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Sphingolipids from Basidiomycetes *Pleurotus ostreatus*

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Mihovil Proštenik (1916–1994) initiated the concept of the isolation of sphingolipids from fungi. We started the isolation of ceramides from Basidiomicetes together, and this paper should be viewed as a kind of tribute to his pioneer work and science devoted spirit.

Summary

Previous studies have shown Basidiomycetes to contain various types of sphingolipids, especially ceramides and cerebrosides. The aim of the study was to investigate the composition of the ceramides from this particular fungus, and to find the optimal method of their isolation. The basidiomycete Pleurotus ostreatus was collected as a wild-growing fungus. Lipid extraction was performed from fresh fungus. Using a combination of chloroform and methanol solvents, lipids were separated applying column chromatography and TLC. The degradation of isolated ceramides was performed by methanolysis, and fatty acids and long-chain bases were isolated and identified by TLC and GLC. Ceramides and cerebrosides of the fungus Pleurotus ostreatus were detected. Eight groups of ceramides were isolated according to their R_f values on TLC. The isolated ceramides contained amide bound fatty acids that were identified by GLC after methanolysis. Saturated and unsaturated, 2-hydroxy and non-hydroxy fatty acids, with chain length of C_{10}–C_{24} were found.

Key words: basidiomycete, sphingolipid, ceramide

Introduction

Sphingolipids and their breakdown products have been found to play a role as intracellular signaling substances *(1)*. With the discovery of protein kinase C inhibition by the action of sphingosine *(2)*, the possibility for the sphingolipids to serve as precursors of some important biological mediators has come into the very focus of interest. The breakdown of sphingolipids results in the formation of ceramides, which modulate many intracellular regulatory activities *(3)*, *e.g.*, cell growth inhibition, induction of cell differentiation, *etc.* Recent discoveries imply that ceramides are potent tumor suppressors. Ceramides are involved as cellular signaling substances in the etiology of many neurologic, hepatologic, nephrologic and immunologic disorders, even carcinoma *(4)*.

Cerebroside-like compounds were discovered in fungi as early as 1905 *(5)* and investigated during the next 15 years *(6,7)*. However, it was much later when their structure was positively determined as N-acyl derivatives of sphingolipid bases (ceramides). Such compounds were discovered in 1972 in *Amanita muscaria* and *Agaricus bisporus (8)*, later also in some other fungi *(9)*. The basidiomycete *Pleurotus ostreatus* (Jacq. ex Fr.) Kummer also contains ceramides. It is a well known and largely grown edible mushroom, naturally found on the trees (poplar-tree, elm-tree, *etc.*). The fungus has previously been studied for the content of phospholipids *(10)*, mycelial fatty acids *(11)* and total lipids, depending on the nutrient medium composition. Ceramides were also found and separated from the fungi *Lactarius deliciosus (12)* and *Hipholoma fasciculare (13)*.

The aim of the present study was to determine the composition of ceramides isolated from the basidiomycete *Pleurotus ostreatus*. Extraction of ceramides from fungi and their identification would serve as a new source of these important substances.

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Experimental

The fruit bodies of *Pleurotus ostreatus* were harvested in the vicinity of Zagreb, Croatia. Fresh material was used in the study. Solvents were redistilled. Lipids were extracted from the fungus with volume ratio of chloroform:methanol:water 8:4:3 by the modificated method of Folch *et al. (14)*; evaporation of the lower phase furnished the total lipid fraction. Total lipids were fractionated on a column filled with 400 g Florisil (60/100 mesh, Floridin Co., Tallahassee, FL) using 1500 mL of chlorofom and 1000 mL each of the following solvent mixtures (volume ratio) chloroform:methanol 98:2, 96:4, 94:6, 92:8, 90:10, 85:15 and 80:20. Similar fractions as monitored by TLC (solvent systems A and B) were pooled into 28 major fractions. Ceramide-containing fractions (10–15) were eluted from the column with chloroform:methanol mixture (95:5). Ceramide-monohexoside fractions (23–26) were eluted with chloroform:methanol (90:10) and (80:20). Creamed fractions were divided by preparative TLC and rechromatography into eight polarity groups (ceramides 1–8), each showing a single spot on TLC.

Acid-catalyzed methanolysis of ceramides and cerebrosides was carried out by refluxing with a mixture of methanol:concentrated HCl (5:1, volume ratio) for 10 h *(15)*. The resulting mixtures of nonhydroxy and hydroxy fatty acid methyl esters were analyzed and quantified by GLC. The long-chain bases and carbohydrate components, in case of cerebrosides, were identified by TLC *(16)* and GLC.

Thin-layer chromatography was carried out on silica gel (E. Merck, Darmstadt, Germany) plates, 20x20 cm (analytical, 0.22 mm; preparative, 0.5 mm). The following solvent systems were used (all expressed in volume ratio) (A) chloroform:methanol (19:1.5) for less polar lipid classes; (B) chloroform:methanol:water (64:25:4) for more polar lipids; (C) chloroform:methanol:2 N ammonia (40:10:1) for long-chain bases; and (D) chloroform: methanol (6:4, plates impregnated with sodium acetate) for sugar components. Detection for lipid classes in solvent systems (A) and (B) was carried out with ammonium molybdate:perchloric acid spray reagent *(17)*, (C) for long chain bases with ninhydrin; and (D) with α-naphthol for sugar components *(18)*. In addition, the plates were visualized successively with ninhydrin, silver-nitrate-ammonium hydroxide and Dragendorff's reagent for selective detection of phosphatidylethanolamines, phosphatidylinositols, and phosphatidylcholines, respectively *(19)*. The preparative TLC plates were developed once or twice, as necessary, in solvent system A, and lipids were visualized by spraying with water or methanol:water (1:1).

Fatty acid methyl esters were analyzed by GLC (Perkin Elmer, model Sigma 2, with FID detector). Separation was performed on a capillary column (AT-WAX, All Tech GmbH, Unterhaching, Germany), 30 m, diameter 0.25 mm, active layer thickness 0.25 μm, under the following conditions: temperature 150–250 °C with a linear program of 5 °C/min, then 250 °C, 20 min; temperature of injector 250 °C, temperature of detector 260 °C; gas carrier, nitrogen, 20 cm^3/min. Data were recorded on an Omega-2 (Perkin Elmer) analytical unit.

Individual fatty acids were identified on the basis of comparison of retention times of authentic standards supplied by Supelco (Gland, Switzerland).

Results

Results of this study refer to the upper, fruiting body of the basidiomycete *Pleurotus ostreatus*. The mushroom was harvested as a wild growth on a tree (elm--tree, *Ulmus*). From 2295 g of fresh mushroom, 15.80 g (0.69 %) of total lipids were extracted. Chromatographically separated fractions were found to contain carbohydrates, triacylglycerols, sterols, free fatty acids, sterol glycosides, ceramides, cerebrosides and phospholipids. Eight ceramide groups (1–8) of a varying polarity were separated by TLC. The remaining lipids undergoing ester-bond cleavage by alkaline hydrolysis, were removed. The total mass of purified ceramides was 0.52 g. Infrared (IR) spectrum showed all features characteristic of ceramides. The absorption characteristic of trans-double bond typical for sphingosine was exhibited by low polarity compounds, *i.e.* those in fractions 1–3, and was absent in high polarity ceramides. Ceramide groups 1 and 2 are viscous oils soluble in cold chloroform. The main fatty acids were 16:0 and 18:X, and sphingosine was the predominant long-chain base. In ceramide groups 3 and 4, the 16:0 and 18:X acids prevail, however, they contained acids with longer chains, 22:0 and 24:0, as well. Sphingosine was the long-chain base. Ceramide groups 5 and 6 are foliaceous crystals, M.p. 136–137 °C. The 22:0 and 24:0 acids predominate, with the presence of 16:0 and 18:0 acids. Ceramide groups 7 and

Table 1. Composition of fatty acids derived from ceramides of *Pleurotus ostreatus* (%)

Fatty acids*	Ceramide groups							
	1	2	3	4	5	6	7	8
10:0	–	0.36	–	0.21	–	–	–	–
12:0	–	0.36	–	1.17	–	–	–	–
14:0	–	tr	–	0.14	4.80	–	–	–
15:0	–	3.20	2.01	2.62	0.76	–	–	–
16:0	48.17	49.27	26.84	37.26	13.91	12.64	–	3.65
16:X	tr	–	–	–	–	–	–	–
h 16:0	–	–	–	–	–	–	–	4.41
18:0	5.08	8.39	5.02	5.17	11.13	10.06	–	–
18:X	42.30	25.55	21.91	43.00	–	–	–	–
h 18:0	–	–	–	–	–	–	1.02	10.95
20:0	–	7.30	3.93	4.76	7.96	4.22	8.32	6.84
h 20:0	–	–	–	–	–	–	–	3.04
h 21:0	–	–	–	–	–	–	9.55	–
h 21:1	–	–	–	–	–	–	6.77	–
22:0	–	5.47	15.05	4.55	28.82	40.87	17.74	3.04
h 22:0	–	–	–	–	–	–	–	18.25
24:0	–	–	20.23	1.10	32.62	28.64	–	–
24:X	–	–	5.02	–	–	–	–	–
h 24:0	–	–	–	–	–	3.57	42.98	28.21
h 24:1	–	–	–	–	–		13.64	21.60

Legend: tr = traces, X = total unsaturated fatty acids, h = hydroxy fatty acids,
(*) fatty acids were quantified as methyl esters by means of GLC

8 are characterized by the highest polarity. They contain phytosphingosine as the base, with the h24:0 (ceramide group 7), and h22:0 and h24:0 (ceramide group 8) hydroxylated acids as the prevailing fatty acids. The substance is crystallized in warm chloroform, M.p. 132–133 °C, and is almost insoluble in cold chloroform. It is soluble in a mixture of chloroform and methanol (2:1). The results of this analysis are shown in Table 1.

Discussion

Animal tissues, especially brain tissue, contain lipids rich in cerebrosides (ceramide monohexosides). Ceramides are detectable in traces, as they obviously are metabolic intermediary products of the synthesis and breakdown of cerebrosides and other sphingolipids. The fungi are relatively abundant in ceramides. The ratio of the proportion of ceramides and cerebrosides vary from species to species. Some basidiomycetes were found to contain no cerebrosides, while ceramide was present. Interestingly enough, there is a similarity in the qualitative composition of the fatty acids of sphingolipids of the animal and vegetable origin. Thus, cerebrosides of the central nervous system contain more than 80 % of fatty acids with a chain length of > 20 C-atoms. Cerebrosides of higher fungi appear to be an exception. However, hydroxy fatty acids are found in sphingolipids irrespective of the tissue origin (except for sphingomyelins). As differentiated from cerebrosides, fungal ceramides are abundant in a variety of fatty acids. The acid composition of ceramides varies both between different ceramides of a single fungus and in comparison with the fatty acid composition of the respective ceramides of different fungi. It should be noted, however, that fungal ceramides contain fatty acids of 10–26 C-atom chain length, whereas fatty acids characteristic for fungal cerebrosides are no longer then those with 20 C-atoms.

The composition of the ceramides investigated is by far more complex compared to the fatty acid cerebroside composition. The physical properties of ceramides, especially their function polarity, are related to their fatty acid composition and long-chain base.

The ceramide group of lowest polarity (1), see Table 1, contains shorter acid chains and unsaturated acids, especially oleic acid, while the amide bound base is sphingosine. Ceramide group (2) exhibits higher polarity, and contains less unsaturated acids, with the presence of longer chain fatty acids. Ceramide group (3) fatty acids exhibit a further decrease of unsaturation, with the presence of longer chain acids. Ceramide group (4) has a similar fatty acid composition, and so does ceramide group (5), however, it is characterized by higher polarity due to the presence of a trihydroxy base, phytosphingosine, and absence of trans-double bond characteristic of sphingosine, as demonstrated by IR spectrum. The bases that have no double bond mostly belong to the family of phytosphingosines *(20)*. Dihydrosphingosines are also present to a lesser extent. This ceramide group, likewise ceramide group (6), matching the former by the polarity as well as by the acid and base composition, occurs in the form of foliaceous crystals. Ceramide groups (7) and (8) contain significantly more hydroxylated fatty acids, and the long-chain base is phytosphingosine. It should be noted that h24:0 accounts for almost a half, and hydroxy fatty acids for three quarter (73.96 %) of all ceramide fatty acids in group (7). In ceramide group (8) exhibiting higher polarity on TLC, the proportion of all hydroxy fatty acids is even higher (86.46 %). Ceramide group (7) has a higher degree of unsaturation than ceramide group (8) (13.64 % *vs.* 21.60 %), which may influence its solubility characteristics. It is well soluble in warm chloroform, thus it can be obtained directly from a mixture of all ceramides. This simple separation procedure appears to suggest that ceramide group (7) is probably identical to the »cerebrin« described in some early studies (*5*) from the beginning of the 20th century.

Some basic information on the elements of the ceramide structure are presented, however, many questions have yet to be answered. Further studies are needed to determine the composition of lipids, especially ceramides, according to the medium of fungal wild or cultured growth. An extremely intriguing issue to study will certainly be the proportion of individual lipid types in total lipids of both the fruiting body and mycelium of the basidiomycete. Fungi in general, including this one, are an important source of ceramides, *i.e.* substances known for quite a long time, whose biological role still has to be elucidated. Also, fungi are a source of a great variety of different lipid types. Future studies should hopefully lead to some concepts that will serve as a basis for considering the value of a fungus from another, its dietary aspect, including their growth, preparation, and use.

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Sfingolipidi bukovače *(Pleurotus ostreatus)*

Sažetak

Dosadašnja istraživanja pokazala su da bazidiomiceti sadržavaju razne vrste sfingolipida, osobito ceramida i cerebrozida. Ovim se radom želio odrediti sastav ceramida ispitivane gljive te naći najpogodniji postupak za izolaciju tih spojeva. Bukovača (Pleurotus ostreatus) ubrana je kao samonikla gljiva. Lipidi su ekstrahirani iz svježe gljive otapalima (kloroform, metanol), a lipidne su vrste odjeljivane kromatografijom na stupcu i tankom sloju. Osam skupina ceramida izolirano je na osnovi različite vrijednosti R_f na TLC. Razgradnjom ceramida metanolizom oslobođene su masne kiseline i dugolančane baze. Njihova identifikacija izvedena je s pomoću TLC i GLC.