GLYCOPROTEINS OF THE GLYOXYSOMAL MATRIX

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1. Introduction

The early postgerminative growth of fat-storing seeds is characterized by a period of gluconeogenic activity during which time stored triglycerides are converted to carbohydrate [1]. Many of the enzymes catalysing this conversion are known to be functionally located within single-membrane bound glyoxysomes [2]. These enzymes are regarded as major components of the granular protein matrix of such organelles which is characteristically observed in electron micrographs [3]. An increasing effort is now underway to sub-fractionate and further characterize proteins of the glyoxysomal matrix [4–7]. Information regarding the nature and origin of such proteins is relevant to the question of glyoxysome biogenesis.

We report here that several glyoxysomal proteins contain bound carbohydrate, and discuss the occurrence of corresponding glycoproteins in the microsomal fraction in relation to the intracellular segregation of glyoxysomal proteins.

2. Experimental

Castor bean (*Ricinus communis* L.) seeds were soaked overnight in running tap water and germinated in the dark in moist vermiculite at 32°C. Endosperm halves excised from 2–3 day old seedlings were homogenized by chopping with a single razor blade in grinding medium [8] contained in a petri dish on ice. The crude homogenate was centrifuged at 20 000 × *g* and 2°C for 15 min. The pellet was gently resuspended in grinding medium and cellular organelles were separated by sucrose density gradient centrifugation. Gradient preparation and centrifugation was exactly as in [8]. After centrifugation, gradients were collected as 1.0 ml fractions using an ISCO model 185 density gradient fractionator.

Gradient fractions which contained the protein bands recovered at mean buoyant densities of 1.12 g/ml and 1.24 g/ml (i.e., the microsomes and glyoxysomes, respectively) were pooled. Two volumes of 50 mM Tricine, pH 7.5, containing 0.2 M KCl were added to each pooled sample. After shaking in a vortex mixer, these suspensions were incubated at 25°C for 30 min to ensure complete osmotic disruption of the organelles. Membranes were removed by centrifugation at 100 000 × *g* for 30 min. The resultant supernatants, containing the released organelle matrix proteins [4], were brought to 80% ammonium sulphate saturation by the addition of solid salt. The precipitated proteins were recovered by centrifugation and redissolved in 0.5 ml 50 mM Tricine, pH 7.5.

Polypeptide profiles of organelle matrix protein preparations thus obtained were produced by incubating aliquots with equivalent volumes of 10 mM sodium phosphate, pH 7.5, containing 2% (w/v) sodium dodecyl sulphate (SDS) and 2% (v/v) β-mercaptoethanol overnight at 37°C. Polypeptides present were separated by electrophoresis in buffers containing SDS as in [9] on gels polymerized from 10% (w/v) acrylamide. Gels were stained for protein using Coomassie brilliant blue, destained and scanned in a Gilford gel scanner at 600 nm. Before staining for carbohydrate, gels were extensively washed to ensure complete removal of SDS and then stained with a periodic acid–Schiff (PAS) reagent as in [10] and scanned at 550 nm. The fidelity of the PAS staining procedure was checked with a positive stain on gels

containing a known glycoprotein (κ-casein, kindly provided by Dr J. V. Wheelock) and negative staining of gels containing proteins known to lack carbohydrate moieties (bovine serum albumin and cytochrome *c*). As an additional control, appropriate amounts of albumin and cytochrome *c* were added to isolated glyoxysome preparations before their osmotic disruption. Following electrophoresis of the matrix protein preparations, albumin and cytochrome *c* were readily identified on Coomassie brilliant blue stained gels, but were not stained by the PAS procedure.

Malate synthase was purified from either microsomal or glyoxysomal matrix protein preparations by sedimentation velocity gradient centrifugation and Sepharose 6B chromatography as in [11].

Organelle matrix protein preparations were analysed for neutral sugar content by the phenol/H_2SO_4 method [12] and for sialic acid by the thiobarbiturate assay [13].

The sugar components present in organelle proteins were identified by gas–liquid chromatography. The preparations (redissolved 80% $(NH_4)_2SO_4$ precipitates) were dialysed for 7 days against several changes of distilled water. At this stage free sugar was not detected in the distilled water by the phenol/H_2SO_4 test. The protein solution was evaporated to dryness under reduced pressure and kept in a dessicator over P_2O_5 for 12 h. The methyl glycosides of the sugars attached to the polypeptides were obtained by refluxing with anhydrous 0.64 M HCl in methanol at 80°C for 4 h. The reaction mixture was neutralized with Ag_2CO_3 and filtered. D-Mannitol (0.1 ml 0.1% w/v solution in methanol) was added to act as an internal standard. The filtrate and methanol washings were evaporated to dryness under reduced pressure. Trimethyl silyl derivatives were prepared from the methylglycosides by adding 0.2 ml Tri-sil Z and heating at 60°C with shaking for 5 min. After centrifugation, aliquots (1 μl or 2 μl) of the supernatant were injected into the column. The column was 150 cm long. The stationary phase was 3% methyl silicone gum (SE 30) supported on Celite (mesh size 85–100). The gas chromatograph was programmed from 120–230°C at 1°C/min. The analyses were performed on a Pye 104 Series Gas Liquid Chromatograph. Calibration was carried out using methyl glycosides prepared from standard free sugars.

3. Results and discussion

Glyoxysomal matrix protein preparations show a characteristic spectrum of protein bands when examined by SDS–polyacrylamide gel electrophoresis [14]; a typical protein-stained gel is shown in fig.1a. When identical gels were stained with PAS reagent, these protein bands were shown to be carbohydrate positive (fig.1b), although the intensity of the glycoprotein stain was considerably less than that observed with the protein stain. Recent studies into the cellular origin of glyoxysomal matrix proteins has provided evidence for their initial segregation by the endoplasmic reticulum (ER). This evidence is based on the

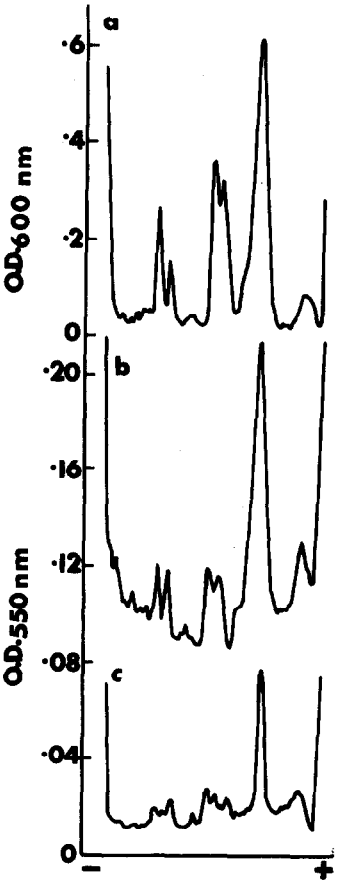

Fig.1. Densitometer scans following SDS–polyacrylamide gel electrophoresis showing: (a) Coomassie blue-stained glyoxysomal matrix proteins; (b) PAS-stained glyoxysomal matrix proteins; (c) PAS-stained microsomal soluble proteins.

findings that identical antigenic determinants are found in both the glyoxysomal matrix and in the soluble components trapped in the microsomal vesicles [14,15], certain characteristic glyoxysomal enzyme activities including malate synthase are found in the microsomal fraction early in germination [15], and the kinetics of protein labelling from radioactive precursors supports a direction of cellular transport from the ER to the glyoxysome [16].

The considerations coupled with the established role of the ER in protein glycosylation [17] suggest that this cellular component may be the site of addition of carbohydrate moieties to the glyoxysomal protein cores in germinating castor bean endosperm cells. PAS-stained gels obtained in the case of soluble proteins from the microsomal fraction revealed a close similarity to the glyoxysomal gels (fig.1c). The conclusion that corresponding glycoprotein components might occur in the microsomal and glyoxysomal fractions was confirmed by examining purified malate synthase. It has been shown that microsomal malate synthase is ultimately sequestered in glyoxysomes [18] SDS–polyacrylamide gel electrophoresis of malate synthase from either microsomes or glyoxysomes shows a single polypeptide band with app. mol. wt 64 000 (fig.2a), these monomers again being shown to be glycoproteins (fig.2b,c).

Hydrolysis of the glyoxysomal protein released several sugars which were identified by gas–liquid chromatography. These include fucose, mannose, galactose and mannosamine (fig.3). Hydrolysis of the microsomal proteins also revealed the presence of similar amounts of fucose and small but significant amounts of the other sugars (not shown). Both glyoxysomal and microsomal protein preparations gave positive tests for the presence of neutral sugars and sialic acid.

The significance of the post-translational modification of glyoxysomal proteins reported here is at present unclear. It may, however, play a role in the association of newly synthesized glyoxysomal enzymes within the ER cisternae and have a directing influence on the subsequent transport of these proteins to their ultimate cellular location.

Acknowledgements

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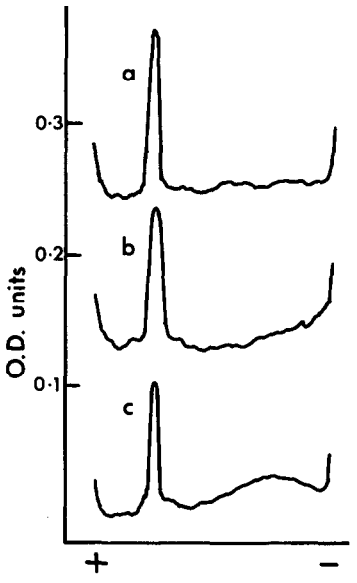

Fig.2. Densitometer scans following SDS–polyacrylamide gel electrophoresis showing: (a) Coomassie blue-stained glyoxysomal malate synthase; (b) PAS-stained glyoxysomal malate synthase; (c) PAS-stained microsomal malate synthase.

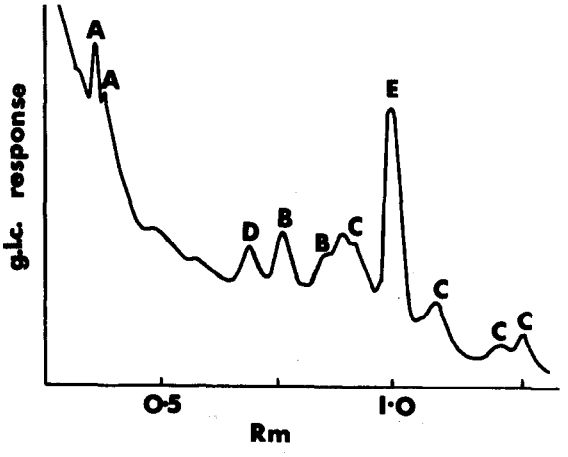

Fig.3. Gas–liquid chromatogram (g.l.c.) of sugars released from glyoxysomal matrix proteins. Identification of peaks: (a) D-fucose; (b) D-galactose; (c) mannosamine; (d) D-mannose; (e) D-mannitol (internal standard).

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