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CYSTEINE AND CROCIN OXIDATION CATALYZED BY HORSERADISH PEROXIDASE

W.F. OSSWALD, W. SCHÜTZ and E.F. ELSTNER

Institut für Botanik und Mikrobiologie, Biochemisches Labor, Technische Universität, Arcisstraße 21, 8000 München 2

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The amino acid cysteine is oxidized by horseradish peroxidase, and the water-soluble carotenoid crocin is bleached by cooxidation. The monophenol p-hydroxyacetophenone stimulates oxygen uptake, cysteine oxidation and crocin bleaching, whereas its concentration does not change. Superoxide dismutase significantly enhances all these oxidative reactions. Addition of H_2O_2 is not required for these peroxidase-catalyzed oxidations.

KEY WORDS: Cysteine oxidation, crocin oxidation, p-hydroxyacetophenone, superoxide dismutase, horseradish peroxidase.

ABBREVIATIONS: HRP: Horseradish peroxidase; pHAP: p-hydroxyacetophenone; SOD: Superoxide dismutase; DTNB: Dithio-bis-nitrobenzoic acid NMBA: 2-nitro-5-mercaptobenzoic acid.

INTRODUCTION

Pigment bleaching and lipid peroxidation are characteristic phenomena of leaf senescence or intoxication after fumigation with air pollutants such as sulfur dioxide or ozone. Chorophyll *a* and *b* may be bleached in the light under conditions of blocked or "overreduced" electron transport.[1] Different reactive oxygen species are discussed to initiate these oxidative bleaching reactions,[2] which may also be catalyzed by enzymes. Kuroki *et al.*[3] described a soluble chlorophyllase from tea leaf sprouts which degrades chlorophyll. Pigments are also frequently cooxidized by alkoxy radicals generated via lipid peroxidation initiated by lipoxygenase.[4] In addition, there are several reports concerning pigment degradation catalyzed by peroxidase.[5,6,7] In most cases hydrogen peroxide and certain phenolic compounds are involved in these reactions. Niinomi *et al.*[8] demonstrated that peroxidase, H_2O_2 and phenolic compounds initiate lipid peroxidation. We recently reported on Indole-3-acetic acid oxidation by horseradish peroxidase (HRP) and simultaneous polyene cooxidation, representing a simplified model for one possible basic reaction involved in the initial processes of plant cell aging.[9]

In this paper we demonstrate that the polyene crocin, a water soluble model substance representing both the groups of plant pigments as well as polyene structures, is oxidized by HRP at the expense of cysteine without added H_2O_2. The stimulation by superoxide dismutase of both crocin and cysteine-oxidation is discussed as the prevention of the $O_2^{\cdot-}$-dependent removal of the final crocin oxidizing species, the sulfenyl- and/or the peroxy-sulfenyl radical of cysteine. These may be intermediarily formed by HRP catalysis as proposed by Harman *et al.*[10].

MATERIAL AND METHODS

Oxygen uptake was measured polarographically at 25°C with an oxygen electrode (Rank Brothers, Bottisham, Cambridge, England). An air saturated phosphate buffer (100 mM, pH 7.8, Chelex treated) was used. Cuvettes contained horseradish peroxidase (10 U/2 ml), cysteine (0.5 mM), pHAP (0.5 mM) and crocin (10 μM). 1, 5, 10, 50 or 100 units of superoxide dismutase were added at times indicated.

Monitoring of crocin oxidation

Crocin oxidation was measured at 436 nm using a Uvikon 810 spectrophotometer (Kontron, Eching).

Monitoring cysteine oxidation

Cysteine was determined according to Ellman[11] modified as follows: 5,5-dithiobis-(2-nitrobenzoic acid) (1 mM) was dissolved in MeOH-NaOH (0.1 N) solution (98:2, v/v). After the times indicated, 100 μl of the reaction mixture was added to 900 μl of the DTNB solution. The precipitated phosphate buffer was centrifuged in a microcentrifuge for 5 min. Formation of 2-nitro-5-mercapto-benzoic acid (NMBA) was quantified spectrophotometrically at 312 nm.

RESULTS

Oxygen uptake during cysteine oxidation by HRP is demonstrated in table I. A small rate of oxygen uptake was measured during the oxidation of cysteine by HRP which

TABLE I
Oxygen uptake during cysteine oxidation by horseradish peroxidase

Experiment	Oxygen uptake (n mol/min)
HRP + Cys	2
HRP + Cys + Crocin	4
HRP + Cys + pHAP	39
HRP + Cys + pHAP + Crocin	2

Oxygen uptake was measured at 25°C with an oxygen electrode. The reaction mixture contained in 2 ml: HRP (10 U), cysteine (0.5 mM), pHAP (0.5 mM) and crocin (10 μM). An air-saturated 100 mM phosphate buffer (Chelex treated) pH = 7.8 was used.

TABLE II
Stimulation of oxygen uptake by SOD during HRP-catalyzed cysteine oxidation

	SOD (U/2 ml)				
	0	5	10	50	100
Experiment	per cent stimulation of oxygen uptake				
Cys + HRP + pHAP	0	0	28	72	76
Cys + HRP + Crocin	0	127	242	255	242
Cys + HRP + pHAP + Crocin	0	290	330	1365	2050

Concentrations for HRP, cysteine, pHAP and crocin were used as indicated in table 1. SOD was added three minutes after the reaction was started with HRP.

was stimulated by crocin as well as by p-hydroxyacetophenone (pHAP). The stimulation of oxygen uptake by pHAP was reduced to the initial rate after crocin addition. In table II it is shown that superoxide dismutase stimulated oxygen uptake during cysteine oxidation by HRP in the prescence of either pHAP, or crocin as well as by both crocin and pHAP.

In figure 1 it is shown that 2-nitro-5-mercaptobenzoic acid also is a substrate for HRP in a pHAP-stimulated reaction. Figure 2 represents the calibration curve for cysteine according to the modified Ellman reaction. Figure 3 shows that pHAP stimulates cysteine oxidation by HRP. Cysteine decomposition in the presence or absence of pHAP is further increased by superoxide dismutase (table III).

During cysteine oxidation by HRP the polyene crocin is bleached (Figure 4). This bleaching reaction is further stimulated by pHAP. Polyene oxidation in the presence or absence of pHAP is enhanced by superoxide dismutase (Table IV).

DISCUSSION

In this paper we demonstrate that the polyene crocin is oxidized at the expense of cysteine catalyzed by horseradish peroxidase in the absence of hydrogen peroxide.

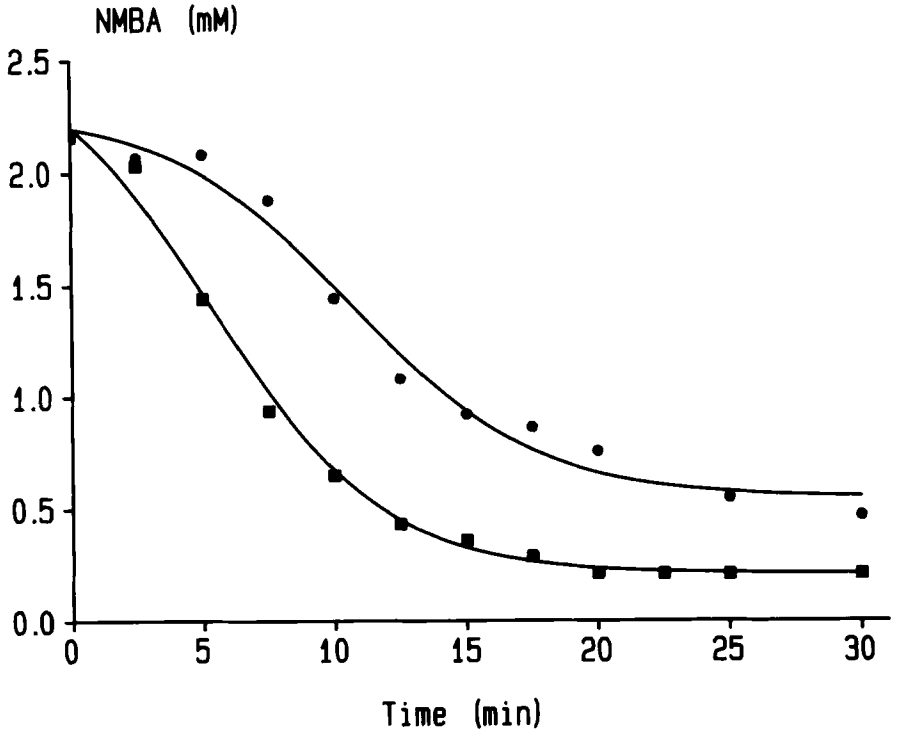

FIGURE 1 Oxidation of 2-nitro-5-mercaptobenzoic acid by horseradish peroxidase. Reaction mixture (2 ml): cysteine (0.5 mM) and DTNB (1 mM) with (■) and without (●) pHAP (0.5 mM). After one minute 10 units HRP were added.

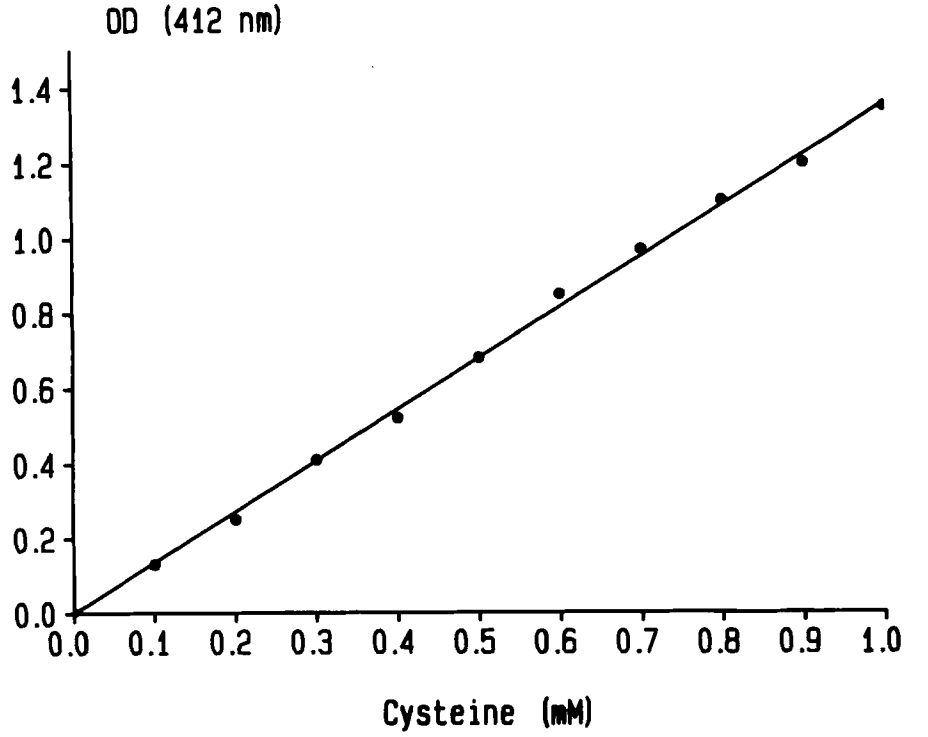

FIGURE 2 Standard calibration curve for cysteine determination. Cysteine concentration was determined according to Ellman[11] modified as described in Materials and Methods.

The monophenol p-hydroxyacetophenone stimulates oxygen uptake, cysteine oxidation as well as crocin oxidation.

Cysteine oxidation by HRP may proceed via the thiyl radical (CyS·).[10] Hydrogen peroxide and superoxide ions may be formed intermediarily by autoxidation of the thiol[12] catalyzed by the heme group of HRP. The oxidation of further thiol groups in the presence of H_2O_2 involves the formation of compound I and II of HRP.[13] Addition of oxygen to the thiyl radical apparently takes place with fast reaction kinetics ($k = 8.1 \times 10^9\ M^{-1} \times sec^{-1}$).[14] Evidence for an oxygen addition product forming a peroxy sulphenyl radical was reported by Al Thannon *et al.*[15] The existence of such a peroxy radical was suggested as an intermediate in pulse radiolysis study of cysteine in the presence of oxygen. In addition Sevilla *et al.*[16] demonstrated that the cysteinyl radical, after the addition of oxygen, forms the highly reactive sulfenyl radical and atomic oxygen.

$$Cys\cdot + O_2 \rightarrow Cys\text{-}OO\cdot \rightarrow Cys\text{-}O\cdot + (O) \qquad \text{(reaction 1)}$$

These sulfenyl radicals might partially be responsible for crocin oxidation. However, the polyene might also be oxidized by the peroxy radical via hydrogen abstraction.[14]

$$Cys\text{-}O\cdot \text{ (or } Cys\text{-}OO\cdot) + Crocin \rightarrow Cys\text{-}OH \text{ or } (Cys\text{-}OOH) + Crocin_{ox}$$

(reaction 2)

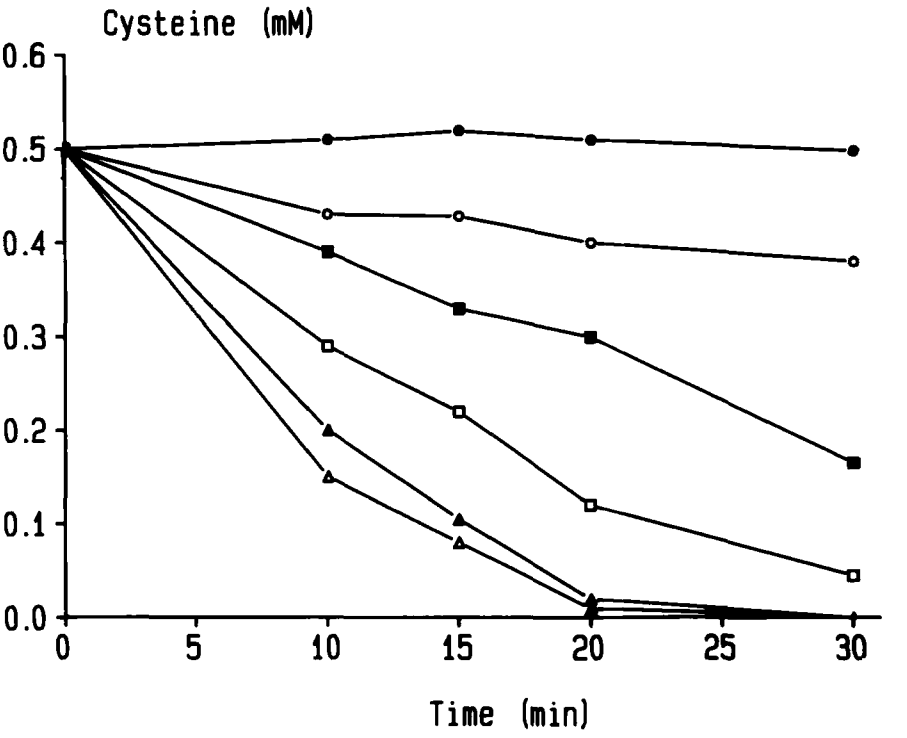

FIGURE 3 Cysteine oxidation catalyzed by HRP and pHAP. Cysteine concentration was measured as described in Figure 2. Reaction mixture (2 ml): cysteine (0.5 mM), HRP (10 U) and different pHAP concentrations. The reaction was repeated three times with identical results. Symbols: Cysteine alone, ●; cysteine and HRP, ○; cysteine, HRP and pHAP at 5 μM (■), 10 μM (□), 25 μM (▲) and 50 μM (△).

The stimulatory effect of monophenolic compounds on HRP catalyzed reactions is well established. p-hydroxyacetophenone is oxidized by compound I forming reactive alkoxy radicals (pHAP-O·) which might be responsible for enhanced crocin bleaching.

TABLE III
Stimulation of HRP-catalyzed cysteine oxidation by SOD

	SOD (U/2 ml)					
	0	1	5	10	50	100
Experiment	per cent stimulation of cysteine oxidation					
Cys + HRP	0	7	35	42	80	81
Cys + HRP + pHAP	0	2	21	27	46	61

Cysteine oxidation was monitored photometrically at 412 nm. The reaction mixture contained in 2 ml: HRP (10 U) and cysteine (0.5 mM). SOD was added after the reaction was started with HRP.

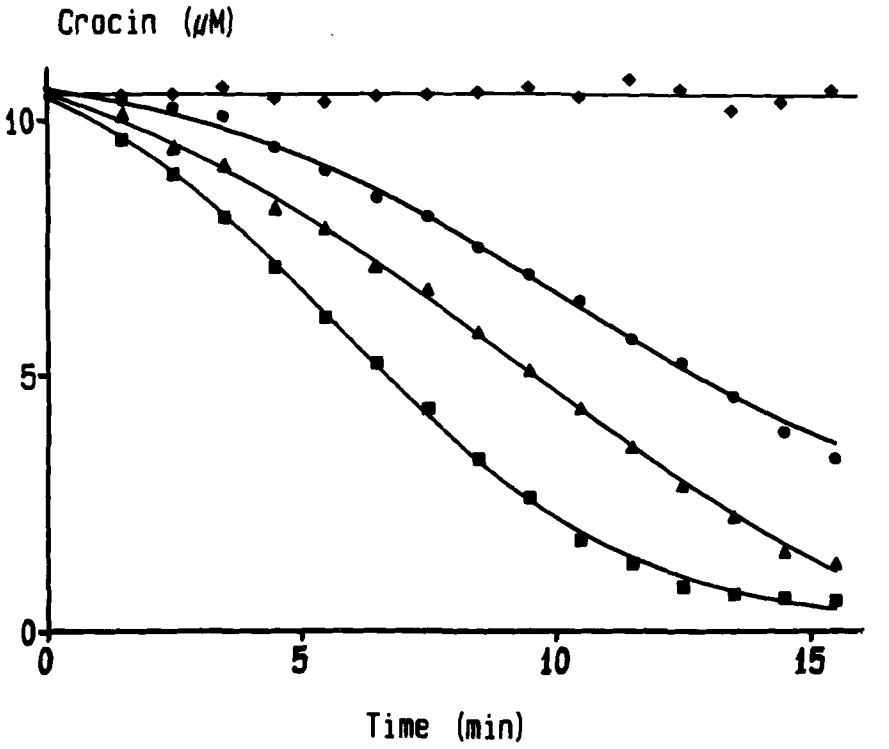

FIGURE 4 Crocin oxidation catalyzed by horseradish peroxidase and cysteine and different concentrations of pHAP. Reaction mixture (2 ml): crocin (10.2 μM), cysteine (0.5 mM), HRP (10 U) and different pHAP-concentrations. The reaction was repeated three times with identical results. Symbols: Cysteine or HRP, ◆; cysteine and HRP, ●; cysteine, HRP and pHAP at 100 μM (▲) and 500 μM (■).

$$\text{pHAP-O}\cdot + \text{Crocin} \rightarrow \text{pHAP} + \text{Crocin}_{ox} \qquad \text{(reaction 3)}$$

This may explain the stimulatory effect of pHAP on cysteine as well as on crocin oxidation.

As shown in tables II, III and IV superoxide dismutase stimulates oxygen uptake, cysteine oxidation and crocin bleaching both in the presence or absence of pHAP. It is postulated that peroxy sulphenyl radicals react with superoxide forming non radical products.[17]

TABLE IV
Stimulation of HRP-catalyzed crocin oxidation by SOD

	SOD (U/2 ml)							
	0	1	2	5	10	20	50	100
Experiment	per cent stimulation of crocin oxidation							
HRP + Cys	0	3	16	20	21	22	22	21
HRP + Cys + pHAP	0	16	–	20	24	42	80	90

Crocin oxidation was monitored photometrically at 436 nm. The reaction mixture contained in 2 ml: HRP (10 U) cysteine (0.5 mM). SOD was added after the reaction was started with HRP.

$$\text{Cys-OO}\cdot \xrightarrow{O_2^-} \text{Cys-OOH} \qquad \text{(reaction 4)}$$

This reaction might diminish the concentration of reactive Cys-OO·- as well as Cys-O·- species (compare also reaction 1). Superoxide dismutase prevents reduction of peroxy sulphenyl radicals via dismutation of superoxide to hydrogen peroxide resulting in higher amounts of Cys-OO· as well as Cys-O· radicals. According to the reaction of GS-OO· with glutathione[17] the Cys-OO· radical might react as follows:

$$\text{CysH} + \text{Cys-OO}\cdot \rightarrow \text{Cys}\cdot + \text{CysOOH} \qquad \text{(reaction 5)}$$

Therefore, cysteine oxidation, oxygen uptake and crocin bleaching are accelerated in the presence of superoxide dismutase.

The *in vitro* reactions presented in this paper may be taken as a further contribution to possible mechanisms of enzyme-catalyzed polyene oxidation such as pigment bleaching and/or lipid degradation. During plant senescence or exposition to air pollutants such as SO_2, O_3 or HF chlorophyll is bleached and fatty acids are degraded. These decomposition reactions are often accompanied by an increase in peroxidase activity.[18,19,20]

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References

1. Elstner, E.F. and Osswald, W.F., Z. Naturforsch., 35c, 129–135, (1980).
2. Elstner, E.F., *Ann. Rev. Plant Physiol.*, **33,** 73–96, (1982).
3. Kuroki, M., Shiioi, Y. and Sasa, *T. Plant Cell Physiol.*, **22,** 717–725, (1981).
4. Grosch, W. and Laskawy, *G. Biochim. Biophys. Acta,* **575,** 439–445, (1979).
5. Huff, A., Phytochemistry, **21/2,** 261–265, (1982).
6. Matile, Ph., Z. Pflanzenphysiol., **99,** 475–478, (1980).
7. Woods, A., *Centralbl. Bakteriol., Parasitenku. Infektionskr.*, **22,** 745–754, (1899).
8. Niinomi, A., Morimoto, M. and Shimizu, S., *Plant Cell Physiol.*, **28/4,** 731–734, (1987).
9. Osswald, W.F., Schütz, W. and Elstner, E.F., *Plant Physiol.*, **86,** 1310–1314, (1988).
10. Harman, L.S., Mottley, C. and R.P. Mason, R.P., *J. Biol. Chem.*, **259,** 5606–5611, (1984).
11. Ellman, G.L., *Arch. Biochem. Biophys.*, **74,** 443–450, (1958).
12. Misra, H.P., *J. Biol. Chem.*, **249,** 2151–2155, (1974).
13. Yamazaki, I., Tamura, M. and Nakajima, R., Molec. *Cell Biochem.*, **40,** 143–153, (1981).
14. Schäfer, K., Bonifac, M., Bahnemann, D. and Asmus, K.D., *J. Phys. Chem.*, **82,** 2777–2780, (1978).
15. Al-Thannon, A.A., Barton, J.P., Packer, J.E., Sims, R.S., CN Trumbore, C.N. and Winchester, *R.V., Int. J. Radiat. Phys. Chem.* **6,** 233–248, (1974).
16. Sevilla, M.D., Becker, D., Stewarts, S. and Herrington, *J., Biochem. Biophys. Res. Com.*, **144/2,** 1037–1042, (1987).
17. Wefers, H., Riechmann, E. and Sies, H., *J. Free Rad. Biol.*, **1,** 311–318, (1985).
18. Keller, T., Schweizer. Z. Forstwesen, **127,** 237–251, (1976a).
19. Curtis, C.R. and Howell, R.K., Phytopathology, **61,** 1306–1307, (1971).
20. Keller, T. and Schwager, *H., Eur. J. Forest Pathol.*, **1,** 6–18, (1971).

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