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IMMUNOCYTOCHEMICAL LABELING OF ENZYMES IN LOW TEMPERATURE EMBEDDED PLANT TISSUE: THE PRECURSOR OF GLYOXYSOMAL MALATE DEHYDROGENASE IS LOCATED IN THE CYTOSOL OF WATERMELON COTYLEDON CELLS

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Abstract

The Lowicryl-technique in combination with protein A gold was used in order to localize the precursor of glyoxysomal malate dehydrogenase in watermelon cotyledons. Preservation of the antigen was evaluated by a preembedding technique in isolated organelles. The glyoxysomal malate dehydrogenase was localized in tissue sections by a postembedding technique. Antigens of glyoxysomal malate dehydrogenase were found in the glyoxysomal matrix and in the cytosol, whereas the endoplasmic reticulum was completely free of labeling. Controls are presented by preimmunserum, by a serum against various proteins of the glyoxysomal membrane and by application of cycloheximide in order to inhibit translation at cytosolic ribosomes. The results are compared with immunocytochemical localizations of other plant microbody enzymes and of plant storage proteins.

Key words: Immunocytochemistry, plant enzymes, transmission electron microscopy, low temperature embedding, glyoxysomes, malate dehydrogenase.

Introduction

Immunocytochemical localization of enzymes is well established in animal tissues. In plant tissues immunocytochemistry was mainly restricted to storage proteins (BAUMGARTNER et al. 1980; CRAIG and MILLERD 1981; CRAIG and GOODCHILD 1982; CRAIG and GOODCHILD 1984 a, 1984 b; GREENWOOD et al. 1984). Comparable few studies dealt with the localization of enzymes (DOMAN and TRELEASE 1985; SAUTTER 1984) regulatory proteins (VERBELEN et al. 1982), phytohormones (ZAVALA and BRANDON 1983) or inhibitors (HORISBERGER and TACCHINI-VONLANTHEN 1983 a, 1983 b).

Plant tissues exibit special difficulties for immunocytochemistry. Most of these difficulties are due to the general differences between animal cells and plant cells. Cell walls usually do not occur in animal tissues. They are a high diffusion barrier for macromolecules (KNOX 1982), although for immunocytochemical localization of microtubules preembedding techniques have been successfully used after cell wall digestion (WICK et al. 1981).

Plants contain a variety of substances, which nonspecifically bind to immunoglobulins. Most important among these substances are lectins and certain glycoconjugates (KNOX 1982). These substances might cause considerable nonspecific labeling and thus severe problems in immunocytochemistry. This difficulty can be completely avoided by preincubations with preimmunserum followed by incubation of protein A not coupled to gold particles (SAUTTER 1984).

Additionally problems are caused by fat storing tissue and heat labile enzymes. Immunocytochemistry of cryosections of fat storing tissue is difficult, because during warming up of the section, the unfixed lipid begins to flow over the section surface. This lipid covers antigenic determinants as well as the ultrastructure. Heat labile enzymes on the other hand prevent embedding in conventional epoxy resins, which need high temperatures for polymerization.

The present study demonstrates localization of a plant enzyme in watermelon cotyledons. In this study the mentioned problems are combined: cell walls are present and do not allow for antibody diffusion (SAUTTER and HOCK 1982);

storage proteins are accumulated in protein bodies and exhibit significant nonspecific binding sites for antibodies; watermelon cotyledons contain a high amount of storage lipid within their cells; glyoxysomal malate dehydrogenase (gMDH) - the enzyme to be localized - is heat labile (WALK and HOCK 1977b); furthermore, the interesting question was the localization of the precursor of this enzyme, which occurs only in low amounts within the cell.

Conventional histochemistry is not useful in localizing gMDH and pre-gMDH because isoenzymes exist and because enzyme precursors may be enzymatically inactive. Thus immunocytochemistry is the technique of choice. Monospecific antibodies against gMDH are available. They are also able to recognize the pre-gMDH (GIETL and HOCK 1982). In order to avoid excessive heat damage of the heat labile gMDH (WALK and HOCK 1977 b) Lowicryl was used as the embedding medium for electron microscopy which is a successful technique in immunolabeling of animal tissues (ROTH et al. 1981). This technique has been used successfully for localization of microbody marker enzymes in watermelon cotyledons (SAUTTER 1984).

Materials and Methods

Plant Material

Watermelon seeds (Citrullus vulgaris Schrad., var. Stone Mountain, harvest 1979) were obtained from Vaughan`s Seed Company (Ovid, Michigan, USA). After removing the seedcoat, the seedlings were germinated at 30°C in the dark under sterile conditions on 0.8% agar as described by HOCK (1969).

Preparation of Protein A Gold Complex (pAg)

Colloidal gold particles (average diameter of 15nm) were prepared according to BENDAYAN et al. (1980). An excess of protein A was applied for coupling. The uncoupled protein A was removed from the conjugate by centrifugation.

Sera

Monospecific anti-gMDH-serum was produced according to the methods of WALK and HOCK (1977 a). Monospecificity was tested by diffusion tests, immunoelectrophoresis and by precipitation from radiolabeled homogenates followed by SDS-gel electrophoresis and fluorography. Polyspecific serum against various proteins of the glyoxysomal membrane was a gift from Dr. M. Conder (University of Warwick, England).

Crude Homogenate

Although 1% glutaraldehyde for 1 hour led to good immunolocalization of microbody marker enzymes (SAUTTER 1984), this fixation protocol was not useful for the preservation of gMDH-antigens. Therefore a preembedding technique was used in order to evaluate a fixation protocol, which preserves enough gMDH-antigens for immunocytochemical detection. 120 cotyledons from 3 day old dark grown watermelon seedlings were homogenized as described earlier (HOCK 1973). The homogenization medium contained 0.25M sucrose, 50mM HEPES, 0.1% (w/v) BSA, 10mM KCl, 1mM $MgCl_2$, 1mM Na_2-EDTA, 10mM DTE at pH 7.5 (WALK and HOCK 1977 b).

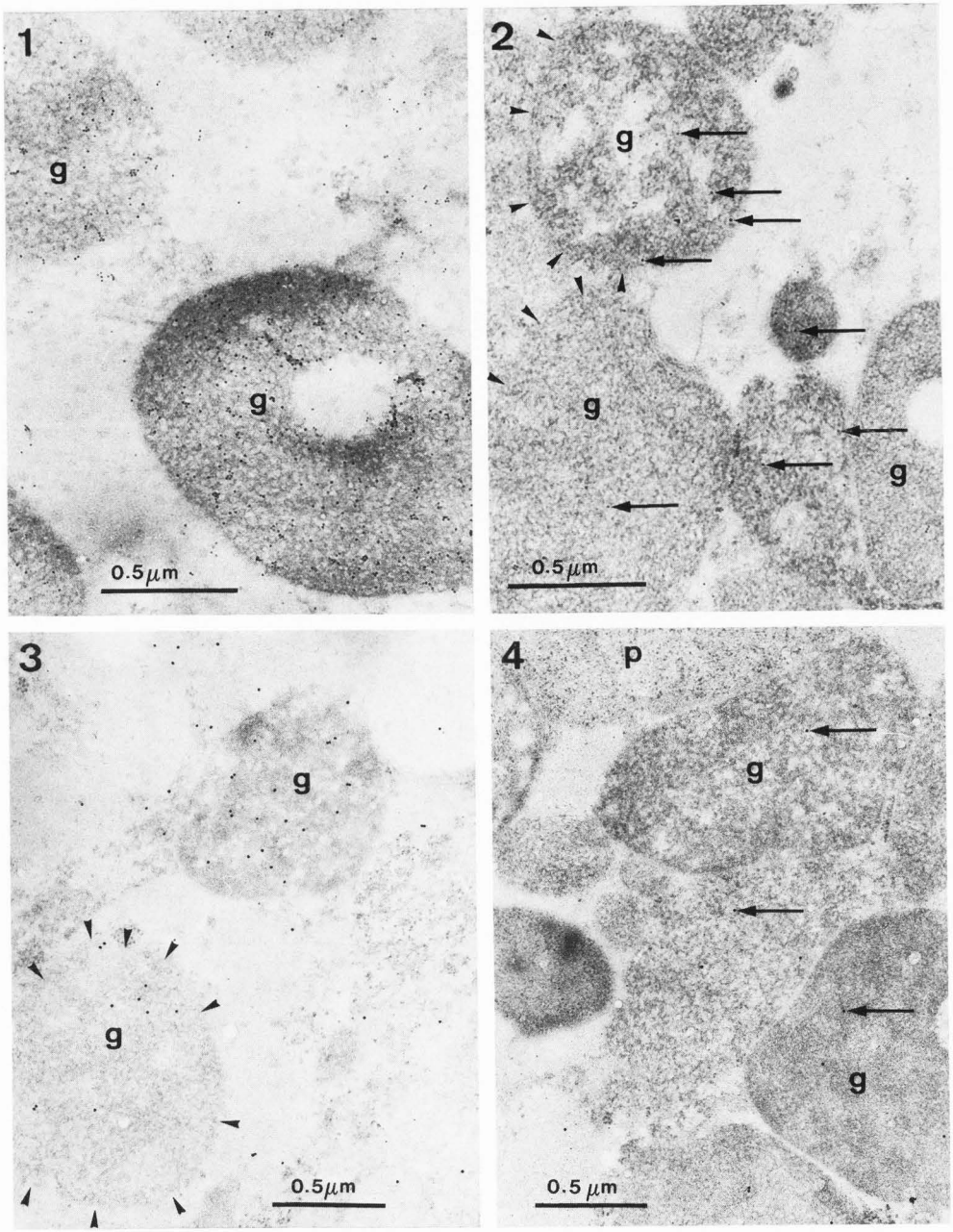
For immunolabeling 80µl of a crude organelle fraction (10,000g pellet) was fixed for 15min at room temperature. The fixative was added to the organelle suspension. The fixed organelles were washed in homogenization buffer for 30min, with several changes; then 20µl anti-gMDH-serum was added. For controls, the anti-enzyme-serum was replaced by preimmunserum. The serum was allowed to precipitate for 1 hour at room temperature and subsequently, for 4 hours at 5°C. Unbound antibodies were washed out with homogenization buffer, which was changed several times at the beginning. The last washing step was overnight at 5°C. A postfixation with 1% glutaraldehyde for 1 hour was followed by washing, dehydration and embedding as described for tissue. Antibodies were labeled at the surface of ultrathin sections with the following protocol: PBST (see below) 5min; incubation of goat anti-rabbit ferritin coupled serum (Paesel, Frankfurt, FRG) diluted 1:20 with PBST for 1 hour at room temperature; washing with PBST (15min, two changes) was followed by a wash with water and counterstaining with uranyl acetate for 7min. Lead counterstaining was omitted in this case. the same immunostaining was done with pAg diluted 1:20 instead of the secondary ferritin coupled antibody.

Under these conditions, fixation in 2.0% formaldehyde and 0.2% glutaraldehyde preserved enough gMDH-antigens for immunocytochemistry. The result is shown on figs. 1-4 and on table 1.

Table 1:
Labeling of gMDH in isolated glyoxysomes.

Sera / Markers	anti-gMDH-serum $\bar{x}_1$ [1]	s.e.m.$_1$ [2]	preimmunserum $\bar{x}_2$ [1]	s.e.m.$_2$	difference $\Delta\bar{x}$ [3]	s.e.m.	s [4]
Ferritin	187.2	± 16.8	36.5	± 3.2	150.7	± 17.1	0.001
pAg	19.9	± 3.9	1.6	± 0.2	18.3	± 3.9	0.001

1) mean from at least 20 organelle profiles in markers per μm^2 section area
2) standard error of the mean
3) $\Delta\bar{x}=\bar{x}_1-\bar{x}_2$
4) significance of $\Delta\bar{x}$



Figures 1-4: Crude organelle fraction.

Fig.1: Preembedding labeling by anti-gMDH-serum, visualization by postembedding ferritin coupled secondary antibodies. Note the high amount of ferritin particles within the glyoxysome (g).

Fig.2: Similar to fig.1, but an experiment with control serum. Only a few ferritin particles (some of them are indicated by arrows) are visible within the glyoxysomes (g). Arrowheads indicate organelle borders.

Fig.3: Preembedding labeling by anti-gMDH-serum, visualization by postembedding pAg technique. Labeling by pAg is clearly significant as compared to the control (fig.4). g: glyoxysome; arrowheads: organelle border.

Fig.4: Similar to fig.3, but an experiment with control serum. Only a few pAg particles are visible (arrows) within the glyoxysomes (g). Not all of the pAg are indicated. At the upper edge a plastid appears (p) which contains ribosomes.

Formaldehyde alone led to severe damage of subcellular structures. According to a rough estimation of cells per cotyledon, the average number of glyoxysomes per cell (unpublished data) and by using the specific enzyme activity of gMDH and the average specific density of proteins, the amount of gMDH molecules present per μm^2 glyoxysome section area can be approximately determined. According to this estimation, indirect immunocytochemical labeling of gMDH after preembedding techniques with ferritin coupled secondary antibodies labeled about 20% of the total number of gMDH molecules present. Under the same conditions, pAg stains about 2% of the gMDH molecules present in the section of glyoxysomal matrix. In spite of its lower binding capacity, I preferred pAg as a marking agent for tissue sections, due to its higher electron density, its larger diameter and its lower nonspecific background.

Preparation of Tissue for Immunocytochemistry

Watermelon cotyledon tissue was cut with a razor blade to cubes of less than 0.5mm length and immediately fixed with 2.0% formaldehyde (freshly prepared from paraformaldehyde) and 0.2% glutaraldehyde either in 0.1M potassium phosphate buffer or in homogenization buffer pH 7.0 (see above). Fixation lasted 1 hour at room temperature under mild vacuum. All the following preparation steps were carried out at $5^{o}C$. A wash in fixation buffer (30min, two changes) was followed by dehydration through a series of increasing concentrations of ethanol (SAUTTER et al. 1981). The samples were embedded in Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg, FRG) according to the manufacturer`s instructions (KELLENBERGER et al. 1980). After polymerization in the cold ($-20^{o}C$) the samples were allowed to polymerize further at room temperature and daylight for at least one week. Ultrathin sections of greyish color were cut on a Reichert Ultracut microtome and mounted on formvar coated nickel grids.

Immunocytochemical Labeling of Tissue Sections

The whole labeling protocol was entirely the same as previously described (SAUTTER 1984). For all labeling protocols, previously air dried sections were used to prevent the sections floating off the grids. Phosphate buffered saline was used (PBST, containing 10mM potassium phosphate pH 7.0, 0.8% NaCl and 0.1% (v/v) Triton X-100) for the first moistening of the sections, for all washing steps (each washing for 15min, two changes) and for all dilutions of the sera. From the first moistening until the last washing step, the grids were prevented from drying. The grids were floated upside down on the respective solutions and moved from one fluid to the next using a platinum loop. The whole protocol was carried out at room temperature: moistening in PBST for 5min; incubation of preimmunserum for 30min at a dilution of 1:20; washing with PBST; incubation of uncoupled protein A (i.e., without gold markers) for 30min at a dilution of 1:20; washing with PBST; incubation of the monospecific antiserum from rabbit at a dilution of 1:10 for 60min; washing with PBST; incubation of the pAg at a dilution of 1:20 for 30min; another washing with PBST completed the immunocytochmical labeling.

In control experiments the specific antiserum was substituted by preimmunserum. The sections were counterstained with uranyl acetate for 7min and lead citrate for 30 sec and finally viewed in a Zeiss EM 10 microscope at 60kV electron acceleration voltage.

Statistical Methods

Immunolabeling is given in particles per section area (μm^2). The section areas were measured by a planimeter (Ott, Kempten, FRG). The means were accounted from at least 20 organelles and were compared by Student`s t-test. Whenever differences are given (e.g., between labeling by specific serum and control serum in the respective compartments), standard errors of the means (s.e.m.) were given following Gauss` error propagation.

Cycloheximide Treatment

In order to inhibit translation at cytosolic ribosomes, cycloheximide was used. Three day dark grown watermelon cotyledons were incubated at $30^{o}C$ in the dark. Incubation was performed in 0.25ml phosphate buffered saline (pH 6.0). After two hours the sample was divided in two halves. One half was additionally incubated for 4.5 hours in 8pM cycloheximide. The other half was also incubated, as a control, without cycloheximide. Fixation and embedding was done as described above.

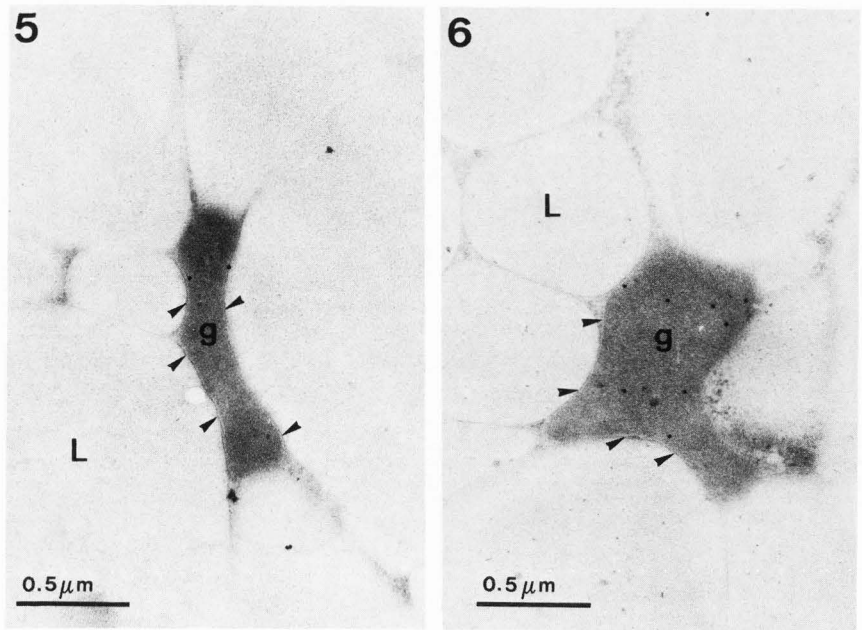
Results

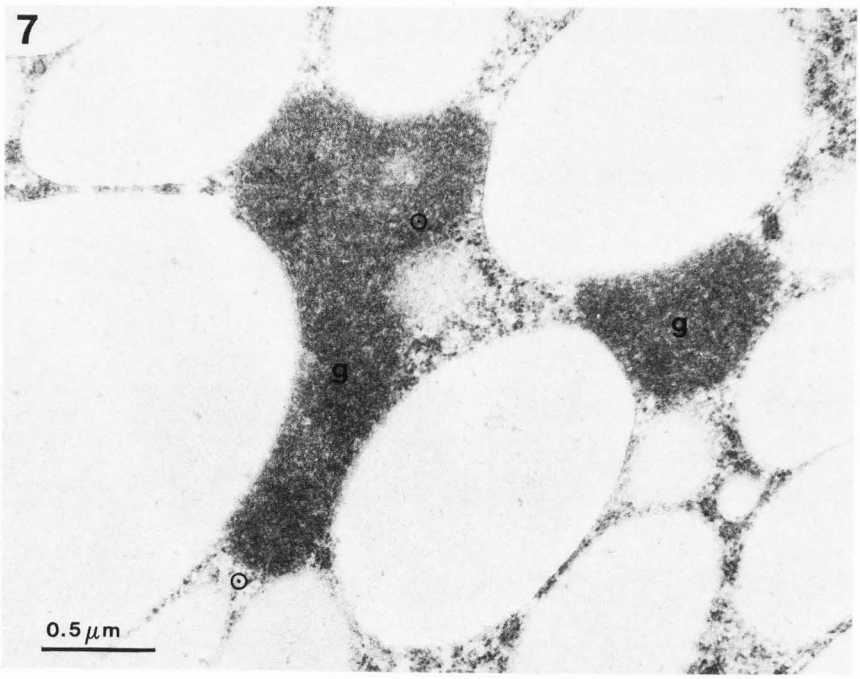
Glyoxysomal Matrix

In watermelon cotyledons the amount of gMDH increases dramatically during early germination according to biochemical results (WALK and HOCK 1977 a). Four day dark grown cotyledons contain much more gMDH-antigens as compared to 2 day dark grown cotyledons. This was confirmed by immunohistochemical experiments (SAUTTER and HOCK 1982). This increase was used additionally as controls by preimmunserum in order to show the specificity of immunolabeling by anti-gMDH-serum.

Two day dark grown cotyledons exhibit a relatively low amount of marker particles within the glyoxysomal matrix (fig. 5, table 2). The labeling of gMDH was not significant as compared to the control, in which monospecific anti-gMDH-serum was replaced by preimmunserum. This was shown after counting the marker particles per μm^2 of at least 20 organelle profiles from the respective incubations. The means were compared by Student`s t-test (table 2).

The 4 day dark grown developmental stage of watermelon cotyledons exhibited a clearly significant amount of marker particles within the glyoxysomal matrix after incubation with anti-gMDH-serum (fig. 6, table 2) as compared to the earlier developmental stage (fig. 5). The significance of the labeling was evaluated by Student`s t-test





Figures 5-7: Localization of gMDH in the glyoxysomal matrix by postembedding technique.

Fig.5: Glyoxysome (g) of a 2 day dark grown watermelon cotyledon after fixation in homogenization buffer. The section was incubated with anti-gMDH-serum. Labeling of gMDH is not significant in this developmental stage. Arrowheads indicate the glyoxysomal membrane. Lipid bodies (L) are completely free of labeling.

Fig.6: Glyoxysome (g) of a 4 day old dark grown watermelon cotyledon after fixation in homogenization buffer. The section was incubated with anti-gMDH-serum. Label of gMDH is significantly present in this developmental stage. Arrowheads indicate the glyoxysomal membrane. Lipid bodies (L) are completely free of pAg.

Fig.7: Glyoxysomes (g) of a 4 day old dark grown watermelon cotyledon after fixation in phosphate buffer. The section was incubated with preimmunserum. Only a few pAg particles are present (encircled).

Table 2:
Labeling by anti-gMDH-serum in watermelon cotyledon tissue

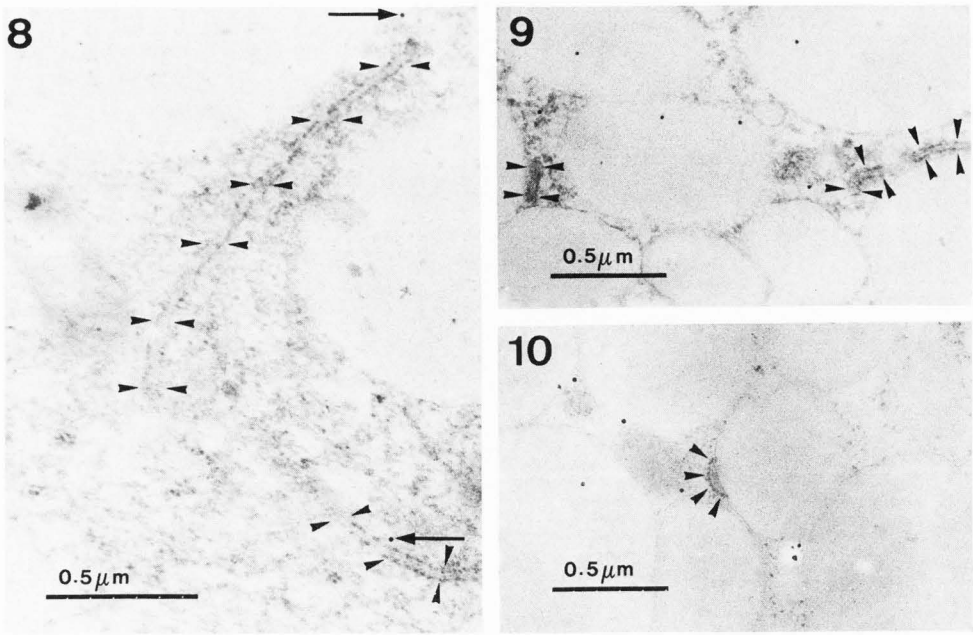
organelle	age of cotyledon	$\Delta\bar{x}$ *)	± Δs.e.m.	significance
glyoxysomal matrix	2 d	2.8	2.9	-
	4 d	42.5	10.7	0.001
endoplasmic reticulum	2 d	0		-
	4 d	0		-
cytosol	2 d	0.2	0.5	-
	4 d	3.2	1.5	0.001
lipid bodies	2 d	0.0	0.1	-
	4 d	0.3	0.3	-

*) difference between labeling after incubation with anti-gMDH-serum and labeling after incubation with preimmunserum in marker particles per μm^2 section area.
s.e.m.: standard error of the mean following Gauss` error propagation.

Table 3
Labeling by anti-gMDH-serum after cycloheximide treatment

	anti-gMDH-serum $\bar{x}_1$ 1)	± s.e.m.$_1$ 2)	preimmunserum $\bar{x}_2$ 1)	± s.e.m.$_2$	difference $\Delta\bar{x}$ 3)	± Δs.e.m.	S 4)
glyoxysomes							
control	9.82	1.72	0.57	0.33	9.05	1.75	0.001
cyclohex.	3.06	0.55	0.72	0.22	2.34	0.59	0.001
cytosol							
control	2.59	0.38	0.58	0.23	2.01	0.44	0.001
cyclohex.	0.78	0.18	0.57	0.13	0.21	0.22	-
relative cytosol area in %							
control	0.32	0.03	0.32	0.02	0.00	0.04	-
cyclohex.	0.27	0.01	0.30	0.03	-0.03	0.03	-

1) mean from at least 20 organelle profiles. Labeling given in markers per μm^2 section area. Relative cytosol area given in % of total area of micrograph (without cell wall).
2) standard error of the mean
3) $\Delta\bar{x} = \bar{x}_1 - \bar{x}_2$
4) significance of $\Delta\bar{x}$.



Figures 8-10: Localization of gMDH in the endoplasmic reticulum by postembedding technique. Samples fixed in phosphate buffer.

Fig.8: Endoplasmic reticulum in 2 day dark grown cotyledon cells (arrowheads). None of the pAg (arrows) is associated with the endoplasmic reticulum.

Fig.9: Endoplasmic reticulum in 4 day dark grown cotyledon cells (arrowheads). None of the pAg is associated with the endoplasmic reticulum.

Fig.10: Proglyoxysome (arrowheads) in 4 day dark grown cotyledon cells. Protein A gold is not associated with the proglyoxysome.

after counting the marker particles per μm^2 (table 2). The control in which monospecific anti-enzyme-serum was replaced by preimmunserum showed negligible nonspecific background (fig. 7).

Endoplasmic Reticulum

The endoplasmic reticulum was completely free of marker particles after incubation with anti-gMDH-serum. This holds true for the 2 day old (fig. 8) as well as for the 4 day old (Figs. 9,10) cotyledon tissue. Even those parts of the endoplasmic reticulum, which are proglyoxysomes according to WANNER et al. (1982) were entirely free of gMDH-labeling (fig. 10).

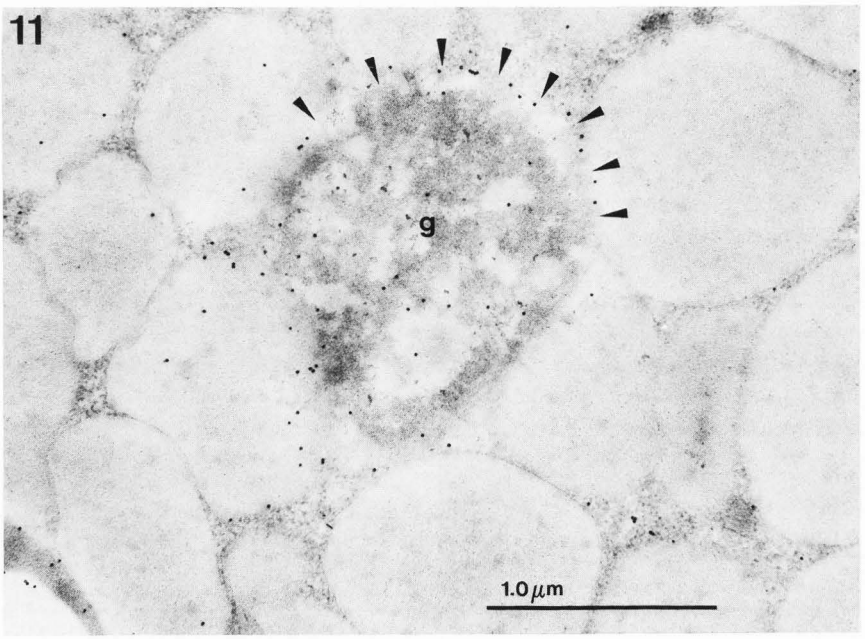
The absence of an antigen under given circumstances never can be proven due to logical reasons (PETRUSZ 1983). However in order to exclude a simple technical reason for the lack of labeling in the ER a control experiment was carried out, in which the monospecific anti-enzyme-serum was substitiuted by a polyspecific serum against various proteins of the glyoxysomal membrane. This serum led to significant accumulation of marker particles along the glyoxysomal membrane (fig. 11). Furthermore, a significant number of particles were found clearly associated with the membrane of the endoplasmic reticulum including the membrane of proglyoxysomes (fig. 12). Labeling of the ER-membrane in watermelon cotyledons is comparable with other immunocytochemical labeling experiments in the literature (BENDAYAN and SHORE 1982; CRAIG and GOODCHILD 1984 a). Thus technical reasons for the lack of gMDH-labeling within the ER can be excluded.

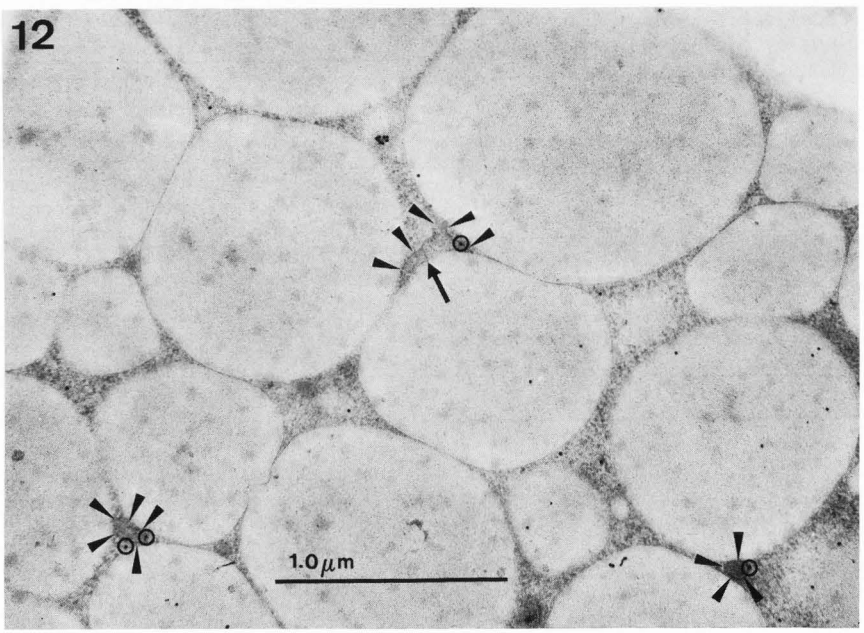
Cytosol

In contrast to the endoplasmic reticulum marker particles were found in the cytosol after labeling with monospecific anti-gMDH-serum. This labeling was too low to be detectable by usual comparison of the micrographs from incubation with anti-enzyme-serum and control incubation with preimmunserum. In this case counting of marker particles was essential.

Two day old cotyledon cells showed no significant labeling of gMDH-antigens in the cytosol (table 2). In contrast 4 day dark grown cotyledons exhibited clearly significant anti-gMDH-labeling in the cytosol (table 2). In order to exclude a technical artifact for this labeling in the cytosol (e.g., incomplete washing steps) the labeling in the lipid bodies was counted. The lipid bodies should be essentially free of binding sites. Indeed the labeling in these organelles was comparably low in 2 day old an in 4 day old cotyledons (table 2). Thus a sole technical artifact for the labeling in the cytosol can be excluded.

Cycloheximide inhibits the translation of gMDH-precursors at cytosolic ribosomes (WALK and HOCK 1977 a). The gMDH-precursor should therefore disappear from the cytosol by a cycloheximide





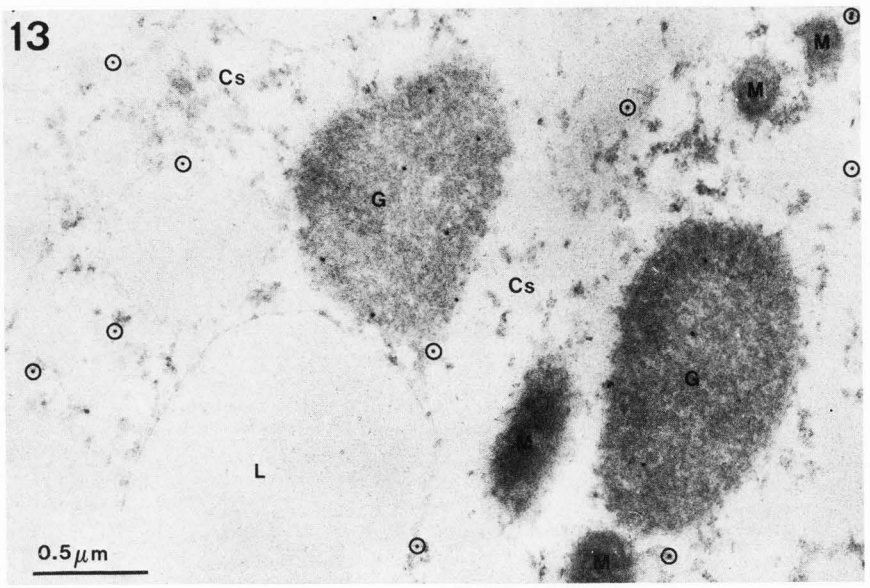
Figures 11 and 12: Labeling of various glyoxysomal membrane proteins in 4 day dark grown watermelon cotyledons after fixation in phosphate buffer.

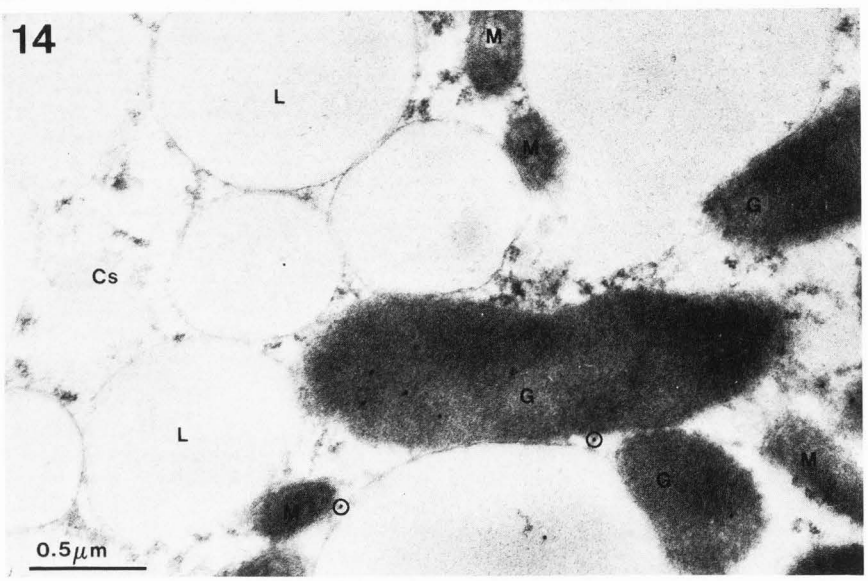
Fig.11: Glyoxysome (g) which exhibits a clearly membrane associated labeling (arrowheads).

Fig.12: Parts of the endoplasmic reticulum (arrowheads) which show an overall low, however, clearly membrane associated labeling (encircled). One of these parts of the endoplasmic reticulum represents a proglyoxysome (arrow).

treatment, and should be detectable by immunocytochemistry. Therefore an experiment was designed in which 3 day old watermelon cotyledons were treated with cycloheximide. For control, an untreated sample was embedded. Specificity of labeling was checked by preimmunserum substituting the anti-gMDH-serum.

The result is shown on figs. 13, 14 and in table 3. In the untreated cotyledon tissue labeling of gMDH antigens was significantly present in the glyoxysomal matrix as well as in the cytosol (fig. 13, table 3). A different labeling pattern was found in the cycloheximide





Figures 13 and 14: Inhibition of translation at cytosolic ribosomes by cycloheximide. Labeling of gMDH antigens in 3 day dark grown watermelon coytledons after fixation in phosphate buffer.

Fig.13: Untreated control, which exhibits marker particles in the cytosol (encircled). Cs: cytosol; G: glyoxysome ; L: lipid body; M: mitochondria.

Fig.14: Cycloheximide treated cell. Solely two pAg are visible in the cytosol (encircled). Cs: cytosol; G: glyoxysome; L: lipid body; M: mitochondria.

treated cotyledons. Antigens of gMDH were significantly localized within the glyoxysomal matrix (fig. 14, table 3), although the amount of labeling was lower as compared to the untreated control. In the cytosol of the cycloheximide treated cotyledon cells no gMDH-antigens were detectable (fig. 14, table 3). The amount of markers per μm^2 cycloheximide treated cytosol section surface was similarly low regardless whether incubation was carried out by anti-gMDH-serum or by preimmunserum (table 3). This unspecific labeling corresponds to the unspecific labeling in antigen lacking mitochondria (SAUTTER 1984).

Application of cycloheximide might cause additional effects. Simple uptake of water into the cytosol could lead to a volume increase and could dilute the antigen in this compartment. Thus an antigen could simply disappear in the background by dilution. In order to exclude an effect of cycloheximide onto the volume of the cytosol, the relative section area of the cytosol was measured and compared. Neither between the cycloheximide treated and the untreated tissue nor between the sections used for anti-gMDH-labeling and the section used for control incubation was any significant difference found (table 3). This result leads to the conclusion that gMDH-antigens in the cytosol disappeared by inhibited translation and were not simply diluted.

Discussion

Preservation of the Antigen

Different fixation procedures preserve proteins to different degrees. Glyoxysomal MDH is extremely sensitive to aldehyde treatments. In principle gMDH-labeling increases by decreasing fixative concentration. Immunohistochemical localization of gMDH in the light microscope was most effective at the lowest aldehyde concentration applied (SAUTTER and HOCK 1982). However, 0.25% formaldehyde is in no way sufficient for electron microscopy. This situation makes a compromise necessary, which preserves enough antigen as well as ultrastructure. This compromise was found for gMDH.

In contrast, antigen preservation of the glyoxysomal marker enzyme isocitrate lyase increases with increasing fixative concentration. Fixation with 2% formaldehyde and 0.2% glutaraldehyde leads to an average labeling of 7.6 pAg particles per μm^2 glyoxysomal profile after incubation with anti-isocitrate lyase-serum as compared to 17.9 pAg particles per μm^2 after fixation by 1% glutaraldehyde (unpublished). This is in agreement with DOMAN and TRELEASE (1985). These authors labeled immunocytochemically isocitrate lyase in glyoxysomes of cotton seeds. After fixation with 3% glutaraldehyde they found up to 100 marker particles per μm^2 glyoxysomal profile and after additional postfixation with osmium tetroxide up to 170 markers per μm^2. DOMAN and TRELEASE labeled with protein A-gold of 10-15nm diameter for the marker, and used sections from samples embedded in Spurr's epoxy resin.

Incubation Protocol

The technique of labeling the antibodies influences the labeling result. The use of secondary antibodies enhances the amount of marker particles per μm^2 up to tenfold as compared to the use of protein A-gold (15nm) for marker (table 1).

Protein A coupled to a smaller gold particle (5nm) provides also a greater amount of markers per μm^2 section profile as compared to a larger (15nm) pAg complex (unpublished). The lower labeling of the large marker might be due to stereological hindrance (HORISBERGER and TACCHINI-VONLANTHEN 1983 c). However by increasing labeling with smaller markers the overall unspecific background increases also. Therefore, the main reason for this overall higher labeling might be due to poor washing efficiency, because the velocity of the washing buffer decreases drastically towards the section surface. This is in agreement with the inability to avoid this kind of nonspecific labeling by preincubations with preimmunserum, whereas labeling by nonspecific binding of antibodies can be completely eliminated.

Nonspecific Binding Sites

Nonspecific binding was reported from pea seeds by CRAIG and GOODCHILD (1984 a). Furthermore in these authors' experience this nonspecific binding was not prevented by pretreatment of the sections with sugars specific for pea lectin. This is in agreement with our own experience that preincubations with mucin did not lower the nonspecific label within protein bodies. Therefore the nonspecific binding site in the protein bodies is probably not due to lectins. Whatever the nonspecific binding of antibodies might be, a preincubation with preimmunserum followed by incubation with unlabeled protein A inhibited this background completely.

Sensitivity of Labeling

Localization of the precursor of a glyoxysomal matrix enzyme in the cytosol is a unique result for precursors of microbody enzymes. Several microbody enzymes have already been localized in animal and plant tissue (BENDAYAN et al. 1983; Yokota et al. 1983, DOMAN and TRELEASE 1985; SAUTTER 1984). In none of these publications, a localization of the respective precursor was reported. Especially in case of isocitrate lyase (DOMAN and TRELEASE 1985; SAUTTER 1984) developmental stages of the seedlings have been examined, in which this enzyme is synthesized (RADIN and TRELEASE 1976; HOCK 1970; KAGAWA et al. 1973). According to biochemical experiments, the precursor of isocitrate lyase should be present in the cytosol (FREVERT et al. 1980; KINDL et al. 1980; LORD and ROBERTS 1982). This lack of labeling might be caused by several reasons: (1) antigenic determinants of the precursor might not be recognizable for the anti-enzyme-antibodies, although the precursor has the same molecular weight as the mature enzyme (RIEZMAN et al. 1980; ZIMMERMANN and NEUPERT 1980; LORD and ROBERTS 1982); (2) the precursor might have lost its antigenicity to a greater degree than the mature enzyme due to fixation or embedding; (3) the antigenic determinants of the precursor although recognizable by the antibody are hidden by aggregation; (4) the amount of precursor molecules outside the microbodies is too low for detection by immunocytochemistry.

Glyoxysomal MDH is translated as a higher molecular weight polypeptide (41 kDalton) as compared to the subunit of gMDH (33 kDalton) isolated from watermelon cotyledons (WALK and HOCK 1978; GIETL and HOCK 1982). In this respect gMDH is unique in the set of glyoxysomal enzymes that have been localized immunocytochemically. The relatively large extra sequence, the socalled signal sequence (BLOBEL and DOBBERSTEIN 1975), is

probably the reason for the precursor to exhibit more antigenic determinants at the section surface as compared to the mature subunit of gMDH or it may additionally increase the stability of the antigen during preparation.

Conclusion

Glyoxysomal MDH is involved in the glyoxylate cycle and must be imported into glyoxysomes from outside because glyoxysomes lack a translation system. Biochemical data support a direct import from the cytosol (KRUSE et al. 1981; LORD and ROBERTS 1982; ZIMMERMANN and NEUPERT 1980) whereas other investigators propose at least the glyoxysomal membrane to be derived from the endoplasmic reticulum (DONALDSON et al. 1981). Biogenesis of glyoxysomes by budding from endoplasmic reticulum during early seedling development in watermelon is supported by ultrastructural and histochemical results (WANNER et al. 1982). In the present study it was not possible to localize the precursor of gMDH within the endoplasmic reticulum. It cannot be comletely excluded (PETRUSZ 1983) that this compartment contains in vivo antigenic determinants. In comparable studies concerning the pathway of storage proteins in developing cotyledons (CRAIG and GOODCHILD 1984 a, 1984 b; CHRISPEELS 1983) accumulation of antigens was found in the endoplasmic reticulum and Golgi cisternae. At least, however, in proglyoxysomes accumulation of the gMDH antigen should have occurred. On the other hand the precursor of gMDH in the cytosol was significantly detectable although it is equally distributed in this compartment.

This labeling pattern favors a model by which pre-gMDH is translated at free cytosolic ribosomes and is subsequently directly imported into glyoxysomes. Such a transfer of cytosolic translation products was already proposed for some enzymes of the glyoxysomal matrix which lack a signal sequence (KRUSE et al. 1981; ZIMMERMANN and NEUPERT 1980).

Labeling of pre-gMDH in the cytosol shows the suitability of the applied techniques for localization of antigens, which occur only in low amounts and which are not accumulated in a small compartment of the plant cell.

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