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Critical assessment of the applicability of superoxide dismutase as an antioxidant in lipid foods

Anne S. Meyer, Karen Rørbæk* & Jens Adler-Nissen

Department of Biotechnology, Building 221, Technical University of Denmark, DK-2800 Lyngby, Denmark

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The potential antioxidative effect of copper–zinc superoxide dismutase (SOD) from *Saccharomyces cerevisiae* was studied in different lipid food model systems. SOD did not inhibit a haemin-catalysed oxidation of emulsions containing 23% by weight of vegetable oil. In contrast, addition of xanthine oxidase and xanthine to generate superoxide *in situ* unexpectedly lowered the rate of oxidation of these emulsions. However, in 10 mM emulsions of linoleic acid, xanthine oxidase added with its substrate had the expected pro-oxidative effect, and SOD retarded this oxidation. Substitution of linoleic acid by herring oil (0·3% (w/w)) again reverted the effect of xanthine oxidase to an antioxidative one in this system. The results suggest that the superoxide-driven initiation reaction is significantly pro-oxidative in dilute linoleic acid systems, but comparatively insignificant in rapidly oxidizing triglyceride systems, which indicates that it is the rate of propagation and not the amount of new initiations that determines the overall oxidation rate. In turn, this is assumed to explain the lack of antioxidative effect of SOD in the triglyceride emulsions employed in these studies.

INTRODUCTION

Superoxide dismutases (EC 1.15.1.1) catalyze the disproportionation of superoxide radicals to oxygen and hydrogen peroxide and thereby contribute to the antioxidative defence in living cells. The possible application of superoxide dismutase as an antioxidative agent in foods was patented in the 1970s (Michelson & Monod, 1975; Michelson, 1977), but the antioxidative effect of the enzyme has only been demonstrated in model systems. Thus, addition of superoxide dismutase inhibits linolenate oxidation (1 mM linolenate, pH 8·1) initiated by a xanthine–xanthine oxidase system (Kellogg & Fridovich, 1975) (xanthine oxidase (EC 1.1.3.22) catalyses the oxidation of xanthine during consumption of oxygen and production of superoxide and hydrogen peroxide (Bray, 1975)). Superoxide dismutase has also proven effective in retarding haemin or lipoxygenase-induced oxidation of dilute emulsions of linoleic acid (2·66 μM–10 mM) (Richter *et al.*, 1975; Lingnert *et al.*, 1989; Nice & Robinson, 1992), or copper-catalyzed oxidation of cholesterol (Lingnert *et al.*, 1989). Superoxide dismutase added to a milk-related model system containing 2 mM trilinolein also exhibits an antioxidative effect (Allen & Wrieden, 1982).

* Present address, Ministry of Fisheries of Technology Laboratory, Building 221, DK-2800 Lyngby, Denmark.

The potential use of superoxide dismutase as a food antioxidant rests on the hypothesis that lowering of the concentration of the superoxide radical, $O_2^{\cdot}$, in the food will retard the overall oxidation rate. However, $O_2^{\cdot}$ cannot initiate lipid peroxidation directly (Rosenthal, 1985; Kanner *et al.*, 1987), but the conjugate acid of $O_2^{\cdot-}$, i.e. the perhydroxyl radical $HO_2^{\cdot}$ (pK_a 4·7) has been shown to initiate chain oxidation of linoleic, linolenic and arachidonic acid (Gebicki & Bielski, 1981; Bielski *et al.*, 1983). Further, it is generally recognized that superoxide can function as an indirect initiator in the presence of transition metal ions, where the highly reactive hydroxyl radical, ·OH, can be generated from an $O_2^{\cdot-}$ driven, metal-catalysed Fenton reaction (Kanner *et al.*, 1987; Nice & Robinson, 1992). Although the requirement for ·OH radicals to initiate lipid peroxidation is debated (Gutteridge & Halliwell, 1990), the high redox potential of ·OH, $E^{\circ} > 2$ V at pH 7 (Kanner *et al.*, 1987) dictates that ·OH is able to initiate lipid peroxidation directly via abstraction of hydrogen at a number of sites along the fatty acid carbon chain (Gutteridge, 1984; Rosenthal, 1985).

Since hydrogen peroxide, H_2O_2, is a product of the superoxide dismutase-catalyzed reaction, the enzyme should be more effective in conjunction with catalase to remove H_2O_2. This expected cooperative effect between the two enzymes has been found in an Fe^{2+}-accelerated

milk fat system (Valenzuela *et al.*, 1981) and superoxide dismutase and catalase have also been reported to improve the stability of milks rich in linoleic acid (Hill, 1979). However, Lingnert *et al.* (1989) did not see any additional antioxidative effect of adding catalase to their superoxide dismutase preparation.

In recent years our group has been engaged in the study of superoxide dismutase as a potential antioxidant for lipid foods. As part of this study we investigated possible constraints in the applicability of yeast copper–zinc superoxide dismutase in emulsion models simulating lipid foods (Refsgaard *et al.*, 1992). In continuation of this latter work, it was surprisingly found, that the copper–zinc superoxide dismutase from *Saccharomyces cerevisiae* apparently did not inhibit oxidation of emulsions with 23% soybean oil that were used as a general lipid food model. The purpose of the present work was to elucidate these observations more systematically by studying the effect of superoxide dismutase, catalase and xanthine oxidase in different model systems.

MATERIALS AND METHODS

Materials

Superoxide dismutase was a lyophilized preparation of copper–zinc superoxide dismutase from *S. cerevisiae* obtained from Carlbiotech A/S (Copenhagen, Denmark) (specific activity was 14 806 U/mg assayed as described below). Catalase from *Aspergillus niger* (1790 U/mg) was a lyophilized preparation purchased from Serva Feinbiochemica GmbH & Co. (Heidelberg, Germany). Maize oil and soybean oil were from Aarhus Olie A/S (Aarhus, Denmark), and herring oil from Thyborøn Andels Fiskeindustri (Thyborøn, Denmark). Emulsifier Acidan N12 and antioxidant BHA were from Grindsted Products A/S (Brabrand, Denmark). Filtrol$^{(R)}$ was from Filtrol Corporation (Vernon, CA, USA). Xanthine oxidase from buttermilk, xanthine (99–100%), haemin and linoleic acid (95%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetaldehyde (>99%) was bought from Merck (Darmstadt, Germany). All chemicals used were of analytical grade and the water was doubly deionized via a Millipore water purification system (Bedford, MA, USA).

Experimental procedures

Tocopherols were removed from the oils prior to use by adsorption on Filtrol® (Søndergaard & Dam, 1966). Oil in water emulsions were produced as described previously (Refsgaard *et al.*, 1992). In 23% (w/w) emulsions the aqueous phase was 0·1 M succinate buffer pH 6 and, in 0·3% (w/w) emulsions of linoleic acid or herring oil, the buffer used was 0·1 M phosphate pH 6·5. Total homogenization time was 3·5 min for 23% (w/w) emulsions and 4 min for 10m M linoleic acid or 0·3% (w/w) herring oil emulsions (10 mM linoleic acid is approx. equivalent to 0·3% (w/w)). Oxidation of the 23% oil/water emulsions was accelerated by addition of Fe^{3+} in the form of a saturated, ethanolic haemin solution (0·55 mM haemin) to a final concentration of 2 ppm Fe^{3+} (≈37 μM haemin) and storage of the emulsions in the dark at 37°C in pure oxygen (1 atm). Oxidation of emulsions containing 10 mM linoleic acid or 0·3% by weight of herring oil was accelerated by addition of haemin to a final concentration of 0·043 ppm Fe^{3+} (≈0·8 μM haemin) and storage of the emulsions in the dark at 37°C with access to air. All investigations were carried out in two parallel, identical systems.

Activity determinations on superoxide dismutase were made according to a modified pyrogallol assay as described earlier (Refsgaard *et al.*, 1992). Peroxide values were determined according to a colorimetric, microscale method (Asakawa & Matsushita, 1980). In emulsions containing 23% oil by weight, peroxide values were determined on the lipid phase after separation of emulsion samples by centrifugation (16 300 g for 20 min) and in emulsions containing 0·3% (w/w) of lipid, peroxide values were determined directly on unseparated samples. All peroxide values given are an average of four determinations. The investigations were carried out in two parallel, identical systems. Peroxide values were determined by duplicate analyses on each system, giving four determinations. In 10 mM linoleic acid emulsions the antioxidative effects of added enzymes were also calculated from conjugated diene measurements at 234 nm before and after incubation according to a procedure described by Lingnert *et al.* (1979). Dissolved oxygen was measured with a polarographic oxygen electrode (Jenway, Dunmow, UK).

RESULTS AND DISCUSSION

Effect of superoxide dismutase in high lipid emulsions

The effect of adding copper–zinc superoxide dismutase from *S. cerevisiae* to emulsions containing 23% by weight of tocopherol-stripped maize oil was tested in a wide dose spectrum, i.e. addition of 50–6860 U/g emulsion. Typical results are shown in Fig. 1. In no case was it possible to demonstrate a significant effect of the enzyme addition on the rate of oxidation as measured by the increase in peroxide value over time.

Effect of xanthine oxidase in high lipid emulsions

In order to find an explanation for the lack of antioxidative effect of SOD, addition was carried out in the 23% oil/water emulsions to test whether generation of superoxide radicals by xanthine oxidase would promote the rate of oxidation in the emulsions. The oxidation of the 23% (w/w) soybean oil emulsions was accelerated by Fe^{3+} (haemin) at two different levels of addition. It was unexpectedly found that the emulsions containing

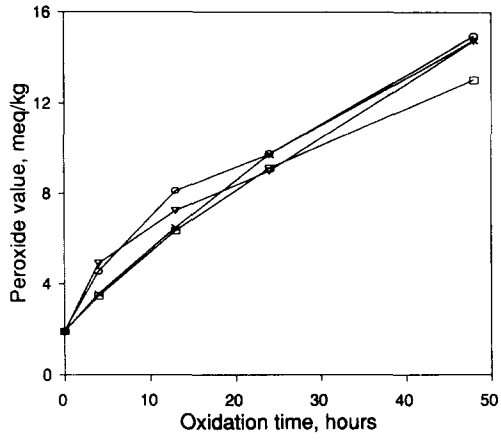

Fig. 1. Change in peroxide values with time measured in duplicate sets of oil/water emulsions containing 23% (w/w) of tocopherol-free maize oil. Labels refer to amount of SOD added per gram emulsion. —□—, control; —×—, 340 U/g; —○—, 685 U/g; —▽—, 1370 U/g.

xanthine and xanthine oxidase did not oxidize as fast as those without (Fig. 2). In fact, a comparison of the rate of oxidation progress in the emulsions by one way analysis of variance showed a strongly significant antioxidative effect of the xanthine/xanthine oxidase addition at both 2 ppm and 0·4 ppm Fe^{3+}. The same pattern was seen in another experiment where the level of Fe^{3+} was 0·2 ppm (results not shown). The measured antioxidative effect of xanthine oxidase in these fast oxidizing, high-lipid systems is probably a result of the xanthine oxidase-mediated oxygen removal, which apparently affects the rate of oxidation progress more significantly than the concomitant production of superoxide radicals. In separate experiments we have observed decreases in the dissolved oxygen concentration in 23% oil/water emulsions from 8 mg O_2/litre to 0·1 mg O_2/litre within 1 h when xanthine oxidase was present, which substantiates this hypothesis.

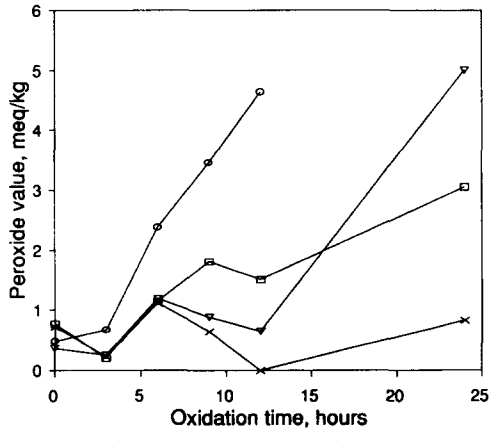

Fig. 2. Change in peroxide values with time measured in oil/water emulsions containing 23% (w/w) of tocopherol-free soybean oil. Labels refer to emulsions to which different amounts of Fe^{3+} (haemin) and xanthine/xanthine oxidase (x/xo) have been added. In all cases 0·27 mmol of xanthine and 3 units of xanthine oxidase have been added per 40 g emulsion. —○—, 2 ppm Fe^{3+}; —▽—, 2 ppm Fe^{3+} + x/xo, —□—, 0·4 ppm Fe^{3+}; —×—, 0·4 ppm Fe^{3+} + x/xo.

Effect of enzymes in dilute linoleic acid emulsions

Since Lingnert *et al.* (1989) had successfully demonstrated an antioxidative effect of the same superoxide dismutase as applied by us, the 23% oil/water emulsion was substituted by the 10 mM linoleic acid system used by Lingnert *et al.* (1989). In this system addition of SOD gave the expected significantly antioxidative effect (Table 1) both when the oxidation was assayed by peroxide value and by conjugated diene measurements. The latter was the method employed by Lingnert *et al.* (1989). Further, the inhibition obtained increased with increased SOD doses. There was also a significant antioxidative effect of SOD plus catalase at any level as compared to the control. However, in agreement with the findings of Lingnert *et al.* (1989), the extra catalase did not enhance the antioxidative effects obtained with SOD alone (Table 1). When catalase was added alone, no significant effects could be obtained from measurements of conjugated dienes but, if judged from the peroxide values, the high catalase addition level (10 U/g) exerted a significantly inhibitory effect.

Addition of xanthine oxidase to the 10 mM linoleic acid emulsions resulted in the expected pro-oxidative effect since the peroxide levels and conjugated diene levels of emulsions containing xanthine oxidase were significantly higher than those without this enzyme addition both when Fe^{3+} was present and when it was not (Table 2). In these experiments xanthine was replaced by acetaldehyde due to interference from xanthine on conjugated diene measurements (Kellogg & Fridovich, 1975). All the results given in Tables 1 and 2 agree very well with those of others (Lingnert *et al.*, 1989; Nice & Robinson, 1992). Likewise, the observed effects of catalase addition are in accordance with those found by Lingnert *et al.* (1989), but do not agree with the proposed $O_2^{\cdot-}$ and H_2O_2-mediated initiation mechanism, i.e. production of ·OH via a metal-catalyzed Fenton reaction (Kanner *et al.*, 1987; Nice & Robinson, 1992) because, according to the latter mechanism, addition of

Table 1. Peroxide values and antioxidative effects in haemin-catalyzed 10 mM emulsions of linoleic acid after 24 h at 37°C

Addition[a]	Peroxide value[b] (mEq/kg)	Antioxidative effects[c]
0 (control)	3·32	(0)
SOD 1 U/g	1·41	0·52
SOD 5 U/g	1·07	0·47
SOD 25 U/g	0·72	0·56
SOD 1 U/g + CAT 0·4 U/g	1·04	0·38
SOD 5 U/g + CAT 2 U/g	1·13	0·59
SOD 25 U/g + CAT 10 U/g	0·84	0·52
SOD 25 U/g + CAT 30 U/g	1·32	0·01
CAT 0·4 U/g	3·93	−0·12
CAT 2 U/g	3·10	−0·01
CAT 10 U/g	2·29	−0·18

[a] SOD, copper–zinc superoxide dismutase from *S. cerevisiae*; CAT, catalase from *A. niger*.
[b] Average SD = 0·39.
[c] Values calculated from conjugated diene measurements. Average SD = 0·11.

Table 2. Peroxide values and antioxidative effects in emulsions of 10 mM linoleic acid after 25 h at 37°C

Addition[a]	Peroxide value[b] (mEq/kg)	Antioxidative effects[c]
0 (control)	0·42	(0)
Fe^{3+}	0·81	−0·33
xo/acetaldehyde	1·60	−0·92
Fe^{3+} + xo/acetaldehyde	1·43	−1·32

[a] Fe^{3+}, 0·043 ppm Fe^{3+} in the form of haemin; xo, xanthine oxidase 0·075 U/ml; acetaldehyde, 10 μl/ml.
[b] Average SD = 0·13.
[c] Calculated from conjugated diene measurements. Average SD = 0·19.

Table 3. Peroxide values in emulsions with 0·3% (w/w) tocopherol-free herring oil after 120 h at 37°C

Addition[a]	Peroxide value[b] (mEq/kg)
0 (control)	0·91
Fe^{3+}	0·56
xo/acetaldehyde	0·22
Fe^{3+} + xo/acetaldehyde	0·23
BHA	0·12
Fe^{3+} + BHA	0·11

[a] Fe^{3+}, 0·043 ppm Fe^{3+} in the form of haemin; xo, xanthine oxidase 0·075 U/g; acetaldehyde, 10 μl/ml; BHA, 0·08 μmol/ml.
[b] Average SD = 0·03.

catalase to remove H_2O_2 ought to retard the oxidation markedly in conjunction with SOD.

Effect of xanthine oxidase in dilute herring oil emulsions

When substituting linoleic acid for tocopherol-free herring oil in 0·3% (w/w) emulsions, the effect of xanthine oxidase addition was now again significantly antioxidative both with and without Fe^{3+} (Table 3) in accordance with the observations in the 23% oil emulsions (Fig. 2). BHA (butylated hydroxyanisole) was included as a positive control. The reason for the peroxide value in the control containing Fe^{3+} being lower than the control without Fe^{3+} is assumed to be due to iron-accelerated breakdown of peroxides. The added iron (haemin) not only directly stimulates new initiations, but also stimulates lipid peroxidation by decomposing lipid hydroperoxides to produce peroxyl and alkoxyl radicals (Chan, 1987). These radicals initiate further oxidation and hence may decrease the relative significance of super-oxide-driven initiations. Early findings by Gutteridge (1984) demonstrate that when iron salts are added to lipids containing variable amounts of lipid peroxides, the decomposition of lipid peroxides to initiating radicals is favoured, while ·OH radicals contribute little to the rate of oxidation.

CONCLUSIONS

The results show that, in the applied 10 mM linoleic acid system, the superoxide-mediated induction of oxidation is sufficiently important to result in significant acceleration at increased levels of $O_2^{\cdot-}$ and in turn inhibition by SOD. However, from the results obtained, the mechanism of superoxide initiation cannot be unequivocally explained, since addition of catalase to SOD did not improve the inhibition obtained. However, the role of H_2O_2 in lipid peroxidation was not investigated further in this study.

The antioxidative effect of xanthine oxidase, which was demonstrated both in dilute emulsions of 0·3% (w/w) herring oil and in concentrated emulsions containing 23% oil by weight, support the assumption that in fast oxidizing triglyceride systems—as opposed to systems of pure linoleic acid—the scavenging of oxygen is comparatively more significant than the pro-oxidative effect of the generated superoxide. The reason for the different behaviour of the two types of system is probably a result of the different oxidative stage of the lipids. Thus, the significant effect of oxygen removal in the employed triglyceride systems is assumed to be due to the importance of oxygen for the propagation step in lipid autoxidation. Hence, it seems that in the employed triglyceride systems, the lipid autoxidation is already progressed to a degree where the propagation, perhaps fuelled by initiations caused by metal-catalyzed breakdown of lipid peroxides, is relatively more important for accelerating the oxidation rate than the amount of new superoxide-driven initiations. In turn, this explains why superoxide dismutase had an antioxidative effect in systems where the lipids were composed of purified, presumably relatively unoxidized, free fatty acids, but could not be demonstrated to have the same antioxidant effect in any of our triglyceride systems. Since the oils applied in our study were of high quality with a very satisfactory oxidative status, as judged from the peroxide values, this negative conclusion may probably hold for most lipid food systems in practice. This conclusion is further substantiated by the finding that the *S. cerevisiae* copper–zinc superoxide dismutase is inactivated by lipid hydroperoxides in emulsions (Refsgaard *et al.*, 1992), and hence this enzyme is particularly unsuitable for application in lipid food systems. Apart from elucidating the potential of superoxide dismutase as an antioxidative agent in lipid-rich food models, the study has also stressed that the different roles played by $O_2^{\cdot-}$, H_2O_2 and ·OH in iron-catalyzed lipid systems are still poorly understood and require further investigation.

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