed and compared saccharide compositions of *P. porrigens* gathered in 2004 and 2007. Moreover, we reported that *P. porrigens* is rich in *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) (Fig. 1), and that NeuGc was present only in *P. porrigens* collected in the years in which encephalopathy occurred.

MATERIALS AND METHODS

Chemicals —— Glucose (Glc, 98%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NeuAc aldolase (≥ 20 units/mg), neuraminidase (≥ 0.1 units/mg), NeuAc, and NeuGc were purchased from Sigma-Aldrich Japan Co., Ltd. (Tokyo, Japan). *p*-aminobenzoyl ethyl ester (ABEE)-saccharide analysis kit was purchased from J-Oil Mills Co., Ltd. (Tokyo, Japan).

Extraction and Fractionation —— *P. porrigens* was taxonomically identified and collected from Ishikawa Prefecture during fall 2004 to 2006, from Yamagata prefecture during fall 2007.

They were stored in a database at the Kanazawa University Graduate School of Science and Technology under registration numbers T2004–2007. Raw mushroom samples were quickly frozen at −80°C until analysis. Fruiting bodies of *P. porrigens* (400 g wet weight) collected in 2004, 2005, 2006, and 2007 were extracted with Milli-Q water (1000 ml) at 90°C for 30 min to yield extracts 1 to 4 (around four gram). Similarly, fruiting bodies of *P. porrigens* (400 g wet weight) collected in 2004 and 2005 were extracted with Milli-Q water (1000 ml) at 4°C for 1 day to yield extracts 5 and 6 (around four gram). Water extracts 1 to 6 were then dialyzed against Milli-Q water by cellulose tubes [Pore size: 40–50 Å. Sankojunyaku Co., Ltd. (Tokyo, Japan)] for 1 day and freeze-dried to yield high-molecular-weight fractions (Fr. 1–6, Table 1). Fraction 1 (Fr. 1; 1.0 g) was separated by Sephadex G-75 column chromatography [2.6 cm $\phi \times 30$ cm, 40–

120 μm; Tosoh Co., Ltd. (Tokyo, Japan)] and fractions were eluted with Milli-Q water. Absorbance of each fraction was monitored at 280 nm. Absorbance was measured using a Power wave HT micro plate reader [Bio Tec Co., Ltd. (Tokyo, Japan)].

Two peaks appeared on chromatography profiles and two groups of high-absorbance fractions were collected to the first-peak-fraction (Fr. 1F) and second-peak-fraction (Fr. 1S) (Table 1). Similarly Fr. 2F, 2S, 3F, 3S, 4F, 4S, 5F, 5S, 6F and 6S were afforded from extracts 2 to 6 (Table 1). The molecules of components were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PGE in the presence and absence of 2-mercaptoethanol using a slab gel was conducted in Tris/glycine buffer system (pH 8.3) as described by Schagger and von Jagow.[9] The composition of slab gel was 4%T stacking gel and 10–20%T resolving gel [Bio-Rad Lab. Co. Ltd. (Tokyo, Japan)]. The composition of Tris/glycine buffer was 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS (Bio-Rad Lab. Co. Ltd.). SDS-PAGE of the first-peak-fractions and second-peak-fractions showed some bands with the apparent mass of 37–200 kDa and 10–37 kDa.

Molecular marker was Preccision Plus Protein Dual Color Standards (Bio-Rad Lab. Co. Ltd.).

Measurement of Saccharide Content —— The saccharide content was estimated by the phospho-phenol method[10] and Glc was used as the standard (correlation, 0.98; range, 0–100 μg/ml)

Analysis of galactose (Gal), mannose (Man), *N*-acetylmannosamine (ManNAc), *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), and fucose (Fuc) —— The saccharide constituents were analyzed by ABEE-saccharide labeling method (ABEE-saccharide analysis kit). A mixed solution of NeuAc aldolase and neuraminidase (10 μl; ABEE-saccharide analysis kit)[10, 11] was added to Fr. 1F–6S (1.0 mg); the samples were then heated to 45°C for 1 hr. Then 4.0 *N*-trifluoroacetic acid (10 μl) was added to the samples and the solution were heated to 100°C for 3 hr. Reaction mixtures were centrifuged under reduced pressure. 2-Propanol (40 μl) was then added and evaporated, and acetic anhydride (10 μl) and pyridine/MeOH (40 μl) was added. The solution was incubated at room temperature for 30 min. After evaporation, a mixture of H_2O (10 μl) and ABEE in a pyridine–borane mixture (40 μl) was added and heated to 80°C for 1 hr.

Finally, each residue was added of a mixture of H_2O (200 μl) and $CHCl_3$ (200 μl). After partition, the compounds in the H_2O layer were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) on an octadecyl silane (ODS) column.

RP-HPLC was carried out using a Waters Delta-600 machine [Waters Co., Ltd. (Tokyo, Japan)]. A SunFire Prep C_{18} column (Particle size, 5 μm; 4.6 mm $\phi \times 250$ mm; Waters Co., Ltd.) and solution solvent of 0.2 M potassium borate: acetonitrile (93 : 7) were used for RP-HPLC. The absorbance wavelength for detection was 305 nm. The identification of Gal, Man, ManNAc, GalNAc, GlcNAc, and Fuc labeled by ABEE were based on the retention time and the concentrations were estimated from the peak values compared to those standards.

Analysis of NeuAc and NeuGc —— A mixed solution of NeuAc aldolase and neuraminidase

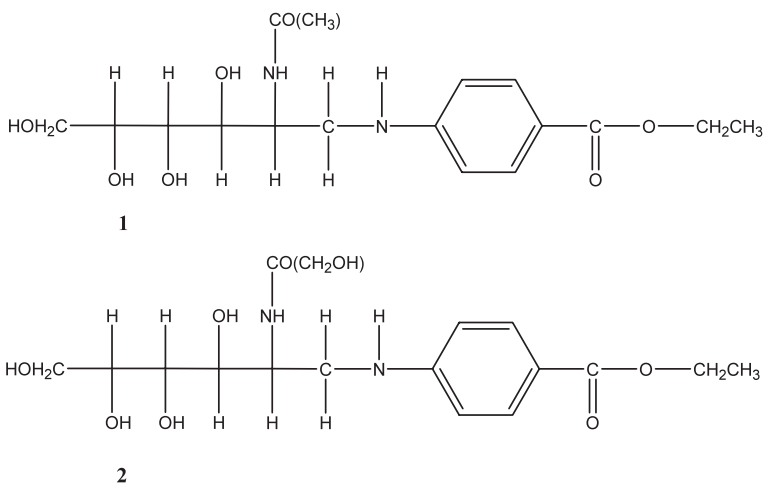

Fig. 2. ABEE-labeled *N*-acetylmannosamine (**1**) and ABEE-labeled *N*-glycolymannosamie (**2**)

(10 μl; ABEE-saccharide analysis kit) was added to Fr. 1F–6S (1.0 mg), and the solution was heated to 45°C for 1 hr. After evaporation of each fraction, H_2O (10 μl) and ABEE in a pyridine–borane mixture (40 μl) were added and the mixture was heated to 80°C for 1 hr. The mixture was added of H_2O (200 μl) and $CHCl_3$ (200 μl). After partition, the compounds in the H_2O layer were analyzed by RP-HPLC with the same conditions as described above.

ABEE-labeled ManNAc, which was the derivative of NeuAc, and ABEE-labeled ManNGc (derivative of NeuGc), were analyzed by HPLC on an ODS column with the same conditions as described above and identification were based on the retention time. The concentrations were estimated from peak area values compared to those of the standards.

Purification and Identification of ABEE-labeled ManNAc and ManNGc by High-resolution-fast-atom-bombardment Mass Spectrometry (HR-FAB-MS) —— Adequate amounts of NeuAc aldolase (≥ 20 units/mg) and neuraminidase (≥ 0.1 units/mg) were dissolved in phosphate buffer saline (50 μl, pH 7.2).

Fr. 1F (10 mg) was dissolved in this enzyme mixture solution and treated using an ABEE-saccharide analysis kit according to the manufacturer's instructions. ABEE-labeled ManNAc and ManNGc were purified by HPLC on an ODS column with the same conditions as described above

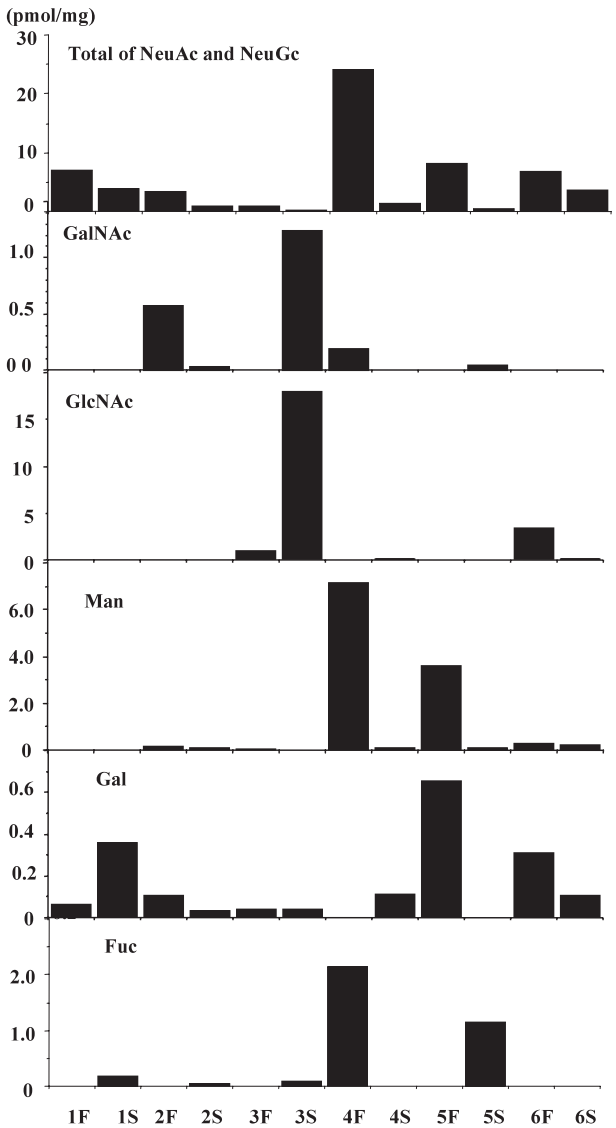

Fig. 3. Constitution of Oligosaccharides of First-peak-fractions and Second-peak-fractions of Water Extracts

All oligosaccharide were labeled by ABEE and the concentration of ABEE-labeled saccharides were estimated from the peak area values compared to those of standards. Concentrations of NeuAc and NeuGc were expressed as those of ABEE-labeled ManNAc.

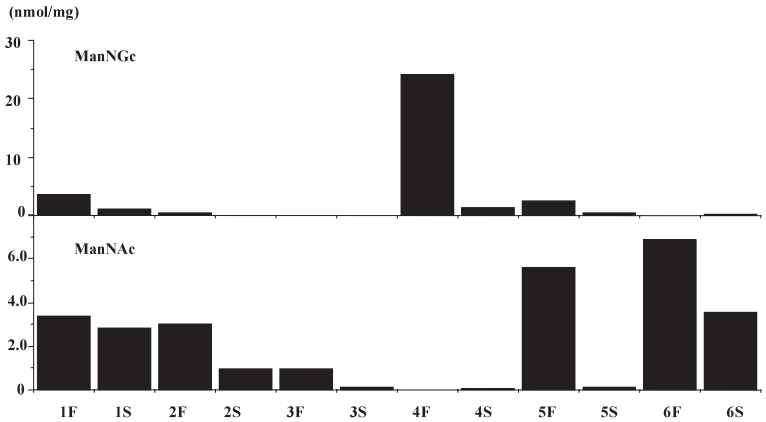

Fig. 4. Constitutions of NeuGc and NeuAc of First-peak-fractions and Second-peak-fractions of Water Extracts
Concentrations of NeuAc and NeuGc were expressed as those of ABEE-labeled ManNAc and ManNGc.

and identified by HR-FAB-MS. Mass spectra were measured on a JEOL SX-102 mass spectrometer [Japan Electron Optics Laboratory Co., Ltd. (Tokyo, Japan)].

Ionization mode was positive mode and matrix was glycerol. ABEE-labeled ManNAc (**1**): HR-FAB-MS, m/z: 371.18127 $[M+H]^+$ (calculated for $C_{17}H_{26}O_7N_2$, 371.18183), m/z 409.13740 $[M+K]^+$ (calculated for $C_{17}H_{26}O_7N_2K$, 409.13771). ABEE-labeled ManNGc (**2**): HR-FAB-MS m/z: 409.15886 $[M+Na]^+$ (calculated for $C_{17}H_{26}O_8N_2Na$, 409.15867).

RESULTS AND DISCUSSION

P. porrigens collected from 2004 to 2007 was extracted with Milli-Q water at 90°C and 4°C, and these extracts were freeze-dried (Table 1). The extracts were dialyzed against Milli-Q water by cellulose tubes and lyophilized to obtain six high-molecular-weight fractions (Table 1). Fr. 1 was subjected to column chromatography on a Sephadex G-75 column with absorbance-guided fractionation to obtain Fr. 1F and 1S (wavelength: 280 nm). We similarly obtained 10 fractions (Fr. 2F–6S) from Fr. 2–6 (Table 1). The saccharide contents of Fr. 1F–6S were shown in Table 1.

Saccharide components were then analyzed by a labeling method. A mixture of *N*-neuraminic acid aldolase and neuraminidase[10, 11] was added to Fr. 1F to release NeuAc and NeuGc. The sample obtained from Fr. 1F was hydrolyzed with trifluoroacetic acid; this was followed by re-acetylation with acetic anhydride. The sample was labeled with ABEE. The reaction mixture of labeled saccharides was analyzed by HPLC with an ODS column and the components were identified from the retention time (Fig. 2). The reaction mixture was rich in ABEE-labeled ManNAc (**1**) and ManNGc (**2**) originating from NeuAc and NeuGc (Fig. 3).

We measured the ratio of NeuAc to NeuGc by modified analytical method where trifluoroacetic hydrolysis nor acetylation were not performed. Yields of compound NeuGc from Fr. 1F, 1S, 4F, 4S, 5F, and 5S were 3.66, 1.07, 24.1, 1.33, 2.41, and 0.40 nmol/mg, respectively (Fig. 4). Yields of NeuGc from Fr. 2F, 2S, 3F and 6S were 0.40, 0.01, 0.01, and 0.13 nmol/mg, respectively (Fig. 4). NeuGc was not detected in Fr. 3S and 6F.

Levels of NeuGc were high in *P. porrigens* gathered in the year in which *P. porrigens*-related encephalopathy was observed (Fig. 4). The labeling protocol is well recognized for saccharide labeling, standards were prepared to calibrate trifluoroacetic acid hydrolysis of each saccharide. Seven saccharides (Man, Gal, NeuAc, GalNAc, ManNAc, Fuc, NeuGc) were analyzed successfully. This analysis performed after dialysis (Pore size: 40–50 Å) and gel-filtration, so 'free ManNAc and ManNGc' and 'low-molecular compounds contain NeuAc and NeuGc' might be removed. On the other hand, if glycoproteins and polysaccharides contain ManNAc and ManNGc, neuraminidase can not release ManNAc and ManGc from them. Consequently, the originally contained ManNAc and ManNGc could not be detected by RP-HPLC in this study.

NeuAc and NeuGc were sialic acids. Concentration of NeuAc in *Arabidopsis thaliana* MM2 cells, fruit of *Malus pumila* and seed of *Vigna radiate* were about 3–10 pmol/g fresh weight.[13] As far as we know, there is no report about NeuAc and NeuGc in the mushroom contain. NeuAc is biosynthesized in human bodies. Humans can not biosynthesize NeuGc because of the loss of

cytidine monophospho-NeuAc hydroxylase (CMP-NeuAc hydroxylase).[14–17] Nevertheless humans daily uptake NeuGc in meat (beef, pork, chicken, mutton and so on) and have the antibody production against NeuGc.[18] On the other hand, other mammals have CMP-NeuAc hydroxylase and NeuGc exist on glycoproteins and glycolipids of the tissues.[14–17] Then tolerization against NeuGc seems to be established in mice. Actually, even 5.0 g/kg of hot water extracts of *P. porrigens* collected in 2004 failed to show the toxicity against normal mice on oral administration and finally higher concentrations of extracts (750 and 1000 mg/kg) shown the toxicity against normal mice by intraperitoneal administration.[5] However, when only 1.0 g/kg of hot waster extracts were treated to the model mice with renal failure on oral administration, the acute severe toxicity was observed.[6]

In conclusion, our results suggested that the *P. porrigens* collected in 2004 and 2007 contained large amounts of NeuGc compared with those collected 2005 and 2006. We suspect that "*P. porrigens*-related encephalopathy" may be caused by combination of NeuGc uptake and renal failure. We will intend to analyze the toxic mechanism of NeuGc in "*P. porrigens*-related encephalopathy" in the future.

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