Enhancement of Emulsifying Properties of Protein by Sonicating with Egg Yolk Lecithin

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Emulsifying properties of several globular proteins except lysozyme and ovomucoid were greatly enhanced by sonicating with egg yolk lecithin. Much enhancement was attained when the sonication was carried out at acidic pH regions such as pH ≤4. The results of gel filtration experiments clearly showed that the formation of protein–lecithin complex was the cause of this enhancement. When ovomucoid and lysozyme were previously incubated with a small amount of dithiothreitol, their emulsifying properties were improved by sonicating with egg yolk lecithin.

In previous work (Mizutani and Nakamura, 1984) on the clarification of characteristics of emulsifying properties of egg yolk low-density lipoprotein (LDL), comparisons were made between the emulsifying properties of egg yolk lecithin and those of bovine serum albumin (BSA). The results showed that emulsifying activity of LDL was much larger than that of egg yolk lecithin or BSA. Furthermore, we also showed that emulsifying properties of apoLDL–egg yolk lecithin complex were superior to those of either lecithin vesicles or lecithin suspension and almost the same as those of LDL (Mizutani and Nakamura, 1987a). These results clearly suggest that superior emulsifying properties of LDL mainly depend on the characteristic structure of its lipid–protein complex and emulsifying properties of protein will be enhanced by complex formation with egg yolk lecithin.

In the present work, several globular proteins were sonicated with egg yolk lecithin and their emulsifying properties were investigated.

MATERIALS AND METHODS

Materials. Both egg yolk lecithin and ovalbumin were prepared from fresh egg of unfertilized White Leghorn hens according to the methods of Singleton et al. (1965) and Marshall and Neuberger (1972), respectively. Purity of egg yolk lecithin was confirmed by thin-layer chromatography. The ovalbumin was recrystallized three or four times, dialyzed until free of salt, and freeze-dried. Bovine serum albumin (BSA, fraction V), conalbumin (type I), β-lactoglobulin (3× crystallized), lysozyme (grade I), and ovomucoid (type III-o) were purchased from Sigma Chemical Co.

Preparation of Protein–Lecithin Complex. A few milliliters of egg yolk lecithin suspension containing 10–100 mg of lecithin was added to 1 mL of protein solution of a definite concentration, usually 2.5 mg/mL. The total volume of this solution was adjusted to 5 mL with an appropriate buffer solution, usually 0.1 M sodium acetate buffer (pH 3.0). Next, the solution was sonicated at 20 °C for 10 min with an Insonator (Model 200 M, Kubota, Tokyo) at maximum output.

Measurement of Emulsifying Properties. The turbidimetric method of Pearce and Kinsella (1978) was used. Peanut oil was added to a protein–lecithin complex solution of a definite concentration, followed by homogenization with a Polytron (Kinematica, Model CH-6010) at 10000 rpm for 60 s. The emulsion was immediately diluted with 0.1% Triton X-100 to final dilutions in the range of 1:500, and the absorbance at 500 nm was recorded. Since the absorbance of diluted emulsions is directly related to the interface area for coarse emulsions (Kerker, 1969), it was used as the index for emulsifying properties in the present work.

Gel Filtration. Protein–lecithin complex solution containing 20 mg of protein in 5 mL of an appropriate buffer, usually 0.1 M sodium acetate buffer (pH 3.0) or 0.1 M sodium phosphate buffer (pH 7.0), was applied on a Sepharose CL-4B column (1.8 × 42 cm) and eluted with the same buffer.

Liquid Chromatography. Properties of ovomucoid and lysozyme were studied with a Jasco HPLC system (JASCO880) using a fine-pack SIL G-18 column (for ovomucoid) and Toyo-Soda phenyl-5PW column (for lysozyme) with UV spectrophotometer detector Jasco UN-IDEC-100V at 280 nm as the monitor. Ovomucoid was eluted with a linear gradient ranging from 99:1 to 20:80 trifluoroacetic acid–isopropyl alcohol over 40 min at a flow rate of 0.5 mL/min while lysozyme was eluted with a linear gradient ranging from 99:1 to 50:50 trifluoroacetic acid–isopropyl alcohol over 15 min at a flow rate of 1 mL/min.

Analytical Procedure. Protein concentration was determined by a modified Lowry method (Markwell et al., 1978) using bovine serum albumin as a standard. Lecithin concentration was determined by Bartlett method (1959).

Table I. Absorbance of Emulsions Prepared from Globular Proteins and Their Complex with Egg Yolk Lecithin[a]

	absorbance of emulsions[b]	
	from proteins	from protein–lecithin complex
bovine serum albumin	0.41 ± 0.01	1.05 ± 0.06
ovalbumin	0.21 ± 0.02	0.75 ± 0.08
conalbumin	0.33 ± 0.0±	0.86 ± 0.07
β-lactoglobulin	0.50 ± 0.02	0.72 ± 0.02
lysozyme	0.18 ± 0.01	0.21 ± 0.02
ovomucoid	0.58 ± 0.04	0.18 ± 0.01

[a] Both protein and lecithin concentrations in protein–lecithin complex solution were adjusted to 2.5 and 20 mg/mL, respectively, and protein–lecithin complex was prepared at pH 3.0. Oil volume fraction of emulsion was 0.5. [b] Mean ± SD (n = 4).

RESULTS AND DISCUSSION

Emulsifying properties of several globular proteins were measured after sonicating with egg yolk lecithin at pH 3.0 (Table I). Emulsifying properties of many proteins were enhanced by sonicating with egg yolk lecithin, but those of ovomucoid and lysozyme were not enhanced by the same treatment. Since the improvement of emulsifying

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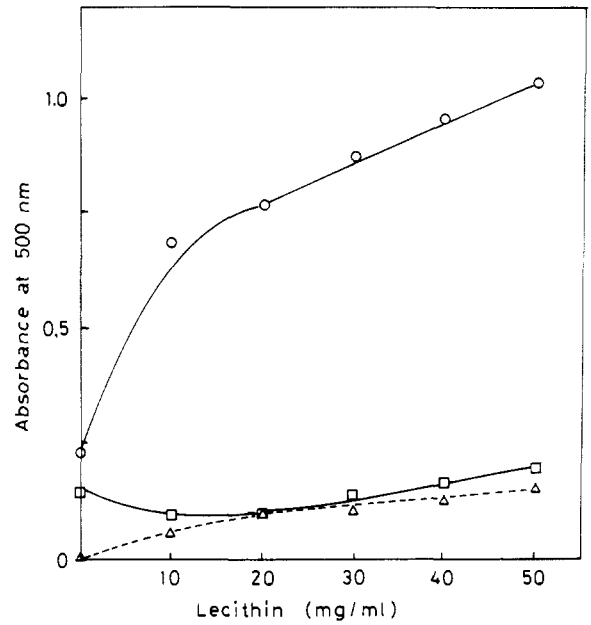

Figure 1. Absorbance of emulsions prepared from a lecithin suspension (△---△), ovalbumin–lecithin complex (○—○), and lysozyme–lecithin complex (□—□). Protein concentration in protein–lecithin complex solution was adjusted to 0.5 mg/mL, and protein–lecithin complex was prepared at pH 3.0. Oil volume fraction of emulsion was 0.5.

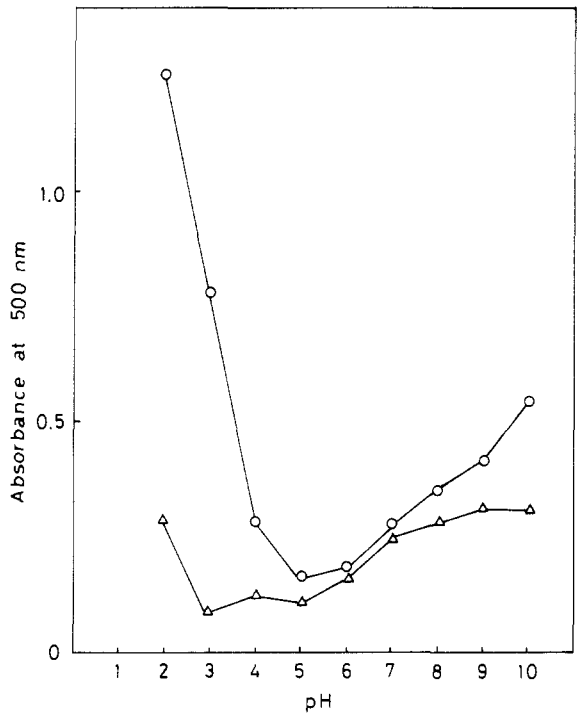

Figure 2. Absorbance of emulsions prepared from ovalbumin–lecithin complex (○—○) and lysozyme–lecithin complex (△—△) made at various pH's. Protein and lecithin concentrations in protein–lecithin complex solution were adjusted to 2.5 and 20 mg/mL, respectively. Oil volume fraction of emulsion was 0.5.

properties of protein is very interesting, further experiments were made about the detailed conditions of protein treatment using ovalbumin and lysozyme.

As shown in Figure 1, emulsifying properties of ovalbumin were gradually enhanced by sonicating with increased amount of egg yolk lecithin, but those of lysozyme were not changed by the same treatment. This enhancement of emulsifying properties of ovalbumin was greatly dependent on pH (Figure 2); when the ovalbumin solution

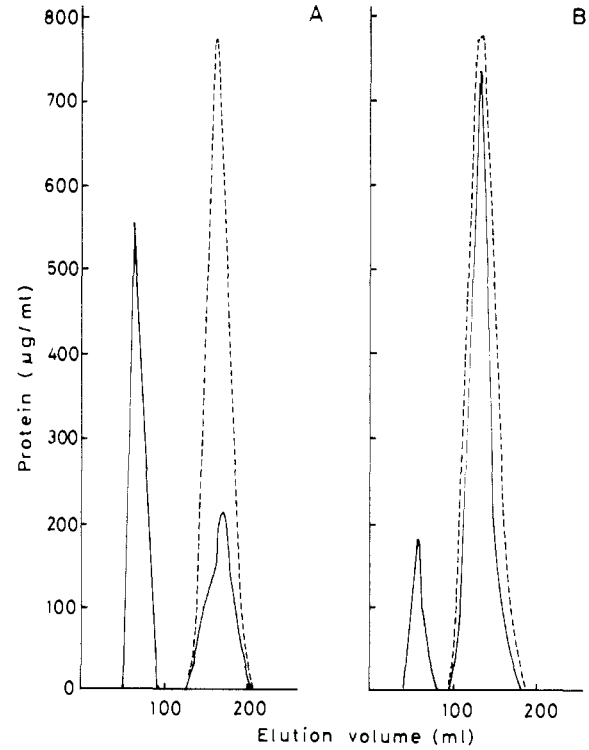

Figure 3. Gel filtration patterns of ovalbumin–lecithin complex prepared at pH 3.0 (A) and pH 7.0 (B). Ratio of egg yolk lecithin to ovalbumin in the complex was adjusted to 8, and the amount of protein in the complex applied to the column was 20 mg. Dotted line shows the elution pattern of ovalbumin.

was below pH 4, much enhancement was observed. However, when the ovalbumin solution was above pH 5, enhancement of the emulsifying properties was rather small. A similar effect of pH on the enhancement of emulsifying properties of protein sonicated with egg yolk lecithin was also observed about BSA, β-lactoglobulin, and conalbumin (data not shown). However, changes in the emulsifying properties of lysozyme were very small when the solution was changed from pH 2 to 10 (Figure 2).

Enhancement of emulsifying properties of proteins sonicated with egg yolk lecithin seems to be caused by complex formation between protein and egg yolk lecithin. To ascertain this assumption, gel filtration was carried out about ovalbumin and lysozyme solution, sonicating with egg yolk lecithin. Figure 3 shows the gel filtration patterns of ovalbumin solution sonicated with egg yolk lecithin at pH 3 and 7. In the gel filtration patterns of ovalbumin sonicated with egg yolk lecithin, two peaks were noted; the first peak was around the void volume of the gel filtration column and almost the same as that of the lecithin vesicle, and the second peak was that of untreated ovalbumin. Since lecithin was only detected in the first peak, the first peak should be that of a protein–lecithin complex formed by sonicating with egg yolk lecithin. when ovalbumin solution was sonicated with egg yolk lecithin at pH 3, a large amount of protein–lecithin complex was formed. This protein–lecithin complex solution, however, was turbid, and some portions of it remained in the gel filtration column. A part of the protein–lecithin complex might interact to give large particles not to be eluted from the column. When ovalbumin solution was sonicated with egg yolk lecithin at pH 7, only a small amount of protein–lecithin complex was formed. On the contrary, when lysozyme solution was sonicated with egg yolk lecithin at pH 3, a very small amount of protein–lecithin complex was formed (Figure 4). The gel filtration pattern of lysozyme solution sonicated with egg yolk lecithin at pH 7 was al-

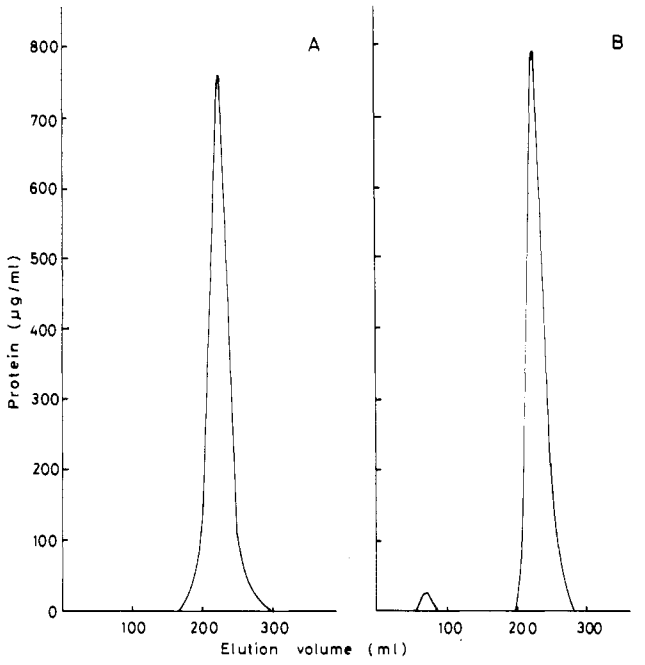

Figure 4. Gel filtration patterns of lysozyme (A) and lysozyme–lecithin complex (B) prepared at pH 3.0. Ratio of egg yolk lecithin to lysozyme in the complex was adjusted to 8, and the amount of protein in the complex applied to the column was 20 mg.

most the same as Figure 4B (data not shown). All these results clearly show that enhancement of emulsifying properties of protein by sonicating with egg yolk lecithin depends on the formation of a protein–lecithin complex.

To investigate the properties of this protein–lecithin complex, sucrose density gradient centrifugation was applied to the protein solution, which was sonicated with egg lecithin. But it gave only one broad zone containing both protein and egg lecithin (data not shown). The protein–lecithin complex may be not so stable as to give a sharp band by this procedure. The emulsifying properties of the protein solution sonicated with egg lecithin, however, were still high after the pH was adjusted to neutral and the solution kept for a few days at room temperature. These seem to show that the protein–lecithin complex is surely present in this solution. These properties of protein–lecithin complex are very useful to modify the functional properties of various proteins. We already showed that the emulsifying properties of both blood globin and wheat gluten were improved by complex formation with soy lecithin (Mizutani and Nakamura, 1988).

It is presumed that both ovomucoid and lysozyme cannot form enough protein–lecithin complex to give excellent emulsifying properties. The reason why these two proteins show different behavior from other globular proteins is not clear. Since ovomucoid and lysozyme have many disulfide bonds (Canfield and Liu, 1965; Kato et al., 1976) and their molecular structure is shown to be rather rigid, this rigid structure of both proteins might suppress the interaction between protein and egg yolk lecithin. On the basis of this assumption, the effect of dithiothreitol, a reducing agent, on the complex formation of both proteins with egg yolk lecithin was studied.

Figure 5 shows the effect of dithiothreitol treatment of ovomucoid and lysozyme on the emulsifying properties of their protein–lecithin complex. The emulsifying properties of the protein–lecithin complexes of both proteins were greatly enhanced by incubating with small amount of dithiothreitol such as 1 mM or less. Dithiothreitol treatment might induce some structural changes of both

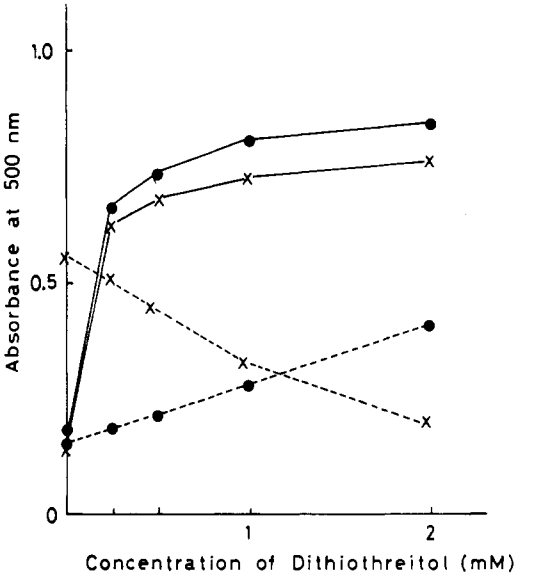

Figure 5. Effect of dithiothreitol treatment of protein on the emulsifying properties of the protein and the protein–lecithin complex: ×—×, ovomucoid–lecithin complex; ●—●, lysozyme–lecithin complex; ×---×, ovomucoid; ●---●, lysozyme. Protein solutions were previously incubated with various concentrations of dithiothreitol (0–20 mM) at 30 °C for 24 h. Protein and lecithin concentrations in the protein–lecithin complex solution were adjusted to 2.5 and 20 mg/mL, respectively, and protein–lecithin complex was prepared at pH 3.0. Oil volume fraction of emulsion was 0.5.

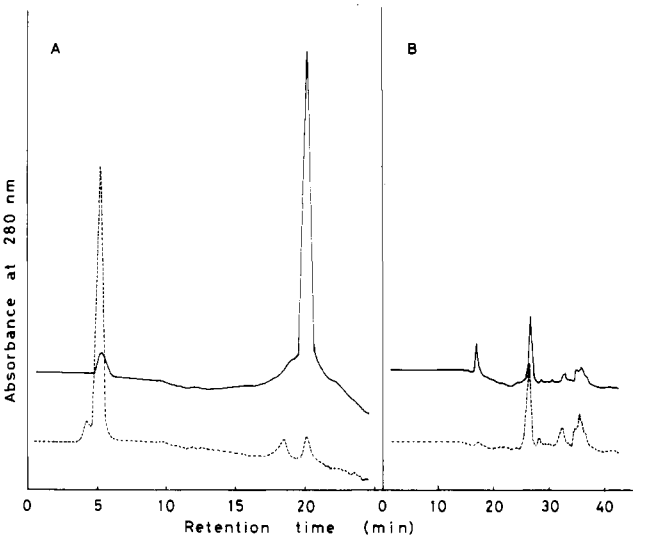

Figure 6. HPLC chromatograms of ovomucoid and lysozyme before (---) and after (—) dithiothreitol treatment: A, lysozyme; B, ovomucoid. Ovomucoid solution (2.5 mg/mL) and lysozyme solution (1 mg/mL) were incubated with 1 mM dithiothreitol at 30 °C for 24 h. Portions of 10 μL of each solution were injected onto the HPLC column. Column: fine-pack SIL G-18 for ovomucoid; Toyo-Soda phenyl-5PW for lysozyme. Effluent and flow rate: linear gradient from 99:1 to 20:80 trifluoroacetic acid–isopropyl alcohol over 40 min at a flow rate of 0.5 mg/min for ovomucoid and linear gradient from 99:1 to 50:50 trifluoroacetic acid–isopropyl alcohol over 15 min at a flow rate of 1 mL/min for lysozyme. Detector: UV 280 nm.

proteins and affect their protein–lecithin complex formation. To confirm this idea, HPLC chromatograms were taken of both proteins before and after dithiothreitol treatment (Figure 6). It is clear that both proteins were changed with dithiothreitol treatment. Judging from the changes in the retention time of both proteins, hydrophobicity of lysozyme was increased by dithiothreitol

treatment, while that of ovomucoid was decreased a little. Although explanation cannot be made about the different behavior of both proteins, some conformational change must have occurred in both proteins with dithiothreitol treatment.

Recently, phospholipid–protein interaction has been studying about various kinds of proteins such as β-lactoglobulin (Brown et al., 1983), α-lactalbumin (Hanssens et al., 1985), BSA (Schenkman et al., 1981), and insulin (Farias et al., 1985). All these proteins interact well with phospholipid vesicles at low pH. These proteins are shown to expose hydrophobic amino acid side chains at low pH. According to the amphipathic helix model of lipid–protein interaction (Segrest and Feldman, 1977), the initial interaction is between positively charged amino groups in the protein and the negatively charged head group of the phospholipid, after which the hydrophobic side of the helix is burried in the hydrocarbon chains of the lipid. Both ovomucoid and lysozyme may be unable to expose the hydrophobic amino acid side chains owing to their rigid structure, but addition of reducing agent cleaves the disulfide bonds and might expose hydrophobic side chains at low pH.

Registry No. Lysozyme, 9001-63-2; dithiothreitol, 3483-12-3.

LITERATURE CITED

Bartlett, G. R. *J. Biol. Chem.* **1959**, *234*, 466.
Brown, E. M.; Carroll, R. J.; Pfeffer, P. E.; Sampugna, J. *Lipids* **1983**, *18*, 111.
Canfield, R. E.; Liu, A. K. *J. Biol. Chem.* **1965**, *240*, 1997.
Farias, R. N.; Vinals, A. L.; Morero, R. D. *Biochem. Biophys. Res. Commun.* **1985**, *128*, 68.
Hanssens, I.; Ceunebroech, J.-C.; Pottel, H.; Preaux, G.; Cauwelaert, F. *Biochim. Biophys. Acta* **1985**, *817*, 154.
Kato, I.; Shrode, J.; Wilson, K. A.; Laskowski, M., Jr. *Protides Biol. Fluids* **1976**, *23*, 235.
Kerker, M. *The Scattering of Light and Other Elecrro-Magnetic* Radiation; Academic: London, 1969.
Kim, J.; Kim, H. *Biochemistry* **1986**, *25*, 7867.
Markwell, M. A. K.; Haas, S. M.; Bieber, L. L.; Tolbert, N. E. *Anal. Biochem.* **1978**, *87*, 206.
Marshall, R. D.; Neuberger, A. *Glycoproteins*; Gottshalk, A., Ed.; Elsevier: Amsterdam, 1972; p 732.
Mizutani, R.; Nakamura, R. *Lebensm.-Wiss. Technol.* **1984**, *17*, 213.
Mizutani, R.; Nakamura, R. *Agric. Biol. Chem.* **1987a**, *51*, 1115.
Mizutani, R.; Nakamura, R. *J. Food Sci.* **1988**, *53*, 527.
Pearce, K. N.; Kinsella, J. E. *J. Agric. Food Chem.* **1978**, *26*, 716.
Schenkman, S.; Arauje, P. S.; Kijkman, R.; Quina, F. H.; Chaimovich, H. *Biochim. Biophys. Acta* **1981**, *649*, 633.
Segrest, J. P.; Feldman, R. J. *Biopolymers* **1977**, *16*, 2053.
Singleton, W. S.; Gray, M. S.; Brown, M. L.; White, J. L. *J. Am. Oil Chem. Soc.* **1965**, *42*, 53.

Received for review October 12, 1987. Revised manuscript received February 26, 1988. Accepted March 16, 1988.

Chemical Studies on Novel Rice Hull Antioxidants. 1. Isolation, Fractionation, and Partial Characterization

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Methanol extracts of rice hull from Katakutara (long-life) and Kusabue (short-life) seeds exhibited antioxidative activity stronger than that of α-tocopherol. Fractionation of the crude extracts on Amberlite XAD-2 column followed by antioxidative assay revealed that both samples possessed strong antioxidative constituents in methanol–water (50:50, 75:25) fractions. HPLC separation of 50:50 methanol–water fractions gave a major peak in Katakutara (retention time 23.6 min), having very strong antioxidative activity, while Kusabue showed a major peak at 18.2 min, which exhibited antioxidative activity comparable to that of α-tocopherol. The 75:25 methanol–water fraction showed the presence of major peaks in Katakutara with retention times 5.6, 6.4, 12.8, and 13.6 min, while in Kusabue they were present at 5.6 and 13.8 min. However, these subfractions were found to become weaker in their antioxidative activity after separation. Both active fractions were richer in total phenolics than water and acetone fractions. The investigation demonstrated that the novel antioxidative defense system in rice hull, being more active than α-tocopherol and oryzanol, could play a vital role in controlling the germination potentials of rice seeds during long storage.

All physiological processes in living systems involve complex combinations of oxidation–reduction reactions governed by a variety of agents such as enzymes, hormones, trace elements, etc. Any change in the normal redox equilibrium established in healthy systems leads to malfunctioning of the cells, thereby to diseases and, in extreme cases, eventually in death. Thus, the antioxidants that regulate the various oxidation reactions and are found naturally in tissues are evaluated as a potential class of longevity determinants. Inadequacies of endogenous synthesis of antioxidants or other components of antioxidant compensatory system could be involved in producing specific types of disease processes, some of which might result in accelerated aging syndromes (Cutler, 1984). Though there are a number of defense and protective mechanisms in a cell that are essential for defending the organism against the toxic effects of normal oxygen metabolism (Fridovich, 1976; Mead, 1976), many others are likely to be discovered. Hence, research on investigation of antioxidative defense mechanism in biological systems has been gaining importance ever since oxidative damage to cell components was assumed to be one of the several factors of aging.

Osawa and Namiki (1981) had postulated that the stability against oxidative degradation of *Eucalyptus* oil could

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0021-8561/88/1436-0732$01.50/0