Plant Physiol. (1980) 65, 1073-1075
0032-0889/80/65/1073/03/$00.50/0

Analysis of Glycoconjugate Saccharides in Organelles Isolated from Castor Bean Endosperm[1]

Received for publication October 30, 1979 and in revised form January 23, 1980

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ABSTRACT

The presence of lipid- and protein-bound sugars in the major organelle fractions isolated from germinating castor bean (*Ricinus communis* L.) endosperm has been established. Microsomes, glyoxysomes and mitochondria were subfractionated into a membrane fraction and a fraction containing peripheral membrane and soluble matrix proteins. The membranes were further subfractionated into monosaccharide lipid, oligosaccharide lipid and lipid-free protein components. The constituent sugars present in the prepared fractions were released and identified by gas-liquid chromatography. While all derived protein fractions contained the *N*-acetylglucosamine and mannose typically found in the inner core region attached to asparagine residues in many glycoproteins, some differences were noted in the organellar distribution of peripheral sugars such as fucose, arabinose, and xylose.

Recently several reports have established that glyoxysomes (1, 14, 16) and microsomes (10, 12, 14) isolated from germinating fatty seeds contain glycoprotein components. In germinating castor bean endosperm, the ER has been identified as the site of protein glycosylation (12, 15). Although organellar glycoproteins are primarily associated with the membrane, matrix proteins are also glycosylated (14). Further, the sugar moieties of the membrane glycoproteins are primarily exposed on the luminal face, rather than the cytoplasmic face, of the membrane (Lord and Mellor, unpublished data). It is therefore possible that interaction between membrane and matrix glycoproteins could occur, and this interaction could have a functional significance with respect to the mechanism of glyoxysome assembly. Such concepts are at present speculative but warrant further investigation. As a preliminary study, we have subfractionated the major organelles of germinating castor bean endosperm into their membrane and matrix fractions. This report describes the analysis of the saccharide components present in glycoconjugates derived from these subfractions.

MATERIALS AND METHODS

The growth of tissue, isolation and subfractionation of organelles and preparation of these fractions for GLC were exactly as described previously (14). After extensive dialysis, fractions were freeze-dried and extracted first with chloroform-methanol (2:1) and then with chloroform-methanol-water (10:10:3). These extracts were evaporated to dryness at 40 C under reduced pressure and were designated monosaccharide lipid and oligosaccharide lipid, respectively. The residue was designated lipid-free protein.

Previously described procedures were used to prepare salt-free glycopeptides from the lipid-free protein (3) and for glyco-conjugate hydrolysis, the preparation of saccharide derivatives and GLC (4).

RESULTS AND DISCUSSION

The major organelle fractions present in homogenates prepared from 3-day-old castor bean endosperm were effectively separated by sucrose density gradient centrifugation. The distribution of microsomes, mitochondria, plastids, and glyoxysomes across a typical gradient is shown in Figure 1 on the basis of the activity of the respective marker enzymes choline phosphotransferase, fumarase, ribulose-1,5-diP carboxylase, and isocitrate lyase. Care was taken while collecting the glyoxysomes to avoid including the less dense part of the visible protein band in order to prevent significant cross-contamination with intact plastids. While it is clear that cross-contamination of the major organelles assayed for marker enzyme activity is not a serious problem, the possibility of contamination with other cellular components must be considered.

The homogenization technique used results in minimum organelle breakage (Fig. 1 and ref. 9) and the crude homogenates were centrifuged at 500*g* for 10 min before being applied to sucrose gradients. This step removed most of the homogenate DNA and presumably most of the intact nuclei. Gradients were centrifuged for 3 h at 24,000 rpm and after this time the ribosome-denuded microsomes (density 1.12 g/ml) were clearly separated from a less dense membranous band at a mean buoyant density of 1.08 g/ml (band A, ref. 9). Further, the microsomal fraction was not contaminated with ribosomes which had sedimented as a single peak of A_{254nm} absorbing material and reached a position in the gradient corresponding to a buoyant density of 1.09 g/ml (data not shown).

The nondividing endosperm tissue apparently does not possess a well developed Golgi apparatus and the low level of latent IDPase which can be detected in similar gradients to those employed have peaks around 1.16 g/ml (13).

At present we do not have reliable markers for the presence of plasma membrane and tonoplast membrane, the gradient distribution of which are therefore unknown. In addition, the proportion of total ribulose-1,5-diP carboxylase found in the soluble fraction at the top of the gradient (Fig. 1) indicates significant breakage of plastids. Once again, the gradient distribution of membranes derived from broken plastids is not known. In spite of these obvious uncertainties, we feel the purity of the major organelle fractions analyzed in the present study is probably as high as we can achieve with the current separation techniques. In support of this, electron micrographs prepared from peak mitochondrial

[1] This work was supported by the Sigrid Jusélius Foundation and the Science Research Council (GR/A 3703.1).

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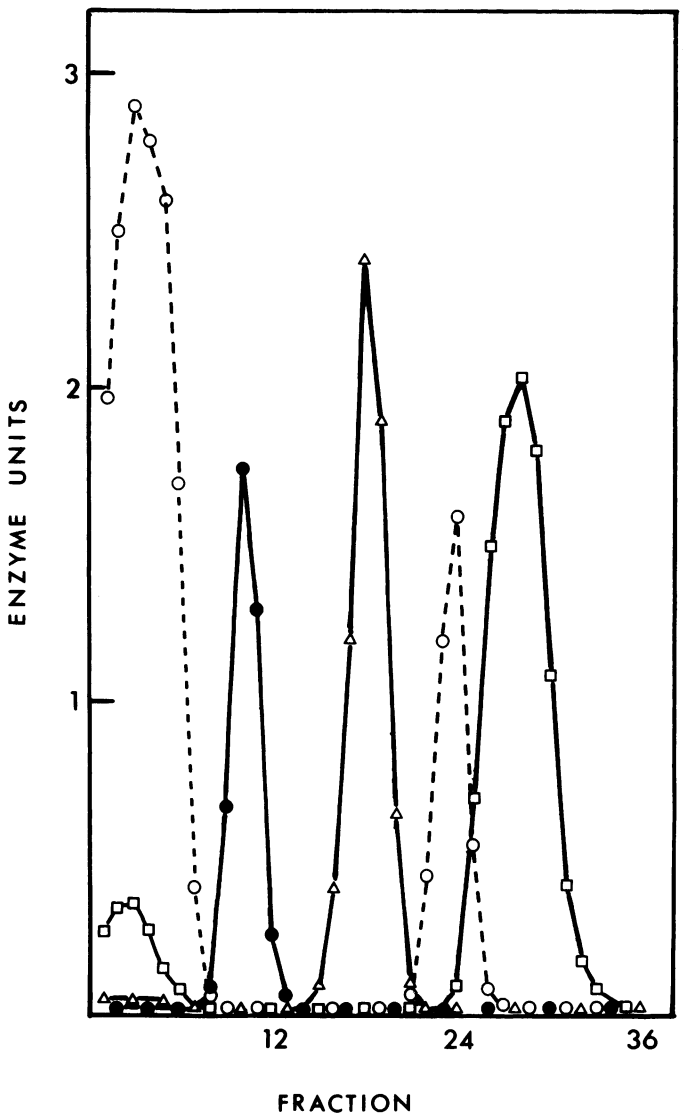

FIG. 1. Sucrose density gradient separation of organelles from castor bean endosperm homogenates. Enzyme units: cholinephosphotransferase activity (●), nmol/h·fraction × 20; fumarase activity (Δ), μmol/min·fraction × 10; ribulose-1,5-diP carboxylase activity (○), μmol/min·fraction × 20; isocitrate lyase activity (□), μmol/min·fraction × 20.

and glyoxysomal fractions have not revealed any obvious significant contaminants (data not shown). Our belief that the microsomal band is also very pure is supported by the observation that virtually all the protein, phospholipid and microsomal marker enzyme activity present in this peak is recovered at higher sucrose densities under conditions which maintain ribosome-membrane attachment (9, 12, and unpublished data).

Peak microsomal, mitochondrial and glyoxysomal fractions were pooled from sufficient gradients to ensure that each protein subfraction prepared for sugar analysis contained at least 200 mg protein. This subfractionation was achieved by osmotically disrupting the intact organelles upon the addition of buffers containing 0.2 M KCl (14). Thus, the membrane fraction would retain the integral membrane proteins, while the released fraction (designated here as the matrix protein fraction) would additionally be expected to contain peripheral membrane proteins. At least one such peripheral membrane protein, microsomal and glyoxysomal malate synthase, is known to be glycosylated (14). It must therefore be considered possible that sugars reported here to be associated with the matrix fraction could be significantly, perhaps largely, associated with peripheral membrane proteins.

The occurrence and relative amounts of saccharides present in membrane-derived glycopeptides is shown in Table I. Each membrane fraction contained the sugars characteristic of the core region of glycoprotein saccharide chains, *N*-acetylglucosamine and mannose (17), together with arabinose, xylose, galactose, and glucose. Certain saccharides appeared to be concentrated in particular membranes: fucose and mannosamine in the ER, arabinose and galactose in the glyoxysomal membrane, and xylose in the mitochondrial membrane. The total percentage of sugars in each fraction was estimated by using a value of 160 as the mean saccharide mol wt. Because the organelles were initially isolated on sucrose gradients, we were concerned that the glucose identified in various subfractions might have derived from contaminating sucrose. However, repeating the analyses on fractions which had been exhaustively dialyzed confirmed that glucose was a glycoconjugate component.

The matrix protein fraction released from osmotically disrupted organelles also contained a range of saccharides (Table II). The membranes were separated from the soluble matrix proteins by centrifugation and studies using phosphatidyl [^{14}C]choline-labeled

Table I. *Constituent Sugars Detected in Glycopeptides Derived from Washed Organelle Membranes*

Sugar	Constituent Sugars		
	ER	Glyoxysome	Mitochondria
	nmol/mg lipid-free protein		
Arabinose	103	644	164
Fucose	148	trace	trace
Xylose	63	25	1090
Mannose	350	555	1080
Galactose	600	1340	1320
Glucose	191	188	319
N-Acetyl glucosamine	313	478	707
N-Acetyl galactosamine	0	trace	0
Mannosamine	1000	trace	0
Total nmol sugar	2768	3230	4680
ng Sugar/mg protein	442,880	516,800	748,800
% Total sugar	30	34	43

Table II. *Constituent Sugars Detected in Glycopeptides Derived from Organelle Matrix Proteins*

Sugar	Constituent Sugars		
	ER	Glyoxysome	Mitochondria
	nmol/mg lipid-free protein		
Arabinose	203	179	0
Fucose	0	0	0
Xylose	47	22	0
Mannose	340	125	77
Galactose	200	439	310
Glucose	102	180	90
N-Acetyl glucosamine	202	170	78
N-Acetyl galactosamine	224	0	0
Mannosamine	98	388	0
Total nmol sugar	1416	1503	555
ng Sugar/mg protein	226,560	240,480	88,800
% Total sugar	18.7	19.3	8.0

membranes (7) established that this step completely removed all membrane fragments from the matrix protein-containing supernatants (data not shown).

Chloroform-methanol (2:1)-soluble lipids were extracted from the membranes and analyzed for sugar content. This extraction is known to remove monosaccharide lipids (11). Monosaccharide lipids were present in the ER and mitochondrial membranes but were not detected in the glyoxysomal membrane (Table III). The ER membrane monosaccharide lipids probably include dolichol monophosphate derivatives since the ER is the site of enzymes catalyzing the formation of lipid-linked sugar intermediates for glycoprotein biosyntheses (5, 11). The organelle membranes also contained chloroform-methanol-water (10:10:3)-soluble oligosaccharide lipids (Table III). The precise nature of the oligosaccharide lipids is not known at present.

N-Acetyl neuraminic acid was not detected among the released saccharides prepared from any of the glycopeptide or glycolipid samples analyzed in the present work.

Previous studies have established a major role for the ER during the biogenesis of glyoxysomes in castor bean endosperm (8). The presence of glycoproteins in both the glyoxysomal membrane and matrix further emphasizes this role. The ER has been identified as the major site of protein glycosylation in this tissue (15), core sugars presumably being cotranslationally attached to nascent polypeptide chains (2, 6).

The glyoxysomal membrane is thought to be derived directly from the ER (8), probably from specialized regions where vesiculation occurs. The association of glycoproteins with unique carbohydrate moieties in such regions may facilitate this vesiculation step and subsequently have a role in the association of glyoxysomal matrix proteins with the luminal surface of the glyoxysomal membrane.

Table III. *Constituent Sugars Detected in Monosaccharide Lipids (ML) and Oligosaccharide Lipids (OL) Extracted from Washed Organelle Membranes*

Sugar	Constituent Sugars					
	ER		Glyoxysome		Mitochondria	
	ML	OL	ML	OL	ML	OL
	nmol/total glycolipid					
Arabinose	0	6.0	0	70.0	0	0
Fucose	0	0	0	0	0	0
Xylulose	0	0	0	trace	0	0
Mannose	2.4	3.9	0	15.0	4.5	trace
Galactose	9.1	8.0	0	13.8	17.6	trace
Glucose	5.1	17.8	0	15.2	0	trace
N-Acetyl glucosamine	trace	trace	0	trace	trace	trace
N-Acetyl galactosamine	trace	trace	0	0	0	0

Acknowledgments—We thank Dr. L. K. Evans, Croda Premier Oils, Hull, England, for kindly supplying the castor beans. The sugar analyses were performed at the University of Helsinki during a visit by R. B. M. which was funded by the British Council.

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