

PRODUCTION OF CEREBROSIDE IN YEAST AND ITS FUNCTIONALITY

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ABSTRACT

*The authors investigated the production of cerebrosides from yeasts grown in whey and beet molasses, by-products from the manufacture of cheese and sugar, respectively. Although there is no cerebroside present in *Saccharomyces cerevisiae*, a yeast used for industrial and research purposes, examination of 33 species of *Saccharomyces* and related genera indicated that cerebrosides are present in eight yeast strains including *Kluyveromyces lactis* and *S. kluyveri*. Cerebrosides were also found to be widely present in lactose-assimilating yeasts (lactic yeasts) at concentrations from <0.1 mg/g to 1.82 mg/g dry cell weight. To develop effective fermentation production of cerebrosides by yeast, clarification should be made of the culturing conditions for achieving a higher yield of cerebrosides in yeast cells. In addition, selecting and breeding of mutants or strains accumulating cerebrosides at high concentration are actively being investigated. Recently, a sphingolipid, essentially a cerebroside, from plant materials has been separated for use as a safe functional foodstuff and supplement. In this study, the authors likewise investigated whether cerebrosides of yeast and plant origins have any effects on human health. Sphingolipids are abundantly present in neural tissue, including the brain, but subsequent to outbreak of BSE (bovine spongiform encephalopathy), their use has become limited. Cerebrosides must thus be obtained primarily from plants and yeasts.*

Keywords: cerebrosides, yeasts, cheese whey, beet molasses, sphingolipids

INTRODUCTION

Sphingolipids are a family of compounds with a sphingoid base and an amide-linked fatty acid as well as a polar head group such as carbohydrates (for cerebroside and other complex glycolipids) and phosphorylcholine (for sphingomyelin), except in the case of free ceramide. Sphingolipids are frequently found in eukaryotic cells, with cerebrosides usually occurring in extremely large amounts in animal brain and nervous tissues. Thus, bovine brain was a potential source of sphingolipids as food and cosmetic ingredients prior to the onset of BSE in cattle, but the safety of using sphingolipids from such sources for these purpose has not yet been confirmed.

Recently, a sphingolipid, essentially a cerebroside (monoglucosylceramide), has been separated from plant materials such as rice bran, maize seeds, and wheat germ for use as a safe functional foodstuff and supplement,

although cerebroside production from plants requires expensive and complicated processing because of the small amounts found in plant materials. We have found some yeast strains in which cerebrosides are accumulated. Based on this, our research project was designed to develop effective fermentation production of cerebrosides by yeast using agricultural by-products such as beet molasses and cheese whey as culture nutrients.

In this paper, we describe the distribution and chemical characterization of cerebrosides in yeasts as well as some properties of plant cerebrosides used as functional foodstuff on the market. In addition, this report is briefly concerned with the present situation and further technological development of yeast production of cerebrosides. Regarding physiological roles of dietary sphingolipids, several studies have demonstrated that intake of cerebroside and sphingomyelin, major dairy sphingolipids,

significantly reduces the appearance of colonic aberrant crypt foci (ACF) in CF1 mice treated with 1, 2-dimethylhydrazine (DMH). A plausible mechanism for this suppression is considered to be bioactive sphingoid bases and ceramides, hydrolyzed from dietary sphingolipids by intestinal enzymes, which play important roles as intracellular mediators of cell apoptosis. Although orally ingested sphingolipids of mammalian origin are considered to be hydrolyzed by intestinal enzymes and taken up by mucosal cells, the fate of dietary sphingolipids of plant and yeast origins is still not well understood. We investigated whether yeast and plant cerebrosides have any effects on human health.

MATERIALS AND METHODS

Yeast strains and culturing

The 33 strains in the genera *Saccharomyces*, *Torulaspota*, *Zygosaccharomyces*, and *Kluyveromyces* were used to analyze cerebrosides. In addition, 11 lactose-assimilating yeasts including *K. lactis*, *K. wickerhamii*, and *K. marxianus* were examined for the concentration and chemical composition of cerebrosides. The strains were basically cultured at 25°C with shaking in YPD medium until the early stationary phase. Harvested cells were lyophilized after being washed twice with distilled water.

For cerebroside production in yeast, cheese whey medium containing 2% lactose, 1% corn steep liquor, 0.5% (NH₄)₂SO₄, 0.075% KH₂PO₄, and 0.075% MgSO₄ (pH 5.5) was used for the growth of *K. lactis*. *S. kluyveri* was cultured in molasses medium containing 5% beet molasses in glucose equivalent, 1% corn steep liquor, 0.3% (NH₄)₂SO₄, 0.19% urea, 0.075% KH₂PO₄, and 0.075% MgSO₄. Based on the media described above, culturing conditions such as nutrient concentrations and culturing period, and effects of different pH and permeation pressure were examined for increasing cerebroside concentrations.

Extraction, separation and analysis of cerebrosides

Total lipids were extracted from lyophilized yeast cells with chloroform-methanol (2:1 and

1:2, v/v) and hydrolyzed in 0.4N KOH in methanol to remove glycerolipids such as triacylglycerol and phospholipids. The purification of plant and yeast cerebrosides was conducted by silicic acid column chromatography and preparative thin-layer chromatography (TLC) on silica gel in conjunction, and their components were analyzed as reported previously (Takakuwa *et al.* 2000a). Differential scan calorimetric (DSC) study and fluorescence depolarization measurement were performed as described previously (Ohnishi *et al.* 1988, Takakuwa *et al.* 2002c).

Alkali-stable lipids prepared above were applied to HPLC equipped with an evaporative light-scattering detector (ELSD) to determine cerebroside concentration (Tanji *et al.* 2004). The cerebrosides were separated and recovered by normal phase HPLC under the same conditions as above. Each cerebroside (about 15 µg) was dissolved in chloroform-methanol (1:4, v/v), which was then injected into reverse phase HPLC equipped with ELSD with an evaporation temperature maintained at 70°C and nebulization gas pressure of 350kPa. A Superspher 100 RP-18 (5µm, 250 x 4mm, i.d., Merck) served as the HPLC column. The mobile phase was methanol-water (95:5, v/v) and the flow rate, 1.0 mL/min. Cerebroside peaks were identified by component analysis.

PCR-based cloning of glucosylceramide synthase

To determine the internal sequence of glucosylceramide synthase (GCS), PCRs were performed using the genomic DNA of yeast strains as the template and degenerate primers: 5'-AAAYCCNAARRTNMRNAAAYHT-3' and 5'-ADRAACATYTCNTCNCNTCNAR-3'. Based on the PCR fragments, the entire region spanning the ORF was sequenced by thermal asymmetric interlace (TAIL)-PCR (Takakuwa *et al.* 2002a).

Quantitative analysis of apoptotic cells

Human colon cancer (Caco-2) cells were cultured in DMEM supplemented with penicillin (100 units/mL), streptomycin (100µg/mL), and 10% FCS. Apoptosis cells were quantified by counting cells with characteristic fragmented-nuclei under a fluorescent microscope (Harada-

Shiba *et al.* 1998). The percentage of apoptotic cells was calculated as the number of apoptotic cells/total number of cells x 100 (Sugawara *et al.* 2002, Aida *et al.* 2004a).

Assays of cerebrosidase and ceramidase activity, and determination for hydrolysis of cerebroside in the intestinal tract

Small intestine and cecum were excised from male Sprague-Dawley rats under ether anesthesia. The mucosa of the small intestine was homogenized in ice-cold sodium phosphate buffer (10 mmol/L), pH 7.2, containing sodium chloride (50 mmol/L). The homogenate was centrifuged at 10,000 x g for 30 min at 4°C. The pellet was used as a crude enzyme source. The cecal contents were washed out, mixed in cold saline, and centrifuged at 100 x g for 20 min. The supernatant was used for the assay. For assays of cerebrosidase, the reaction mixture contained 100 µg of cerebroside, 2 mg of sodium taurodeoxycholate in 0.1 mL of sodium citrate/phosphate buffer (0.1/0.2 mol/L), pH 5.2, and 0.1 mL of the enzyme source. After incubation of the mixture for 2 h at 37°C, the glucose released enzymatically was determined with a commercially available kit. The reaction mixture for determination of ceramidase activity contained 100 µg of ceramide as a substrate, 2 mg of sodium taurocholate in 0.1 mL of sodium phosphate buffer (0.1 mol/L), pH 7.0, and the enzyme source. After incubation for 2 h at 37°C, the amount of liberated sphingoid bases was measured by the fluorometric method using fluorescamine.

Moreover, the rats were fed, by gastrogavage, 4 mg plant cerebroside dispersed in 2 mL PBS containing sodium taurocholate (12 mmol/L). After 1, 3, or 6 h, the entire small intestine was removed and used to assay the hydrolysis of cerebrosides in the intestinal tract of rats (Sugawara *et al.* 2003).

Cellular uptake of sphingoid bases

The different monolayers of Caco-2 cells suspended in serum-free DMEM were supplemented with sphingoid base (5 µmol/L) in serum-free DMEM containing 5 g/L BSA and 5 mmol/L sodium taurocholate, with or

without selective inhibitors such as *N,N'*-dimethylsphingosine and verapamil. After incubation for an indicated time, the cells were washed to remove surface-bonded sphingoid bases. Lipids were extracted from the washed cells and the sphingoid bases obtained were analyzed by HPLC as *O*-phthalaldehyde (Sugawara *et al.* 2003).

Animal and diet used for evaluation of dietary cerebroside on suppression effects on aberrant crypt foci formation

Four-week-old male BALB/c mice were housed in isolator cages (7 mice per cage) at 20°C and 12-h light/dark cycles. The mice were randomly divided into four groups. Subsequent to test diet acclimation for 10 days, each mouse was administered i.p. 1,2-dimethylhydrazine (DMH)-HCl once a week for 10 weeks. Experimental diets for mice were based on AIN-76. Cerebrosides were obtained from maize and yeast (*S. kluyveri*) and added to the diets (0.1% and 0.5%) (Aida *et al.* 2004b).

Identification of aberrant crypt foci

The large intestine was excised from each mouse under ether anesthesia and the portion from the cecum to the vent was removed and rinsed in cold saline. This was followed by cell fixation in 4% paraformaldehyde in phosphate-buffered saline for overnight and staining with 3% methylene blue solution in saline for 30 min at room temperature. Aberrant crypt foci (ACF) were counted throughout the large intestine under a microscope (Aida *et al.* 2004b).

RESULTS AND DISCUSSION

Yeast cerebrosides

Distribution of cerebroside in yeasts.

Cerebroside is a typical membrane lipid of plants, fungi, and animals. It is found in *Candida albicans*, *Pichia pastoris*, *Pichia anomala*, and the *Cryptococcus* species but is lacking in *Saccharomyces cerevisiae*, which instead synthesizes inositol phosphorylceramides as essential components for viable growth (Fig. 1). When alkali-stable lipids

extracted from the cells of 33 strains of genus *Saccharomyces* and its closely related yeasts were separated on TLC and visualized by heating after spraying with an anthrone reagent, eight strains had a component with an *R_f* value identical to the authentic cerebroside preparation. These were *Saccharomyces kluyveri*, *Zygosaccharomyces cidri*, *Z. fermentati*, *Kluyveromyces lactis*, *K. thermotolerans*, *K. waltii*, *K. wickerhamii*, and *K. marxianus* (Fig. 2). In the phylogenetic tree depicted on the basis of the 18S rDNA of the species examined in these experiments, most species of *Saccharomyces*, *Torulasporea*, and *Zygosaccharomyces* exhibited a genetic association within each genus. *S. kluyveri*, *Z. cidri*, and *Z. fermentati*, which were shown to contain cerebrosides, deviated from their accepted genera and rather related to the *Kluyveromyces* species tested. Moreover, cerebrosides were present in all the lactose-assimilating yeasts tested (Table 1).

The evolution of *S. cerevisiae* and close relative has been studied in detail, in which the large duplicated chromosomal regions suggested that *S. cerevisiae* is a degenerate tetraploid generated by genome duplication

approximately 10⁸ years ago. *S. cerevisiae* and *S. kluyveri* are assumed to diverge from the lineage, leading to *K. lactis* before genome duplication in *S. cerevisiae*. Since an ancestral organism for these yeasts selected inositol phosphorylceramide as an essential constituent for growth (Fig. 1), the biosynthetic pathway of cerebroside may narrow in *K. lactis* and *S. kluyveri* and finally be lost in *S. cerevisiae* during the process of divergence.

Sequence of glucosylceramide synthase genes in yeasts. Cerebroside concentration in yeasts tested ranged from <0.1 mg/g to 1.82 mg/g cell weight, differing significantly according to the yeast. To discuss these differences, entire regions spanning sequences homologous to GCS (glucosylceramide synthase) genes, responsible for cerebroside formation, were amplified from the genomic DNA of the eight strains that contain cerebroside. Concerning *S. kluyveri* and *K. lactis*, the nucleotide sequences of the obtained fragments demonstrated a single ORF of 1662 bp and 1659 bp, sufficiently long to encode a polypeptide of 553 and 552 amino acids, with a deduced molecular mass of 62.7 kDa and 62.9 kDa, respectively. However, the

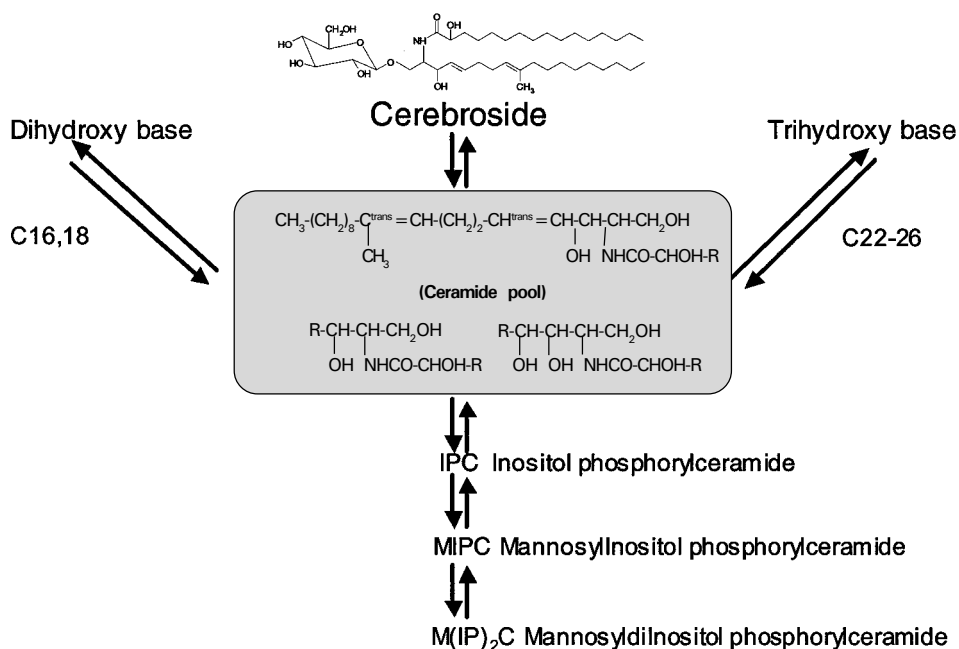


Fig. 1. Metabolic pathway of sphingolipids in fungi

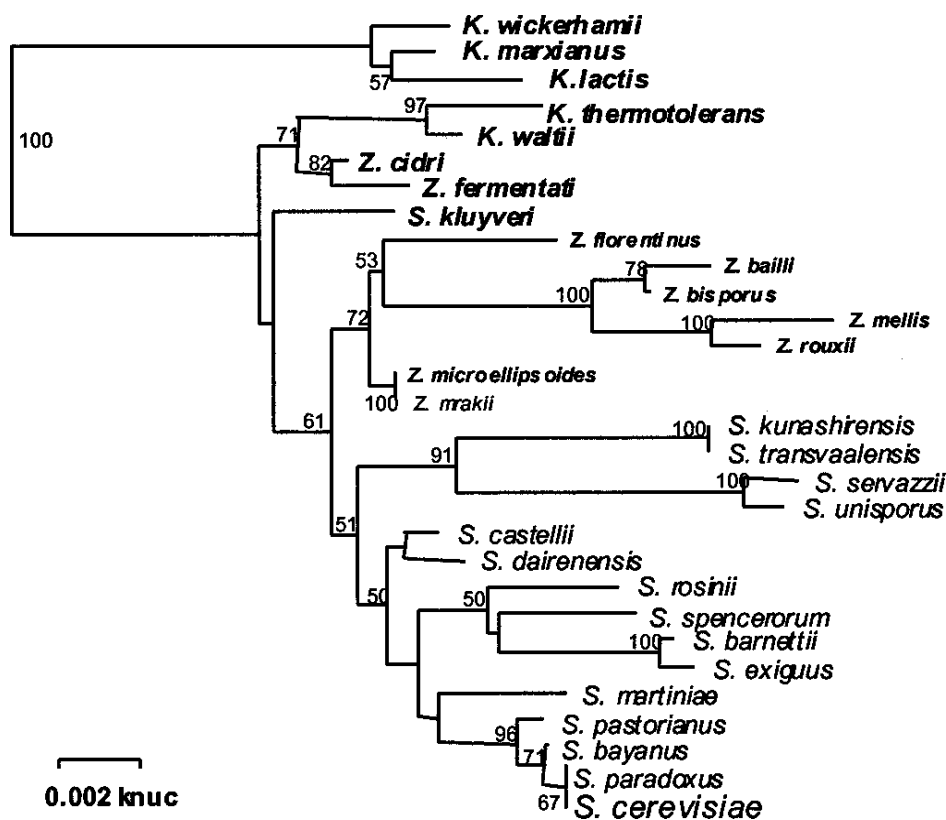


Fig. 2. Phylogenetic tree constructed for the *Saccharomyces* and relatives by the neighbor-joining methods from 18S rDNA sequences. The strains that accumulated cerebroside are shown in bold letters.

Table 1. Cerebroside content and molecular species composition (%) in lactose-assimilating yeasts grown in YPD medium

Ceramide moieties	<i>B. anomalus</i> NBRC 0796 (0.31) ^a	<i>C. intermedia</i> NBRC 0761 (1.02)	<i>C. kefir</i> NBRC 10287 (<0.1)	<i>C. tenuis</i> NBRC 10315 (1.82)	<i>D. hansenii</i> NBRC 0083 (0.29)	<i>K. aestuarii</i> NBRC 10597 (1.46)	<i>K. lactis</i> NBRC 1090 (1.17)	<i>K. wickerhamii</i> NBRC 1675 (0.98)	<i>R. minuta</i> NBRC 0387 (0.48)	<i>Y. farinosa</i> NBRC 1163 (1.80)
14h:0-d18:2	37	-	-	-	-	-	-	-	-	-
14h:0-9-Me d18:2	55	-	-	-	-	-	-	-	-	-
16h:0-d18:2	5	<1	46	25	6	<1	4	<1	6	-
16h:0-9-Me d18:2	3	8	54	60	14	<1	7	<1	64	<1
18h:0-d18:2	-	11	<1	<1	12	23	21	27	-	14
18h:0-9-Me d18:2	-	81	<1	<1	68	77	68	73	7	86
Unidentified	-	<1	-	15	-	-	-	-	23	-

^a Cerebroside content (mg/g dry cell).

deletion of cerebrosides in certain *Saccharomyces*-related genera such as *S. bayanus* was shown to be due to a defective gene encoding GCS.

The presumed proteins of GCS from *S. kluyveri* and seven other yeasts had a conserved structure with 53%-60% similarity, which was higher than that for GCSs from *C. albicans*, *P. pastoris*, and other organisms. D1, D2, D3, and the Q/RXXRW motif, which are assumed to be involved in enzyme activity, were commonly found, as in other yeasts. However, no distinct relation between the phylogenetic tree constructed from GCS sequences and cerebroside concentration in yeasts could be detected.

Chemical characterization of yeast cerebrosides. It has been reported that fungal cerebrosides show close structural similarity with the component sphingoid bases and fatty acids of which being 9-methyl-*trans*-4, *trans*-8-sphingadienine, 9-Me d18:2 (Fig. 3), and 2-hydroxyl acid with carbon chain length of 16 or 18. However, reverse phase HPLC analysis indicated significantly different cerebroside components to be present in yeasts, as summarized in Table 1. The cerebrosides in *K. aestuarii*, *K. wickerhamii*, *S. kluyveri*, and *Yamadazyma farinosa* were all shown for the most part to be only the 18h:0-containing species, the greater part of which combined with 9-Me d18:2. In *Candida intermedia*, *Debaryomyces hansenii*, and *K. lactis*, predominant cerebrosides were all the 18h:0-9-Me d18:2 species (68-80%) while that

containing 16h:0 was present only in small amounts (8-20%). Cerebrosides containing 16h:0 were predominant in *C. kefyri*, *C. tenuis*, and *Rhodotorula minuta*. Cerebrosides with 16h:0-d18:2^{4t,8t} (2-hydroxypalmitic acid-*trans*-4, *trans*-8-sphingadienine) as the ceramide moiety were present at the same concentration as those possessing 16h:0-9-Me d18:2 in *C. kefyri*. On *C. tenuis* and *R. minuta*, the major cerebrosides were those containing 16h:0-9-Me d18:2 (60-64%). 14h:0-containing cerebrosides (more than 90%) were detected in *B. anomalus*, though not in the other yeasts. Cerebrosides with 9-Me d18:2 were present at a relatively high concentration in *B. anomalus*, as also the case for *C. kefyri* and *C. tenuis*. Thus, cerebrosides containing d18:2^{4t,8t} were found present in certain yeasts at significantly different concentrations. In *C. kefyri*, the species containing d18:2^{4t,8t} accounted for nearly half of the cerebroside content. The cerebroside with 14h:0 was predominant in *B. anomalus*.

The component sugar for any given cerebroside in certain yeast strains in this study was only glucose in nearly all cases, which is the same found in plant cerebrosides.

Properties of cerebrosides as functional foodstuffs of plant origins

The ceramide moiety constituents of plant cerebrosides, which have been used as foodstuffs (or considered potential sources), are shown in Table 2. Concerning sphingoid base components, the most common base of

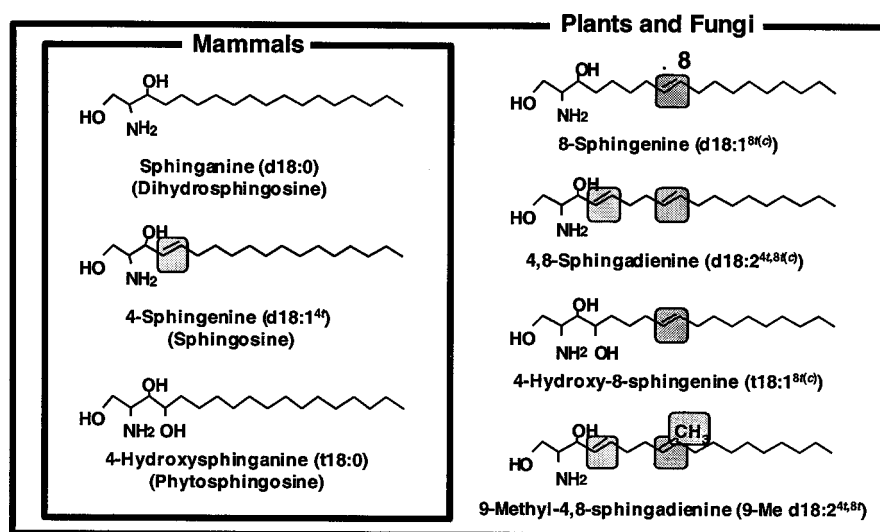


Fig. 3. Structures of major sphingoid bases in mammals, plants and fungi

mammalian sphingolipids is *trans*-4-sphingenine, d18:1^{4t} (sphingosine); and smaller amounts of others, such as sphinganine, d18:0 (dihydrosphingosine) and 4-hydrosphinganine, t18:0 (phytosphingosine), are frequently present (Fig. 3). In higher plants, the structures of the sphingoid bases of sphingolipids are more complicated than those in mammals, as shown in Fig. 3. The major sphingoid bases of cerebrosides from soybean, maize, rice bran, and "Konnyaku" (*Amorphophallus konjak*) tuber were *trans*-4, *cis*, or *trans*-8-sphingadienine (d18:2), and those from wheat and rye grains were mainly 8-sphingenine (d18:1⁸) with the *cis*-configuration (Table 2). Major 2-hydroxy fatty acids also showed species differences. The principal 2-hydroxy fatty acids from rice bran and maize were 20h:0 and 24h:0; 18h:0, 16h:0, and 22h:0 in "Konnyaku," 16h:0 in soybean, and 16h:0 and 20h:0 in wheat and rye grain cerebrosides

(Table 2). Unsaturated 2-hydroxy fatty acids were only found in wheat and rye grain cerebrosides.

In DSC analyses of cerebrosides, the sharp peak of the phase transition temperature in bovine brain cerebroside (major components are 24h:0 and d18:1^{4t}) was observed near 60°C. However, plant cerebrosides showed broad peaks at a significantly lower temperature compared with that of bovine brain cerebrosides. The reason for this would be the diversity of molecular species of cerebrosides especially in terms of sphingoid base and the existence of 8-*cis* configuration in the ceramide moiety. The fluorescence depolarization values of liposome composed of asolecthin (a mixture of phospholipids in soybean) were increased by the addition of plant and fungus cerebrosides. However, the existence of the *cis*-8 double bond or 9-methyl group in the sphingoid base suppressed increases in the depolarization.

Table 2. 2-Hydroxy fatty acid and sphingoid base compositions of plant cerebrosides (mol%)

Constituents	Wheat grain	Rye grain	Rice bran	Maize seed	Soybean seed	"Konnyaku" tuber
2-Hydroxy fatty acid						
16h:0	39	26	1	6	82	19
18h:0	7	5	6	16	<1	32
20h:0	38	36	50	39	<1	14
22h:0	4	8	14	12	7	17
24h:0	4	7	21	21	8	6
24h:1	1	8	-	-	-	-
26h:0	2	2	3	3	1	1
26h:1	1	1	-	-	-	-
Others	4	7	5	3	2	11
Sphingoid base						
d18:0	5	4	1	1	<1	<1
d18:1 ^{4t}	1	<1	4	3	<1	1
d18:1 ^{8t}	21	21	1	<1	<1	1
d18:1 ^{8c}	50	50	<1	<1	5	3
d18:2 ^{4t,8t}	4	3	13	17	49	11
d18:2 ^{4t,8c}	9	5	45	54	24	51
t18:0	1	2	7	2	<1	1
t18:1 ^{8t}	1	2	3	2	9	1
t18:1 ^{8c}	8	12	26	21	13	31

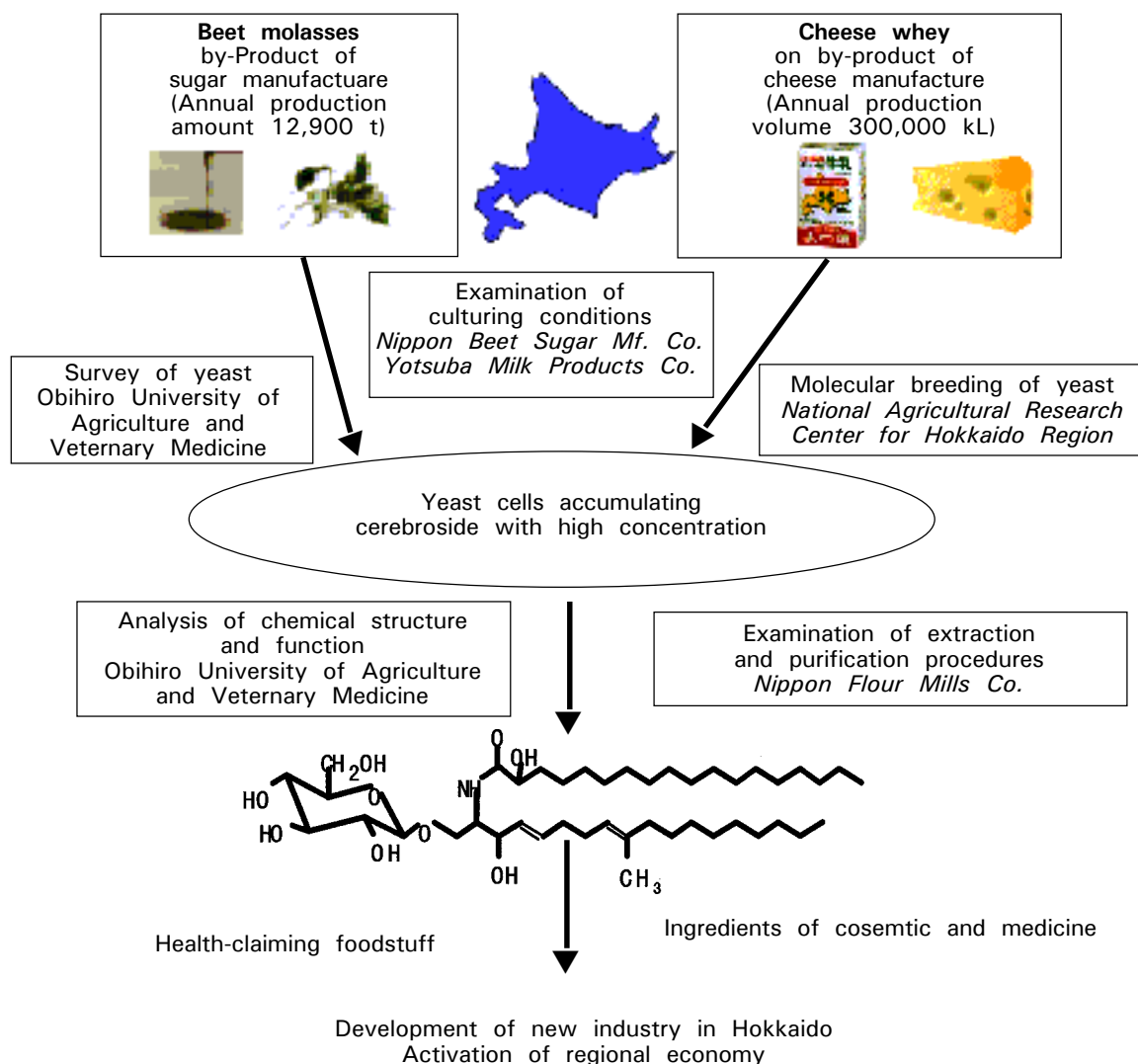


Fig. 4. Outline of the research project on cerebroside fermentation production from by-products of food industry

Yeast production of cerebroside: the present situation and further subject

In Hokkaido (northern Japan), as by-products of sugar and cheese production, about 13 thousand tons of molasses and 300 thousand kiloliters of cheese whey are produced annually. To enhance competitiveness of the agricultural industry, effective utilization of these by-products should be promoted; for instance, by conversion to value-added foodstuffs with certain physiological functionality. From this perspective, we are attempting yeast production of cerebrosides

using beet molasses and cheese whey as culture nutrients. This project is carried out as a cooperative study by various specialists from universities, national research organizations, and private food companies (Fig. 4). In this study, among yeast species capable of synthesizing cerebrosides, *S. kluyveri* and *K. lactis* were selected for culturing in the medium based on beet molasses and cheese whey, respectively, because the former yeast can hydrolyze raffinose by its invertase and α -galactosidase activities, while the latter yeast can assimilate lactose.

Concerning *S. kluyveri*, 22 strains from NBRC and CBS were cultured in the beet molasses medium and cerebroside was determined by HPLC. A maximal cerebroside concentration of 1.5 mg/g dry cell was obtained for *S. kluyveri* NBRC 10847. The alteration of nitrogen sources and other factors such as time of incubation and pH on the production of cerebroside, and the optimization of culture conditions for cerebroside production by yeast have gradually become clearer. In an identical weight basis, the starvation culture was found to be notably effective for accumulating cerebroside at a high concentration (two and a half times). Moreover, some haploids producing cerebroside with higher concentrations have been selected by the spore separation. However, examination to determine the optimization of culture conditions for cerebroside production in whey medium used *K. lactis* NBRC 1267. Application for patents, based on these results, is being planned.

The dry biomass particles (yeast cells) were extracted with hot ethanol to yield crude lipids. Followed by subsequent steps, the cerebroside fraction (ca. 40% purity) was prepared on the laboratory level with the recovery of more than 70%. The final product has been confirmed as safe by acute toxicity and mutagenic tests.

The current commercial demand for cerebroside is dominated by their dietary application as an ingredient in supplements. For this purpose, the final cerebroside concentration in functional foodstuffs is usually adjusted to 3%-5% of the bulk product weight by addition of cyclodextrin or other substances. Since the demand for cerebroside in the health-food market continues to grow, yeast cerebroside is likely to be used as functional foodstuffs in the future. However, the key to the success of yeast production of cerebroside would be the coupling of proven technology in fermentation to increase cerebroside concentration and facilitation downstream processing, and separation of mutants producing cerebroside at an extremely high concentration as well as new applications of sufficiently high added value.

Food functionality of cerebroside

Apoptosis inducement by plant and yeast sphingoid bases in human colon cancer cells. Examination was made of the apoptosis-inducing activity of sphingoid bases from plant and fungus sphingolipids. Morphological criteria of nuclei of Caco-2 cells treated with sphingoid bases followed by DAPI staining indicated aggregated and fragmented nuclei. Subsequent to nuclear staining by TUNEL, only fragmented DNA could be seen. Sphingoid bases from maize cerebroside were clearly shown to induce apoptosis in Caco-2 cells (Fig. 5). Apoptotic cells increased time-dependency and their inducement was also noted to be dose-dependent. But at high doses, sphingoid bases (50 μ M) induced necrosis-like cell death and the apoptotic cell concentration varied.

Apoptosis-inducing activity for plant and fungus sphingoid bases and those of animal origin (d18:1^{4t} and t18:0) were then compared. Plant sphingoid base induced apoptosis in Caco-2 cells was as follows: d18:2^{4t,8t}, 11.5% \pm 1.4; d18:2^{4t,8c}, 13.0% \pm 1.8; and 9-Me d18:2, 13.4% \pm 0.3 (means \pm SD). The values for d18:2^{4t,8c} and 9-Me d18:2 were significantly higher than for d18:1^{4t} (11.5% \pm 1.1). It thus clearly follows that sphingoid bases from plants and fungi express apoptosis-inducing activity in human colon cancer cells. It would thus appear that sphingolipids in edible plants and fungi may be considered functional lipids.

No apoptosis by sphingoid bases was noted to occur in differentiated Caco-2 cells, the model for normal intestine cells. Thus, possibly, sphingoid bases from various biomaterials may induce apoptosis only in active cancer cells.

Digestion of plant cerebroside in rats and uptake of its component sphingoid bases by Caco-2 cells. We investigated the digestion in rats of dietary cerebroside prepared from maize. Cerebroside activity in the small intestinal mucosa and cecal contents toward cerebroside of plant origin did not differ from that toward glucocerebroside of mammalian origin. Similarly, the ceramidase activity, which hydrolyzes the amide linkage of ceramide, toward ceramide derived from maize cerebroside, was the same as that toward ceramide of mammalian origin in both sites.

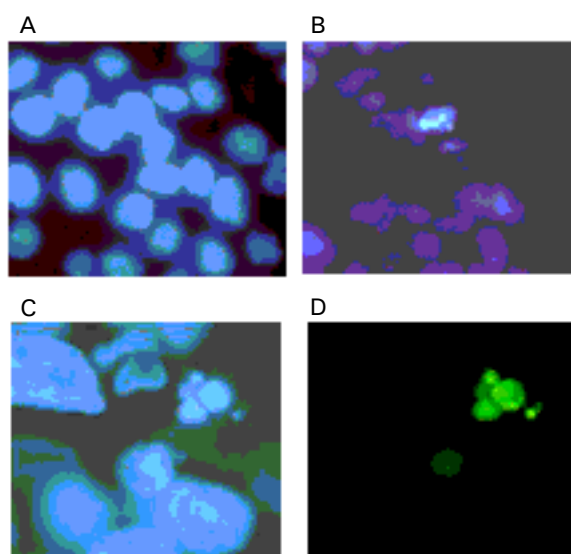


Fig. 5. Apoptosis induced by plant sphingoid bases in human colon cancer Caco-2 cells. A, B and C cells stained with 4', 6-diamidino-2-phenylindole (DAPI); D, and TUNEL assay. A, cells cultured without maize sphingoid bases for 24 h (control); B, cells with maize sphingoid bases for 24 h; C and D, same cells at higher magnification

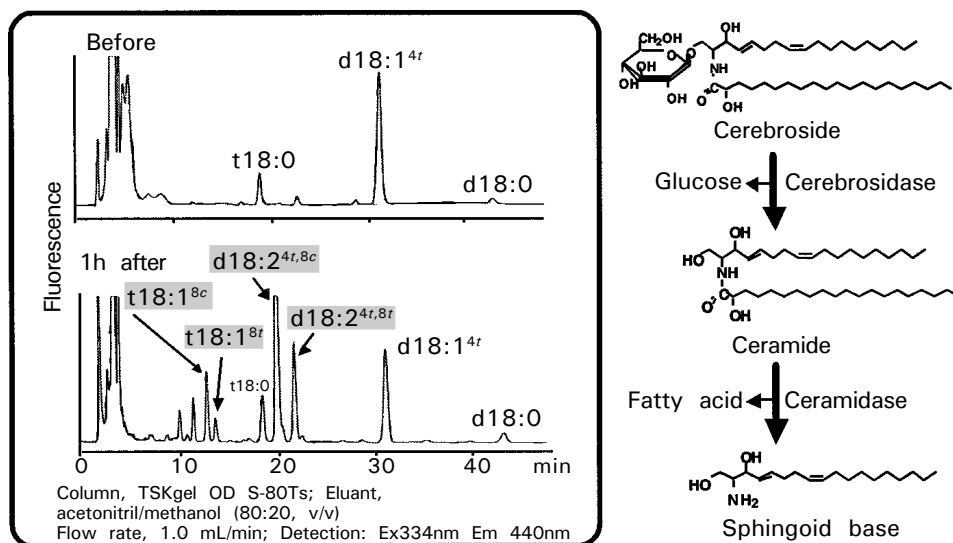


Fig. 6. HPLC of the alkali-stable fraction of extracts from intestinal contents of rats before and 1 h after a single oral dose of maize cerebroside

In examination of hydrolysis of plant cerebroside, which was given by gastrogavage, in the intestinal tract of rats, the formation of free sphingoid bases such as d18:2 was found in the intestinal lumen and mucosa 1 h after a single dose of maize cerebroside (Fig. 6).

However, in both the lumen and the tissue, d18:2 levels peaked 1 h after ingestion and rapidly decreased after 3 h. The total amount of d18:2 recovered from the entire small intestine 1 h after the single oral dose of maize cerebroside was about 3% of the dose given.

When uptake of sphingoid bases by Caco-2 cells was assayed, the accumulation of d18:2 in Caco-2 cells peaked between 0.5 h and 1 h of incubation and declined gradually (Fig. 7). However, in the case of d18:1^{4t}, the predominant sphingoid base in most mammals, the cellular accumulation peaked after 1 h of incubation, and had not declined after 6 h. The cellular d18:1^{4t} level was significantly higher than cellular levels of d18:2 of plant origin at each time point. The accumulation of d18:1^{4t}, but not of d18:2, in Caco-2 cells after 3 h of incubation was increased by the presence of *N,N'*-dimethylsphingosine (an inhibitor of sphingosine kinase). However, in the presence of an inhibitor of P-glycoprotein (verapamil), the cellular d18:2 concentration was increased, but not that of d18:1^{4t}. P-glycoprotein, a member of the ATP-binding cassette transporter superfamily, transports a wide variety of hydrophobic compounds such as natural products and toxicants, and contributes to the barrier function of the gut.

These results described above that plant cerebroside was hydrolyzed to ceramide and free sphingoid bases in the digestive tract. In addition, sphingoid bases such as d18:2 derived from plant cerebroside were not substrates for sphingosine kinase, a key enzyme of sphingolipid catabolism; rather the P-glycoprotein promoted their efflux in differentiated Caco-2 cell monolayer. This

implies plant d18:2 degraded from dietary cerebroside would be absorbed poorly from the digestive tract and pass into the lower digestive tract. Thus, the preventive effect of dietary plant-origin cerebroside on carcinogenesis should occur in the large intestine because various sphingoid bases induced apoptosis in cancer cells as described above.

Prevention of aberrant crypt foci formation by dietary plant and yeast cerebroside in 1,2-dimethylhydrazine-treated mice. After 10 weeks of feeding (without cerebroside), ACF (aberrant crypt foci) were found in the large intestines of the control group treated with DMH (1,2-dimethylhydrazine). With administration of the AIN-76 diet containing 0.1% yeast or plant cerebroside, ACF formation significantly decreased compared with that in the control (Fig. 8). Dietary yeast cerebroside is thus shown to potently inhibit ACF-inducing activity. TLC of alkali-stable lipids of feces from mice acclimatized to diet containing yeast and plant cerebroside demonstrated substantially no cerebroside, indicating that cerebroside underwent degradation by intestinal enzymes and/or microflora. However, determination of liver sphingoid base composition in the yeast and plant cerebroside-administered groups showed that there was no single sphingoid base from yeast and plant cerebroside that could be detected.

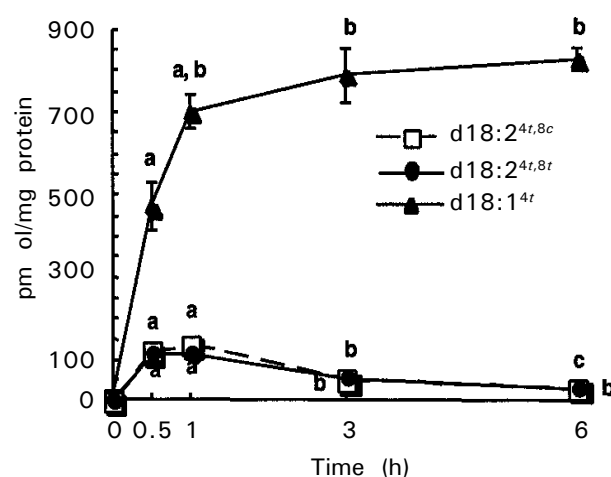
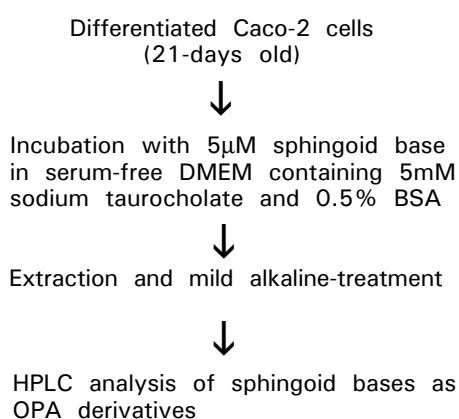


Fig. 7. Cellular accumulation in differentiated Caco-2 cell cultures incubated with sphingoid bases

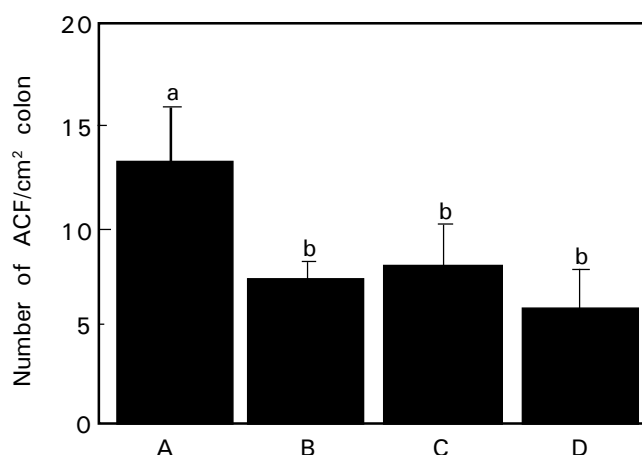


Fig. 8. Effects of dietary plant and yeast cerebrosides on DMH-induced aberrant crypt foci formation in the large intestine of mice A, feeding by AIN-76; B, AIN-76 with 0.1% maize cerebroside; C, AIN-76 with 0.1% yeast cerebroside; D, AIN-76 with 0.5% maize cerebroside. Values are means S.D. of a twice-conducted experiment in quadruplicate. a,b Values with different superscript letters in a column differ significantly ($P < 0.01$).

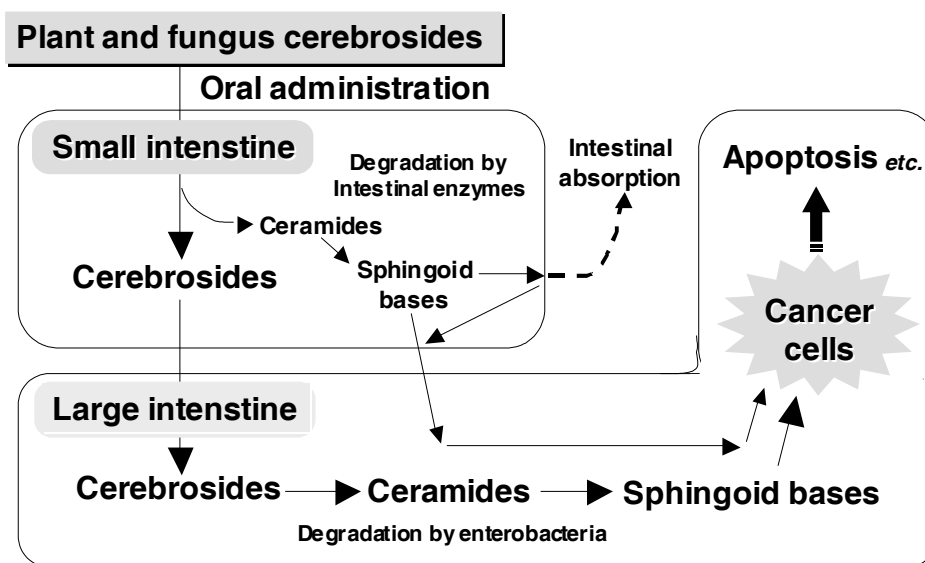


Fig. 9. Physiological effects of dietary sphingolipids (speculation) Dietary sphingolipids from plants and fungi are shown to be degraded into sphingoid bases by intestinal enzymes and/or microflora and are not absorbed by the small intestine. Dietary sphingolipids may thus be considered to have significant anti-carcinogenic effects on the large intestine.

These considerations would support the degradation of dietary sphingolipid by intestinal enzymes and microflora and that the metabolites suppress ACF formation in the large intestine in DMH-treated mice (Fig. 9).

CONCLUSIONS

Cerebrosides from plant materials are now widely used as functional foodstuffs, but the price remains extremely high. In this study, among the genus *Saccharomyces* and its

related yeasts, some yeast strains including *S. kluyveri* and *K. lactis* were shown to contain cerebrosides. Since both yeasts are known to assimilate raffinose or lactose, beet molasses and cheese whey, by-products of the food industries, are utilized as their culture nutrients. Yeasts seem to be suited for cerebroside production because of their relatively fast growth, general absence of toxicity, and ability to be genetically manipulated. However, increasing the cellular level of yeast cerebrosides will be essential to develop effective fermentation production of cerebrosides. Judging from the cost performance of cerebroside production from plants, it is expected that cerebrosides can be accumulated at a level at least five times greater in yeasts (5 mg/g dry yeast cells), based on cerebroside concentration in various type strains. This can be achieved by examining culturing conditions, and surveying and selecting mutants or/and strains with the higher cerebroside biosynthesis ability .

We have demonstrated that the oral intake of cerebrosides from yeast and plant origins significantly reduce the appearance of colonic ACF in DMH-treated mice. A possibility for the application of cerebrosides to skin care has also been discussed, since cerebrosides play an essential role in establishing the permeability barrier of the skin. Concerning the effects of dietary cerebrosides on human skin, the mechanism is still not well understood.

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