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EVIDENCE FOR THE INVOLVEMENT OF SUPEROXIDE ANION IN THE ETHYLENE-INDUCED CHLOROPHYLL *a* CATABOLISM OF *RAPHANUS SATIVUS* COTYLEDONS

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Key Word Index—*Raphanus sativus*; Cruciferae; ethylene-induced chlorophyll *a* catabolism; superoxide anion.

Abstract—We have studied the involvement of superoxide anion in the reactions catalysed by chlorophyll *a* bleaching enzyme purified from cotyledons of ethylene-treated radish (*Raphanus sativus*) seedlings. The enzyme catalyses the bleaching of chlorophyll *a* in the presence of H_2O_2 and 2,4-DCP. SOD and scavengers of superoxide anions of a lower molecular weight, such as copper salicylate, NBT, Tiron, hydroquinone, and Mn^{2+}, inhibited the enzymatic bleaching of chlorophyll *a*. The reaction was also inhibited by radical scavengers, such as *n*-propyl gallate and ascorbate. It is suggested that superoxide anions play important roles in the enzymatic chlorophyll *a* bleaching reactions.

INTRODUCTION

Chlorophyll catabolism has been characterized as a biological enigma [1]. Over the entire globe, some 1.2 billion tonnes of chlorophyll are broken down every year to colourless fragments [2]. Surprisingly, the mechanisms for the catabolism of pigments are poorly understood and there is more information about the biosynthesis of chlorophyll in the greening mechanism. Moreover, the biochemical mechanism responsible for the oxidative cleavage of chlorophyll–porphyrin in ethylene-induced and senescent leaves is still largely unknown. This is largely due to the fact that catabolites of chlorophyll catabolism are hardly detected in *in vivo/in vitro* systems. However, knowledge of the intermediate products of chlorophyll catabolism has increased markedly in the last five years [3–7]. This is due chiefly to the development of suitable systems for chlorophyll catabolism studies, and the use of analytical techniques, such as HPLC, HPTLC, FAB-MS and ^{1}H-/^{13}CNMR in the identification of the catabolites.

In the past few years, considerable progress has been made by Matile's group [8–14]. Recently, the chemical structures of some colourless catabolites that accumulate in senescent leaves have been established [11, 12], and such results indicated the involvement of a dioxygenase in the cleavage of chlorophyll–porphyrin [15].

We have studied ethylene-induced chlorophyll catabolism as *in vivo/in vitro* chlorophyll-degrading system [5, 16–22]. Recently, Shimokawa and Uchida [19] and Shimokawa *et al.* [20] reported the possible involvement of superoxide anions in the reactions of chlorophyllide *a* bleaching enzyme purified from ethylene-induced degreening fruits (*Citrus unshiu*). The enzyme catalyses chlorophyllide *a* catabolism in the presence of H_2O_2 and the phenols, 2,4-dichlorophenol or *p*-coumaric acid.

The ethylene-induced chlorophyll *a* bleaching enzyme from cotyledons of radish (*Raphanus sativus*) seedlings also exhibits the same enzymatic properties of H_2O_2 and 2,4-DCP-dependence. Evidence for the involvement of superoxide anions in the enzymatic chlorophyll *a* bleaching reaction is reported in this paper.

RESULTS AND DISCUSSION

Ethylene-induced chlorophyll catabolism was reported in detail [16]. Previous data showed that chlorophyllase plays an important role as a key enzyme in the initial step of chlorophyll catabolism during ethylene-induced degreening of *Citrus unshiu* fruits. In contrast with the fruit system, there was no chlorophyllase activity in *Raphanus* in spite of degreening with ethylene-treated radish cotyledons (data not shown). The chlorophyll content measured at 670 nm absorbance decreased with time in unboiled extracts, but not in boiled preparations. These results show that the catabolism of chlorophyll is not by a nonenzymic reaction but may be catalysed by heat labile enzymes. Typical results from four replicates are shown in Fig. 1 and indicate that chlorophyll *a* bleaching reaction

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Abbreviations: 2, 4-DCP, 2, 4-dichlorophenol; SOD, superoxide dismutase; NBT, nitroblue tetrazolium; Tiron, 1, 2-dihydroxybenzene-3, 5-disulphonic acid.

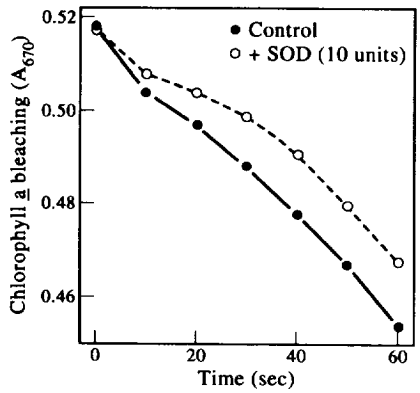


Fig. 1. The effect of superoxide dismutase (SOD) on chlorophyll *a* bleaching. The reaction was started by adding crude enzyme, (●) without SOD, (○) with SOD (10 units/3 ml). The reaction mixture contained the following: 2.46 or 2.47 ml (control) of 0.02M KPi (pH 5.2, with 0.2% Triton X-100), 19.6 μM H_2O_2, 1.333 mM 2,4-DCP, 0.1 mM EDTA, SOD (10 units/3 ml), 13.5 μM chlorophyll *a*, and crude enzyme (0.419 mg protein) in a total volume of 3 ml. Data are typical results from 4 replicates.

was inhibited by SOD. The inhibitory effect was significant at the early stage. SOD (10 units 3 ml^{-1}) inhibited 40% of the control (chlorophyll *a* bleaching without SOD addition) 30 sec after SOD addition. The inhibitory rate of the reaction gradually declined with time. SOD inhibition was not observed in a boiled preparation (for 30 min at 100°). In this chlorophyll *a* bleaching system, H_2O_2 plays a role as a key factor. Its optimum concentration for the reaction was 65.3 μM. When H_2O_2 concentration exceeded 65.3 μM, chlorophyll *a* bleaching activity decreased sharply in the reaction system (data not shown).

To avoid the oxidative reaction catalysed by Cu, Zn-SOD in the presence of H_2O_2 at the high concentrations (4×10^{-5}–1×10^{-4}M) [23, 24], H_2O_2 was added to the reaction mixture at a final concentration of 19.6 μM. The inhibitory effect of SOD for chlorophyll *a* bleaching activity can be reduced by overproduction of H_2O_2.SOD is a ubiquitous enzyme [25] that catalyses the dismutation of superoxide anions to hydrogen peroxide and molecular oxygen [25]. Indeed, SOD is a powerful inhibitor of superoxide-dependent reactions. However, superoxide anion radicals produced at the active site of an enzyme may be inaccessible to a large molecule, such as SOD. Therefore, SOD showed no or weakly inhibitive effects on the enzyme reaction. It may be erroneously concluded that superoxide anions do not participate in the reaction [26].

In order to elucidate the involvement of superoxide anions in the reaction, the inhibitory effect of scavengers of a lower M_r than SOD was examined. Copper chelates, such as copper salicylate, and the copper salts of tyrosine and lysine, copper sulphate, NBT, and Tiron are scavengers of superoxide anions [27, 28]. Thus, copper salicylate, NBT, and Tiron are useful inhibitors in several enzymatic reactions even when SOD is ineffective [29, 30].

Even though copper salicylate is not as catalytically-efficient as SOD, it is of a low molecule relative to the enzyme, which has a M_r of 34 000. deAlvare *et al.* [31] have reported that the copper complex is *ca* 3000-fold less active than the SOD, and the proposed reaction is as follows:

$$Cu(II) + O_2^- \rightarrow Cu(II)O_2^- \rightarrow Cu(I)O_2 \rightarrow Cu(I) + O_2$$

$$Cu(I) + O_2^- \rightarrow Cu(I)O_2^- \rightarrow Cu(II)O_2^{2-} \xrightarrow{2H^+} Cu(II) + H_2O_2.$$

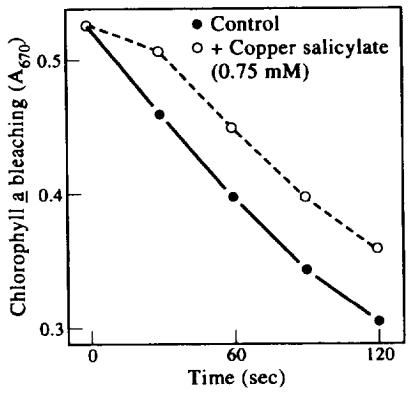
In the case of propyl-/lysyl-hydroxylases, SOD does not inhibit the enzymes, while copper salicylate, copper lysine and copper tyrosine do inhibit them [27]. Younes and Weser [26] have also reported that the activity of pig kidney diamine oxydase was not affected by SOD but was suppressed by copper tyrosine, copper lysine and copper sulphate. Similarly, the activity of catechol dioxygenase is not inhibited by SOD but is inhibited by copper salicylate [29].

We have investigated the involvement of superoxide in the 'chlorophyll *a*-oxidative cleavage reaction' with copper salicylate (complexes) as well as with SOD. The copper complexes have been shown to act as SOD with catalytic efficiencies. Indeed, the involvement of superoxide has been reported in the reaction mechanisms of diamine oxidase [26], propyl hydroxylase [27], and lysyl-hydroxylase [27] with the use of copper complexes.

As shown in Fig. 2A, the inhibitory effect of copper salicylate (0.75 mM) in the reaction was the most prominent at the early stage, as in the SOD-inhibited reaction (Fig.1). As the reaction proceeded, the inhibitory rate of the reaction decreased from 70 to 24%. Fig. 2B shows the effect of copper salicylate concentration on the chlorophyll *a* bleaching reaction for 1 min. There was a linear correlation between the copper salicylate concentration and its inhibitory effect on the reaction. Furthermore, the reaction was completely inhibited by copper salicylate at a concentration higher than 1 mM for 30 sec.

Superoxide anions can be measured based on their reductive effect on NBT [32, 33]. Superoxide anions effectively reduce NBT to the blue formazan, measured at 560 nm and, therefore, NBT is considered an effective scavenger. Myllyla *et al.* [27] have shown that SOD does not inhibit the activity of lysyl-/propyl-hydroxylases, while NBT will. They suggested that SOD cannot inhibit propyl-hydroxylase activity due to the inability of SOD to come into contact with the active site of the hydroxylase. In the case of diamineoxydase, a similar phenomenon was reported [26].

As shown in Fig. 3A, the amount of blue formazan (expressed as absorbance at 560 nm) increased linearly proportional to the NBT concentration in the chlorophyll *a* bleaching reaction. Although H_2O_2 included in the reaction mixture also decreases NBT, its effect was found to be smaller compared with that of superoxide anion by the absorbance of 0.02 in the reaction mixture without chlorophyll *a* bleaching enzyme. In the inhibition of copper salicylate, the inhibitory effect of NBT correlated with the NBT concentration in a linear fashion (Fig. 3B).



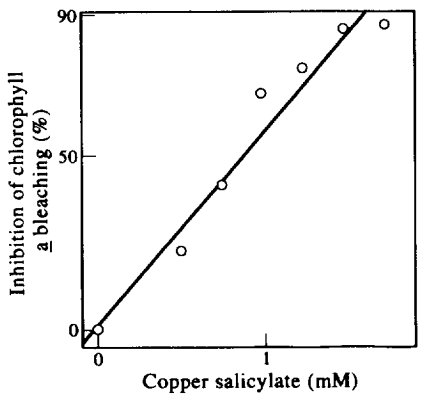
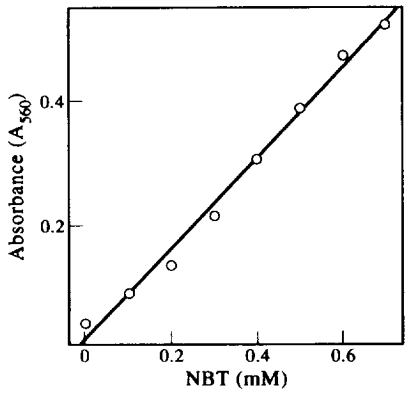


Fig. 2. A. The effect of copper salicylate on chlorophyll *a* bleaching. The reaction was started by adding crude enzyme, (●) without copper salicylate, (○) with copper salicylate (0.75 mM). The reaction mixture contained the following: 2.3 or 2.4 ml (control) of 0.02 M KPi (pH 5.2, with 0.2% Triton X-100), 65.3 μM H_2O_2, 1.333 mM 2,4-DCP, 0.1 mM EDTA, 0.75 mM copper salicylate, 13.5 μM chlorophyll *a*, and crude enzyme (0.419 mg protein) in a total volume of 3 ml. Data are the average of six replicates. B. The effect of copper salicylate concentration on the inhibition of chlorophyll *a* bleaching. The reaction mixture and conditions were as indicated in Fig. 2A. Reaction time was 60 sec.



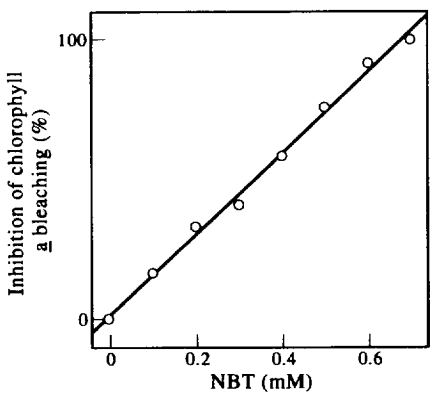


Fig. 3. A. The reduction of nitroblue tetrazolium (NBT) in the chlorophyll *a* bleaching system. The reaction was started by adding crude enzyme. Reduced NBT was expressed as the increased absorbance at 560 nm for 5 min. The reaction mixture was as indicated in Fig. 2A. B. The inhibition of chlorophyll *a* bleaching by NBT reduction. The reaction time was 5 min. The reaction mixture was as indicated in Fig. 2A.

The inhibitory effects of other scavengers on the chlorophyll *a* bleaching reaction are shown in Table 1. The chlorophyll *a* bleaching reaction was inhibited by Tiron, hydroquinone, $Mn^{2+}(MnCl_2.4H_2O)$, *n*-propyl gallate and ascorbate.

These results indicate the involvement of superoxide anions in the chlorophyll *a* bleaching reaction catalysed by ethylene-induced enzyme(s). Our system described here is especially suitable for the study of 'chlorophyll *a*-oxidative cleavage enzyme' because the kinetics of disappearance of chlorophyll *a*-catabolities of red-fluorescing compounds are concomitant with the loss of chlorophyll *a* (HPLC/HPTLC data not shown). In the chlorophyll *a* breakdown system, even the low M_r fragments have not been identified or isolated *in vitro*. We are now trying to purify the chlorophyll *a*-oxidative cleavage enzyme.

Table 1. Effect of various inhibitors on chlorophyll *a* bleaching activity

Inhibitors*		Chlorophyll *a* bleaching activity (% of control)
Control		100
Tiron	(1 mM)	− 0.5
Hydroquinone	(1 mM)	5
n-Propyl gallate	(1 mM)	− 5.5
Ascorbate	(1 mM)	17.5
Mn^{2+}	(1 mM)	3
	(0.1 mM)	60
	(0.01 mM)	90

*The reaction mixture contained the following: 2.1 or 2.4 ml (control) of 0.02 M KPi (pH 5.2, with 0.2% Triton X-100), 65.3 μM H_2O_2, 1.333 mM 2,4-DCP, 0.1 mM EDTA, inhibitors (0.01–1 mM), 13.5 μM chlorophyll *a* and crude enzyme (0.419 mg protein) in a total volume of 3 ml.

EXPERIMENTAL

Materials. Six-day-old intact radish (*Raphanus sativus* L.) seedlings were obtained from a local farm and treated within 2 hr.

Ethylene treatment. The seedlings were exposed to 100 ppm ethylene for 12 hr in a moistened sponge in a 30 l plastic box and also incubated with a beaker containing 50 ml of 5% KOH to absorb CO_2. The seedlings were further incubated in fresh air for 12 hr. All expts were carried out in the dark at 25°.

Prepn of Me_2CO *powder.* The excised cotyledons were immersed in cold (− 20°) Me_2CO, homogenized in a Waring blender, then filtered through a Buchner funnel. The ppt. was dried *in vacuo* and ground to a powder using a mill.

Prepn of enzyme extracts. The crude enzyme extract was prepd as described previously [17]. The Me_2CO powders (5 g) were extracted by stirring with 400 ml of 0.02M KPi (pH 7.0, with 0.2% Triton X-100) for 30 min at 25°. The filtrate was centrifuged at 12 000 *g* for 20 min. The supernatant was used as the crude enzyme extract.

Chlorophyllase activity. The chlorophyllase activity was determined according to the method of ref. [17].

Measurement of chlorophyll a bleaching activity. It was based on spectrophotometric recording of chlorophyll *a* bleaching at 670 nm. The standard reaction mix. contained the following: 2.4 ml of 0.02 M KPi (pH 5.2, with 0.2% Triton X-100), 65.3 μM H_2O_2, 1.333 mM 2, 4-DCP, 0.1 mM EDTA, 13.5 μM chlorophyll *a*, and crude enzyme (0.419 mg protein) in a total volume of 3 ml. Activity is expressed as the decreased absorbance at 670 nm for 10 min per mg protein. The enzymatic assay was carried out under green light at 25°.

Prepn of copper salicylate. Copper(II) salicylate was prepd as described [31].

Measurement of NBT reduction. NBT reduction was determined by measuring the increase in absorption at 560 nm [32].

Protein content. Protein was determined as described previously [34], using bovine plasma γ -globulin as the standard.

Reagents. Cu, Zn-superoxide dismutase was purchased from Toyobo Co., Ltd. (Osaka, Japan). Chlorophyll *a* (pure, 99.0%) from *Chlorella* was obtained from Wako Pure Chemical Industries Co., Ltd.

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