SUBSTRATE DEPENDENT FORMATION OF GLYOXYSOMES IN CELL SUSPENSION CULTURES OF ANISE (*PIMPINELLA ANISUM* L.)

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1. Introduction

Reports on microbodies of plants in cell suspension culture are rare [1–5] and essentially restricted to the biochemical or cytological proof of their occurrence. The de-repression and repression of the glyoxysomal enzyme complements by substrates such as C_2-compounds or glucose as sole source of carbon, respectively, have only been studied in algae [6] and fungi [7–9]. In higher plant tissues exogenous sugars seem to have minor effects on glyoxysomal enzyme activities [10–12]. Here, we report that in cell suspension cultures of anise (*Pimpinella anisum* L.) that possess no typical microbodies functional glyoxysomes develop after removal of sucrose from the growth medium.

2. Material and methods

Cell suspension cultures of anise (*Pimpinella anisum* L.) were grown in batch culture in B-5 medium [13] in 2 liter Fernbach flasks containing 500 ml medium with 5 μmol 2,4-dichlorophenoxy acetic acid/l. The cultures were shaken on a gyratory shaker (150 rev./min) at 27°C in white fluorescent light (~8 W/m^2). Fresh media were inoculated to give 10^5 cells/ml and cultivated for 7 days (late logarithmic phase of growth). Cell numbers per culture were determined as in [14]. The cell aggregates were harvested by filtration, rinsed with glass distilled water, weighed and homogenized. Anise seeds were surface sterilized [15] and germinated for 4 days at 28°C in the dark in petri dishes lined with wet filter paper that was exchanged daily.

Preparation of crude homogenates and crude particulate fractions and sucrose density gradient centrifugation were performed as in [8,15]. Enzyme activities were determined as reported in the literature with minor modifications: citrate synthase (EC 4.1.3.7) [16]; $NADH_2$–cytochrome *c* reductase (EC 1.6.2a) [17]; fumarase (EC 4.2.1.2) [18]; $NADH_2$–isocitrate dehydrogenase (EC 1.1.1.41) [19]; isocitrate lyase (EC 4.1.3.1) [16]; malate synthase (EC 4.1.3.2) [16]; catalase (EC 1.11.1.6) [20,21]; malate dehydrogenase (EC 1.1.37) [22]. Protein contents and sucrose concentration were estimated as in [15,23].

3. Results

The anise cell suspension cultures [14] grow heterotrophically in liquid medium as dedifferentiated small cell aggregates that are pale yellow. Inoculated to 10^5 cells/ml the cultures enter the stationary phase of growth after 9 days reaching ~4 × 10^6 cells/ml. Seven days old cultures were either transferred to fresh sucrose medium for 3 days or to sucrose-free medium for 48 h followed by the addition of 20 mmol/l acetate for subsequent 24 h. As listed in table 1 in both crude homogenates and crude particulate fractions of cells cultivated in sucrose medium catalase activity (peroxisomal marker) is present in addition to enzyme activities of mitochondria and of the endoplasmic reticulum. But the key enzyme activities for glyoxysomes, isocitrate lyase and malate synthase, are entirely missing. After substitution of acetate for sucrose the key enzyme activities of the glyoxylate cycle appear in the cells (table 1), while catalase activity is not changed remarkably. The amount of specific enzyme activities in the derepressed cultures is comparable to that found in preparations of anise

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Table 1
Specific enzyme activities in the crude homogenate (CH) and crude particulate fraction (CPF) of anise endosperm after 4 days of germination, and of 7-day-old suspension cultures after transfer to sucrose or acetate medium (per mg protein)

Enzyme	Cell suspension culture transferred to				Anise endosperm	
	Sucrose-medium (72 h)		Sucrose-free (48 h) and acetate medium (24 h)		CH	CPF
	CH	CPF	CH	CPF		
Catalase (μkat)	0.65	1.1	0.5	1.75	0.11	0.46
Isocitrate lyase (pkat)	0	0	18	60	20	90
Malate synthase (pkat)	0	0	80	160	90	260
Malate dehydrogenase (nkat)	9.0	8.0	6.0	5.0	n.m.[a]	
Citrate synthase (nkat)	0.15	0.29	0.10	0.27	n.m.	
Isocitrate dehydrogenase (nkat)	0.04	0.1	0.01	0.1	n.m.	
$NADH_2$–cytochrome *c* reductase (nkat)	2.0	1.8	1.0	1.8	n.m.	

[a] n.m. = not measured

endosperm where during early germination glyoxysomes occur when the storage fat is mobilized (table 1).

When the crude homogenate of an anise cell suspension culture grown in sucrose medium to the stationary phase of growth (14 days), is resolved by sucrose density gradient centrifugation the distribution profiles of protein and enzyme activities shown in fig.1 are obtained. The mitochondrial markers cosediment in the major protein band at 1.185 kg/l and show a minor peak at 1.14 kg/l. The endoplasmic reticulum membranes band at 1.13 kg/l as reflected by the distribution of $NADH_2$–cytochrome *c* reductase activity. All of the particulate catalase activity is recovered from this band, and no activity is detected at higher densities (1.22–1.25 kg/l) where usually peroxisomes of plant and animal cells sediment.

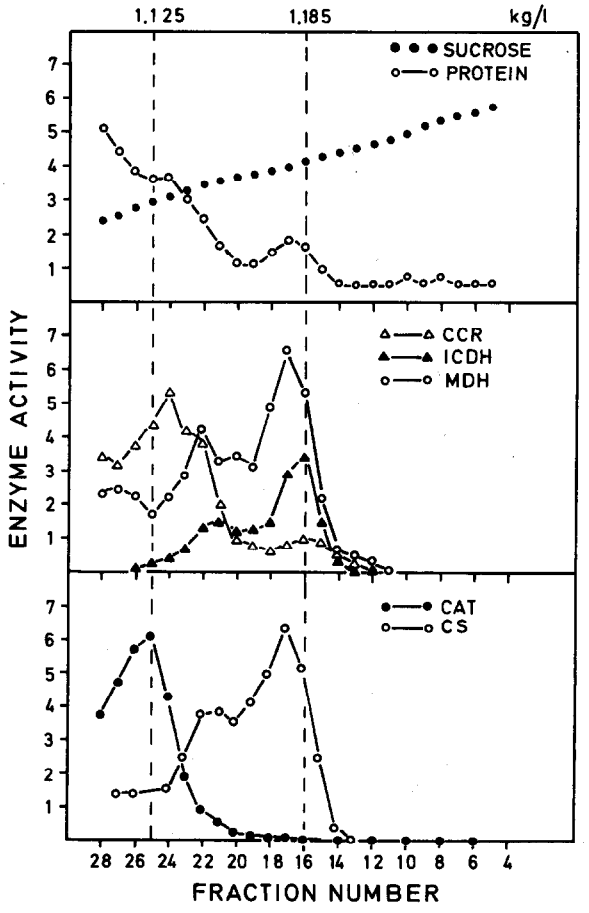

Fig.1. Distribution of protein and marker enzyme activities of mitochondria and microbodies after sucrose density gradient separation of a crude homogenate of 4 g (fresh wt) anise cell suspension cultures grown in sucrose medium to the stationary phase of growth. Units are (per g fresh wt): protein, 0.12 mg; NADH-cytochrome *c* reductase (CCR), 40 nkat; NADH-isocitrate dehydrogenase (ICDH), 0.14 nkat; malate dehydrogenase (MDH), 3.3 nkat; catalase (CAT), 0.11 μkat; citrate synthase, 0.06 nkat.

For derepression of the glyoxylate cycle enzymes the growth medium of 7-day-old cell suspension cultures was replaced by sucrose-free medium to give an identical cell density. After 24 h, 20 mmol/l acetate was added and the cultures were incubated for a subsequent 24 h. Such treatment causes the appearance of particulate activities of isocitrate lyase and malate synthase (fig.2). Both the glyoxysomal markers and catalase activity are recovered in a broad band sedimenting throughout the gradients severely overlapping with the peak of mitochondrial fumarase activity. When 7-day-old suspension cultures were starved for 48 h in fresh sucrose-free medium followed by treatment for 24 h with acetate, the distribution of the marker enzyme activities in the gradients shown in fig.3 was obtained. It illustrates that under such conditions a sharp band showing activities of both catalase and glyoxylate cycle enzymes is fairly well separated from the mitochondrial band and sediments with a buoyant density of 1.225 kg/l. The peak of catalase activity at lower densities as observed for

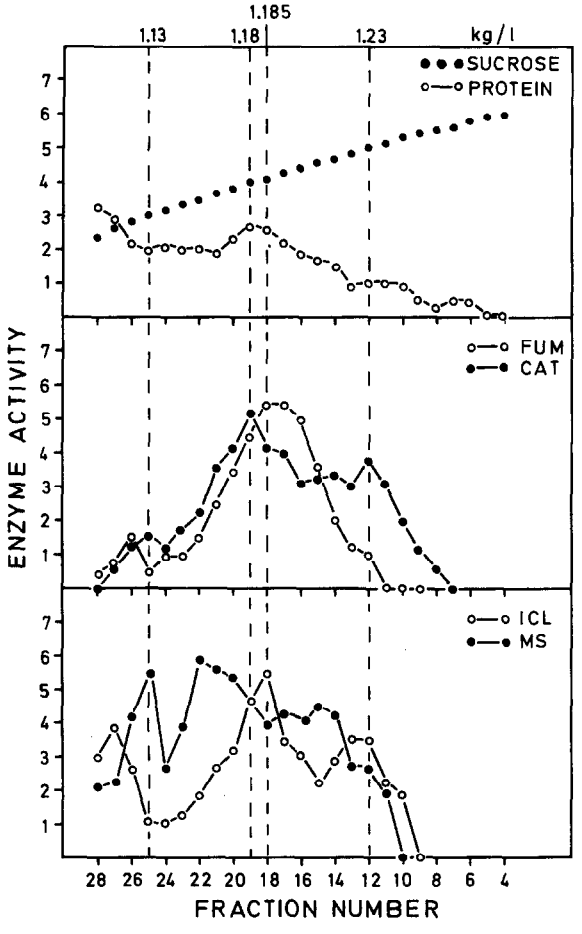

Fig.2. Distribution in a sucrose density gradient of protein and enzyme activities after centrifugation of a crude homogenate of 6 g (fresh wt) anise suspension culture cells. The cultures were grown for 7 days (late logarithmic phase of growth) in sucrose medium, starved for 1 day in fresh sucrose-free medium and incubated for one additional day in 20 mmol/l acetate added to the medium. Units are (per g fresh wt): protein, 39 μg; fumarase (FUM), 0.27 nkat; catalase (CAT), 0.25 μkat; isocitrate lyase (ICL), 20 pkat; malate synthase (MS), 6.5 pkat.

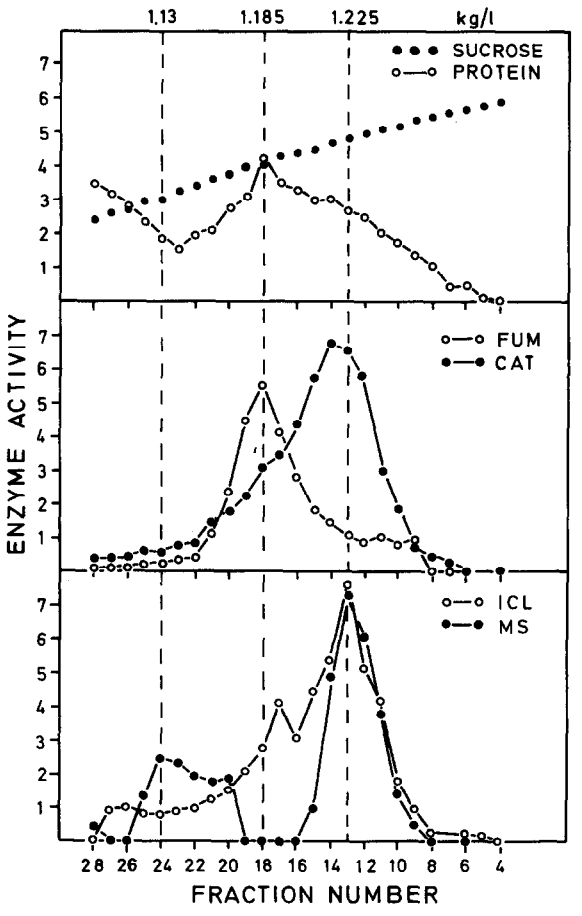

Fig.3. Distribution of protein and enzyme activities in a sucrose density gradient after centrifugation of a crude homogenate of anise suspension culture cells; 7-day-old cultures were starved in sucrose-free medium for 2 days and then incubated in acetate medium for 24 h. Units are (per g fresh wt): protein 20 μg; fumarase (FUM), 0.18 nkat; catalase (CAT), 0.17 μkat; isocitrate lyase (ICL), 8.0 pkat; malate synthase (MS), 5.0 pkat.

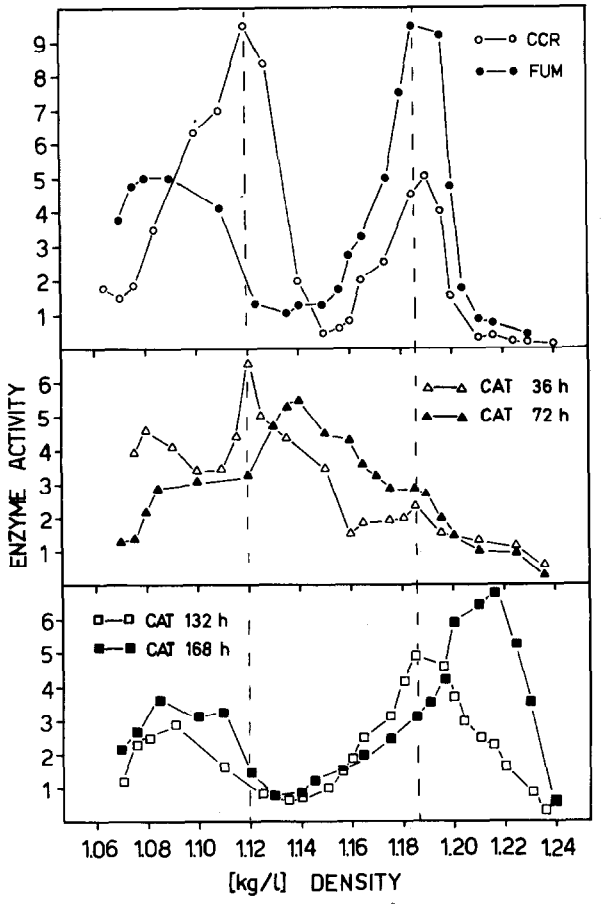

Fig.4. Change in the distribution profile of catalase activity in sucrose density gradients after centrifugation of crude homogenates of suspension culture cells; 7-day-old cultures were resuspended in fresh sucrose medium and incubated for the time indicated. The distribution profiles of catalase activity in 4 typical gradients are superimposed. The buoyant densities of the peaks of fumarase and $NADH_2$–cytochrome *c* reductase activity were identical in all gradients; here the distributions obtained with homogenates of cells 72 h after transfer are given. Units of enzyme activity are: CCR = 30 nkat; FUM = 0.15 nkat; CAT = 0.12 μkat. No ICL or MS activity was measureable.

untreated cell suspension cultures (fig.1,4) is no longer present.

As a control, 7-day-old cell suspension cultures were transferred into fresh sucrose-containing medium. After 1.5, 3, 5.5 and 7 days crude homogenates of such cells were resolved by sucrose density gradient centrifugation. The only microbody enzyme discovered was catalase; particularly, no activities of glyoxysomal markers such as isocitrate lyase or malate synthase were detected. The distribution profiles of catalase activity in the different gradients are superimposed in fig.4. It clearly illustrated that the buoyant density of the catalase bearing structures increases gradually with the incubation time of the cells in fresh sucrose medium. Evidently, these organelles are subject to some process of maturation and reach after 7 days a buoyant density of 1.22 kg/l which is similar to that of the glyoxysomes formed in the cells after 3 days in sucrose-free medium (fig.3). Such changes in density are not observed for other cell components such as mitochondria or membranes of the endoplasmic reticulum (fig.4).

4. Discussion

The studies show that in dedifferentiated anise cells in suspension culture glyoxysome formation can be initiated by removal of sucrose from and addition of acetate to the medium. So far, similar data were obtained only with lower eukaryotes [6–9]. The mechanism of glyoxysome formation in the cell culture is not revealed by the data presented. But it is hypothesized that the catalase bearing structures recovered from the gradients together with membrane material of the endoplasmic reticulum (fig.1,4), represent precursor vesicles of microbodies similar to the structures recently described as intermediary pool of catalase in maturing cucumber seeds [24]. After transfer of the cells to acetate medium these structures mature within 3 days into glyoxysomes presumably by acquiring the derepressed glyoxysomal enzymes. When transferred to fresh sucrose medium the cells eventually form 'non-specialized' microbodies which differ from the glyoxysomes in their enzyme complement (fig.4). Experiments designed to examine this hypothesis are currently under way in this laboratory.

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