Antioxidative Mechanism of Maize Zein in Powder Model Systems against Methyl Linoleate: Effect of Water Activity and Coexistence of Antioxidants

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Food proteins such as soybean protein, milk casein, maize gluten, and zein were examined against methyl linoleate autoxidation in powder model systems. A gas chromatographic (GC) method was employed to estimate antioxidant activity by measuring oxygen consumption of head-space gas. Although strong antioxidant activities were observed for gluten and zein, stored at 60 °C without humidity regulation, they resulted from phenol compounds contaminating in the proteins such as tocopherols and not from the antioxidant action of proteins themselves. Water activity affected the antioxidative efficacy when α-tocopherol was added to the proteins: the tendency that the higher the A_w, the stronger was the antioxidant activity was found. But the prominent antioxidant activity of defatted zein at A_w 0.9 could not be contributed only to the activity of traces of unextracted phenolics contained in zein, as zein's hydrolysate almost lost activity at the same condition. A remarkable decrease in hexane-extractable efficiency of substrate oil at A_w 0.9 compared to that at A_w 0.3 implied a possible mechanism which suggested a delayed binding to protein or physical shielding by protein resulting in a reduced tendency of the lipid to autoxidize.

INTRODUCTION

Autoxidation of fats and oils in foods is responsible for the deterioration of flavors and tastes as well as nutritional values. The deterioration of lipids in foods is a very complicated process and involves various factors. Several approaches are available to retard lipid peroxidation in foods. Addition of phenolic antioxidants is most popular among them. Certain proteins are likewise expected to act as antioxidants under suitable conditions (Laakso et al., 1982, 1984). For example, wheat gliadin has been proven to be effective under conditions of 40 °C and water activity (A_w) 0.6, and its effectiveness continued longer at higher water activity than at lower one (Iwami et al., 1987). Although Iwami et al. noted that the gliadin they used was heterogeneous in its purity, they negated the possibility that other active compounds could be responsible for the antioxidant activity because the original source gluten did not show any antioxidant activity. There has been only a cursory understanding of the antioxidative protein species and their acting mechanisms. Corn is one of the three major crops in the world, and its main protein, zein, plays important roles both in foods and in animal feeds. Its amino acid composition and physiochemical properties have been well investigated. Zein possesses properties similar to those of wheat gliadin in amino acid composition and solubility. On the other hand, water content in foods is generally accepted to affect their resistance to lipid peroxidation. In this paper, the effect of various A_w values on the antioxidant activity of zein and other proteins has been compared by gas chromatographic and ferric thiocyanate methods, and the mechanisms of antioxidant action by food proteins under different circumstances have been discussed.

MATERIALS AND METHODS

Materials. Methyl linoleate (>99%) was prepared from the methyl ester of safflower seed oil by urea-adduct formation (Keppler et al., 1959). A trace amount of impurities, such as tocopherols and peroxides, was eliminated by silicic acid column chromatography and vacuum distillation. Methyl stearate was from Tokyo Kasei Kogyo Co., Ltd., which gave one peak in gas-liquid chromatography (GLC) analysis. Milk casein and soybean protein were purchased from Wako Pure Chemicals Industries Ltd., Osaka. Maize gluten, a byproduct during wet milling of corn, was provided by Institute Japan Food Chemicals Industries Ltd., Tokyo. Two kinds of zein preparations were used in the experiment, i.e., a commercial zein purchased from Nakarai Chemicals Ltd., Kyoto, and the prepared one from maize gluten which had been previously defatted with ethyl acetate, by extraction with 70% ethanol followed by lyophilization. Glutelin was prepared from the residue of zein after extraction by distilled water (pH 10.0, adjusted by 0.5 N NaOH) followed by precipitation in 0.05 N HCl and freeze-drying. Standard α-, γ-, and δ-tocopherols were obtained from Tama Biochemicals Ltd., Tokyo. Papain, from *Carica papaya*, was a product of Merck. Other reagents also were commercially obtained and used without further purification.

Preparation of Powder Models Systems. Each protein was mixed with 10% (w/w) methyl linoleate dissolved in ethyl ether, and the residual solvent was evaporated in cabinet drier. Aliquots of the mixture were put into either a 15-mL glass test tube directly (without humidity regulation) or a 4 cm high polypropylene vial placed in a humidity-controlled 15-mL glass test tube. These test tubes were sealed with a W-type rubber cap and stored at 60 °C in the dark.

Oxidation Stability. Oxygen absorption in the head space of test tubes was measured by gas chromatography with a thermoconductivity detector (Shimadzu GC-4C) under the following conditions: column, a stainless steel column (0.3 × 200 cm) packed with molecular sieve 5A (30/60 mesh); column temperature, 70 °C; detector temperature, 90 °C; injection temperature, 90 °C; carrier gas, helium, at a flow rate of 25 mL/min. Ten microliters of the head space was subjected to GC. Peroxide value (POV) was determined by the ferric thiocyanate method (Iwami et al., 1987). Water activity was adjusted with different concentrations of sulfuric acid: 55, 44, 33, and 22% (w/w) for A_w = 0.3, 0.5, 0.7, and 0.9, respectively. Each 15-mL test tube contained 3 mL of the sulfuric acid solution.

Recovery Test. Extracts from gluten and zein were remixed with the defatted gluten and zein at ratios identical with their original contents. On the other hand, α-tocopherol was added instead of extracts at ratio of 0.55% (w/w) to substrate oil.

Determination of Tocopherols. The tocopherol concentrations of the ethyl ether extract of gluten and the ethyl acetate

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extract of zein were determined by high-performance liquid chromatography (HPLC) (Abe et al., 1976).

Detection of Other Antioxidants. Ethyl acetate extract from zein was applied to TLC using various solvent systems and spray reagents to compare with some known phenolic antioxidative standards: TLC plate, 5714 Kieselgel 60 F_{254} 5 × 20 cm (0.25 mm); solvent I, toluene–methyl formate–formic acid (5:4:1); solvent II, toluene–chloroform–acetone (40:25:35); solvent III, benzene–pyridine–formic acid (36:9:5); indicator I, Folin–Ciocalteu's phenol reagent; indicator II, anisaldehyde–sulfuric acid; indicator III, 0.5% Fast Blue B. Methods in detail were described by Stahl (1969).

Hydrolysis of Zein. Zein (10 g) was dissolved in 300 mL of 70% ethanol, and to this ethanolic solution was added 200 mL of distilled water to precipitate zein as a fine powder. After this, ethanol and water were removed in vacuo until the final volume was about 200 mL. The concentrate obtained was adjusted to pH 5.0, and papain 0.5 g (1/20 w/w) was added followed by incubation in a 35 °C water bath for 48 h with stirring. After filtration with a Whatman No. 2 filter paper, the supernatant was lyophilized for 48 h. The extent of hydrolysis was assessed as follows: 2 mL of 12% (w/w) trichloroacetic acid (TCA) was added to 1 mL of hydrolysate, and the absorbance of the supernatant after removal of the precipitate was read at 280 nm. The bright yellowish powder of hydrolysate was stored with methyl linoleate as described before.

Extraction Efficiency. A mixture of methyl linoleate and methyl stearate (7:3 w/w) was used as the substrate oil instead of methyl linoleate only. Samples stored at A_w 0.3 and 0.9 were extracted three times with 1 mL of hexane and filtered through an Advantec 5C filter to separate the hexane-extractable portion from protein, and the filter was washed twice with hexane. The combined hexane solution was evaporated to dryness and dissolved in 500 μL of hexane; 2 μL was directly injected into the column inlet of a Shimadzu GC-8A apparatus equipped with hydrogen flame ionization detector (230 °C). Analytical conditions were as follows: a glass column (0.3 × 200 cm) was packed with silar 10C (10%) on a Uniport HP (100/120 mesh); column temperature, 180 °C; injection temperature, 230 °C; carrier gas, N_2 pressure, 1 kg/cm²; H_2 pressure, 0.8 kg/cm²; air pressure, 0.5 kg/cm².

Extraction of Bound Oil. Zein separated from the hexane-extractable portion was dissolved by 5 mL of 70% ethanol with warming to get a transparent solution; 1 mL of hexane was added and mixed vigorously by vortex. The supernatants after centrifugation were combined by three extractions, evaporated to dryness, and dissolved in 500 μL of hexane for GLC analysis. For zein's hydrolysate, distilled water was used to dissolve the sample instead of 70% ethanol.

Statistical Analysis. Approximately 400 mg/sample was used for oxygen absorption analysis (triplicate), 20 mg/sample for POV determination (triplicate), and 100 mg/sample for GLC analysis of extraction efficiency (duplicate). The results shown represent the averages of two or three independent experiments, with coefficient of variation of less than 19.60% for oxygen absorption, 19.86% for POV value, and 32.32% for extraction efficiency.

RESULTS AND DISCUSSION

Figure 1a shows the oxygen uptake by stored methyl linoleate in powder model systems without humidity regulation. The oxygen absorption was inhibited strongly by maize gluten as well as two kinds of zein preparations, namely a commercial zein and another zein prepared from gluten, in comparison with the contrast, in which methyl linoleate contained 0.55% α-tocopherol. With maize glutelin, milk casein, and soybean protein, oxygen absorptions progressed very rapidly and reached a plateau after 2 days of incubation. The antioxidant activities of these proteins were suggested to be weak. Lipid extraction with ethyl ether resulted in a total loss of the antioxidant activity of gluten. Nevertheless, zein prepared from this gluten retained a substantial antioxidant activity just the same as that of the gluten before lipid extraction. This was

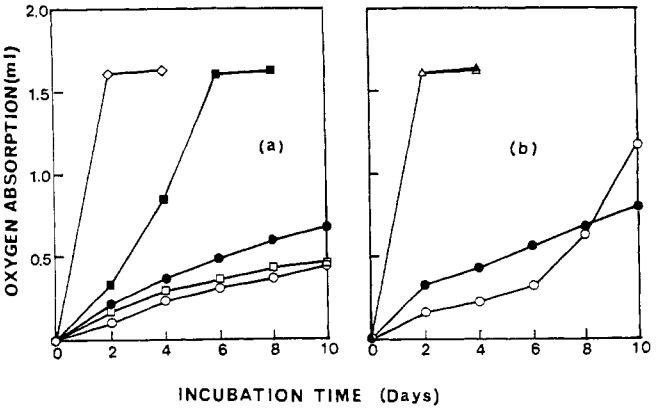

Figure 1. Antioxidant activities of food proteins in powder model system during storage at 60 °C without humidity regulation. (a) Each 2.7 g of proteins, glutelin (◇), gluten (●), commercial zein (○), and zein prepared from corn gluten (□), was added to 0.3 g of methyl linoleate. On the other hand, 0.55% α-tocopherol (■) was added to 0.3 g of methyl linoleate. (b) Each 2.7 g of defatted commercial zein (△) and defatted gluten (▲) was added to 0.3 g of methyl linoleate, and 0.028 g of ethyl acetate extract from commercial zein and 0.101 g of ethyl ether extract from gluten were readded to 2.672 g of defatted commercial zein (○) and 2.599 g of defatted gluten (●), respectively.

Table I. Tocopherol Content of Gluten and Commercial Zein (Mean ± SD, n = 3)

	α-tocopherol, μg/g	γ-tocopherol, μg/g	δ-tocopherol, μg/g	total tocopherol, μg/g
zein	0.86 ± 0.03	4.70 ± 0.16	0.23 ± 0.01	5.79 ± 0.16
gluten	0.19 ± 0.00	0.75 ± 0.02	0.09 ± 0.00	1.03 ± 0.02

similar to that reported by Iwami et al. (1987) for wheat gliadin. They reported that wheat gliadin showed a prominant antioxidant activity, while zein was almost ineffective during storage at 40 °C in A_w 0.6. Contradictory in our experiments, the relationship between the antioxidative effect of gliadin (used as a control but data not shown) and of zein was the reverse. Such a difference may be due to changes in the antioxidative components present in prepared sample which could be derived from differences of raw material, manipulating method, and so on. We could verify this by the lipid extracting experiment which showed that both gluten and zein lost their antioxidant activities after extraction with ethyl ether or ethyl acetate (Figure 1b). However, we cannot deny the possible cooperation of these active components with proteins and/or the possible loss of effectiveness due to protein denaturation during solvent extraction. To make this question clear, we tried to recover the original antioxidant activity of gluten and zein, by remixing the extract with the defatted protein (Figure 1b). As a result, gluten recovered antioxidant ability similar to the original one (Figure 1a). Zein also showed a remarkable antioxidant effect after recombination with the ethyl acetate extract, although its activity was not so strong as that of the original. The decomposition of some antioxidant compounds during the extraction may be responsible for it.

The antioxidant compounds in the organic solvent extracts were analyzed by TLC. Three antioxidative spots with the identical R_f values with authentic α-, γ-, and δ-tocopherols were respectively observed. The HPLC assay also showed the occurrence of tocopherols in which γ-tocopherol was the major constituent (Table I). In the case of zein, the amount of total tocopherols corresponded to about 0.005% relative to methyl linoleate. Besides tocopherols, other medium polar phenolic antioxidants such as ferulic acid and catechol seem to exist together (Table

Table II. Characteristics of Medium Polar Compounds in Ethyl Acetate Extract of Zein by TLC

	solvent[a] I			solvent[a] II			solvent[a] III		
	1[b]	2[b]	3[b]	1[b]	2[b]	3[b]	1[b]	2[b]	3[b]
ferulic acid									
authentic	0.40	0.33	0.32	0.11	0.11	0.08	0.44	0.39	0.44
	blue	blue	yellow	blue	blue	brown	blue	blue	brown
isolated	0.40	0.32	0.34	0.10	0.12	0.05	0.43	0.40	0.43
	blue	blue	yellow	blue	blue	brown	blue	blue	brown
catechol									
authentic	0.38	0.29	0.31	0.35	0.28	0.36	0.49	0.47	0.51
	blue	pink	gray	blue	blue	gray	blue	pink	gray
isolated	0.38	0.29	0.31	0.34	0.26	0.36	0.50	0.49	not determined
	blue	pink	yellow	blue	blue	yellow	blue	pink	

[a] Solvent I, toluene/methyl formate/formic acid (5:4:1); solvent II, toluene/chloroform/acetone (40:25:35); solvent III, benzene/pyridine/formic acid (36:9:5). [b] Spray reagent 1, Folin–Ciocalteu's phenol reagent; spray reagent 2:anisaldehyde–sulfuric acid reagent: spray reagent 3, Fast Blue B.

Table III. Effect of Water Activity (A_w) on Antioxidant Strength of Food Proteins[a]

	no α-tocopherol addition					with α-tocopherol addition				
	casein	soybean protein	gluten[b]	zein[b]	hydrolysate	casein	soybean protein	glutein[b]	zein[b]	hydrolysate
A_w 0.3	2	2	2	2	2	8	10	6	10	10
A_w 0.5	2	2	2	2	4	8	16	8	12	>30
A_w 0.7	2	2	4	2	10	8	16	12	16	>30
A_w 0.9	2	2	8	>30	10	10	18	>24	>30	>30

[a] Oxygen uptake curves (as shown in Figure 1) were obtained without or with the addition of α-tocopherol (0.55% w/w, added to the substrate oil). Each value indicates the number of days required to reach a plateau on the oxygen uptake curve. [b] Samples were defatted before use as described under Materials and Methods.

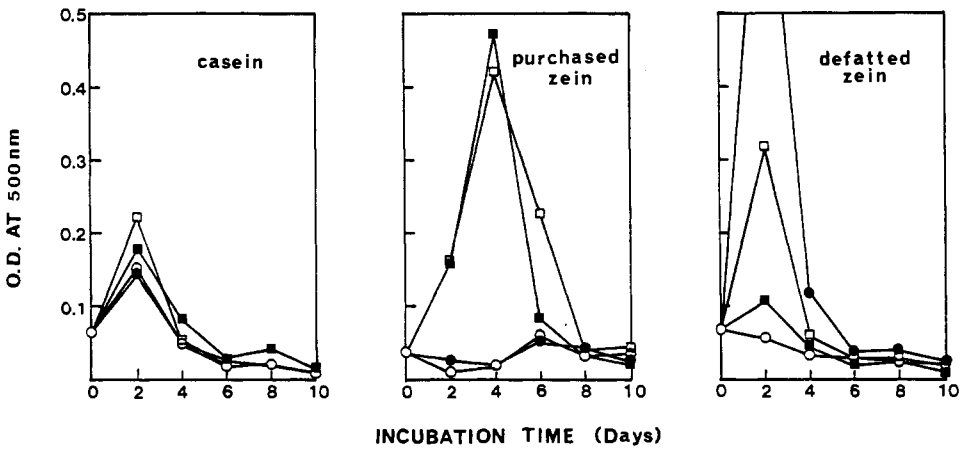

Figure 2. POV changes in commercial zein, defatted zein, and milk casein containing 10% methyl linoleate and stored at 60 °C and various A_w values. (○) A_w 0.9; (●) A_w 0.7; (□) A_w 0.5; (■) A_w 0.3.

II). From these results, the antioxidant activities observed by gluten and zein during storage at medium water activity were derived from cooperative action of tocopherols and other phenolic compounds, not from the antioxidant functions of proteins themselves.

It is known that A_w can affect the function of antioxidants added in the food model systems (Labuza et al., 1969; Chou and Labuza, 1974). We then investigated the antioxidant properties of casein, soybean protein, gluten and zein under different A_w conditions. The days that oxygen absorption reached a plateau were used to compare the antioxidant activity. As shown in Table III, methyl linoleate stored with soybean protein or milk casein without α-tocopherol supplementation was rapidly oxidized within 2 days, independently of differences in A_w. On the other hand, an extraordinary strong antioxidant effect was observed for defatted zein at A_w 0.9. Under this condition, the induction period lasted for more than 30 days of incubation compared with 2 days at lower A_w values. The influence of A_w on protein antioxidant activities became clear when 0.55% α-tocopherol in methyl linoleate was added: the higher the A_w, the stronger was the antioxidant activity. According to this, the longer induction period of defatted gluten without α-tocopherol addition at higher A_w could be attributed to insufficient removal of antioxidants. But it was not applicable to defatted zein because the differences of antioxidative strength derived from A_w in α-tocopherol supplemental samples were far smaller than that in defatted zein. The addition of α-tocopherol does increase the storage period of defatted zein at lower A_w, but does not affect its antioxidant activity at the A_w of maximum effectiveness. This suggests that zein protein may have antioxidant activity itself under a proper A_w condition. Moreover, the stable low POV at A_w 0.9 throughout the experimental period with both untreated and defatted zein (Figure 2) also suggests the protective effect of zein. The antioxidant effectiveness of the untreated zein at A_w 0.7 may be mainly due to tocopherols remaining in the systems, as defatted zein lost the activity, which was in accordance with the result shown in Figure 1.

We considered the special antioxidative activity of zein

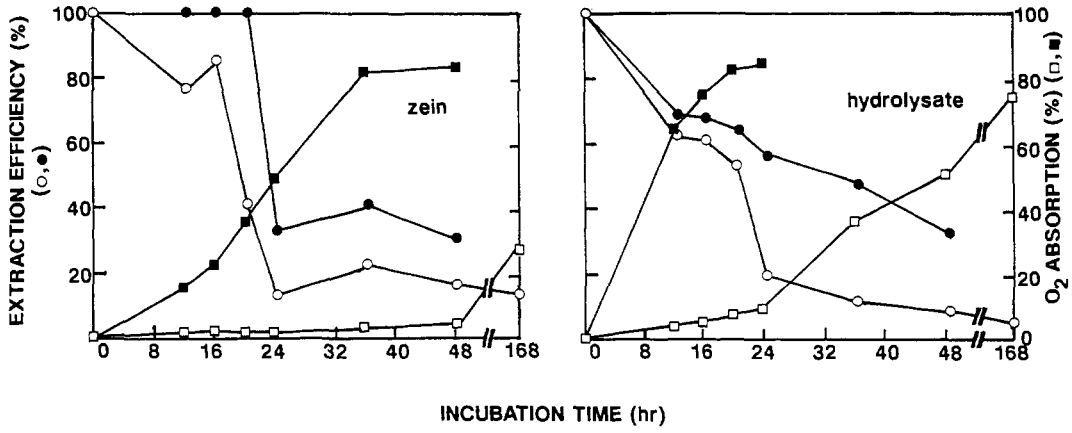

Figure 3. Extraction efficiency of methyl fatty acids (○, ●) and corresponding oxygen uptake (□, ■) in zein and its hydrolysate-based model systems which were stored at A_w 0.3 (●, ■) and A_w 0.9 (○, □) together with a methyl linoleate–methyl stearate (7:3 w/w) mixture.

at A_w 0.9 as due to its macromolecular structure and hydrophobicity of the protein. We then modified the properties of zein by hydrolyzing partially with papain to make it water soluble. The partially hydrolyzed zein did not show any remarkable antioxidant activity as was observed in the untreated zein, although some weak effect was observed in high A_w values (Table III). The possible autoxidation of unextracted phenolics contained in defatted zein may occur during the 48-h hydrolysis, but it would not be so notable because the same efficacies (from 2 to 10 days in Table III) were obtained for both zein and its hydrolysate at A_w 0.3 when 0.55% α-tocopherol was added. On the other hand, the antioxidative property of zein hydrolysate at higher A_w values could be pronounced by the addition of 0.55% α-tocopherol (>30 days in Table III). It seems that the hydrolysate showed antioxidant activity synergistically with α-tocopherol, since at higher A_w values (0.5, 0.7, 0.9) the formation of brown products was observed, which have been reported to have hydroperoxide decomposing ability and synergistic effect with antioxidants like tocopherols (Yamaguchi, 1980). The antioxidant activity of peptides, especially synergistic effects with phenolic antioxidants, has been reported many years (Bishov and Henick, 1972; Kawashima et al., 1979; Hatate et al., 1990).

Figure 3 compares the efficiency of methyl fatty acid extraction by hexane and corresponding oxygen absorption between A_w 0.3 and 0.9 in zein and its hydrolysate model systems. High A_w values caused greater decreases in extraction efficiency with both samples. A pronounced fall after 24 h of incubation with zein was observed, and there was little change until 7 days, while a consecutive decline was observed with its hydrolysate at both A_w values. The hexane-extractable recovery after 7 days was about 15% for zein and 6% for its hydrolysate at A_w 0.9. Zein that was free of hexane-extractable oil was destroyed by 70% ethanol, and about 23% recovery of the substrate oil was obtained after 7 days of storage, while only 0.67% was obtained for zein's hydrolysate. The oxygen uptake curves show the delay of oxidation at higher A_w which correlated with their lower efficiency of substrate oil extraction by hexane. A possible explanation is that the specific antioxidant activity observed in zein at A_w 0.9 may be responsible for its hydrophobic property derived from the high levels of hydrophobic amino acids such as glutamine, leucine, proline, alanine, and phenylalanine (Gianazza et al., 1977). This hydrophobicity, which is provoked in high A_w environments, could either include the hydrophobic substrate oil to protect it from oxygen attack or make it bind to protein to delay the oxidation and result in a decrease of hexane-extractable oil. The consecutive decline in hexane extraction efficiency for zein's hydrolysate may be derived from the formation of methyl linoleate hydroperoxide–amino acids and peptide complexes (Gardner, 1979), although it is not clear which would occur first between oxidation and the decline of extraction efficiency.

In conclusion, maize zein presents prominent antioxidant activity at high water activity in powder model systems either by synergizing the activity of traces of unextracted phenolics or by binding and physically shielding the lipid. The former may apply to enzymatically digested zein; the latter is more likely for zein, but in neither case is the mechanism certain. We suspect that other prolamines present in crops could offer similar activities to zein, since their amino acid compositions and other physiochemical properties are similar. These proteins may be antioxidative in food processing.

ABBREVIATIONS USED

GC, gas chromatography; A_w, water activity; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; POV, peroxide value; TLC, thin-layer chromatography; TCA, trichloroacetic acid.

LITERATURE CITED

Abe, K.; Yuguchi, Y.; Katsui, G. Quantitative Determination of Tocopherols by High-Speed Liquid Chromatography. *J. Nutr. Sci. Vitaminol.* **1975**, *21*, 183–188.

Bishov, S. J.; Henick, A. S. Antioxidant Effect of Protein Hydrolyzates in a Freeze-Dried Model System. *J. Food Sci.* **1972**, *37*, 873–875.

Chou, H. E.; Labuza, T. P. Antioxidant Effectiveness in Intermediate Moisture Content Model Systems. *J. Food Sci.* **1974**, *39*, 479–483.

Gardner, H. W. Lipid Hydroperoxide Reactivity with Proteins and Amino Acids: A Review. *J. Agric. Food Chem.* **1979**, *27*, 220–229.

Gianazza, E.; Viglienghi, V.; Righetti, P. C.; Salamini, F.; Sosve, C. Amino Acid Composition of Zein Molecular Components. *Phytochemistry* **1977**, *16*, 315–317.

Hatate, H.; Nagata, Y.; Kochi, M. Antioxidative Effect of Bovine Serum Albumin Hydrolyzates and Their Synergistic Effect with Antioxidants. *Yukagaku* **1990**, *39*, 42–46.

Iwami, K.; Hattori, M.; Ibuki, F. Prominent Antioxidant Effect of Wheat Gliadin on Linoleate Peroxidation in Powder Model Systems at High Water Activity. *J. Agric. Food Chem.* **1987**, *35*, 628–631.

Kawashima, K.; Itoh, H.; Chibata, I. Synergistic Ternary Antioxidant Compositions Comprising Tocopherol, Partially Hydrolyzate of Gelatin and Organic Acid. *Agric. Biol. Chem.* **1979**, *43*, 827–831.

Keppler, J. G.; Sparreboom, S.; Stroink, J. B. A.; Mikusch, J. D. V. A Note on the Preparation of Pure Oleic and Linoleic Acid. *J. Am. Oil Chem. Soc.* **1959**, *36*, 308–309.

Laakso, S. Inhibition of Lipid Peroxidation by Casein Evidence of Molecular Encapsulation of 1,4-Pentadiene Fatty Acids. *Biochim. Biophys. Acta* **1984**, *792*, 11–15.

Laakso, S.; Lilius, E. M. Milk Casein: Inhibitor of Lipoxygenase-Catalyzed Lipid Peroxidation. *J. Agric. Food Chem.* **1982**, *30*, 913–916.

Labuza, T. P.; Tsuyuki, H.; Karel, M. Kinetics of Linoleate Oxidation in Model Systems. *J. Am. Oil Chem. Soc.* **1969**, *46*, 409–416.

Stahl, E. Hydrophilic Plant Constituents and Their Derivatives; Spray Reagents. In *Thin-Layer Chromatography*; Springer-Verlag: Berlin, 1969.

Yamaguchi, N.; Naito, S.; Yokoo, Y.; Fujimaki, M. Oxidative Stability of Dried Model Food Consisted of Soybean Protein Hydrolysate and Lard. *Nippon Shokuhin Kogyo Gakkaishi* **1980**, *27*, 51–55.

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Registry No. α-Tocopherol, 59-02-9; γ-tocopherol, 7616-22-0; δ-tocopherol, 119-13-1; ferulic acid, 1135-24-6; catechol, 154-23-4; water, 7732-18-5.