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ACTIVITY AND HEMATIN CONTENT OF CATALASE FROM GREENING SUNFLOWER COTYLEDONS

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Key Word Index—*Helianthus annuus*; Compositae; sunflower; catalase; hematin; peroxisomes.

Abstract—The specific activity of catalase purified from the peroxisomes of sunflower cotyledons declines in parallel with the total cotyledonary catalase activity during the transition of peroxisomes from glyoxysomal to leaf peroxisomal function. The hematin content of the purified catalase however, remains constant at 4 hematin groups per catalase molecule. The absorbance coefficients of catalase at 404 and 280 nm were determined to be 372 and 540/mM/cm, respectively.

INTRODUCTION

The activity, per cotyledon, of the peroxisomal marker enzyme catalase (EC 1.11.1.6) declines considerably during the transition of peroxisomes from glyoxysomal to leaf peroxisomal function in greening fatty cotyledons [1, 2]. In pumpkin cotyledons, this decline in total catalase activity is accompanied by a decline in the specific activity of the enzyme [3]. As an explanation of this change in the catalytic activity of catalase, Yamaguchi and Nishimura [3] suggested that the hematin content in the catalase molecule changes during peroxisomal transition. It was assumed that the pumpkin glyoxysomal catalase contains four hematin groups per molecule since the absorbance ratio A_{404}/A_{280} corresponds to that of mammalian catalase which contains four hematin groups per molecule.

Both Schiefer *et al.* [4] and Esaka and Asahi [5] suggested that plant catalase may contain only two hematin groups per molecule since the absorbance ratio of their electrophoretically homogeneous preparations was *ca* half of that of mammalian catalase. To date, plant catalase hematin contents have only been determined for the enzyme from spinach leaves. According to Galston *et al.* [6] it contains two, but according to Gregory [7] the enzyme contains four hematin groups per molecule.

The present paper reports on the hematin content and the absorbance ratio A_{404}/A_{280} of catalase which was purified from the peroxisomes of sunflower cotyledons and exhibited declining specific activity during the transition of peroxisomal function.

RESULTS

Purification of catalase

Catalase was purified from the peroxisomes of sunflower cotyledons at day 1.5 through day 7.5 of seedling development. The transition of peroxisomal function takes place during the development in the light beginning at day 2.5 [8]. Independent of the developmental stage of the cotyledons, 15–20% of the total extractable cotyledonary catalase activity was recovered in the isolated peroxisomal fractions and 15–25% of this catalase activity was recovered following purification of the enzyme. At all stages of cotyledon development, analytical electrophoresis of the peroxisomal fraction and the final enzyme preparation resulted in identical patterns of catalase activity on the gel when equal amounts of catalase activity of both samples were applied to the gel (Fig. 1). Moreover, the patterns of catalase activity do not change during cotyledon development [8].

According to analytical electrophoresis the catalase purified at different stages of cotyledon development was free from other proteins (Fig. 1a). Protein bands on the gel were only localized in identical positions to the two strongest bands of catalase activity. Table 1 summarizes the purification. Ammonium sulphate fractionations were not performed since precipitation of catalase resulted in a partial inactivation of the enzyme. The catalase also lost activity in the presence of Cl^- ions. Activities of 1 μkat/ml decreased by 50% in the presence of 100 mM Cl^- within 1 hr. Therefore, Cl^- ions in buffers and salt gradients were replaced by SO_4^{2-} ions.

Compared to the specific activity of the enzyme in the cotyledonary extracts, a 206-fold (s.d. ± 26; $n = 5$) purification of catalase was achieved at days 1.5 to 2.5 of seedling development. The purification factor increased up to 1.5 times that value during greening of the cotyledons. The specific activity of the purified catalase was 1230 (s.d. ± 159; $n = 5$) μkat/mg during seedling development in the dark. It steadily declined to *ca* 25% of that value after 5 days of seedling development in the light (Table 2).

Hematin assay

The sensitivity of published spectrophotometric assays for hematin [9–11] proved to be insufficient for the determination of catalase hematin in the Sephadex G-25 and Sephadex G-200 eluates. Therefore, a hematin assay of markedly improved sensitivity was developed (see Experimental). The colour development due to

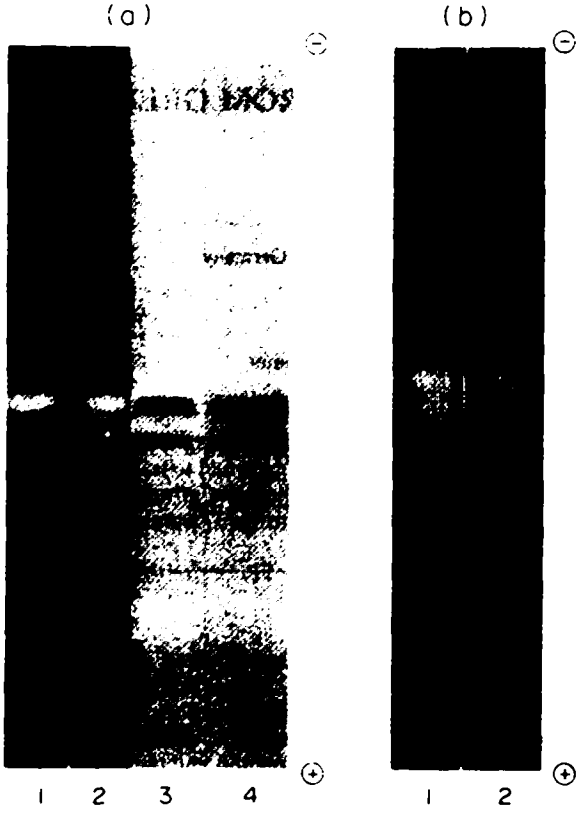

Fig. 1. Non-denaturing polyacrylamide gel electrophoresis of catalase. The peroxisomal fraction from 4.5-day-old sunflower cotyledons and catalase purified from this peroxisomal fraction were used. Lanes 1 and 2: staining for catalase; (a) 20 nkat applied, (b) 50 nkat applied; lane 1: peroxisomal fraction; lane 2: purified catalase. Lane 3: staining for protein; 10 μg of purified catalase were applied. Lane 4: staining for hematin; 10 μg purified catalase containing 150 pmol hematin were applied.

the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) is proportional to the quantity of hematin up to 50 pmol/test volume. The slope of the standard curve prepared with hemin chloride amounts to 0.173 ΔA_{655} per 10 pmol hematin (s.d. ± 4.9%; $n = 13$).

When performing the hematin assay with catalase, it is necessary to use the denatured protein because of the catalatic (on H_2O_2) and/or peroxidatic (on TMB) activity of catalase. The complete denaturation of catalase following the addition of the acid TMB solution is confirmed by the following observations: (i) the absorbance of reaction mixtures containing bovine liver catalase standards was independent of the time interval between TMB and H_2O_2 addition; (ii) following denaturation of catalase by 4 M or 8 M urea [12], the 1.24- and 1.52-fold, respectively, increase in A_{655} of the catalase standards corresponded to that resulting from the influence of urea on the assay with hemin chloride; (iii) catalase treated with 5% acetic acid did not exhibit catalatic activity (the activity assay is more sensitive than the hematin assay); its peroxidatic activity was identical to that obtained in the non-enzymatic reaction with hemin chloride.

When samples, containing 5–50 pmol hematin/ml of a catalase stock solution which contained 3.26 nmol hematin/ml as determined from A_{404} were used in the hematin assay a hematin concentration of 3.57 nmol/ml was calculated for the catalase stock solution. Thus, the hematin content of catalase is quantitatively determined by the hematin assay. The hematin content of hemoglobin and myoglobin, which contain 4 and 1 mol hematin/mol protein respectively, was determined by the hematin assay to be 4.06 and 1.06 mol hematin/mol protein.

Hematin content of catalase

The catalase purified at different stages of cotyledon development was not contaminated by non-catalase hematin. Following analytical electrophoresis hematin bands on the gel were only localized in positions coincident with the two strongest bands of catalase activity (Fig. 1a). The profile of catalase activity obtained following chromatography on Sephadex G-200 coincided with that of hematin. More than 90% of the catalase activity and the hematin applied to the Sephadex G-200 column was recovered. The ratio of catalase activity to hematin (μkat/nmol hematin) differed by less than 10% between the collected fractions.

In contrast to the specific activity of catalase, the specific hematin content (nmol hematin/mg protein) of the enzyme remains nearly constant during the functional

Table 1. Purification of catalase from cotyledonary peroxisomes of sunflower seedlings

Fraction	Volume (ml)	Activity (μkat)	Yield (%)	Protein (mg)	Sp. act. (μkat/mg)	Hematin (nmol)
(a) Broken peroxisomes	13	1193	100	21.1	57	
Peroxisomal matrix	12.5	1157	97	19.0	61	
DEAE eluate	10.5	796	67	1.05	758	
Sephadex G-25 eluate	10.5	758	64	0.99	766	10.16
Sephadex G-200 eluate	14	279	23	0.251	1112	3.82
(b) Broken peroxisomes	19.5	355	100	13.3	27	
Peroxisomal matrix	19	326	92	11.3	29	
DEAE eluate	10.5	204	57	0.46	443	
Sephadex G-25 eluate	10.5	195	55	0.45	433	2.81
Sephadex G-200 eluate	17.5	70.9	20	0.109	650	1.76

(a) Seedlings grown for 2.5 days in darkness; peroxisomes isolated from 150 pairs of cotyledons.
(b) Seedlings grown for 2.5 days in darkness and thereafter for 2 days in continuous light; peroxisomes isolated from 100 pairs of cotyledons.

Table 2. Activity and hematin content of catalase from sunflower cotyledons at different developmental stages of the seedlings

Day of development	Catalase activity (μkat/cotyledon)	Sp. act. (μkat/mg)	Sp. hematin content (nmol/mg)	Hematin groups per catalase molecule
1.5	21.1	1252	16.2	4.3
2.5	22.5	1179	16.0	4.2
3.5	15.6	981	15.6	4.1
4.5	9.36	650	16.1	4.2
5.5	6.83	546	14.3	3.8
6.5	4.62	416	15.3	4.0
7.5	3.15	334	14.1	3.7

The seedlings were grown for 2.5 days in darkness and thereafter in continuous light. Specific activity and specific hematin content were determined on the purified enzyme.

transition of the cotyledonary peroxisome population (Table 2). On the average, the specific hematin content amounted to 15.8 (s.d. ± 0.4; $n = 4$) and 15.5 (s.d. ± 1.2; $n = 6$) nmol/mg, respectively, during the first 2.5 days of seedling development in the dark and the following 5 days of seedling development in the light.

An M_r 280 000 (by ultracentrifugation) and 250 000 (by gel filtration), respectively, has been determined for catalase purified from etiolated as well as green sunflower cotyledons [13]. Based on the mean value of 265 000 it follows that 1 mg catalase corresponds to 3.8 nmol. From these data and the specific hematin content, 4.2 and 4.1 mol hematin/mol catalase were calculated for the enzyme purified from the peroxisomes of etiolated and greening/green cotyledons, respectively. We thus conclude that catalase contains four hematin groups per molecule independent of the functional stage of the cotyledonary peroxisome population.

Absorbance ratio A_{404}/A_{280} of catalase

The absorbance of catalase at 404 and 280 nm was determined following concentration of the Sephadex G-200 eluate *in vacuo*. The absorbance ratio A_{404}/A_{280} was 0.69 and was independent of the developmental stage of the cotyledons from which the catalase was purified. From the A_{404} and the hematin concentration, determined by the hematin assay, an absorbance coefficient of 93/mM/cm at 404 nm for catalase hematin (corresponding to 372/mM/cm for catalase) was calculated. The absorbance coefficient of catalase at 280 nm was calculated to be 540/mM/cm based on the absorbance at 280 nm, the protein content (determined by the Lowry method) and an M_r of 265 000. When liver catalase was treated in the same way as the cotyledonary catalase, an absorbance coefficient of 105/mM/cm at 404 nm for hematin and of 264/mM/cm at 280 nm for catalase was determined.

DISCUSSION

Degradation of catalase hematin has been shown to occur during purification of rat liver catalase from crude liver extracts, but did not occur if the enzyme was purified from an organelle fraction [14]. It has also been suggested that loss of catalase hematin may occur during purification of the enzyme from crude plant extracts [4]. Due to its sensitivity in the pmol range, the optimized hematin assay allows the determination of the hematin content of catalase purified from a limited amount of isolated plant peroxisomes. Four hematin groups per molecule were determined for the cotyledonary sunflower catalase independent of the developmental stage of the cotyledons (Table 2). Since catalase, as a tetrameric enzyme, does not contain more than four hematin groups per molecule we conclude that (i) no loss of catalase hematin occurred during the purification of catalase and (ii) the purified catalase did not contain contaminating protein (as also indicated by the electrophoretic analysis of the enzyme; Fig. 1a). The multiple forms of catalase detectable in the peroxisomal fractions were also present in the preparations of the purified enzyme (Fig. 1), that is, a specific loss of one form seems not to occur during the enzyme purification. Thus, the hematin content of the purified catalase is considered to reflect the hematin content of the catalase in the peroxisomes.

The absorbance coefficients at 404 and 280 nm of catalase purified from various sources are summarized for comparison in Table 3. The absorbance coefficients at 404 nm do not vary greatly between plant and mammalian catalases. Those at 280 nm, however, are generally higher for plant than for mammalian catalases, resulting in a low absorbance ratio A_{404}/A_{280} for plant catalases. Catalase of sunflower cotyledons exhibits an absorbance ratio A_{404}/A_{280} of *ca* 0.7 despite containing four hematin groups per molecule. Thus, correspondingly low absorbance ratios reported for plant catalases [4, 5] may not indicate that the hematin content of the catalases is lower than that of mammalian catalase.

With the beginning of the light-stimulated transformation of peroxisomes (glyoxysomes to leaf peroxisomes) in greening fatty cotyledons, the catalase activity per cotyledon starts to decline [2]. In sunflower cotyledons, the catalase activity decreases by 80–90% during 5 days of illumination. This development-dependent decline in catalase activity is paralleled by a decrease in the specific activity of the purified enzyme (Table 2). A similar result has been reported for greening pumpkin cotyledons and explained by a development-dependent change in the hematin content of catalase [3]. However, the hematin content of cotyledonary sunflower catalase does not change during greening of the seedlings (Table 2). Thus, we conclude that the development-dependent decrease in the specific activity of catalase in greening fatty cotyledons

Table 3. Absorbance coefficients and hematin content of plant and mammalian catalases

Source	404 nm (1/mM/cm)	280 nm (1/mM/cm)	A_{404}/A_{280}	Hematin groups per catalase molecule	Reference
Helianthus annuus (etiolated and green cotyledons)	372	540	0.69	4	
Spinacia oleracea (leaves)	n.d.	n.d.	0.65	2	[6]
	450	440	1.01	4	[7]
Ipomoea batatas (root tubers)	430	620	0.67	n.d.	[5]
Cucurbita sp. (etiolated cotyledons)	300	300	1.0	n.d.	[3]
Liver	430	397	1.08	4	[14]
	340	280	1.21	4	[15]
Blood	380–420	280	1.36–1.50	4	[16]

n.d., Not determined.

is not caused by a change in the hematin content of the enzyme.

EXPERIMENTAL

Plant material. Achenes of sunflower (*Helianthus annuus* L. cv. Spanners Allzweck) were soaked for 16 hr and then germinated in moist Vermiculite at 30°. After 2.5 days of growth in darkness the seedlings were exposed to continuous white light. The counting of days of development started with the planting of the soaked achenes.

Preparation of peroxisomal fractions and cotyledonary extracts. For isolation of peroxisomes, organelle fractions were prepared as described [17] and layered on discontinuous sucrose density gradients. The discontinuous sucrose density gradients were composed of 4 ml 60%, 5 ml 57%, 6 ml 51%, 6 ml 47%, 6 ml 42% and 5 ml 35% sucrose soln. All sucrose soln (percentages expressed as w/w and adjusted refractometrically) contained 1 mM EDTA (pH 7.5). The gradients were centrifuged at 82 500 g_{av} for 1.5 hr. The peroxisomal fractions at the interphase between the 57% and 51% sucrose soln were collected as whole fraction. Cotyledonary extracts were prepared with minor modifications as described in ref. [17]. The grinding medium (1 ml per cotyledon) consisted of 50 mM Tris–H_2SO_4, pH 8.

Purification of catalase. All steps were carried out at 2–4°. Tris–H_2SO_4 buffer, pH 8, was used throughout. Peroxisomal fractions diluted 1:2.5 with 20 mM buffer were stirred for 15 min and then centrifuged at 30 000 g for 10 min. The supernatant containing more than 90% of the catalase activity of the peroxisomal fraction was applied to a column (15 × 1.5 cm) of DEAE cellulose. The column was washed with 100 ml 20 mM buffer to remove the sucrose. The protein was then eluted (3.5 ml fractions) with a convex Na_2SO_4-gradient from 0 to 0.5 M (Na_2SO_4 dissolved in 20 mM buffer; 100 ml mixing chamber; flow rate 80 ml/hr). The three fractions with the highest catalase activity were pooled (DEAE-eluate). The DEAE-eluate was loaded onto a column (22.5 × 1.5 cm) of Sephadex G-25 and the protein was eluted with 5 mM buffer (flow rate 45 ml/hr; 3.5 ml fractions). The three fractions with the highest catalase activity were pooled (Sephadex G-25 eluate) and applied to a column (80 × 3 cm) of Sephadex G-200. After elution with 5 mM buffer (flow rate 20 ml/hr; 3.5 ml fractions) the fractions (3–6) with the highest catalase activity were pooled (Sephadex G-200 eluate).

Electrophoresis. Analytical disc electrophoresis (200 V, 14 hr, 4°) was performed on 6% polyacrylamide gel slabs (16 × 12 × 0.15 cm) using Maurer's system 1 [18]. The applied samples contained 10 mM dithioerythritol and a catalase activity of 10–50 nkat when the activity was to be detected. For detecting proteins and hematin, concentrated samples containing 10 mM dithioerythritol and up to 20 μg of protein and up to 300 pmol of hematin, respectively, were applied. Catalase activity was detected according to ref. [19]. The staining method for hematin was as described in ref. [18] with minor modifications and using tetramethylbenzidine instead of benzidine. Proteins were stained according to the procedure A described in ref. [20].

Assays. Catalase was assayed with some modifications as described in ref. [21] following the destruction of H_2O_2 at 230 nm (ε_{230} = 0.067/mM/cm; [22]). The reaction mixture (1 ml) contained 10 mM KPi-buffer, pH 7 and 12.5 mM H_2O_2. Peroxidatic activity of catalase was assayed according to ref. [23].

Hematin was determined by a method based on the ability of hematin to catalyse a redox reaction between H_2O_2 and TMB. The method [9, 10] was modified and optimized to increase its sensitivity 1000-fold. TMB-soln (100 mg/50 ml 10-vol. % HOAc) was freshly prepared every day and kept at 4°. TMB soln (1 ml) was added to 1 ml of sample and the mixture left to stand for 15 min at room temp. Then 0.05 ml of H_2O_2 soln (30-vol. %) was added. After incubating the reaction mixture for 20–25 min at room temp. the A_{655} of the blue coloured reaction product(s) was measured. For reagent blanks the sample was replaced by 5 mM Tris–H_2SO_4, pH 8. A standard curve was prepared with hemin chloride (5–50 pmol/ml 5 mM Tris–H_2SO_4, pH 8).

Protein was determined by the method of ref. [17]. The standard curve was prepared with bovine liver catalase.

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