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Characterization of glucosylceramide from plasma membranes of plant root cells

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Plasma membranes of oat root cells were isolated from intracellular membranes by subfractionation of the microsomal fraction using an aqueous polymer two-phase system. The plasma membranes originated from oat plants which were acclimated to dehydration by exposure to a repeated water-deficit stress program. Glucosylceramides was a major component of the plasma membrane lipids and amounted to 9% of the lipid of control plants and 5% of the lipid of acclimated plants. Structural analysis using FAB-MS showed only one type of glucosylceramides. The constituent monosaccharide was exclusively glucose and the sphingosine base was 4,8-sphingadienine. The fatty acid composition was determined to 24:1-OH, with only trace levels of non-hydroxy acids. The decrease in the level of glucosylceramides during acclimation to dehydration was accompanied by a corresponding decrease in phospholipids and increase in free sterols.

Introduction

Glucosylceramides were first reported in higher plants by Carter et al. [1]. Since then several reports have described the chemical composition of plant sphingolipids mostly from seeds and leaves [2–6]. They appear to constitute a minor component of the isolated lipids from plant organs. Compared to the cerebroside composition in animals the ceramide moiety usually exhibits a remarkable variation [2,3].

Sphingolipids are known to occur widely in organisms as components of the membranes. Recently several methods have been developed or modified for isolation of plant organelles and membrane vesicles of high purity [7]. It has now been possible to isolate plasma membranes from different parts of the plant for characterization [8]. However, so far very few reports of the lipid composition of isolated plasma membranes have been reported [9–16].

In tonoplast and plasma membranes glucosylceramides seem to be major lipid components besides phospholipids. However, these sphingolipids are often only tentatively identified in the membrane preparations.

In a study of stress induced dehydration tolerance of oat root plasma membranes we found high levels of glucosylceramides which changed during the induction (unpublished data).

In this paper we describe the composition and structures of the glucosylceramides from root plasma membranes of oat plants acclimated to water deficit stress.

Material and Methods

Plant cultivation and water deficit stress treatment

Oats (*Avena sativa* L. cv. Seger) were sown in gravel in plastic pots with holes in the bottom. The grains were evenly distributed over the surface and then covered with a thin layer of gravel. The pots filled with gravel up to a height of 9 cm were placed in plastic bins containing nutrient solution to a depth of 6 cm. The plants were grown in growth chambers for 11 days (16 h day 18 °C and 8 h night 12 °C). After 5 days the plants were exposed to a water deficit stress program [17].

By placing the pots on a coarse stainless steel net, excess nutrient solution was drained off and gravel and

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roots were dried effectively. The stress program consisted of three cycles with 24-h stress and 24-h rewatering. After the final rewatering period the plants were harvested.

Harvest and membrane preparation

The root system was washed in distilled water, cut in one cm pieces and homogenized with a Waring blendor in an isolation medium; 10 mM Tris-HCl buffer (pH 7.5) with 0.25 M sucrose and 1.0 mM EGTA. The homogenate was filtered through two layers of nylon cloth and centrifuged for 15 min at $10000 \times g$. The supernatant containing the membranes was then centrifuged at $60000 \times g$ for 30 min. The microsomal membrane pellet was resuspended in 5 mM potassium phosphate buffer (pH 7.8) containing 0.25 M sucrose and 6.0 mM KCL.

Plasma membranes were prepared by partitioning the microsomal suspension in a 24 g aqueous polymer two-phase system containing 6.5% Dextran T500 (Pharmacia Fine Chemicals, Uppsala, Sweden) and 6.5% polyethylene glycol 3350 (Carbowax Union Carbide Corp., CT, U.S.A.) in 5 mM potassium phosphate buffer (pH 7.8) with 0.25 M sucrose and 6.0 mM KCL. For further detailed information about aqueous polymer two-phase systems see Sommarin et al. [18].

The upper phase containing the plasma membrane was diluted 5–10 times in 10 mM Tris-HCl buffer (pH 7.5), 0.25 M sucrose, centrifuged for 1 h at $100000 \times g$ and resuspended in a smaller volume of the same solution. A defined portion of the different phases were frozen in liquid nitrogen and stored in a freezer ($-18°C$) until further use, e.g. protein and enzymatic activity assays.

Lipid extraction, isolation and determination of cerebrosides

The lipids were immediately extracted from the membrane suspension by adding two volumes of boiling isopropanol followed by two volumes of chloroform/methanol (2:1, v/v), two volumes of chloroform and one volume of distilled water. After agitation and phase separation the chloroform phase was removed and the aqueous phase was washed once more with two volumes of chloroform. The combined chloroform phases were taken to dryness, dissolved in chloroform and stored at $-18°C$.

The total lipid extract was run on a silicic acid column (Mallinckrodt, Silicar cc-4). The lipids were eluted with chloroform, acetone and methanol in that order [19]. The acetone fraction containing the cerebrosides was taken to dryness and dissolved in a small defined volume of chloroform. The cerebrosides were then further separated from the glycolipids of the acetone fraction by another silica gel chromatography step (Merck, LiCroprep Si-60, 15–25 μm). The column was eluted with chloroform, 10 ml/g silicic acid, followed by chloroform/methanol (9:1,v/v), 15×1 ml/g silicic acid.

The composition of each fraction from the column chromatography was examined by HPTLC (Merck, High performance silica gel 60, 10×20 cm) with chloroform/methanol/water (80:20:2, v/v) as solvent system. The ceramides were visualized on the HPTLC-plates by spraying with orcinol [20]. Cerebroside containing fractions were pooled and taken for structural analyses.

The cerebroside fractions were permethylated according to Månsson et al. [21]. Positive FAB-MS of the permethylated fractions and GC-MS of the partially methylated alditol acetates were performed as described previously [21,22].

Result and Discussion

Plasma membranes were islolated from oat roots by partitioning in an aquous polymer two-phase system. Cerebroside determination from highly purified plasma membranes, microsomes and total lipid fractions gave a uniform answer. Regardless of material used for the determinations, mass spectra showed the same composition i.e. the oat roots contained only one type of cerebrosides.

The constituent monosaccaride of oat root sphingolipids was a neutral sugar, exlusively glucose. Permethylation of the monoglucosylceramide gave an unsubstituted sugar, namely 2,3,4,6-Me$_4$-glucose. Mass spec-

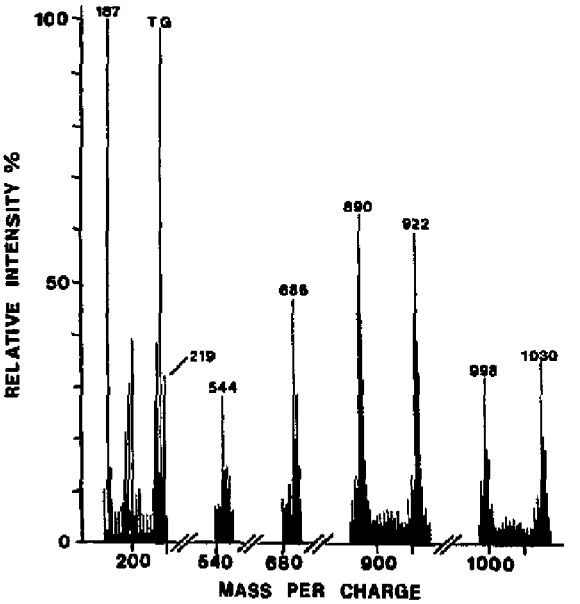

Fig. 1. Positive ion FAB MS of the permethylated oat root cerebroside. Thioglycerol was used as matrix.

tra on intact permethylated substance showed a m/z 219, 187 (219–32), which matched with a terminal unsubstituted hexose (Fig. 1).

Degradation of the molecular ion gave cleavage at m/z 544, appearing as $M+2H$ minus the acylgoup and corresponding to a hexose connected to a d18:2 sphingosine base.

The ceramide fragment at m/z 686 gave two possibilities; a ceramide with a fatty acid 26:0 or with 24:1-OH. Degradation showed a dominating degree of hydroxy fatty acids and only trace amounts of non-hydroxy fatty acids (< 10%). No attempt to determine the substitution position of the hydroxyl group was made.

Further, molecular ions appeared at m/z 922 (M + H) and m/z 890 (M + H–32). These correspond to a hexose-ceramide with 24:1-OH/d18:2. Moreover, two additional ions were obtained. The FAB-MS matrix, thioglycerol, is bound during certain conditions to unsaturated fatty acids like 24:1. In this case m/z 1030 (922 + 108) and m/z 998 (890 + 108). For comparison the mass spectrum from permethylated glucosylceramide isolated from human spleen was used (Fig. 2).

The physiological effects of the dehydration acclimation were several. The ion leakage was reduced and so was the relative as well as the absolute water content. Furthermore, if the oat plants were exposed to a long term water-deficit stress the roots of the acclimated plants showed a lower osmotic potential than the non-acclimated. They maintained a positive hydrostatic pressure of the root cells, with possibilities for continued metabolism and growth, much longer than the non-acclimated plants (to be published).

Dehydration acclimation did not imply any alteration of the glucosylceramide compostion. However, there was a large decrease in the level of cerebrosides (unpublished data).

In the present work highly purified plasma membranes from oat root were analysed. In earlier work the material used for the characterisation of plant glucosylceramides have been seeds and leaves [2–6] and in reports on the lipid composition of plant membranes ceramides are often only tentatively identified as cerebrosides [19,23]. In a few cases a more careful determination of the cerebrosides have been made from well characterized membrane fractions [9,10,24].

Cerebrosides usually have a high transition temperature like, for example, bovine brain galactosylceramide model membranes which exhibit an acyl chain order-disorder transition at 67 °C. Moreover, cerebrosides in aqueous dispersions are shown to form lamellar phases and exhibit polymorphism in the gel state [25]. It has been reported that the high degree of order the cerebrosides exhibit, are achieved through intermolecular hydrogen bonding with the hydroxyl groups [25].

The molecular species of glucosylceramides of oat root plasma membranes were few and the acyl chain

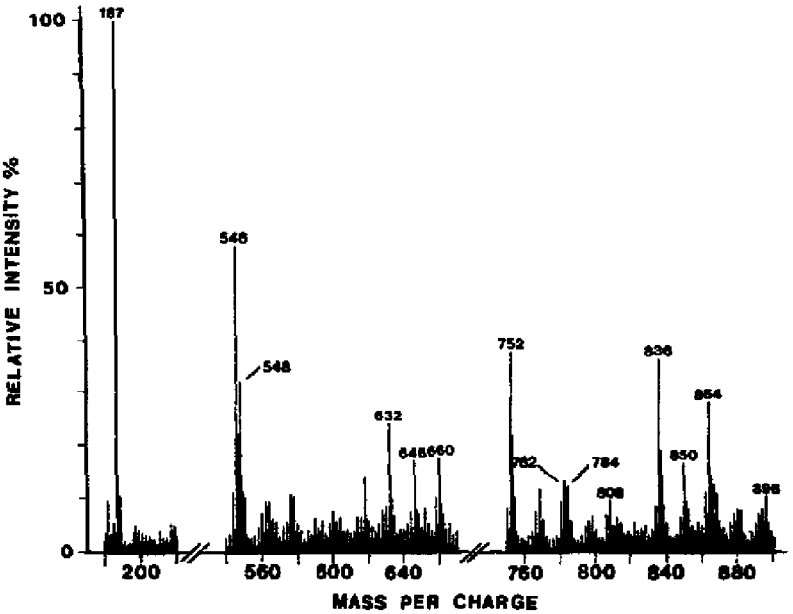

Fig. 2. Positive ion FAB-MS of permethylated reference glucosylceramide from human spleen. Hexose sphingosine d18:1 with a 24:0 acyl chain. The molecular ion (M + H) observed at m/z 896 corresponded to hexose-ceramide with 24:0 fatty acid and d18:1 sphingosine and m/z 864 corresponded to 896–32. The corresponding ions with 16:0 instead were found at m/z 784 and 752. M + 2H-acyl was found at m/z 546 and terminal hexose at m/z 187 (219–32).

24:1-OH was clearly dominant (> 90%). This makes the amide-linked chain significantly longer than the 4,8-sphingadienine moiety. When adding a minor fraction long-chained lipids to a system of otherwise uniform chain composition, this could relax packing constraints, and thereby mean a lowering of the lamellar to reversed hexagonal transition temperature [26]. Small changes in the cerbroside content of the natural membrane could have the same effects.

Studies of model systems with phosphatidylcholine/cerebroside mixtures showed different liposome morphology depending on hydrocarbon chain length of phospholipid and cerebroside as well as the ratio phospholipid/cerebroside [27]. Potential phase separation of the phosphatidylcholine-cerebroside system was eliminated by the presence of cholesterol [28].

The plant material used in this study were both control plants and plants which had been exposed to a stress cycle inducing dehydration tolerance. The glycosylceramide level of the control (non-acclimated cells) was 9% of total lipids and the value was decreased to 5% in the dehydration acclimated cells (unpublished data). A corresponding decrease of rye leaf plasma membrane cerebrosides was shown following cold acclimation, from 17% to 7% [10]. However, no changes was observed in the cerebroside composition between dehydration acclimated and non-acclimated plants.

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