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Inhibition of lipid autoxidation by bovine superoxide dismutase

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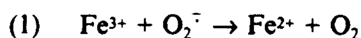
For autoxidation, the initiation reactions, which are believed to be responsible for the formation of hydroxyl radicals, are inhibited by superoxide dismutase (SOD). Soybean lipoxygenase types I, IV and V were not inhibited by SOD in model systems containing linoleic acid.

Autoxidation in a low-iron model system was further reduced by SOD. Autoxidation in the presence of haemin was rapid but also inhibited by SOD. These observations indicate that SOD mainly prevents the formation of hydroxyl radicals via the Haber–Weiss reaction during the iron-catalysed oxidation of linoleic acid, although it is possible that the enzyme might also inhibit haemin-catalysed oxidation by scavenging an oxy–haem complex.

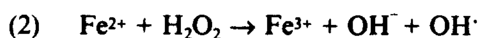
INTRODUCTION

Lipid peroxidation is a major cause of quality deterioration in foods and is a significant factor affecting the stability and nutritional value of many foods (Chan, 1987). It can be initiated either by enzymes such as lipoxygenases (LOX), which catalyse the formation of hydroperoxides, by metal ions and other constituents or possibly by irradiation, which gives rise to free radicals.

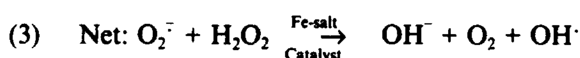
Free radicals of oxygen are often proposed to be the causative agents of unsaturated fatty acid peroxidation, with the hydroxyl radical ($OH^{\cdot}$) considered to be ultimately responsible for the initiation of peroxidation reactions (Miller & Aust, 1989). In the presence of trace amounts of metal ions, such as iron, $OH^{\cdot}$ can be formed from the superoxide radical ($O_2^{\bar{\cdot}}$) and hydrogen peroxide (H_2O_2) via the metal-catalysed Haber–Weiss reaction (3). The reaction involves the coupling of the reduction of Fe^{3+} by $O_2^{\bar{\cdot}}$ and the reoxidation of Fe^{2+} by H_2O_2 (the Fenton reaction) (2).



(1) $Fe^{3+} + O_2^{\bar{\cdot}} \rightarrow Fe^{2+} + O_2$



(2) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\cdot}$



(3) Net: $O_2^{\bar{\cdot}} + H_2O_2 \xrightarrow[\text{Catalyst}]{\text{Fe-salt}} OH^{-} + O_2 + OH^{\cdot}$

Thus, $O_2^{\cdot}$ may have an important role during the peroxidation of unsaturated fatty acids and possibly other susceptible substances.

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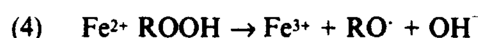
$OH^{\cdot}$ is a strong oxidising agent and is believed to initiate fatty acid peroxidation by the abstraction of a hydrogen atom, from a molecule of unsaturated fatty acid. Once initiation has occurred, a free radical chain process of peroxidation continues autocatalytically until two radicals combine to terminate the reaction (Chan, 1987).

Metal ions, such as iron, play an important role in fatty acid peroxidation. They are involved not only in initiation reactions via the formation of $OH^{\cdot}$ (reactions (1) and (2)) but also in propagation reactions by the decomposition of hydroperoxides:

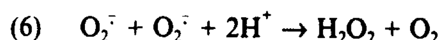


(4) $Fe^{2+}\ ROOH \rightarrow Fe^{3+} + RO^{\cdot} + OH^{-}$

(5) $Fe^{3+} + ROOH \rightarrow Fe^{2+} + H^{+} + ROO^{\cdot}$

where ROOH represents the fatty-acid hydroperoxide. In general, the reductive pathway (reaction (4)) is favoured; however, the two reactions can operate in a cycle to produce the alkoxy radical ($RO^{\cdot}$) and the peroxy radical ($ROO^{\cdot}$).

Superoxide dismutase (SOD) (EC 1.15.1.1) catalyses the dismutation of $O_2^{\bar{\cdot}}$:



(6) $O_2^{\bar{\cdot}} + O_2^{\bar{\cdot}} + 2H^{+} \rightarrow H_2O_2 + O_2$

It is present in almost all living cells exposed to oxygen and is considered to constitute an essential defence against oxygen toxicity (McCord *et al.*, 1971). The use of SOD as an antioxidant in food was first discussed by Michelson and Monod (1975), who showed that SOD inhibited peroxidation in anchovies and browning in

fruits and vegetables and that a number of model oxidation reactions were also inhibited. Other workers have shown the antioxidant effect of SOD in milk (Aurand *et al.*, 1977; Hill *et al.*, 1977) and in fatty acid model systems containing xanthine oxidase (Kellogg & Fridovich, 1975) and lipoxygenase (Richter *et al.*, 1975). Recently, Lingnert *et al.*, (1989) have confirmed the inhibitory effect of SOD from yeast (*Saccharomyces cerevisiae*) on the oxidation of ascorbic acid, autoxidation of linoleic acid and Cu^{2+}-catalysed oxidation of cholesterol.

However, little has been published on the mode of action of SOD as an antioxidant in model food systems. The present study investigated the role of bovine SOD both in the LOX-catalysed oxidation of linoleic acid and during the autoxidation in model systems containing different amounts of iron and haemin. Unlike Richter *et al.*, (1975), the inhibition of the peroxidation of linoleic acid with bovine SOD catalysed by LOX types I, IV or V was not observed in the present study.

MATERIALS AND METHODS

Linoleic acid (99%), Tween 20, lipoxidase (LOX) type I, IV and V, conalbumin and haemin, were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Bovine superoxide dismutase was obtained from BCL (Lewes, East Sussex, UK). All other chemicals were analytical reagent grade.

LOX-catalysed oxidation of linoleic acid

LOX solutions were made by dilution with buffer to give activities of 3660 units/ml.

Bovine SOD solutions were made by dilution with distilled water to give concentrations of 0·2, 0·5 and 4 mg/ml.

The reaction mixture contained 1 ml of substrate, 0·1 ml of LOX type I, IV or V, 0·1 ml of SOD and 0·8 ml of buffer. The substrate was 2·66 μM of linoleic acid, emulsified by shaking with ethanol (Richter *et al.*, 1975) or Tween 20, 0·5% in sodium-borate buffer (0·2 M, pH 8·6). This solution was stable for the duration of the test.

The reaction was started by the addition of the LOX, and the increase in $OD_{234\,nm}$ was followed using a Pye Unicam spectrophotometer.

Autoxidation of linoleic acid and the effect of bovine SOD (5 μg/ml)

A solution of linoleic acid (2·5 mM) was prepared by emulsification with Tween 20 (0·5%) in sodium-phosphate buffer (50 mM, pH 7·8). Aliquots (20 ml) were placed in clean, disposable plastic bottles, and SOD was added to give a final concentration of 5 μg/ml. The bottles were incubated open to the air in the dark at 20°C. The oxidation of linoleic acid was followed by measuring the $OD_{234\,nm}$ of a 100-μl sample of the incubation solution diluted to 2 ml with distilled water. A corresponding control was performed in the absence of SOD.

Autoxidation of linoleic acid in a low-iron buffer

The previous experiment was repeated, except that low-iron buffer, made by dialysis against conalbumin (Gutteridge, 1987), was used to prepare the solution of linoleic acid. Controls were performed in the absence of SOD, in normal and low-iron buffer.

Haemin-catalysed oxidation of linoleic acid

Autoxidation was carried out in the presence of haemin at a final concentration of 20 μM. SOD was added to give a final concentration of 5 μg/ml. A corresponding control was performed in the absence of SOD.

RESULTS AND DISCUSSION

Effect of SOD on LOX-catalysed oxidation

It was observed that SOD (10–200 μg/ml) did not inhibit the rate of LOX-catalysed oxidation of linoleic acid emulsified with Tween 20 (0·5%) for each type of soybean LOX (Table 1). Surprisingly, when emulsified with ethanol, the LOX-catalysed oxidation of linoleic acid was increased significantly in the presence of 200 μg/ml of SOD for LOX type I, IV and V by 19, 47 and 26%, respectively (Table 2). These results differ from the findings of Richter *et al.* (1975), who reported that SOD (4 μM, 132 μg/ml) reduced the formation of

Table 1. LOX-catalysed oxidation of linoleic acid emulsified with 0·5% Tween 20[a]

	$OD_{234\,nm}$ (min^{-1})	SD
LOX type I	0·261	0·038
LOX type I + 10 μg/ml bovine SOD	0·265	0·012
LOX type I + 25 μg/ml bovine SOD	0·256	0·01
LOX type I + 200 μg/ml bovine SOD	0·271	0·011
LOX type IV	0·392	0·006
LOX type IV + 10 μg/ml bovine SOD	0·355	0·03
LOX type IV + 25 μg/ml bovine SOD	0·385	0·01
LOX type IV + 200 μg/ml bovine SOD	0·372	0·007
LOX type V	0·234	0·016
LOX type V + 10 μg/ml bovine SOD	0·221	0·007
LOX type V + 25 μg/ml bovine SOD	0·219	0·005
LOX type V + 200 μg/ml bovine SOD	0·245	0·011

[a] Values are the mean of three experiments; none differ significantly from their respective control.

Table 2. LOX-catalysed oxidation of linoleic acid emulsified with ethanol[a]

	$OD_{234\,nm}$ (min^{-1})	SD
LOX type I	0·276	0·008
LOX type I + 10 μg/ml bovine SOD	0·268	0·01
LOX type I + 25 μg/ml bovine SOD	0·271	0·01
LOX type I + 200 μg/ml bovine SOD	0·329[b]	0·021
LOX type IV	0·546	0·1
LOX type IV + 10 μg/ml bovine SOD	0·523	0·14
LOX type IV + 25 μg/ml bovine SOD	0·506	0·09
LOX type IV + 200 μg/ml bovine SOD	0·807[c]	0·02
LOX type V	0·279	0·008
LOX type V + 10 μg/ml bovine SOD	0·289	0·008
LOX type V + 25 μg/ml bovine SOD	0·285	0·02
LOX type V + 200 μg/ml bovine SOD	0·352[d]	0·02

[a] Values are the mean of three experiments.
[b] <1% significance.
[c] <2% significance.
[d] <0·2% significance.

lipid peroxide to 39% of the control value catalysed by the LOX type I isoenzyme. However, the present investigations (Table 3) have shown that bovine serum albumin (BSA) reduces the oxidation of linoleic acid catalysed by LOX type I. It is possible that, in 1975, BSA may have been inadvertently present as a contaminant in Richter's SOD; thus, it may account for the differences between the results.

It is generally accepted that the 'native' lipoxygenase-I (LOX-I) contains iron predominantly in the Fe(II) state (Slappendel *et al.*, 1981), where it cannot co-ordinate dioxygen and is, therefore, unlikely to catalyse hydroperoxide formation (Feiters *et al.*, 1985). Veldink *et al.* (1977) has proposed a mechanism for the LOX-catalysed reaction (Fig. 1) where iron, which is essential to the catalytic role of the enzymes, alternates between the Fe(II) and Fe(III) oxidation states. It has been proposed that the small amount of LOX–Fe(III) (1%) present in purified LOX-I catalyses the initial formation of hydroperoxide. The native 'resting' enzyme in the inactive LOX-Fe(II) form could be activated by oxidation of the iron to LOX–Fe(III), by the resulting hydroperoxide. Despite the existence of numerous examples of iron-containing proteins in which the Fe(II)-form reversibility binds oxygen, as yet there is no evidence to show that this occurs for LOX. Therefore, for the mechanism proposed by Veldink *et al.* (1977), it seems unlikely for SOD to inhibit LOX because the lipid radical–enzyme complex is formed before the involvement of oxygen.

Table 3. LOX-catalysed oxidation of linoleic acid emulsified with Tween 20 and the effect of BSA[a]

	$OD_{234\,nm}$ (min^{-1})	SD
LOX type I	0·371	0·007
LOX type I + 10 μg/ml BSA	0·326	0·01
LOX type I + 25 μg/ml BSA	0·335	0·019
LOX type I + 200 μg/ml BSA	0·299	0·003

[a] Values are the mean of three experiments; all differ significantly from the control.

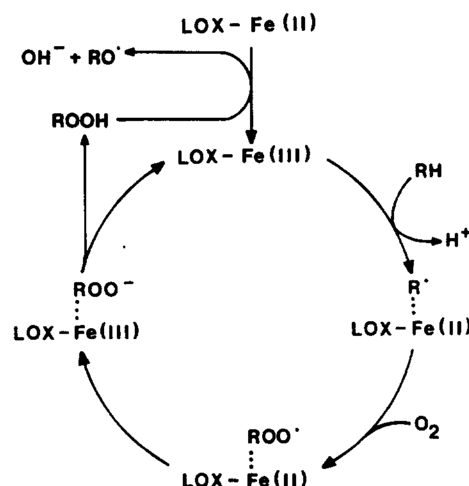


Fig. 1. Proposed reaction scheme for soybean LOX-1 under aerobic conditions (Adapted from Veldink *et al.*, 1977).

The surprising apparent increase of 26% in the rate of LOX oxidation of linoleic acid in the presence of a high concentration of SOD (Table 2) is probably due to the production of H_2O_2. Dismutation of $O_2^{\cdot-}$ by SOD produces O_2 and H_2O_2, which absorbs at 240 nm. Therefore, it is suggested that the increase in absorbance at 234 nm is not due to additional diene formation but to the H_2O_2 production at the higher concentration of SOD.

Effect of SOD on autoxidation

Autoxidation was carried out in separate normal analytical-grade buffer, low-iron and haemin systems. The autoxidation of linoleic acid in buffer containing trace amounts of iron at a level normally present in laboratory AnalaR chemicals and reagents is shown in Fig. 2. This amount of iron and other trace metals is sufficient to initiate and maintain the autoxidation of the linoleic acid via reactions (3)–(5). The addition of SOD is seen to lengthen the lag phase, but it has no effect on the maximum rate of diene formation as measured at 243 nm during the later propagation phase of autoxidation.

Lingnert *et al.* (1989) reported that either bovine SOD or yeast SOD (*Saccharomyces cerevisiae*) can act as antioxidants on the autoxidation of linoleic acid. They measured the absorption at 234 nm after 24 h. However, they did not study the antioxidant effect of SOD after this time or at which stage of the autoxidation process SOD acts.

During the early stages of trace-metal-catalysed autoxidation the Haber–Weiss production of OH·

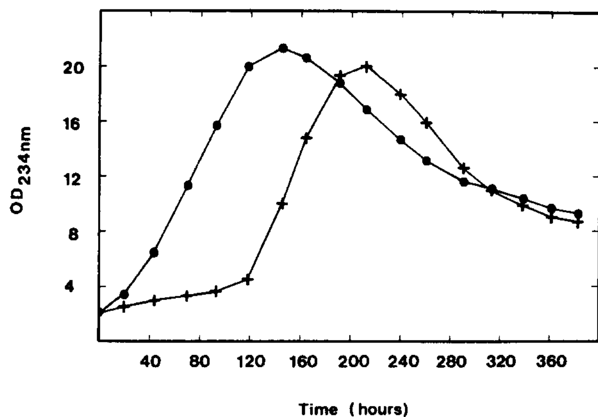


Fig. 2. Autoxidation of linoleic acid and the effect of bovine SOD (5 μg/ml): linoleic acid, (●), linoleic acid plus bovine SOD (+).

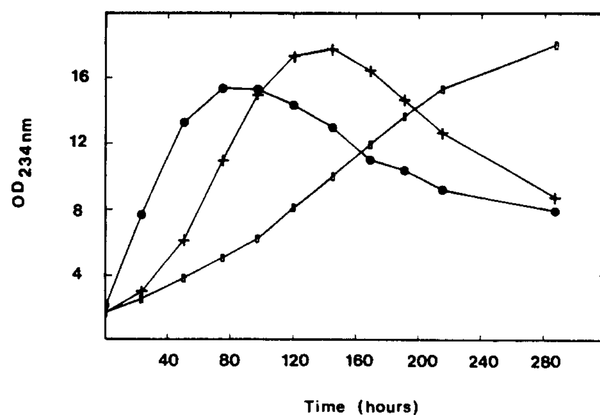


Fig. 3. Autoxidation of linoleic acid in low-iron buffer and the effect of bovine SOD (5 μg/ml): linoleic acid in normal buffer (●), linoleic acid in low-iron buffer (+), linoleic acid in low-iron buffer plus bovine SOD (□).

(reaction (3) appears important in the initiation of lipid autoxidation, and it is here that SOD probably exerts its effect. Dismutation by SOD of O_2^{-} removes one of the reactants in the Haber–Weiss reaction and may be responsible for decreased formation of OH·. As the concentration of hydroperoxide increases, reactions (4) and (5) become more important in the initiation of new radical chains and eventually become the predominant initiation reactions. Once this occurs, the Haber–Weiss reaction would be overshadowed and inhibition by SOD would no longer be observed. It has been claimed that O_2^{-} can react with lipid hydroperoxides (Sutherland & Gebicki, 1982; Thomas *et al.*, 1982). However, this mechanism of oxidation is only likely to be important when the hydroperoxide concentration is high, at which time the metal-catalysed decomposition of hydroperoxide predominates (reactions (4) and (5)). The present results showed no change in the maximum rate of hydroperoxide formation with the addition of SOD, and it is suggested that the reaction of O_2^{-} with hydroperoxide is insignificant, because no inhibition was observed during the later stages of autoxidation.

The autoxidation of linoleic acid in the low-iron system in buffer, dialysed previously against conalbumin to remove iron, is shown in Fig. 3. However, the presence of very small amounts of iron or other metal ions cannot be ruled out, as the linoleic acid and Tween 20 were not treated and are likely to contain some metal contaminants. For this reason, the system is referred to as 'very-low-iron' rather than 'iron-free'. As shown in Fig, 3, removal of iron from the buffer lengthened the lag phase but did not affect the maximum rate of hydroperoxide formation. The initial initiation reactions driven by the Haber–Weiss reaction (3) appeared to be most affected by the removal of iron from the buffer, as evidenced by the lengthened lag phase of autoxidation. Furthermore, it seemed that once the hydroperoxide concentration had reached a significant level, hydroperoxide decomposition still occurred at a sufficient rate for the reaction to be propagated.

When SOD is present in the very-low-iron system, the shape of the oxidation curve was changed (Fig. 3). The lag phase of autoxidation had no clear end and the maximum rate of hydroperoxide formation for the model system was not achieved. If SOD is able to inhibit the Haber–Weiss reaction sufficiently at very low iron concentrations, it might be that what is observed in Fig. 3 is a very long, extended lag phase and that the hydroperoxide-dependent initiation had not occurred to any great extent. Alternatively, or in addition, the direct reaction of O_2^{-} with the hydroperoxides may be important at very low iron concentrations, and SOD may inhibit this reaction indirectly by dismutation of the O_2^{-}.

In the presence of haemin, the oxidation of linoleic acid proceeded at a rapid rate with no initial lag phase (Fig. 4). On addition of SOD, there was a small decrease in hydroperoxide formation compared to the control. However, after 24 h, SOD appeared effective in delaying the further rapid increase in hydroperoxide concentration.

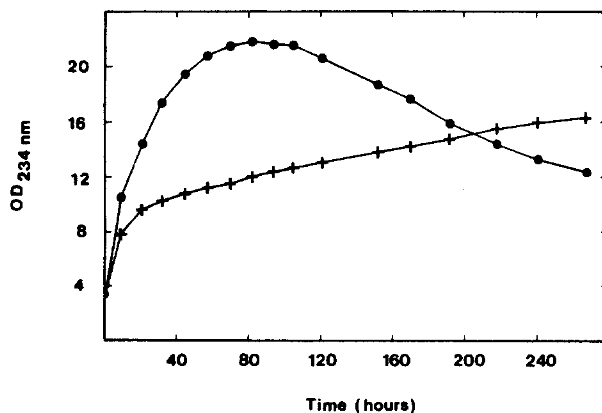


Fig. 4. Haemin-catalysed oxidation of linoleic acid and the effect of bovine SOD (5 μg/ml): linoleic acid plus haemin (●), linoleic acid plus haemin plus bovine SOD (+).

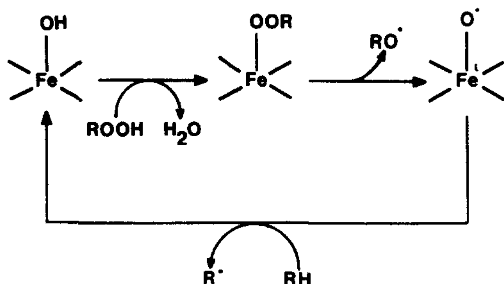


Fig. 5. Proposed mechanism for haem-catalysed oxidation of fatty acids, where RH and ROOH represent the fatty acid and fatty-acid hydroperoxide, respectively.

It has been proposed that haemin catalyses lipid oxidation by forming a complex with the hydroperoxide, which then undergoes homolytic cleavage into RO· and an oxy–haem radical. The oxy–haem radical can then proceed to abstract a hydrogen from a substrate molecule, regenerating the haem compound and producing another chain-initiating free radical (Fig. 5).

The delay of the further rapid increase in hydroperoxide concentration by SOD suggests that either $O_2^{\cdot-}$ is involved in the haemin-catalysed oxidation or that SOD is able to scavenge oxy–haem radicals and prevent them from initiating new radical chain reactions. Bors (1989) has recently claimed that CuZn–SOD reacts with ROO· with a rate constant comparable to its value for dismutating $O_2^{\cdot-}$, and therefore the function of SOD might include the scavenging of other radicals beside $O_2^{\cdot-}$. It has even been suggested that the biological role of SOD is not dismutation of $O_2^{\cdot-}$ but some other activity not yet determined and the dismutase activity is a result of the occurrence of the transition metal in the enzyme (Fee, 1982). If SOD is able to dismutate the oxy–haem radical, this would explain the observed antioxidant effect once autoxidation has started.

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