EFFECTS OF WASHING AND OSMOTIC SHOCK ON CATALASE ACTIVITY OF INTACT CHLOROPLAST PREPARATIONS

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1. Introduction

When oxygen is the terminal electron acceptor for photosynthetic electron transport, hydrogen peroxide is the first stable product of the reaction (review [1]). Since catalase causes release of oxygen from hydrogen peroxide, an enhancement of net oxygen evolution on addition of catalase to illuminated chloroplasts indicates that photosynthetic reduction of oxygen has occurred. This effect has been demonstrated both in broken, washed chloroplasts [2] and in intact chloroplasts capable of CO_2 fixation [3–5]. In the latter case endogenous catalase activity presents a difficulty [4] which cannot satisfactorily be overcome by addition of the catalase inhibitors, cyanide and azide, since these compounds also inhibit photosynthetic CO_2 fixation [6].

In the present investigation it is shown that repeated washing of chloroplast preparations results in a loss of catalase activity which is not accompanied by a corresponding decrease in the proportion of intact chloroplasts present. It is concluded that catalase is not a chloroplast enzyme but a contaminant of unwashed chloroplast preparations. Osmotic shock is shown to stimulate chloroplast-associated catalase activity. It is also shown that shock-induced stimulation of catalase activity decreases as the chloroplasts are washed; this finding is inconsistent with the assumption that the effect of osmotic shock can be attributed to release of a putative chloroplast catalase. It is also shown that the inhibitory effect of washing on CO_2-dependent oxygen evolution cannot be a result of contaminant catalase loss, since addition of excess catalase does not prevent the inhibition. It follows that, even in the absence of catalase, accumulation of inhibitory hydrogen peroxide concentrations does not occur during chloroplast photosynthesis under the experimental conditions described.

2. Experimental

Intact chloroplasts were isolated from outdoor-grown spinach in an isotonic sorbitol medium using a conventional technique based on [7] and described in detail [17]. Each washing of a chloroplast suspension consisted of resuspension of the pellet (approx. 1 mg chlorophyll) in an ice-cold medium (25 ml) containing sorbitol (0.33 M), EDTA (2 mM), $MgCl_2$ (5 mM), NaCl (5 mM), sodium ascorbate (10 mM) and Hepes-NaOH (50 mM) at pH 7.6, and subsequent centrifugation of the suspension at 5000 × *g* for 10 s (acceleration 20 s; deceleration 75 s) in an MSE 6L centrifuge. Final resuspension of each pellet in a small volume of the same medium gave a suspension with a chlorophyll concentration of approx. 1 mg ml^{-1} [8]. The proportion of intact chloroplasts present was estimated by measuring the increase in ferricyanide-dependent oxygen evolution that resulted from osmotic shock [9].

Oxygen evolution was measured at 20°C in twin oxygen electrodes (Hansatech, King's Lynn, England) each illuminated (for photosynthetic measurements) by a 300 W (tungsten filament) slide projector incorporating an orange filter. Light intensity was saturating for uncoupled non-cyclic electron transport.

Catalase activity was measured as oxygen evolution on addition of hydrogen peroxide (final conc. 4 mM)

to a reaction mixture (final vol. 1.00 ml) containing sorbitol (0.33 M), Hepes–NaOH (50 mM, pH 7.6) and chloroplasts (20 μg chlorophyll). The chloroplasts were shocked by adding them 2 min before the addition of sorbitol (0.1 ml of 3.3 M).

For measurements of photosynthetic oxygen evolution the reaction mixture (final vol. 1.00 ml) contained sorbitol (0.33 M), EDTA (2 mM), $MnCl_2$ (1 mM), $MgCl_2$ (1 mM), Hepes–NaOH (50 mM, pH 7.6), sodium ascorbate (2 mM), K_2HPO_4 (0.5 mM), $NaHCO_3$ (10 mM) and chloroplasts (50 μg chlorophyll). For ferricyanide-dependent oxygen evolution, ascorbate, K_2HPO_4 and $NaHCO_3$ were replaced by glyceraldehyde (10 mM), $K_3Fe(CN)_6$ (5 mM) and NH_4Cl (5 mM). Ferricyanide-dependent oxygen evolution proceeded in osmotically shocked chloroplasts at rates of approx. 300 μmol (mg chl)$^{-1}$ h^{-1}.

Bovine catalase (crystalline suspension) was purchased from Boehringer, Mannheim, FRG.

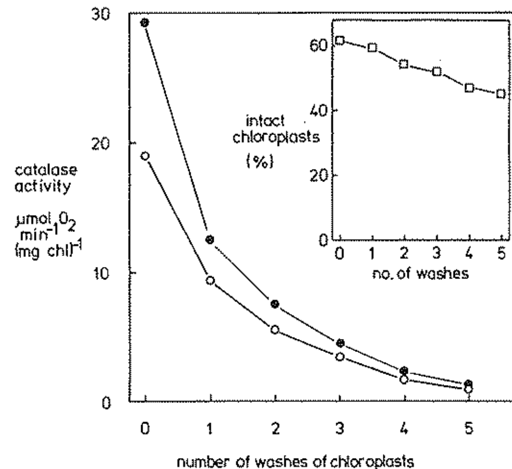

Fig.1. Effect of washing on catalase activity of chloroplast preparations. (●——●) Shocked chloroplasts (a). (○——○) Unshocked chloroplasts (b). (□——□) Percentage of intact chloroplasts.

3. Results and discussion

Figure 1 shows the effect of successive washes of a chloroplast suspension on its associated catalase activity per unit chlorophyll, the catalase assay having been carried out with both shocked and unshocked chloroplasts. In both cases each washing decreased the relative catalase activity to approx. 50% its previous value, so that after five washes less than 5% of the catalase activity of the unwashed preparation remained. This reduction in catalase activity was not accompanied by a corresponding drop in the proportion of intact chloroplasts in the preparation (inset fig.1). The percentage of intact chloroplasts decreased by a similar amount (3.3%) at each wash.

If the increase in catalase activity on osmotic shock of the chloroplasts (fig.1) had been caused by release of an endogenous catalase from the chloroplast stroma on rupture of the chloroplast envelope, then the difference (a – b) between the shocked (a) and unshocked (b) catalase activities should either be unaffected by washing or should decline no more rapidly than does the proportion of intact chloroplasts in the unshocked preparation. Figure 2 shows that this is not the case; the catalase activity apparently released by shock was roughly halved at each wash and therefore declined with successive washes in the same way as the total catalase activity. Similarly, if the effect of osmotic shock is to release catalase from the chloroplast then the effect of washing should be to increase the released catalase when expressed as a proportion of the total catalase present ((a – b)/a). No such increase is seen in fig.2.

These results are therefore consistent with the

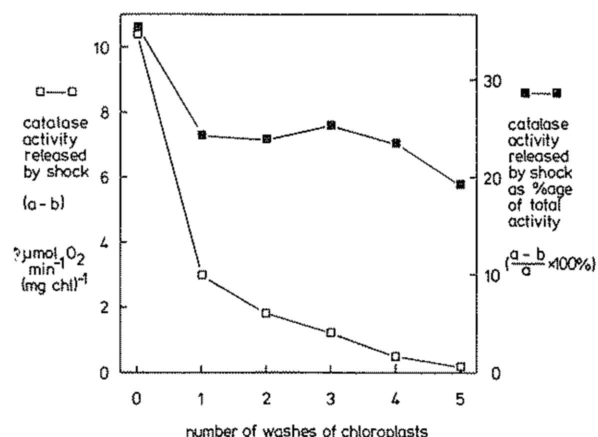

Fig.2. Effect of washing on the catalase activity that is released by osmotic shock of chloroplast suspensions. The values have been calculated from the data of fig.1.

catalase of chloroplast preparations being wholly a contaminant from the cytosol or from another organelle. Absence of catalase from chloroplasts has been indicated by cell fractionation studies [10–12] and by cytochemical localization of catalase in the peroxisome [13]. Partial elimination of catalase activity by washing chloroplast preparations has been reported [14], and although these authors were unable completely to dissociate catalase activity from chloroplasts by this treatment, they also concluded that 'endogenous' catalase is a cytoplasmic contaminant.

Catalase is predominantly if not exclusively a peroxisomal enzyme [10–13]. Thus it is conceivable that the increase by osmotic shock of effective catalase activity is caused by release of catalase on rupture of contaminating peroxisomes. This alternative explanation is ruled out by the results presented in table 1, where it is shown that on centrifugation of a chloroplast suspension (approx. 1 mg chl ml^{-1}) the resulting colourless supernatant contains a catalase which, unlike that of the original suspension, is insensitive to osmotic shock. The recovery of about 75% of the original catalase activity (per unit volume of solution) in the supernatant is additional evidence that there is no necessary connection between catalase and intact chloroplasts. The insensitivity of the catalase of the supernatant to osmotic shock suggests that the effect of osmotic shock observed in the presence of chloroplasts must in some way result from activation of contaminant catalase by a chloroplast component. The nature of this activation is at present unexplained, and the results in table 1 serve merely to indicate that it is osmotic shock of chloroplasts themselves which is responsible for enhancement of catalase activity.

Table 1
Catalase activity of a sample of unwashed chloroplast suspension and of an equal volume of colourless supernatant obtained by centrifugation of the same suspension

Exp. Sample	Catalase activity (nmol O_2 min^{-1})	
	Shocked	Unshocked
1. Chloroplast suspension (20 μg chl)	580	327
Equal volume colourless supernatant	254	251
2. Chloroplast suspension (20 μg chl)	541	320
Equal volume colourless supernatant	236	239

52% of the chloroplasts were intact

Figure 3 shows that the photosynthetic capacity of intact chloroplast preparations declines as the chloroplasts are washed. Though for fig.3 no Calvin cycle intermediates had been added to the reaction, similar results (not shown) have been obtained in the presence ribose-5-phosphate (1 mM). The important feature of fig.3 is that addition of excess catalase does nothing to arrest the decline in CO_2-dependent oxygen evolution which results from washing. It follows that the decline cannot be attributed to depletion of contaminant (or 'endogenous') catalase. The effect of addition of catalase (an increase in rate of about 3 μmol O_2 (mg chl)$^{-1}$ h^{-1}) is small enough to be accounted for by abolition of oxygen uptake in a Mehler reaction in the broken chloroplasts of the preparation. Thus, even where catalase activity has been largely eliminated by washing, illuminated, intact chloroplasts, with CO_2 as electron acceptor, do not

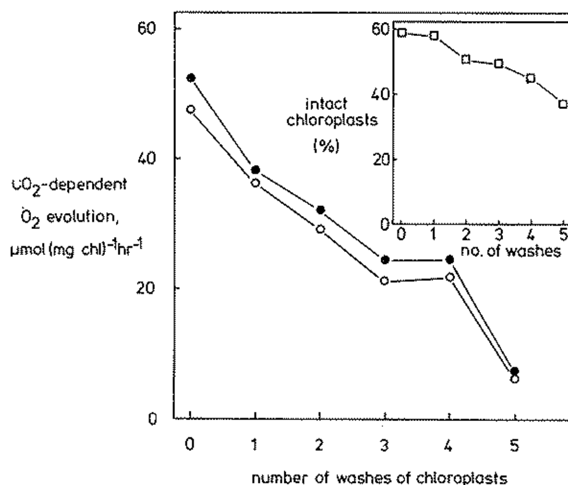

Fig.3. Effect of washing on CO_2-dependent oxygen evolution by a chloroplast preparation containing intact chloroplasts (see inset), in the presence (•——•) and absence (o——o) of added catalase (2 × 10^3 units).

accumulate inhibitory concentrations of hydrogen peroxide.

This conclusion is contrary to that of [4], [6], where catalase inhibitors were used to demonstrate production of hydrogen peroxide during photosynthesis by intact chloroplasts. A possible explanation of this inconsistency is that production of hydrogen peroxide may in fact be induced by cyanide or azide which are known to be inhibitors of CO_2 fixation and which cannot be expected to act as inhibitors of catalase alone.

Production of hydrogen peroxide during photosynthesis by intact chloroplasts in the absence of such inhibitors has nevertheless been reported [3], using washed chloroplast preparations apparently free of catalase. Inhibitory concentrations of hydrogen peroxide could be produced as a result of overloading of the peroxidase reactions [15,16] which under physiological conditions may ensure that the product of photosynthetic oxygen reduction is water rather than hydrogen peroxide. It is possible that hydrogen peroxide is produced only under particular conditions such as low light intensity, and if this is so, then the apparent inconsistency of the present findings with [3] can be resolved, and understood merely to reflect differences in experimental conditions.

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