Incomplete Glyoxysomes Appearing at a Late Stage of Maturation of Cucumber Seeds

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Seeds of cucumber fruits at a late stage of ripening were analyzed for microbodies and microbody components. On isopycnic density gradient centrifugation of homogenates in the presence of EDTA, several particulate fractions were obtained: a light membraneous fraction (density $d = 1.09 - 1.11$ kg × l^{-1}), a mitochondria-enriched fraction ($d = 1.21$ kg × l^{-1}), a microbody-enriched fraction ($d = 1.23$ kg × l^{-1}), and a protein body fraction ($d = 1.26 - 1.29$ kg × l^{-1}). Microbodies were revealed by exactly coinciding peaks of malate synthase, catalase and crotonase; small proportions of citrate synthase and malate dehydrogenase were also present in this zone. Isocitrate lyase activity, however, did not occur in the seeds at this stage. The examination of enzyme activities indicated the presence of microbodies which cannot function as competent glyoxysomes because of the lack of isocitrate lyase. Moreover, *de novo* synthesis from [^{3}H]leucine could be demonstrated for malate synthase by means of immunoprecipitation of newly synthesized malate synthase and subsequent electrophoretic analysis.

Introduction

Two main categories of plant microbodies have been characterized: glyoxysomes, found always in germinating seeds actively transforming fatty acids into dicarboxylic acid via β-oxidation and glyoxylate cycle, and leaf peroxisomes, catalyzing several steps of photorespiration [1].

While two current models for microbody biogenesis at a definite stage of development are subject of contraversy [2 – 5], little information is available about the timing of translation of glyoxysomal proteins: either continuously during seed formation, or at a defined step of germination, or both.

Findings that enzymatic activities of glyoxysomal proteins increase from zero level to maximal values at day 4 – 5 of germination [6 – 8] have led to the hypothesis that these proteins are synthesized *de novo* exclusively during the first period of germination [1]. That seemed to be further substantiated by germination experiments carried out in the presence of $^{15}NO_3^-$ or ^2H_2O. High density-labelling observed in the case of catalase and isocitrate lyase was taken as evidence that glyoxysomal proteins are predominantly, if not exclusively [9, 10], synthesized *de novo* during germination.

Abbreviations: EDTA, Na_2-ethylenedinitrilo-N,N,N′,N′-tetraacetate; Tricin, N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; TCA, trichloroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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In contrast to these results, we could demonstrate that dry seeds of cucumber already contain considerable amounts of the total set of glyoxysomal proteins [11]. The cells of cotyledons must have, therefore, the capacity for both transcription *and* translation of information responsible for the biosynthesis of glyoxysomal proteins prior to the stage of dry seeds. We report here about the occurrence of several glyoxysomal proteins and describe a microbody form, partially competent glyoxysomes, in ripening seeds of cucumbers.

Methods

Plant material

Seeds of cucumber (*Cucumis sativus*) were prepared from fruits of 5 – 6 months old plants. They were selected for uniformity, *i. e.* according to size (7 – 8 mm length). This was the raw material for preparations of cell structures occurring at the late stage of ripening. Seeds with 4 – 5 mm length obtained from younger fruits were used for investigations concerning the somewhat earlier stage, biochemically defined by the presence of catalase, but the absence of malate synthase.

Preparation of extracts

Hundred seeds were homogenized by chopping for 30 min with a stainless steel razor blade in 4 ml of grinding medium on a polystyrene block kept on

ice. The grinding medium contained 150 mM Tricin-NaOH, pH 7.5, 10 mM KCl, 1 mM EDTA, and 15% (w/w) sucrose. The homogenate was squeezed through two layers of miracloth and the filtrate was directly applied on a sucrose gradient.

Sucrose gradient separation

The sucrose gradient consisted of 4 ml 60% (w/w) sucrose, 24 ml of a linear gradient 60–25% sucrose, and 4 ml 20% sucrose. The sucrose solutions contained 50 mM Tricin-NaOH, pH 7.5, and 1 mM EDTA. 4 ml of the crude homogenate were layered on top of the gradient. After centrifugation at 27000 rpm (Beckman SW-27 rotor) for 2.5 h at 5 °C, 1.2 ml fractions were collected across the gradient using an ISCO density gradient fractionator, model 640.

Application of [^{3}H] leucine

0.5 mCi L-[4,5-^{3}H]leucine (60 Ci/mmol) were applied to 50 seeds. Incubation was in a beaker at 23 °C in darkness for 20 h. Subsequently, seeds were washed with cold grinding medium and homogenized as described above.

Electrophoretic analysis

Slab gel electrophoresis in the presence of sodium dodecyl sulfate was performed with minor modifications [11] in a discontinuous system described by Laemmli [12].

Enzyme assays

Malate synthase [13], citrate synthase [13], catalase [14], malate dehydrogenase [15] were tested according to well described procedures. The assay of crotonase [16] and isocitrate lyase was modified as reported earlier [11]. Cytochrome oxidase was determined by the method of Schnarrenberger *et al.* [17], and NADH: cytochrome c reductase was measured photometrically at 560 nm [18].

Immunoprecipitation of malate synthase

Labelled malate synthase, released from glyoxysome-like microbodies, was incubated with antibodies raised against purified glyoxysomal malate synthase from 5 day old germinating cotyledons. Precipitation, washing and further analysis was exactly as described [11, 19].

Other methods

Radioactivity was measured in a liquid scintillation spectrometer (Berthold-Friesecke, BF 5001) equipped with an Alphatronic computer (Diehl). Absolute radioactivity was calculated by a program based on channel ratio methods. The scintillation cocktail was Quickszint 212 (Zinsser, Frankfurt). Gel slices were incubated with 1% SDS for 25 h prior to the addition of the cocktail. Sucrose concentrations were determined refractometrically. Protein was determined according to Lowry *et al.* [20].

Results

Preliminary studies had indicated that several glyoxysomal proteins could be detected in ripening seeds, when seeds were analyzed in a very late stage of maturation. We made every effort to utilize seeds from fully ripened cucumbers which were grown for at least 5 months. We used seeds as large as possible, in order to demonstrate and to investigate as many glyoxysomal proteins as possible. Catalase, but not malate synthase, could be detected in early stages of maturation, while both enzymes were present in a late stage of development.

Fig. 1 summarizes the relevant findings with respect to glyoxysomal components present in the late stage: a) occurrence or lack of enzyme activities, b) association of enzymes with distinct cell structures, and c) the quantitative amount of enzyme activities related to fresh weight. Assessments of the last point can be made as the gradient profiles given represent the entire amount of enzyme activities extractable under the conditions used; the total homogenate was applied onto the gradient without any preceding enrichment step. Repeated controls have shown, that the yield of enzyme activities obtained by gentle homogenization with razor blades and the yields which were possible by exhaustive homogenization differed by a factor of 1.8–2.1. If this factor of 2, the size of fractions, and the absolute activities of peaks are taken into account, the occurrence of the enzymes can be quantified.

It is evident from Fig. 1 that, besides catalase, enzymes of the glyoxylate cycle (malate synthase, citrate synthase, malate dehydrogenase) as well as of β-oxidation (crotonase) are present. But isocitrate lyase was not detectable, although it was looked for throughout the gradient.

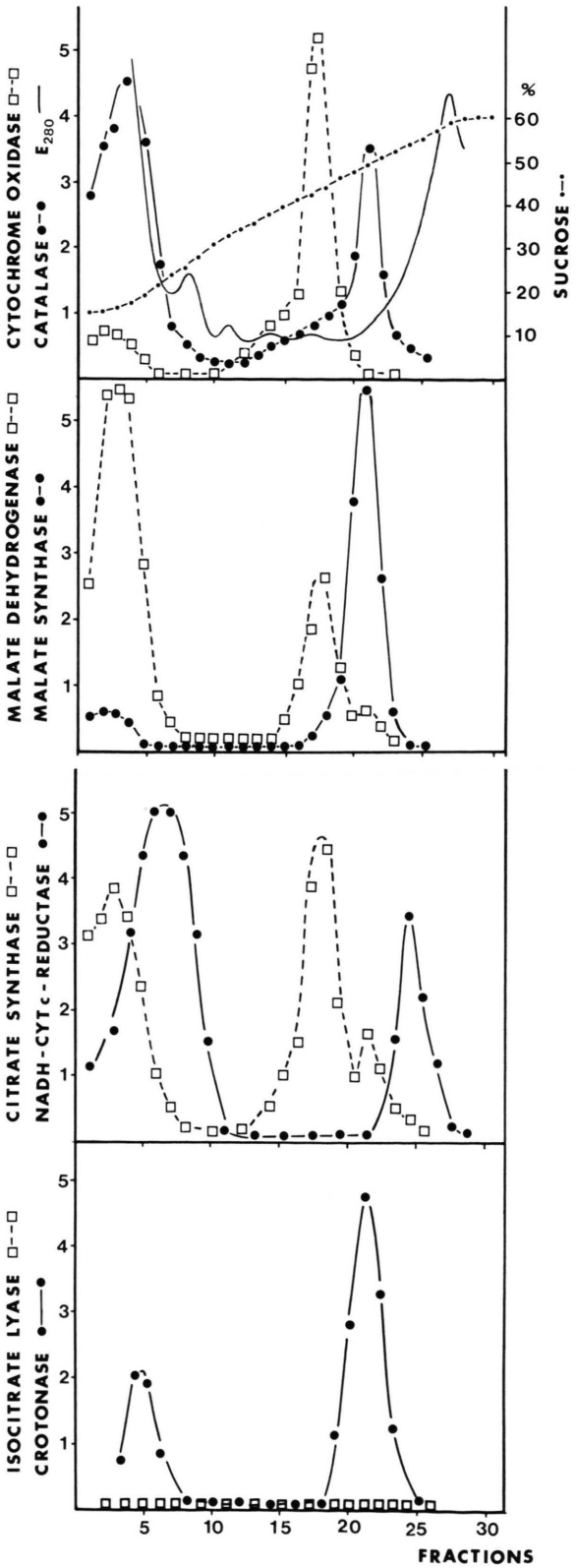

Fig. 1 demonstrates, moreover, that the profiles of catalase and malate synthase coincided at 50% sucrose. This was a good indication for the occurrence of microbodies. Crotonase and small proportions of malate dehydrogenase and citrate synthase also seem to be attributable to these microbodies. The position of mitochondria at 40% sucrose, evidenced by the sharp maximum of cytochrome oxidase and citrate synthase, is clearly separated from the one of microbodies.

Although microbodies and mitochondria were well separated from each other and also from endoplasmic reticulum, identified by NADH-dependent cytochrome c reductase, the profiles do not allow an estimation of how much the fractions were contaminated by other proteins. It could be anticipated that globulins dominated in the seeds already at this stage of development. Fig. 2 indicates that constituents of protein bodies were already present at this stage and that they heavily contaminated all fractions throughout the gradient. As evidenced in track F, globulins occurred which were identical – according to their subunit M_r – to the one previously described for dry cucumber seeds [11]. Here again, globulins were found as components of the protein peak in the high density range. As centrifugation time was only 2.5 h, only particles larger than 10^3 S could migrate to this position which therefore is indicative of protein bodies.

It was probable, but it could not be taken for granted, that enzymes were synthesized when they are needed. Examples are known where enzymes are activated by mobilization of proforms or by change of aggregation forms. As for the present problem, we showed that malate synthase was not built as proform, analog with catalase and globulins in an earlier stage of development. But rather it was synthesized *de novo* at the same period when the enzyme activity could be observed. We asked the question whether the seeds have the capacity of synthesizing

Fig. 1. Analysis of cell structures in ripening seeds by zonal centrifugation. Qualitative determination and quantitative measurement of the following enzymes in total homogenates prepared from 100 seeds in the late stage of ripening: catalase (1 relative unit = 220 nkat × ml^{-1}), cytochrome oxidase (1 relative unit = 110 nkat × ml^{-1}), malate dehydrogenase (1 relative unit = 33 nkat × ml^{-1}), malate synthase (1 relative unit = 3.3 nkat × ml^{-1}), NADH: cytochrome c reductase (1 relative unit = 0.03 nkat × ml^{-1}), citrate synthase (1 relative unit = 0.11 nkat × ml^{-1}), crotonase (1 relative unit = 0.83 nkat × ml^{-1}), E_{280} (1 relative unit = 0.1), one fraction: 1.2 ml.

Stage of seed development	Fresh weight of seeds [g]	Radioactivity applied [^{3}H]leucine [mCi]	Radioactivity incorporated into malate synthase [nCi]
Early stage of ripening	3	0.5	<0.05
Late stage of ripening	8.5	0.5	0.85
Early stage of germination (taken from [21])	9	1.0	0.40

Table I. Comparison of amounts of malate synthase synthesized *de novo* in ripening and germinating seeds. [^{3}H]Leucine of the same high specific radioactivity was used as precursor.

malate synthase during one of both stages: a) at the step where catalase activity but no malate synthase activity could be demonstrated, or b) at the step where both enzyme activities attained a significant level. From Table I it becomes evident that [^{3}H]leucine was incorporated into malate synthase at the late stage only.

Microbodies were isolated by gradient centrifugation at 51.5% sucrose, and malate synthase was solubilized. Electrophoretic analysis of immunoprecipitated malate synthase showed a peak of radioactivity at a subunit M_r of 63000. The peak had more than twice the height compared with a peak of analogous experiments with germinating seeds (following paper [21]). Comparison was made on the basis of fresh weight of seeds.

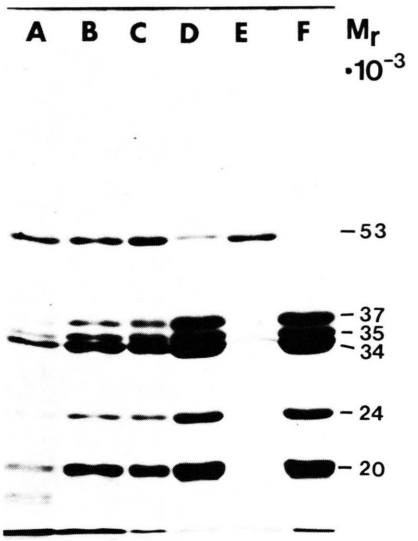

Fig. 2. Electrophoretic analysis of gradient fractions in the presence of sodium dodecyl sulfate and mercaptoethanol. Proteins were separated according to their subunit molecular weight. The fractions were taken from gradient described in Fig. 1. A: gradient supernatant. B: zone at density $d = 1.10\ \text{kg} \times \text{l}^{-1}$ (endoplasmic reticulum), C: zone at density $d = 1.20\ \text{kg} \times \text{l}^{-1}$ (mitochondria), D: zone containing maximal glyoxysomal activity, (soluble fraction), E: zone containing maximal glyoxysomal activity, sedimentable proteins, F: zone at density $d = 1.27\ \text{kg} \times \text{l}^{-1}$ (protein bodies).

Discussion

The present studies provide evidence for the occurrence of glyoxysome-like cell structures in ripening cucumber seeds. Microbodies found at this late stage of seed maturation lack isocitrate lyase, but contain enzymes of fatty acid β-oxidation and enzymes of glyoxylate cycle. Most probably a complementary step is necessary to afford microbodies with the complete set of glyoxysomal proteins. There are indications that prior to the stage investigated here microbodies occur but with an even smaller set of components, perhaps only with catalase.

The picture which can be derived from these data is similar to that reported by Choinski and Trelease [22]. With cotton embryos, they observed that catalase was already present before malate synthase appeared. In contrast to our results obtained with cucumber seeds, isocitrate lyase was not detectable in dry cotton seeds. Earlier reports claim the occurrence of isocitrate lyase in dry cotton seeds [23], and emphasize the importance of mitochondrial enzymes [24, 25] which are already present in dry seeds and which are, therefore, available at an early stage of germination.

We postulate that distinct microbody species, but differing in enzyme composition, are synthesized in ripening seeds. During progress of maturation more and more enzymes are translated and then associated with microbodies, until the complete set of glyoxysomal proteins is at disposal in the dry seed.

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