Antioxidative Effect of Superoxide Dismutase from *Saccharomyces cerevisiae* in Model Systems

Hans Lingnert,* Göran Åkesson, and Caj E. Eriksson

Active oxygen species are possible initiators of oxidative reactions in foods, and their elimination could provide one mechanism of antioxidative effect. In this work we studied the antioxidative properties of superoxide dismutase (SOD) from yeast (*Saccharomyces cerevisiae*) in various model systems. The crude yeast extract was partially purified by ammonium sulfate precipitation. The purified fraction was found to inhibit the oxidation of emulsified linoleic acid, emulsified cholesterol, and ascorbic acid. Catalase had a prooxidative effect in the linoleic acid system, while at high concentrations it protected ascorbic acid against oxidation. No synergistic effect was observed between catalase and SOD. In order to evaluate possible applications of yeast SOD as an antioxidant in food, the influence of pH and heat treatment on the antioxidative effect was studied. Within the range pH 4.5–9.0 the antioxidative effect of the SOD increased with increasing pH. In heat treatment experiments the SOD showed unusually high thermal stability and an interesting inactivation–reactivation behavior. The enzyme activity was for instance retained at about 60% of its original value after heating at 70 °C for 15 min. A few percent of the original enzyme activity remained even after 30 min at 100 °C. The possible technological implications of these results are discussed.

The role of oxygen radicals in nature has attracted much attention in recent years. Several symposia have been held on the subject (Bors et al., 1984), and the field of study has been reviewed in a number of books (Halliwell and Gutteridge, 1985). The increasing research on oxygen radicals in biological systems has lead to an increased interest in oxygen-converting enzymes such as superoxide dismutase (SOD) and catalase.

The role of activated oxygen species in oxidative reactions in food is also widely discussed. A comprehensive review was published by Korycka-Dahl and Richardson (1978). Oxidation of various food constituents (lipids, vitamins, flavor compounds, etc.) is a major problem and influences the sensory as well as the nutritional quality of foods and may even produce toxic substances. The possibility that active oxygen species may be responsible for the initiation of oxidation in food systems has generally been overlooked. Consequently, inactivation of active oxygen as a possible antioxidative mechanism has been studied very little. Such inactivation could be achieved by using nature's own protective systems, such as oxygen-converting enzymes. The idea of using SOD was discussed in a patent application (Michelsen and Monod, 1974). A protective effect of SOD against oxidation of milk has also been reported (Aurand et al., 1977; Hill, 1979; Allen and Wrieden, 1982), but to our knowledge, no other investigations have been published on SOD or similar enzymes as antioxidants in other food systems.

For large-scale production of enzymes it is often favorable to choose enzymes of microbial origin. Enzymes produced for use in food should preferably be obtained from microorganisms traditionally used in food technology, such as yeasts, lactic acid bacteria, etc. Hansson and Häggström (1983, 1984) studied the production of SOD from *Saccharomyces cerevisiae* var. ellipsoideus and *Streptococcus lactis* and the influence of various growth conditions. The enzyme preparation from *Saccharomyces* was used in our work, and in this paper we report on the antioxidative effect of the SOD in model systems of linoleic acid, cholesterol, and ascorbic acid, respectively, after partial purification.

SIK—The Swedish Food Institute, P.O. Box 5401, S-402 29 Göteborg, Sweden.

However, if an enzyme such as SOD is to be used in foods, its effectiveness will depend on various factors such as the pH of the food, its water activity, storage temperature, thermal processes applied to the food, etc. These factors have to be investigated to make it possible to identify food products in which the enzyme could possibly be used. Reported here is the influence of pH and heat treatment on the antioxidative properties of the yeast SOD.

MATERIALS AND METHODS

SOD Preparation from Yeast. The SOD preparation from *S. cerevisae* var. ellipsoidus ATCC 560 was provided by the Department of Applied Microbiology, The Chemical Center, Lund, Sweden. Growth conditions have previously been described by Hansson and Häggström (1983). The cells were collected by centrifugation at 30000*g* for 15 min at 4 °C and washed twice in a 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA. The cells were then resuspended in one-fifth of the original volume in the same buffer and disintegrated in a ball mill. Cell debris was removed by centrifugation at 30000*g* for 30 min at 4 °C. The supernatant was dialyzed against the same buffer for about 16 h. The cell-free crude SOD preparations were stored at −20 °C until use.

SOD from bovine erythrocytes (Sigma Chemical Co.) and catalase from bovine liver (Sigma) were used for comparison.

SOD Assay. The SOD activity was assayed by measuring the ability to inhibit the reduction of cytochrome *c* by xanthine/xanthine oxidase, according to McCord and Fridovich (1969) with the modifications described by Hansson and Häggström (1983).

Catalase Assay. Catalase activity was assayed by a spectrophotometric method, as described in *Worthington Enzyme Manual* (1972).

Measurement of Protein Content. The protein content of the crude SOD preparation and of various fractions thereof was measured according to Lowry et al. (1951), using bovine serum albumin (Sigma) as the standard.

Oxidation of Linoleic Acid. The antioxidative effect of SOD in an emulsified linoleic acid model system was measured by a spectrophotometric method described by Lingnert et al. (1979), with the modification that 20 °C was used as the incubation temperature instead of 37 °C. The

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Table I. Ammonium Sulfate Precipitation of Yeast SOD

fraction	vol, mL	protein content, mg/mL	SOD act., U/mL	sp SOD act., U/mg protein	total SOD act., U	AE[a]
crude SOD preparation	100	8.4	564	67	56400	0.41
precipitate, 20% $(NH_4)_2SO_4$	40	1.3	59	45	2360	−0.03
precipitate, 40% $(NH_4)_2SO_4$	40	3.2	28	9	1120	−0.08
precipitate, 60% $(NH_4)_2SO_4$	40	5.4	154	29	6160	0.45
precipitate, 80% $(NH_4)_2SO_4$	20	3.4	1340	394	26800	0.61
precipitate, 90% $(NH_4)_2SO_4$	11	2.7	856	317	9420	0.62
supernatant, 90% $(NH_4)_2SO_4$	117	0.5	118	376	22000	−0.10

[a] AE (antioxidative effect) was measured at a level of 0.04 mg of protein/mL of emulsion.

absorption at 234 nm was measured before starting the incubation and after 24 h. When the influence of pH was studied, both an acetate buffer (pH 3.5, 4.5, 5.5) and a phosphate buffer (pH 5.5, 7.0, 9.0) were used. The buffer strength was in all cases 0.1 M. Comparisons were always made with controls at the same pH and with the same buffer as in the sample. The antioxidative effect (AE) was expressed in values ranging from 0 to 1, where 0 represents no antioxidative effect and 1 complete inhibition of the oxidation. (Negative values indicate a prooxidative effect.)

Oxidation of Cholesterol. A cholesterol emulsion was prepared according to Bergström and Wintersteiner (1942). Cholesterol (Sigma) (1.5 g), recrystallized twice in methanol, was dissolved in 60 mL of ethanol. The ethanol solution was slowly added to a vigorously stirred mixture (75 °C) of 0.3 g of stearic acid (Sigma), 300 mL of distilled water, and 5 mL of 0.1 M potassium phosphate buffer, pH 7.9. Immediately after preparation, the emulsion was cooled to 37 °C. $CuCl_2$ was added to the emulsion to a final concentration of 10^{-6} M, and the emulsion was incubated in darkness at 37 °C in 70-mL portions containing various concentrations of SOD. The oxidation during 24 h was followed by measurement of the peroxide value by a method described by Yanishlieva et al. (1978). One drop of concentrated HCl was added to 10 mL of emulsion to break the emulsion. The lipids were extracted with 10 mL of chloroform, which was then evaporated on a rotating vacuum evaporator.

Oxidation of Ascorbic Acid. Various amounts of yeast SOD and bovine catalase (Sigma) were added to solutions of ascorbic acid (Merck) in 0.05 M phosphate buffer, pH 7.0 (0.2 mg of ascorbic acid/mL of buffer). Incubation was performed by shaking the solutions in a water bath, thermostated at 25 °C. Samples of 0.1 mL were withdrawn at various times during 24 h of incubation, and their content of ascorbic acid was measured by a spectrophotometric method according to Boehringer Mannheim (Catalog No. 409 677).

Heat Treatment. Heat inactivation studies were performed with the SOD solutions enclosed in thin-walled glass capillary tubes in a tube immersion apparatus, as described by Svensson (1977).

RESULTS AND DISCUSSION

Purification of the Crude Preparation. Oxidation studies with linoleic acid emulsion showed the crude SOD preparation from yeast to be only moderately antioxidative, as compared to commercial bovine SOD at the same level of enzyme activity. The yeast preparation did probably also contain prooxidative or inhibiting components, for which reason purification to some extent was needed prior to further studies of the antioxidative effect. Ammonium sulfate precipitation was used for the purification. To the crude SOD preparation was added ammonium sulfate, giving a final concentration of 20% of saturation. After centrifugation, the precipitate was collected and the ammonium sulfate concentration in the supernatant was increased to 40% of saturation. This step-by-step procedure provided precipitates from solutions containing ammonium sulfate at 20%, 40%, 60%, 80%, and 90% of saturation. Protein content, SOD activity, and antioxidative effect were measured in each fraction. The results are given in Table I. The specific SOD activity of the precipitate obtained at an ammonium sulfate concentration of 80% of saturation was almost 6 times as high as that of the original crude SOD preparation. The total SOD activity in the precipitates and the final supernatant in the table sum up to a value higher than the total SOD activity in the initial, crude preparation. This is probably due to some lack of precision in the measurements of SOD activity. Nevertheless, it is obvious that most of the total activity was recovered in the precipitates obtained at 80% and 90% of saturation. The two precipitates with the highest specific SOD activity were also found to be the most antioxidative. However, no antioxidative effect was found in the final supernatant in spite of the high SOD activity. The high ammonium sulfate concentration in this sample did possibly affect the measurement of antioxidative effect. Table I also shows some of the fractions to be prooxidative (negative values of AE), which supports the assumption that crude yeast extract also contained prooxidants.

Table II. Antioxidative Effect of SOD on the Oxidation of Linoleic Acid

SOD act., U/mL emulsion	yeast SOD: crude preparation	yeast SOD: precipitate	bovine SOD
1	−0.04	0.40	0.45
5	0.53	0.72	0.81
25	0.22	0.60	0.96

For further studies the crude yeast extract was purified by first removing the precipitate at an ammonium sulfate saturation of 60% and then increasing the ammonium sulfate concentration to 90% of saturation. The precipitate obtained was used in the studies of antioxidative properties in model systems and in heat inactivation experiments.

Effect of SOD in Various Oxidation Systems. *Linoleic Acid Oxidation.* In Table II the antioxidative effects of the crude yeast preparation, the purified yeast SOD, and the commercial bovine SOD are compared at three different levels of enzyme activity. The bovine enzyme showed a strong antioxidative effect, which increased with increasing concentration. As mentioned, the crude yeast preparation was considerably less antioxidative or, at the lowest concentration, even somewhat prooxidative. The antioxidative effect did also decrease when the concentration was increased from 5 to 25 U/mL. The precipitated fraction showed an antioxidative effect comparable with that of the bovine SOD at the two lowest concentrations. However, the increase of the SOD concentration from 5 to 25 U/mL still led to a decrease of the anti-

Table III. Influence of Bovine Catalase on the Oxidation of Linoleic Acid

catalase act., U/mL emulsion	AE[a]
1	0.13
5	0.13
25	−0.12
125	−0.46

[a] AE = antioxidative effect.

Table IV. Antioxidative Effect (AE) of Bovine SOD Alone and in Combination with Bovine Catalase on the Oxidation of Linoleic Acid

SOD act. U/mL emulsion	AE	
	SOD	SOD/catalase[a]
1	0.51	0.40
5	0.94	0.82
25	0.99	0.97

[a] 0.4 U of catalase/1.0 U of SOD.

oxidative effect. This suggests that the precipitated fraction still contained prooxidative components.

Since SOD dismutates superoxide radicals into oxygen and hydrogen peroxide, an effective protective system would preferably also degrade the hydrogen peroxide. In theory, the combination of SOD/catalase would therefore be a good antioxidative system. For this reason, catalase alone was investigated with regard to its influence on linoleic acid oxidation. As can be seen in Table III, catalase was only slightly antioxidative at low concentrations, while a strong prooxidative effect was obtained at higher concentrations. The probable reason for the prooxidative properties of catalase is its hemoprotein nature. Hemoproteins are well-known as lipid oxidation catalysts.

Considerable catalase activity was also observed in the yeast fractions. The crude yeast preparation contained as much as 3.4 U of catalase/U of SOD. After the ammonium sulfate precipitation, 12% of the original catalase activity remained in the SOD fraction (0.4 U of catalase/U of SOD). This could be one reason for the poorer effect of the yeast preparations, as compared with the pure bovine SOD (Table II). Even the precipitated yeast SOD contained considerable amounts of catalase at the highest concentration tested (10 U of catalase at 25 U of SOD).

To test the influence of catalase on the antioxidative effect of SOD, combinations of the two enzymes were studied in the linoleic acid system. The bovine enzymes were used in the proportion 0.4 U of catalase to 1.0 U of SOD, as in the precipitated yeast fraction. The results are given in Table IV. The addition of catalase reduced the antioxidative effect of SOD, but the effect of the catalase could not fully explain the low antioxidative effect of the yeast precipitate at the highest concentration (see Table II). (It should be observed that the absolute values in Tables II and IV cannot be directly compared since the measurements were performed on different occasions with two different linoleic acid emulsions.)

Cholesterol Oxidation. The influence of the precipitated yeast SOD fraction on the Cu-catalyzed oxidation of emulsified cholesterol was studied. As can be seen in Figure 1, the oxidation was retarded by the SOD. The antioxidative effect increased with increasing concentration of SOD.

Ascorbic Acid Oxidation. Yeast SOD was also found to offer effective protection against the oxidation of ascorbic acid in a neutral solution (Figure 2). Catalase had no antioxidative effect at corresponding levels of enzyme activity, while at higher concentrations catalase was also found to retard the oxidation. The combination of SOD and catalase did not result in any major improvement of the antioxidative effect, as compared to the effect of the two enzymes separately.

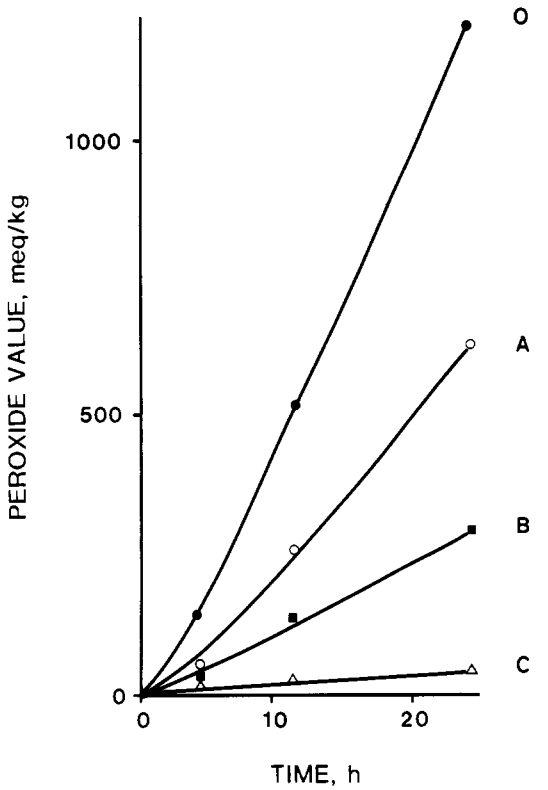

Figure 1. Oxidation of emulsified cholesterol in the presence of various concentrations of yeast SOD: A = 20 U of SOD/L of emulsion; B = 40 U of SOD/L of emulsion; C = 100 U of SOD/L of emulsion; O, control without SOD.

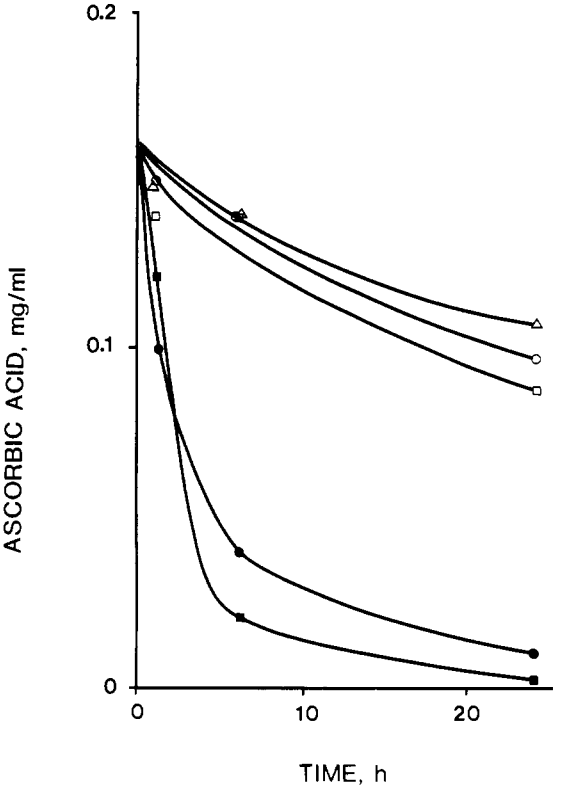

Figure 2. Oxidation of ascorbic acid, as influenced by addition of yeast SOD and bovine catalase: ●, control; △, 7.5 U of SOD/mL; ■, 7.5 U of catalase/mL; □, 75 U of catalase/mL; ○, 7.5 U of SOD + 75 U of catalase/mL.

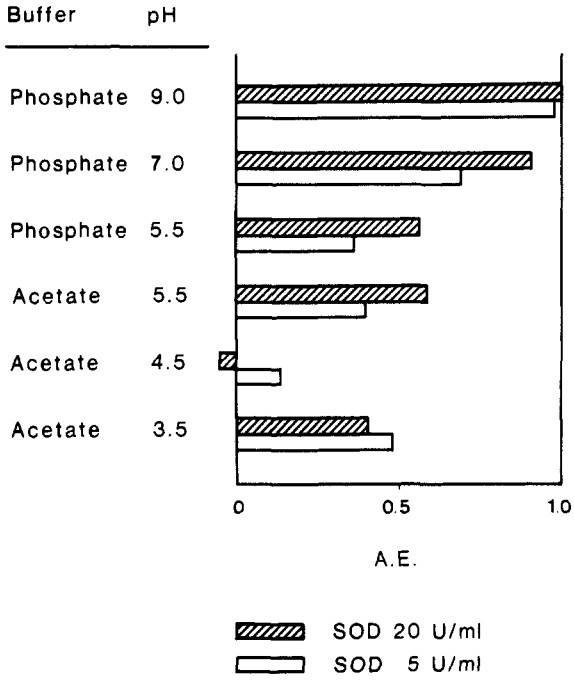

Figure 3. Influence of pH on the antioxidative effect (AE) of yeast SOD.

Role of SOD as an Antioxidant. Oxidation of ascorbic acid by the superoxide anion has previously been demonstrated (Nishikimi, 1975; Nanni et al., 1980). Using xanthine/xanthine oxidase as the superoxide anion generator, Nishikimi also showed that SOD inhibits the oxidation. SOD has also been shown to inhibit the oxidation of linoleic acid in model systems, using lipoxygenase (Richter et al., 1975) or xanthine/xanthine oxidase (Thomas et al., 1982) as the catalyst. However, the mechanism of superoxide anion initiated linoleic acid oxidation has been the subject of debate. It has been claimed that the oxidation results from a reaction between superoxide anion and lipid hydroperoxide (Thomas et al., 1982; Sutherland and Gebicki, 1982).

Postulating that the only mechanism behind the SOD effect is the dismutation of superoxide anions, this oxygen radical should play an important role in the oxidation of our model systems, since SOD was found to inhibit the oxidation in all three systems. The question then arises as to how the superoxide anions were formed in our systems. No catalysts were added to the linoleic acid or ascorbic acid systems, while copper ions were used in the cholesterol system. Metal ions are known to be involved in oxygen radical formation. The generation of superoxide anions has also been demonstrated at the decomposition of linoleic acid hydroperoxides (Yamashoji et al., 1979). However, regardless of the possible mechanisms of superoxide anion generation, the fact remains that the purified yeast SOD acted as an antioxidant in the three model systems.

Influence of pH. The influence of pH on the antioxidative effect of yeast SOD in the linoleic acid system was investigated in the range pH 3.5–9.0. The antioxidative effect was measured at two levels of SOD addition: 5 U/mL of linoleic acid emulsion and 20 U/mL of linoleic acid emulsion. The results are shown in Figure 3. The antioxidative effect decreased with decreasing pH values in the range pH 9.0–4.5. The yeast SOD showed almost no antioxidative effect at pH 4.5. Some antioxidative effect was observed again at pH 3.5. There was good agreement between the measurements at pH 5.5 in samples with acetate and phosphate buffer, respectively,

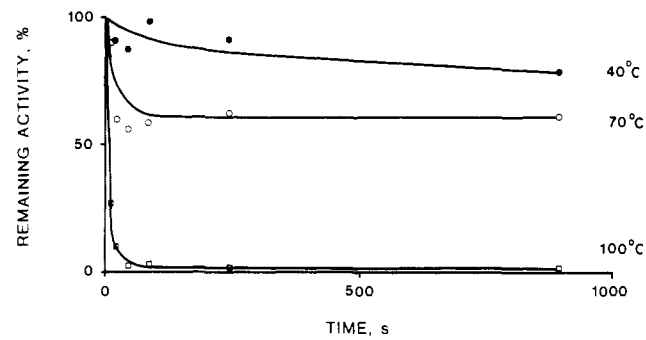

Figure 4. Influence of temperature on the heat inactivation of yeast SOD: ●, heat inactivation at 40 °C; ○, heat inactivation at 70 °C; □, heat inactivation at 100 °C.

implying that the choice of buffer did not influence the measure of antioxidative effect.

The antioxidative effect of yeast SOD was obviously highly dependent on the pH. This may be due to at least two different factors: the pH dependence of the enzyme on the one hand and the pH dependence of the spontaneous dismutation reaction on the other.

At acidic pH values, where the superoxide radical is protonated to form hydroperoxyl radicals ($HO_2^{\bullet}$), spontaneous dismutation takes place far more rapidly than at higher pH values. Halliwell and Gutteridge (1985) reported that the dismutation reaction in an aqueous solution will have an overall rate constant of about 10^2 M^{-1} s^{-1} at pH 11 and about 5×10^5 M^{-1} s^{-1} at pH 7. This means that the importance of the SOD-catalyzed dismutation will decrease with decreasing pH values.

Regarding the pH dependence of the enzyme, the reaction catalyzed by bovine erythrocyte CuZnSOD is claimed to be relatively independent of the pH in the range 5.3–9.5, the rate constant being about 1.6×10^9 M^{-1} s^{-1} (Halliwell and Gutteridge, 1985). The activity of MnSODs, on the other hand, is reported to decrease at alkaline pH values. Marmocchi et al. (1983) studied CuZnSODs from bovine and porcine erythrocytes and from yeast. For all three enzymes they found the rate constant to be higher at pH 7.6 than at pH 10.0. In a later study they found the activity of the porcine CuZnSOD to decrease almost linearly with increasing pH values between pH 7.5 and 12.0 (Argese et al., 1984).

Accordingly, if the SOD activity is pH dependent, it will decrease, if anything, with increasing pH, at least at values above pH 7. A more probable explanation of the decreasing antioxidative effect with decreasing pH is therefore the lessening importance of the enzymatic dismutation relative to the spontaneous dismutation. However, none of these factors explain the greater antioxidative effect observed at pH 3.5 as compared to pH 4.5.

Influence of Heat Treatment. The yeast SOD preparation was heated for varying periods of time at 40, 70, and 100 °C whereafter the remaining enzyme activity was measured. Results are shown in Figure 4, in which the remaining activity is plotted against heating time. When the SOD was kept at 40 °C, the activity slowly decreased to approximately 80% of the original activity. At 70 °C the activity decreased rapidly during the first 20 s, after which time the activity remained relatively constant (at about 60% of the original value) during the rest of the heating period. At 100 °C the activity rapidly decreased to a level of about 2% of the original, but not even at this temperature was the enzyme completely inactivated. Similar leveling at a constant remaining activity was obtained also at other temperatures, the level being dependent on the temperature. The activity was found to

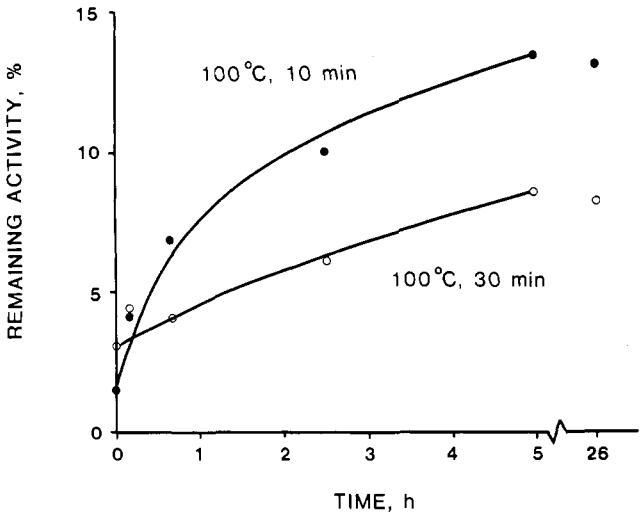

Figure 5. Reactivation of yeast SOD at 0 °C after inactivation at 100 °C: ●, heating time 10 min at 100 °C; ○, heating time 30 min at 100 °C.

Table V. Antioxidative Effect (AE) of Yeast SOD after Heat Treatment at 100 °C

time of heat treatment, s	AE[a]
0	0.64
60	0.21
600	0.25

[a] 1.5 U of SOD (before heat treatment)/mL of linoleic acid emulsion.

be maintained at a constant level also at longer heating times (up to 40 min). We are unable to explain this peculiar behavior of the enzyme upon heat treatment. We also found that the enzyme was reactivated after heating. This is shown in Figure 5 and could possibly explain why the remaining activity measured was never zero, even after the SOD was kept at 100 °C for a considerable length of time. Some reactivation could occur during the few minutes that elapsed between the termination of the heat treatment and measurement of the enzyme activity.

From Figure 5 it can be seen that samples heated at 100 °C for 10 min and kept at 0 °C after the heat treatment recovered approximately 13% of their original activity within 5 h. This level of activity remained also after 1 day. A lower degree of reactivation was obtained when the enzyme solution was heated for a longer period of time.

In some experiments, the antioxidative effect of heat-treated yeast SOD was measured as well, to ensure that the results obtained regarding heat influence on enzyme activity also had a bearing on the antioxidative properties. Table V shows the antioxidative effect of yeast SOD heated at 100 °C for 0, 60, and 600 s. The heat treatment resulted in considerable loss of antioxidative effect. Increase of the heating time from 60 to 600 s had little influence on the antioxidative effect, which is in accordance with the results obtained from enzyme activity measurements. However, the antioxidative effect appears to be less influenced than the enzyme activity by heat treatment. One possible explanation of this is reactivation of the SOD during the 24 h needed for the assay of the antioxidative effect.

Forman and Fridovich (1973) have previously reported bovine erythrocyte SOD to lose 50% of its activity after heating at 77 °C for 10 min. Korycka-Dahl et al. (1979) reported purified erythrocyte SOD to be less heat stable than crude preparations of bovine milk SOD. However, both SOD preparations were reported to be completely inactivated after heating at 100 °C for 10 min. Low-temperature pasteurization (63 °C, 30 min) did not decrease the milk SOD activity to any great extent. About 96% of the original activity remained.

Hicks et al. (1979) carried out further studies of heat inactivation of bovine milk SOD. Purified milk SOD was found to be more heat sensitive than SOD in the milk serum. No inactivation in milk serum was observed after heating to 70.0 °C for up to 30 min. At 71.7 °C, less than 20% of the SOD activity was lost after 30 min. At 75.5 °C, a linear relationship was obtained between heating time and inactivation. More than 70% inactivation was attained after 20 min. Thus, the course of the inactivation of the milk enzyme was not similar to that observed for yeast SOD in the present study.

Reactivation of yeast SOD has previously been noticed by Arnold and Lepock (1982). In their experiments, approximately 50% of the original activity remained after heating at 78 °C for 10 min. One hour after heating, during which time the samples were maintained at 37 °C, the activity had returned to about 70% of the original. These results are in accordance with the findings in the present report.

CONCLUSIONS

Purified yeast SOD was found to act as an antioxidant in oxidative systems of emulsified linoleic acid, emulsified cholesterol, and ascorbic acid. Catalase had a prooxidative effect in the linoleic acid system.

The present study was also carried out in order to evaluate possibilities and limitations in the use of yeast SOD as an antioxidant in food systems. It can be concluded that the pH of the food will greatly affect the antioxidative efficiency of added yeast SOD. The greatest antioxidative effect was obtained at alkaline pH values, but a considerable antioxidative effect is also likely to be obtained in neutral and moderately acidic foods. The pH dependence was probably due to the decreasing importance of enzymatic dismutation at lower pH values.

Yeast SOD could be a potential antioxidant for use also in heat-treated foods. Even after the enzyme was kept at 100 °C for 10 min, more than 10% of the original enzyme activity was restored after reactivation. Comparisons with reports in the literature indicate a greater heat stability of yeast SOD as compared to bovine erythrocyte SOD and bovine milk SOD, possibly owing to the ability of the yeast SOD to reactivate.

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Registry No. SOD, 9054-89-1; catalase, 9001-05-2; linoleic acid, 60-33-3; cholesterol, 57-88-5; ascorbic acid, 50-81-7.

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Effects of Alfalfa Leaf Juice and Chloroplast-Free Juice pH Values and Freezing upon the Recovery of White Protein Concentrate

Amelia Hernández,* Carmen Martínez, and Carmen Alzueta

Juice extracted from alfalfa was frozen at −25 °C and stored for different periods of time. After the juice was adjusted to pH 8.0, 8.5, and 9.0 and pH 6.0 as control, the green proteins were separated by heat treatment. Soluble or white proteins were coagulated in the resulting chloroplast-free juice at two different pH values, 3.5 and 4.0. The results show that greater amounts of dry matter and nitrogen are recuperated in the white protein concentrate at alkaline pH values than at the natural pH value, 6.0, and the amount of concentrate recovered is also greater when soluble proteins are coagulated at pH 3.5 instead of 4.0, although the resulting concentrate has less protein. With regard to the effect of freezing, it was possible to conclude that freezing of the juice was not a desirable storage method because it resulted in the coagulation and subsequent loss of most of the soluble proteins.

The separation of two protein fractions, insoluble or green protein from soluble or white protein, is necessary to prepare chlorophyll-free protein concentrates. The most common method described in the literature is the separation of green proteins by heat treatment, followed by acid precipitation of the white proteins in the resulting chloroplast-free juice.

The main variables affecting heat treatment are temperature, heating time, and juice pH. Although the first two have been sufficiently studied, with regard to the optimum juice pH there is some disagreement in the literature, previously described in detail (Hernández et al., 1988) on whether alkaline or natural pH is more suitable.

The optimum pH value for coagulating the soluble proteins in chloroplast-free juice was studied by Miller et

Departamento de Farmacia, Nutrición y Bromatología, Facultad de Farmacia, Universidad de Alcalá de Henares, 28871 Alcalá de Henares, Madrid, Spain.

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