Application of enzymes as food antioxidants

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The trend to avoid synthetic additives has resulted in a large increase in efforts to find new natural antioxidants. Enzymes represent a new type of potentially applicable natural antioxidants. Three types of enzymatic antioxidative principles are outlined in this article, and the applicability of relevant enzymes in all three categories are discussed.

During the past 10–15 years, the investigation of natural antioxidants for food preservation has received much attention[1]. The interest in natural antioxidants is fuelled by the general resistance both of consumers and the food industry to synthetic food additives, coupled with concerns about the possible carcinogenic effects of the most commonly used synthetic antioxidants, BHA and BHT (butylated hydroxyanisole and butylated hydroxytoluene, respectively)[1,2]. The application of enzymes represents one of several alternatives for exploiting natural substances as antioxidative agents in foods. Enzymes can act as antioxidants by promoting three types of reaction: the removal of oxygen, the removal of active oxygen species such as hydrogen peroxide and superoxide radicals, and the reduction of lipid hydroperoxides. The commercial use of enzymes as food antioxidants is currently limited to the removal of oxygen using glucose oxidase (EC 1.1.3.4) in conjunction with catalase (EC 1.11.1.6) but, as will be discussed, all three antioxidative principles have been investigated for the enzymatic prevention of lipid peroxidation in foods.

Enzymes as food additives

Apart from the ability to catalyse the relevant reaction (i.e. one of the three reaction types mentioned above), an important criterion for the use of enzymes in foods is, of course, that the relevant enzyme system is permitted legally. In the USA, the use of enzymes in foods is regulated by the US Food and Drug Administration (FDA), which lists permitted substances intended to process foods or added to foods as food additives in Title 21 of the US *Code of Federal Regulations* (21 C.F.R.)[3]. Alternatively, the FDA approves food ingredients and processing aids, including enzymes, by granting them GRAS (generally recognized as safe) status. In the European Union, all enzymes, except lysozyme, are treated principally as processing aids, and the intended use of a particular enzyme in foods and food processes has to be specifically approved (J. Fredsted, Danish Food Agency, pers. commun.). Enzymes considered for food use have to be extracted from microorganisms (or plant or animal tissues) that are GRAS; moreover, the enzymes must be toxicologically safe and neither require (e.g. as cofactors) nor generate toxic compounds.

From a practical point of view, it is obvious that enzymatic food antioxidants must not, by their catalytic action, adversely affect the organoleptic properties of the foodstuff in question. Although it is beyond the scope of this article to discuss a cost–benefit analysis for enzymes versus other antioxidants, it has to be stressed that to facilitate their widespread use, the relevant enzymes must be readily available and inexpensive. Furthermore, an important prerequisite for the successful application of enzymes as antioxidants is that the enzymes are resistant to inactivation by factors present in the food microenvironment. In particular, the retention of activity at low pH values, high temperatures, high ionic strengths (NaCl) and in the presence of other additives is important. In addition, the stability of enzymatic antioxidants in the presence of water–lipid interfaces, free fatty acids and lipid hydroperoxides must be considered. Stability in the presence of lipids cannot always be taken for granted because enzymes are prone to inactivation in lipid food systems, such as emulsions (e.g. mayonnaise and salad dressing), as a result of interfacial denaturation[4], and may also be inactivated by free fatty acids and lipid hydroperoxides that are extracted from the lipid phase into the aqueous phase. For example, it is known that lipid hydroperoxides, the primary oxidation products of lipid oxidation, are able to inactivate some enzymes via free-radical reactions resulting in oxidation, fragmentation or crosslinking[5,6]. Unfortunately, the stability of an enzyme in the presence of lipids is difficult to predict, and this trait is rarely included in the fundamental enzyme investigations available or in data sheets on commercially available enzymes. The final evaluation of an enzyme as a potential food antioxidant must therefore always include tests in realistic food systems, and preferably also include investigations of enzyme stability against denaturation in the food microenvironment. Thus, as will be expounded on below, data on the antioxidative efficacy of enzymes in aqueous model systems containing only millimolar quantities of lipid or fatty acids have to be carefully and critically interpreted.

Enzymatic oxygen removal

The oxidation of lipids leading to rancidity in foods requires oxygen to be present in order for the reaction to proceed[7]. Thus, it is easy to infer that the removal of oxygen retards lipid oxidation. However, because lipid oxidation is a self-catalysing, free-radical chain reaction,

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the exact quantitative effect of oxygen removal is not directly interpretable[8]. The rate of lipid peroxidation can be described by simplified rate expressions (modified from Karel[8]):

$$\text{General expression:} \quad R = A\,[\text{LH}]\,\frac{[O_2]}{b + c[O_2]} \qquad (1)$$

$$\text{High oxygen pressure:} \quad R = A'[\text{LH}] \qquad (2)$$

$$\text{Low oxygen pressure:} \quad R = A''[O_2] \qquad (3)$$

where $[O_2]$ and [LH] represent the concentration of oxygen and lipid molecules, respectively, R designates the overall rate of lipid peroxidation, and A, A', A'', b and c are constants composed of multiples of rate constants for the initiation (only A, A' and A''), propagation and termination steps of lipid peroxidation.

When oxygen is present at a relatively high concentration, the rate of oxidation is independent of the oxygen concentration (Eqn 2), whereas the rate of oxidation is directly proportional to the oxygen concentration when it is low (Eqn 3). Thus, in order to obtain a significant antioxidative effect when using oxygen-scavenging enzymes as antioxidants, they necessarily must be capable of lowering the concentration of oxygen to a level where lipid peroxidation follows first-order kinetics with respect to oxygen concentration (Eqn 3) (the numerical limits of high and low oxygen concentrations depend on the type of lipid that is oxidized[8]). As will be discussed below, the glucose oxidase–catalase system has been shown to exert a significant antioxidative effect, even in food systems containing high levels of lipid. This demonstrates that this enzyme system, at least, is able to remove oxygen to such an extent that the lipid peroxidation rate becomes dependent on the oxygen concentration.

Glucose oxidase plus catalase

Glucose oxidase coupled with catalase is both the oldest and best-studied oxygen-scavenging enzyme system with food applications[9,10]. Glucose oxidase is a flavoprotein that catalyses the oxidation of β-D-glucose to δ-D-gluconolactone, utilizing oxygen, O_2, as the electron acceptor and resulting in the production of hydrogen peroxide, H_2O_2 (Fig. 1). The δ-D-gluconolactone produced spontaneously hydrolyses to D-gluconic acid, while the H_2O_2 generated can be removed by catalase. Thus, in the net reaction catalysed by the glucose oxidase–catalase system, one mole of O_2 is removed for every two moles of glucose that are oxidized[10] (Fig. 1). Glucose oxidase–catalase, extracted from *Aspergillus niger*, is commercially available, and permitted for food uses in several countries, including the USA, where it has GRAS status (E. Flamm, US FDA, pers. commun.). Moreover, both glucose oxidase and catalase from *A. niger* are relatively stable to interfacial denaturation for 24 hours in emulsions containing 1–90% (w/w) oil[11].

The glucose oxidase–catalase system has been known for a long time to be a workable antioxidative principle, preventing off-flavour development in various food products, including fruit juices[9,10,12], mayonnaise[9,13] and salad dressing (B.S. Mistry, PhD thesis, The Ohio State University, Columbus, OH, USA, 1988). Recently, it was shown that the addition of an antioxidant system incorporating glucose oxidase–catalase (and glucose) was superior to all other antioxidant combinations tested in increasing the shelf life of mayonnaises made from fish oil[13]: refrigerated mayonnaise containing propyl gallate and citric acid in the oil phase, and a combination of EDTA, ascorbic acid and the glucose oxidase–catalase system in the aqueous phase had a shelf life at refrigeration temperatures that was ~50% longer than that of the same mayonnaise without the glucose oxidase–catalase system (132 days versus 89 days, respectively).

In soybean and fish oil emulsions containing 23% (w/w) lipid, the rate of both oxygen transport and lipid peroxidation have been shown to have a major influence on the antioxidative efficacy of the glucose oxidase–catalase system[14]. Thus, at extremely high oxidation rates, the glucose oxidase–catalase system did not function antioxidatively in emulsions containing 23% (w/w) herring oil from which the endogenous tocopherol had been removed[14]. Based on these findings, investigations into how the antioxidative performance of the glucose oxidase–catalase system is affected by the oxygen availability as well as by factors influencing the oxidation rate are currently under way in our laboratory, using mayonnaise as the model food system. The aim is to gain a better understanding of the antioxidative kinetics and efficacy of the glucose oxidase–catalase system in lipid food systems.

Other oxidases

As many enzymes belonging to the oxidoreductase class can use oxygen as an acceptor of electrons, some of them ought to be applicable as antioxidative agents in foods by catalysing the removal of oxygen. However, as stated above, important requirements for the use of

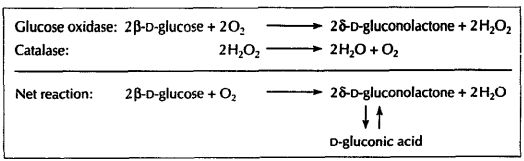

Fig. 1
Individual reactions catalysed by glucose oxidase and catalase, and the overall net reaction catalysed by the glucose oxidase–catalase enzyme system.

enzymes as oxygen scavengers include the toxicological and organoleptic inertness of their substrates and products. Inert, acceptable substrates (and products) are, in theory, certain sugars, vitamins, amino acids and a few other types of compounds. However, in practice, unwanted organoleptic or other unfavourable effects are surprisingly difficult to avoid. For example, the enzyme-catalysed oxidation of amino acids by amino acid oxidases often leads to the production of carbon dioxide[15]; this may generate bubbles, which look unappetizing in some food products and may be mistaken for unwanted microbial growth. Likewise, many dioxygenases that catalytically incorporate oxygen into the substrate require Fe^{2+} for activity[15]. However, because Fe^{2+} is a potent initiator of lipid peroxidation these types of enzymes are inapplicable as lipid antioxidants. In spite of these constraints, other oxygen-scavenging enzymes besides glucose oxidase can be envisaged as potentially usable food antioxidants. These include alcohol oxidase (EC 1.1.3.13), thiol oxidase (EC 1.8.3.2), galactose oxidase (EC 1.1.3.9), pyranose oxidase (EC 1.1.3.10) and hexose oxidase (EC 1.1.3.5).

Alcohol oxidase catalyses the oxidation of primary alcohols by oxygen, forming aldehydes and hydrogen peroxide as reaction products[15]. The enzyme is included in two patented enzyme systems that are designed to retard off-flavour development in emulsions containing fish oil[16], but it is not known if they are used by the food industry. Thiol oxidase or sulfhydryl oxidase, naturally present in milk, catalyses the oxidation of thiols to disulfides during the transfer of electrons from O_2 to H_2O_2. The enzyme has been suggested[17] to exert an antioxidative effect in milk together with lactoperoxidase (EC 1.11.1.7), but the effect of adding this system to other food products appears to be untried. Galactose oxidase catalyses the oxidation of galactose (at C-6) and of several other compounds, including lactose, raffinose and stachyose, by consuming oxygen and forming hydrogen peroxide[18]. Galactose oxidase, purified from the fungus *Dactylium dendroides*, is commercially available for research purposes. Galactose oxidase inhibits the bleaching of β-carotene during cooxidation of β-carotene and linoleate in an antioxidant assay system both with and without the addition of catalase (M. Ibsen and A.S. Meyer, unpublished), but the enzyme's efficacy in realistic food systems has not, to our knowledge, been investigated. Pyranose oxidase, also called glucose 2-oxidase, catalyses the oxidation of the C-2 atom of D-glucose and several other monosaccharides, consuming oxygen and producing hydrogen peroxide[15]. Pyranose oxidase can be extracted from a number of Basidiomycetes, and may be used to make high-purity food sugars (e.g. fructose, mannitol and sorbitol)[19]. However, the applicability of pyranose oxidase as an oxygen scavenger in foods has not, as far as is known, been reported. Hexose oxidase catalyses the oxidation of β-D-glucose in the same way as glucose oxidase, but it can also catalyse the oxidation of, for example, galactose, maltose, lactose and cellobiose[15]. From a food application point of view, this enzyme seems promising because of its broad specificity towards substrates that are widely present in foods. However, to our knowledge, the enzyme can be extracted only from various algae[15], and information about its applicability in food systems is not available in the literature.

Removal of active oxygen species

Neither hydrogen peroxide nor superoxide, $O_2^{\cdot -}$, can directly initiate lipid peroxidation. However, it is generally accepted that both are able to function indirectly as initiators in the presence of transition metal ions such as iron and copper, where the highly reactive hydroxyl radical, ·OH, can be generated via an $O_2^{\cdot -}$-driven, metal-catalysed Fenton reaction[20,21]:

$$M^{(n+1)+} + O_2^{\cdot -} \rightarrow M^{n+} + O_2$$

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^- + \cdot OH$$

Typically, the $M^{(n+1)+}$ is the ferric ion Fe^{3+}, and M^{n+} the ferrous ion Fe^{2+}, and both ions are almost ubiquitously present in foods. The hydroxyl radical is able to initiate lipid peroxidation directly via abstraction of hydrogen from fatty acids[21], although the requirement for ·OH radicals to initiate lipid peroxidation via the Fenton reaction is debatable[20].

Superoxide dismutases (EC 1.15.1.1) catalyse the dismutation of superoxide radicals to oxygen and hydrogen peroxide, and are part of the antioxidative defence of living cells. As discussed above (Fig. 1), catalase breaks down hydrogen peroxide into oxygen and water. The potential use of superoxide dismutase and catalase either individually or together as food antioxidants rests on the hypothesis that lowering the concentration of $O_2^{\cdot -}$ and/or H_2O_2 inhibits the generation of ·OH radicals and thus retards the rate of lipid peroxidation by decreasing the number of new initiations.

Superoxide dismutase was patented as an antioxidative agent in foods some 20 years ago[22,23], but the antioxidative effect of this enzyme has been demonstrated only in model systems, and superoxide dismutase has never been used in the food industry. In several studies, superoxide dismutase has been shown to inhibit the oxidation of dilute emulsions of linoleic acid accelerated by lipoxygenase, haemin or xanthine oxidase[21,24–26], and the oxidation of linolenate mediated by xanthine oxidase[27]. Superoxide dismutase has also proven effective in retarding oxidation in a milk model system containing 2 mM trilinolein[28].

Because hydrogen peroxide is not only a product of superoxide dismutase catalysis but also a reactant in the Fenton reaction, superoxide dismutase ought to be a better antioxidant when used in conjunction with catalase to remove the H_2O_2. This expected greater antioxidative effect of the two enzymes together has been demonstrated in an Fe^{2+}-catalysed milk-fat system[29], and it has also been reported that the addition of both superoxide dismutase and catalase improves the stability of high-linoleic-acid milk[30]. However, neither Lingnert *et al.*[25] nor ourselves[26] have been able to demonstrate an

enhanced antioxidative effect for superoxide dismutase used in conjunction with catalase, nor observe an antioxidative effect with catalase alone. Rather, we have recently demonstrated that neither superoxide dismutase nor catalase acts antioxidatively either individually or together in more realistic oxidizable food emulsion systems containing 23% (w/w) edible oil[26]. Apparently, this lack of antioxidative activity is due to the fact that the rate of propagation, not the number of $O_2^{\cdot-}$- and H_2O_2-driven Fenton initiations, determines the overall oxidation rate in these systems[26]. More advanced studies are required to substantiate this assertion, however. Further, the activity of the Cu,Zn-superoxide dismutase from yeast has been shown to be unstable in emulsions owing to inactivation caused by lipid hydroperoxides[6], making this enzyme particularly unsuitable for application as an antioxidative agent in lipid food systems.

From the information available on the enzymatic removal of active oxygen species for antioxidant purposes, it can be concluded that model systems containing dilute emulsions of (comparatively unoxidized) free fatty acids do not always provide valid models for the oxidative reactions prevailing in more realistic lipid food systems. Furthermore, the significance of $O_2^{\cdot-}$ and H_2O_2 (and ·OH) in iron-accelerated food emulsion systems obviously deserves further investigation.

For completeness, it should be added that it was claimed earlier[31] that superoxide dismutase could catalytically scavenge singlet oxygen, $^1O_2$, which is a very potent initiator of lipid peroxidation mainly involved in photosensitized oxidation reactions[7]. It was also erroneously thought that superoxide removal circumvented the generation of $^1O_2$, which was assumed to be formed during the spontaneous dismutation of superoxide radicals[32]. However, both claims have later been rejected[33]. Although superoxide dismutase (and other proteins) may be able to remove singlet oxygen via the oxidation of histidine, tryptophan and methionine present in the protein molecule[33], no enzymes are known that can enzymatically scavenge $^1O_2$.

Enzymatically catalysed reduction of lipid hydroperoxides

Glutathione *S*-transferase (EC 2.5.1.18) and glutathione peroxidase (EC 1.11.1.9) are able to catalyse the reduction of lipid hydroperoxides to hydroxides during the oxidation of glutathione (Fig. 2), and both enzymes are known to contribute to the antioxidative defence in biological systems. A large amount of work has been published on the biological function, subcellular localization, etc. of these two types of enzymes that exhibit glutathione peroxidase activity[34], but very little is known about how these enzymes might work as natural antioxidants in foods. Glutathione *S*-transferase purified from lean pork and from lamb muscle has been shown to reduce linoleic acid hydroperoxides (0.1 mM solution)

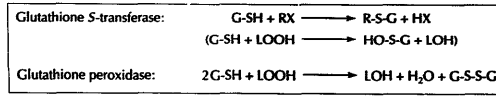

Fig. 2

Reactions catalysed by glutathione *S*-transferase and glutathione peroxidase.

Abbreviations used: G-SH, glutathione; RX, peroxide; R-S-G, G-S-S-G, oxidized glutathione; HX, reduced peroxide; LOOH, lipid hydroperoxide; HO-S-G, glutathione hydroxy product; LOH, reduced lipid hydroperoxide.

and to inactivate *trans,trans*-2,4-decadienal, a secondary product of lipid peroxidation[35,36]. Furthermore, the lamb muscle transferase has been demonstrated to inhibit copper-stimulated peroxidation of dilute emulsions of arachidonate (10 mM) in the presence of glutathione[36]. Thus, based on these observations, it was suggested that endogenous glutathione *S*-transferase may function as an antioxidant that retards off-flavour development in muscle foods during storage[36]. However, this conclusion needs to be experimentally substantiated. The effect of adding glutathione *S*-transferase or glutathione peroxidase to food systems has not been reported, nor has the stability of these enzymes in systems simulating foods been investigated.

Conclusions

In this review article we have attempted to summarize the knowledge available on the application of enzymes to prevent lipid peroxidation in foods. For several reasons, antioxidative enzymes are not currently of commercial significance in the food industry. First, the information available has shown promising results for only a few enzyme systems, with the glucose oxidase–catalase system standing out, to date, as the best-studied and most effective enzymatic antioxidant system. Second, only a few of the many suggested applications of this system have proven technically successful, indicating that there is still a lot to learn about the application of the glucose oxidase–catalase system and/or that other enzyme systems may, in fact, be better suited for antioxidative oxygen scavenging in food systems. From the knowledge available on the antioxidative efficacy of the enzymatic removal of active oxygen species like superoxide and hydrogen peroxide, it has to be concluded that this line of action cannot be immediately recommended. However, the enzymatic reduction of lipid hydroperoxides seems to be a feasible strategy deserving further investigation. In conclusion, the successful and more widespread application of enzymes as antioxidative agents in foods requires much more research. The availability of cheaper, well-defined preparations of the relevant enzymes would help.

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