Isolation and Characterization of Inositol-Containing Glycosphingolipids from Aspergillus niger

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The glycosphingolipids are a group of sphingolipids characterized by the presence of equimolar amounts of long-chain base and fatty acid (ceramide) and containing either one or two mol of inositol phosphate per mol of lipid. From one to several mol of sugar are also present. Glycosphingolipids have been isolated from both plant and fungal sources. Work in this area had for years been the province of Herbert Carter and colleagues. They showed that such glycolipids occurred in seeds from corn, flax, soya beans and bean leaves (Carter et al., 1958; Carter & Koob, 1969). The major glycolipid from corn seed had the structure (Carter et al., 1969):

\[ \text{Ceramide-1-phosphoryl-1'-'O-[2'-O-mannosyl-6'-O-d-glucosaminyl-(1\rightarrow4)-d-glucuronosyl]inositol} \]

Another major component had the trisaccharide (fucosylarabinosylgalactosyl) attached to glucosamine. Kaul & Lester (1975) re-discovered these glycolipids and described a \( N \)-acetylglycosaminylglucuronosylinositolphosphorylceramide and its non-acetylated derivative from tobacco leaves.

Lester and colleagues (Steiner et al., 1969; Smith & Lester, 1974) showed that the fungus Saccharomyces cerevisiae contains large quantities of an unusual glycosphingolipid with the probable structure:

\[
\text{Ceramide-O-P-O-inositol-O-P-O-inositol}
\]

\[
\text{O- mannose O-}
\]

Wagner & Zofcsik (1966) and Smith & Lester (1974) have shown that S. cerevisiae also contains small amounts of the simpler

\[
\text{Ceramide-O-P-O-(inositol-d-mannose)}
\]

\[
\text{O-}
\]

Large quantities of the latter glycolipid and more glycosylated varieties are present in the basidiomycete Agaricus bisporus (P. J. Brennan, M. Mulligan, J. A. Hackett & P. F. S. Byrne, unpublished work).

Little is known about the metabolism and function of these unique glycolipids. We have selected Aspergillus niger, which contains sizeable quantities of glycosphingolipids (Brennan & Roe, 1975), to explore these topics and as a preliminary step we have developed simple procedures for the extraction, purification and analysis of the glycolipids.

A. niger (waste mycelium from Pfizer Chemical Corp., Ringaskiddy, Co. Cork, Ireland) was extracted with 1.5 litres of industrial ethanol at 50°C and filtered while hot (Fig. 1). During storage of the filtrate at 2°C a flocculent white precipitate formed. The residual mycelium was further extracted with two 1.5 litre lots of chloroform/methanol (2:1, v/v). When these extracts were concentrated and an excess of methanol was added a further 6.0 g of a slightly tan precipitate was recovered. To remove glycerides from the precipitates they were exposed to weak alkali, dialysed, freeze-dried and the powder was extracted with acetone.
Aspergillus niger (350g dry wt.)

Ethanol extraction at 50°C; cool
White precipitate (3.5g)

Chloroform/methanol (2:1, v/v) extraction at 50°C; addition of excess of methanol
Pigmented precipitate (6.0g)

0.2M-NaOH in methanol; dialysis; acetone extraction

Sphingolipid preparation I (1.5g; white)
Sphingolipid preparation II (1.6g; pigmented)

Fig. 1. Extraction of sphingolipids from Aspergillus niger

Both sphingolipid preparations were similar in most respects. The constituent sugars were shown by paper chromatography (Brennan & Roe, 1975) to be solely galactose and mannose. Inositol was also evident in this system. About 25% of the weight of the sphingolipid preparation was carbohydrate; 14% was inositol; 12% was long-chain base and 0.6% was Pi. It therefore appeared that much of the preparation was not sphingolipid in nature. T.l.c. of the sphingolipid preparations in chloroform/methanol/8% (v/v) NH₃ (sp.gr. 0.880) (9:7:2, by vol.)(solvent 1) showed six to eight individual glycosphosphosphingolipids, with considerable quantities of free ceramides and immobile material. Ceramides and glycosphosphosphingolipids were recognized by using a modified periodate–Schiff reagent (Hackett & Brennan, 1976). The sphingolipid preparations showed solubility properties atypical of lipids. They were sparingly soluble in chloroform/methanol/water (3:1:1, by vol.) and 30% (v/v) pyridine, and insoluble in anhydrous chloroform/methanol mixtures.

Of several methods used for isolation of individual glycosphosphosphingolipids, including DEAE-cellulose (Brennan & Roe, 1975), silica-gel H (Merck) column chromatography (Svennerholm, 1972) was the most satisfactory. A column (35cm×4cm) was made from a slurry of activated gel in chloroform/methanol (9:1, v/v). The sphingolipid preparation I (1.15g) suspended in chloroform/methanol/water (30:8:1, by vol.) was applied to the column, which was irrigated with 550ml of the same solvent. The majority of the eluted lipids were free ceramides, accounting for about 30% of the applied lipid. Subsequent eluates arising from the application of 2.5 litres of chloroform/methanol/6% (v/v) NH₃ (sp.gr. 0.880) (60:32:7, by vol.) to the column were collected in four equal fractions and examined by t.l.c. in solvent 1. Six individual glycosphosphosphingolipids were present in the second fraction, with Rₓ values between 0.35 and 0.65. They represented 12% of the applied lipid. A very polar glycosphospholipid (Rₓ 0.25 in solvent 1) was present in the fourth fraction and comprised about 4% of the total lipid (45mg). This glycolipid (called GPSL G) presents a broad, long and uniform spot in solvent 1 and in chloroform/methanol/water (6:4:1, by vol.), but it appears as a double spot in the acidic solvent, chloroform/acetic acid/methanol/water (40:27:3:7, by vol.) after multiple development. Ammonolysis (Ballou et al., 1963) of GPSL G and descending paper chromatography of the neutral products in butan-1-ol/pyridine/water (6:4:3, by vol.) for 2 weeks showed only one major oligosaccharide. Hence GPSL G was considered sufficiently pure for analysis (Table 1). The results strongly suggest that GPSL G is a trigalactosyldimannosylinositolphosphorylceramide.
Table 1. Quantitative analyses of purified glycosphingolipid G from Aspergillus niger

Total carbohydrate was measured colorimetrically by the phenol/H₂SO₄ method of Hough & Jones (1962). The individual hexoses and inositol were quantified by g.l.c. by using mannitol as an internal standard (Sweeley et al., 1963). Phosphorus was measured by the method of Bartlett (1959). Long-chain bases were determined by the method of Lauter & Trams (1962).

<table>
<thead>
<tr>
<th>Composition of freeze-dried glycolipid (µmol/mg)</th>
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<tbody>
<tr>
<td>Galactose</td>
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<tr>
<td>Mannose</td>
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<tr>
<td>Inositol</td>
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<tr>
<td>Phosphorus</td>
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<td>Long-chain base</td>
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The extraction procedure is apparently effective for solubilizing all glycosphingolipids from several fungi; continued extraction of mycelium with solvents containing HCl or pyridine removed little precipitable material. The usual silicic acid-chromatography procedures, using anhydrous solvents, were entirely ineffective for resolving these fungal glycolipids. The introduction of aqueous solvents allowed good resolution between polar and non-polar sphingolipids. However, further modifications are necessary for adequate resolution of the less polar variety.

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