Antioxidant Activities of Zeins from Different Maize Varieties Against Docosahexaenoic Acid Ethyl Ester

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ABSTRACT

To investigate the antioxidant effects of zeins on highly unsaturated fatty acids, the deterioration of docosahexaenoic acid ethyl ester in a powder model system of zein and oil was determined by gas-chromatographic analysis. The antioxidant activity of laboratory-prepared zeins from three maize varieties (Popcorn, Golden cross bantam, Koshu) and one commercial variety were compared. Although no zein could completely protect docosahexaenoic acid ethyl ester from oxidation, the laboratory-prepared zeins exhibited higher antioxidant activities than did the commercial zein. Of the laboratory-prepared zeins, the zein from Golden cross bantam showed the highest antioxidant activity, and the zein from Popcorn showed the lowest activity. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and reversed-phase high-performance liquid chromatography of zeins were used to determine the reason for differences in antioxidant activity. These analyses revealed that β- and γ-zein subunits are present only in the laboratory-prepared zeins and are absent in the commercial zein. There was a correlation between the contents of these zein subunits and the antioxidant activities of zeins. These results suggest that β- and γ-zein subunits should contribute to the improvement of the antioxidant activity of zein against docosahexaenoic acid ethyl ester.

Zein, the major storage protein in maize endosperm is characterized as a prolamin by its solubility in aqueous alcohol solutions (Osborne 1924). Unlike legume proteins, the poor solubility of zein in water and salt solutions makes it difficult to use this protein as a gelling substrate, an emulsifier, or an emulsion stabilizer. It is necessary to find a new functionality of zein in order to increase its consumption as food protein.

Wang et al (1991a,b) found that zein had antioxidant activity against the peroxidation of methyl linoleate in a powder model system. Previously, Iwami et al (1987, 1988) demonstrated that gliadin, a prolamin fraction of wheat protein, functioned as an antioxidant against linoleate peroxidation in powder model systems or spray-dried products. Therefore, it is probable that prolamin fractions in crops have such antioxidant activities.

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Recently, highly unsaturated fatty acids (HUFA), such as eicosahexaenoic acids (EPA) and docosahexaenoic acids (DHA), also have been attracting much interest in relation to reducing the risk of cardiovascular disease (Leaf and Weber 1988), thrombosis (Fischer et al 1986), and cancer (Braden and Carroll 1986). The supplementation of processed foods by HUFA is considered seriously.

The peroxidation of HUFA, such as EPA and DHA, is more likely to occur than polyunsaturated fatty acids, such as linoleic and linolenic acids (Cho et al 1987). It is uncertain, therefore, whether zein would function as an antioxidant against EPA or DHA as well as against linoleic acid. The first objective of the present study was to check the antioxidant effect of zein on docosahexaenoic acid ethyl ester (DHE) oxidation.

Zein fractions are composed of many kinds of subunit polypeptides (Wilson 1986). Varieties of maize have different zein subunit compositions. It is expected that zein fractions from different varieties have different physicochemical and functional properties. Our second objective was to investigate the difference in antioxidant activities of zein fractions from different varieties.

MATERIALS AND METHODS

The whole seeds of three maize varieties (commercial names: Popcorn, Golden cross bantam, and Koshu) were purchased from Takii Seed Co., Ltd. (Kyoto, Japan). Commercial zein, milk casein, stearic acid methyl ester, and other reagents were purchased from Nakarai Chemicals Ltd. (Kyoto, Japan). DHE (99% purity) was donated by Nippon Chemical Feed Co. Ltd. (Hakodate, Japan).

Zein Preparation

Maize seeds were ground in a coffee mill (Carioca Mill, Mk-52M, Matsushita Electronics Co., Ltd., Osaka, Japan). Zein was extracted from the ground powder according to a modification of Osborne's method (Tsai 1980) with 70% ethanol + 1 m*M* mercaptoethanol (ME) at 60°C, following extraction with distilled water and sodium phosphate buffer (pH 7) at room temperature. The zein solution was concentrated by evaporation under vacuum at room temperature and then dialyzed against water. Precipitated zein was lyophilized. All zein preparations were washed with ethyl acetate for 24 hr before use.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on slab gels using Laemmli's continuous system (Laemmli 1970). Gels were stained with Coomassie Blue R-250 and destained with trichloroacetic acid to avoid destaining of zeins by an alcoholic solution (Wilson 1986). Relative amounts of the bands on each gel were determined densitometrically with a Shimadzu Dual-Wavelength flying-spot scanner CS-900 (Shimadzu Co. Ltd., Kyoto, Japan) using a reference wavelength of 750 nm and a sample wavelength of 590 nm.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

A 250 × 4.6 mm YMC Pack ODS-AP (C_{18}) column (5 μm particle diameter, 300 Å pores) was used for RP-HPLC. The column was equilibrated with 0.1% trifluoroacetic acid (TFA) + 38 % acetonitrile (ACN) buffer (pH 2.1). Protein elution from the column was accomplished by several segmented gradients of increasing ACN with 0.1% TFA. The gradient time program was similar to that of Wilson (1991): starting at 35% ACN, increasing at 0.5%/min for 14 min, at 1.98%/min for 3 min, at 0.11%/min for 13 min, at 0.20%/min for 18 min, 2.25%/min for 4 min, ending at 65%. The column was then eluted with 0.1% TFA + 65% ACN for 10 min. Operating temperature was 25°C. The eluate was monitored at 210 nm. Protein was injected as a 200-μl aliquot (1.25 mg/ml in 55% isopropanol with 5% 2-ME).

Powder Model System for Antioxidant Activity

A mixture of DHE and stearic acid methyl ester (7:3, w/w) or linoleic acid methyl ester and stearic acid methyl ester (7:3, w/w) dissolved in ethyl ether was added to zeins and casein to a ratio of 1:9 by weight. Samples in powder states were subdivided into small portions and stored in a humidity-controlled plastic vessel at 40°C. Water activity (A_w) was adjusted to 0.9 with 22% (w/w) sulfuric acid (Troller and Christian 1978). Samples were removed from the vessel at stated intervals, and oxidation of DHE was checked by gas chromatography.

Gas Chromatography

Model powder (50 mg) was extracted three times with 1 ml of hexane. After centrifugation, the supernatants were combined and filtered through a cosmonice filter S, No. 440-85 (Nakarai Chemicals Ltd., Kyoto, Japan) to remove insoluble materials. The clarified solution was evaporated to dryness under a stream of nitrogen gas and dissolved in 250 μl of hexane. An aliquot (1 μl) was directly injected into the column inlet of a Shimadzu GC-9A PTF gas chromatograph (Shimadzu Co. Ltd., Kyoto, Japan) equipped with a hydrogen flame ionization detector. Analytical conditions were: a glass column (0.32 × 210 cm) packed with Silar 10C (10%) on chromosorb W (AW-DMCS, 60~80 mesh); temperature program, 160~240°C at 4°C/min; injection temperature, 250°C; carrier gas (N_2) at a flow rate of 60 ml/min; N_2 pressure, 6 kg/cm^2; H_2 pressure, 0.6 kg/cm^2; air pressure, 0.5 kg/cm^2.

Attenuated Total Reflectance (ATR)-Fourier Transform Infrared Spectroscopy (FT-IR) Measurement

ATR-FT-IR spectra of model powders were recorded on a Shimadzu FT-IR-8100 (Shimadzu Co. Ltd, Kyoto, Japan). For ATR measurements, ATR-8100 H type equipment (ZnSe prism) was used. The angle of incidence was 45°, and 150 interferograms were accumulated to yield spectra with resolution of 4 cm^{-1}. By this measurement, the lipid in the surface layer ~2 μm deep was detected.

Measurement of Peroxide Value (POV)

POV was determined by the ferric thiocyanate method (Iwami et al 1987).

Data Analysis

Preparation of powder model system, extraction of oil from powder, gas-chromatographic analysis of extracted oil, and POV measurement of powder were repeated five times. ATR-FT-IR measurement of powder was repeated three times. SDS-PAGE and the densitometric determination of relative amounts of the bands were repeated two times. Analysis produced significant *F* values by analysis of variance, followed by Duncan's multiple range test for comparison of means.

RESULTS

Antioxidant Activity of Zeins Against DHE in Powder Model Systems

To examine the antioxidant activities of zeins against DHE, the amount of DHE remaining in the model powder system was analyzed after storage at 40°C and A_w 0.9. Oil in the powder model system was hexane-extracted from the powder before gas chromatographic analysis. More than 80% of oil was extractable after seven days.

Typical gas-chromatographic patterns of hexane-extractable oil fractions from powders are shown in Figure 1. For commercial zein, the peak corresponding to DHE was very small compared with that of the authentic stearic acid methyl ester peak after three days of storage. This means that the commercial zein had no, or very weak, antioxidant activity against DHE. The zein from Golden cross bantam, on the other hand, exhibited improved antioxidant activity (Fig. 1). The amount of DHE remaining after three days is higher when compared to the commercial zein.

The amounts of DHE remaining in the powder model systems prepared from other zeins were similarly analyzed by gas chromatography. The ratio between the peak areas corresponding to DHE and stearic acid methyl ester (D/S ratio) was calculated. Results are shown in Table I. Proteins such as caseins are generally capable of retarding the progress of the lipids' peroxidation under suitable conditions (Karel et al 1975, Laakso 1984). Therefore, the antioxidant activity of casein was also examined. In the powder model system prepared from casein and oil, D/S ratio decreased to less than 10% of the original value after three and seven days. Compared to casein, all zeins demonstrated significant antioxidant effects ($P = 0.05$) on DHE peroxidation. In Table I, the commercial zein showed the lowest antioxidant activity. The D/S ratio decreased from 2.15 to 0.30 after seven days of storage. The highest antioxidant effect was found with the zein from Golden cross bantam. The D/S ratio was 0.81 and 0.64, after three and seven days of storage, respectively. This means that twice the amount of DHE can be protected from oxidation if we use the zein from Golden cross bantam instead of the commercial zein. The zeins from Koshu and Popcorn varieties ranked second and third, respectively, in antioxidant activity.

In spite of the significant difference in antioxidant activity among zein samples, no zein was able to stop the diminishing of DTE completely. As a result, peroxide values in powder model

systems increased to 250–800 meq/mg DHE after three days of storage, according to zein samples. On the other hand, there was only a small increase of POV (<30 meq/mg of oil) in the powder model system with commercial zein and linoleic acid methyl ester. The decrease of linoleic acid methyl ester in a powder model system with commercial zein was also very small (data not shown). These results are consistent with those of Wang et al (1991a), which indicated the complete protection from oxidation of linoleic acid methyl ester by commercial zein.

ATR-FT-IR Measurement of Powder Model System

Oil emerging onto the powder surface of model systems was analyzed by an ATR-FT-IR method. Figure 2 shows the absorbances of lipid bands in powder model systems of zeins and casein. Bands around 1,740 cm^{-1} correspond to the ester C=O stretching band. Bands around 2,925 and 2,955 cm^{-1} are assigned to antisymmetric stretching band of CH_2 and asymmetric stretching band of terminal CH_3 in lipid hydrocarbon, respectively. The larger absorbance indicates greater emerging or leaking of oil onto or near the surface in the model powder.

Figure 2 clearly demonstrates higher absorbance of lipid bands in the casein-oil powder system. The absorbance of the ester C=O stretching band was higher than those of zein-oil powder systems. Such low encapsulating activity of casein may lead to the low antioxidant activity demonstrated in Table I. On the other hand, all zeins exhibited higher oil-encapsulation or shielding activities. There were also significant differences ($P = 0.05$) in absorbances of lipid bands among zein samples. The absorbances of the commercial zein were higher than those of other laboratory-prepared zeins. Among the laboratory-prepared zeins, the zein from the Popcorn variety protected oil most effectively.

Analyses of Subunit Compositions of Zeins

The differences in antioxidant activity among zein samples demonstrated above may be related to the subunit compositions of zeins that were analyzed by RP-HPLC and SDS-PAGE.

The results of RP-HPLC are shown in Figure 3. Elution patterns are similar to those demonstrated by Wilson (1991) and Dombrink-Kurtzman and Bietz (1993), although their results gave slightly different retention times and better peak separations when compared with our results, probably because of different column temperature and starting concentration of ACN. In the pattern of the commercial zein (Fig. 3D), most peaks appeared only at the retention times from 25–40 min. These peaks are assignable to *A* and *B* or α-type subunits according to previous results (Thompson and Larkins 1989, Wilson 1991, Dombrink-Kurtzman and Bietz 1993). These peaks should also contain low contents of δ subunits. Contrastingly, in the patterns of laboratory-prepared zeins (Fig. 3 A–C), the first cluster of peaks appeared before 20 min, in addition to large amounts of peaks eluted at ~25–40 min. Minor peaks were also found at ~50–60 min. It is thought that peaks eluting before 20 min (front fractions) are composed of β- and γ-type subunits, according to previous results.

Since we used a different HPLC column and got slightly different patterns from those of previous results (Wilson 1991, Dombrink-Kurtzman and Bietz 1993), we examined the identification of peak proteins by SDS-PAGE analysis. The SDS-PAGE patterns of zeins are presented in Figure 4. Only α and

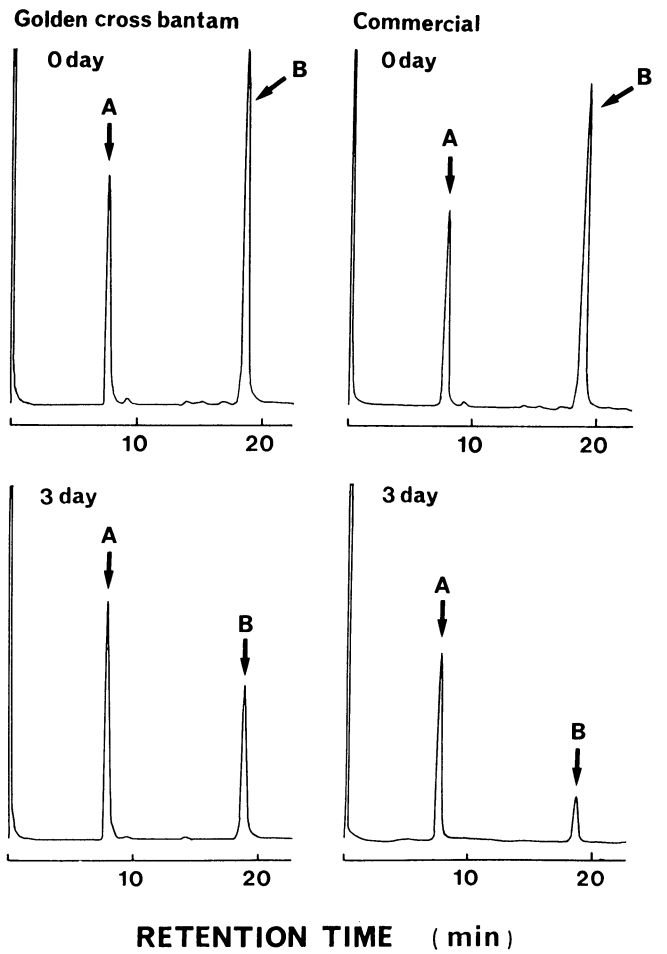

Fig. 1. Typical gas-chromatographic patterns of docosahexaenoic acid ethyl ester remaining in powder model systems of zein from Golden cross bantam and commercial zein after storage. The peaks (A) and (B) in each pattern correspond to the stearate methyl ester and docosahexaenoic acid ethyl ester, respectively.

TABLE I
Ratio of Docosahexaenoic Acid Ethylester to Stearic Acid Methylester Remaining in the Zein- or Casein-Based Powder Systems During Storage[a]

	Storage Time (days)		
	0	3	7
Popcorn	2.10 a	0.45 c	0.42 c
Golden cross bantam	2.12 a	0.81 a	0.64 a
Koshu	2.13 a	0.66 b	0.55 b
Commercial	2.15 a	0.31 d	0.30 d
Casein	2.10 a	0.18 e	0.19 e

[a]Means followed by the same letters in columns are not significantly different at $P = 0.05$ according to Duncan's multiple range test. Values represent the means of five replicates.

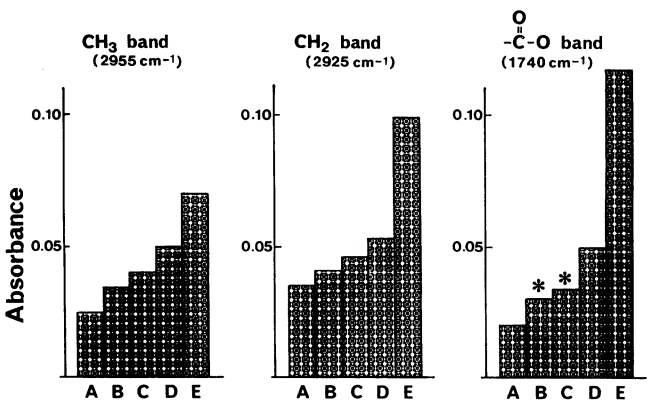

Fig. 2. Attenuated total reflectance-Fourier transform infrared spectroscopy analyses of lipids in powder model systems of zeins and caseins. Assignments of lipid bands are: 2,955 cm^{-1}, asymmetric stretching band of terminal CH_3 of lipid hydrocarbon; 2,925 cm^{-1}, antisymmetric stretching band of CH_2 of lipid hydrocarbon; 1,740 cm^{-1}, ester C=O stretching band. A, B, and C represent zein from Popcorn, Golden cross bantam, and Koshu, respectively. D and E represent commercial zein and casein, respectively. Absorbance values represent the means of three replicates. Means are significantly different at $P = 0.05$ according to Duncan's multiple range test, except B (Golden cross bantam) and C (Koshu) in ester C=O stretching band. * = No significant difference at $P = 0.05$.

trace amount of δ subunits were present in the commercial zein fraction. It should be valid, therefore, to identify the peaks appearing at retention times of 25–40 min in the HPLC patterns (Fig. 3) as α subunit with a minor content of δ-type subunits.

On the other hand, β- and γ subunits, in addition to α- and δ subunits also appeared in the SDS-PAGE patterns of the laboratory-prepared zeins (Fig. 4), although β-subunit bands are not well separated in these electrophoretic patterns. Front fractions in HPLC patterns of the laboratory-prepared zeins (retention time before 20 min in Fig. 3A–C) were separated and reanalyzed by SDS-PAGE (Fig. 5) to identify these fractioned proteins. None of the front fractions from the laboratory-prepared zeins contained α-subunit bands, but they did contain β- and γ bands. This result clearly shows that β- and γ-type subunits are components of front fractions from the laboratory-prepared zeins.

Thus, from results of HPLC and SDS-PAGE, it is obvious that there are significant differences in the subunit compositions between commercial zein and laboratory-prepared zein from three varieties. Commercial zein lacks β- and γ-type subunits, and its major components are α-type subunits. Laboratory-prepared zeins have β- and γ-type subunits in addition to α subunits. These differences in the subunit compositions are expressed quantitatively by the densitometric determination of SDS-PAGE patterns of Figure 4.

Table II shows β subunits are not well separated in the electrophoretic patterns; they are involved with γ-2 subunits. More than 99% of the total amount of protein is composed of α-1 and α-2 subunits in commercial zein. On the other hand, the relative amounts of α-1 and α-2 are lower in the laboratory-prepared zeins. Total amounts of these two bands are 68.8, 51.6, and 55.2%, respectively, for the zeins from Popcorn, Golden cross bantam, and Koshu. Conversely, relative amounts of γ- and β-subunits are higher in laboratory-prepared zeins. Total amounts of these bands are 28.4, 45.5, and 43.1%, respectively for the zeins from Popcorn, Golden cross bantam, and Koshu. The differences in subunit composition, particularly the differences in the amounts of β- and γ-subunit bands between the commercial and the laboratory-prepared zeins, with respect to the different antioxidant activities of the zeins, are discussed below.

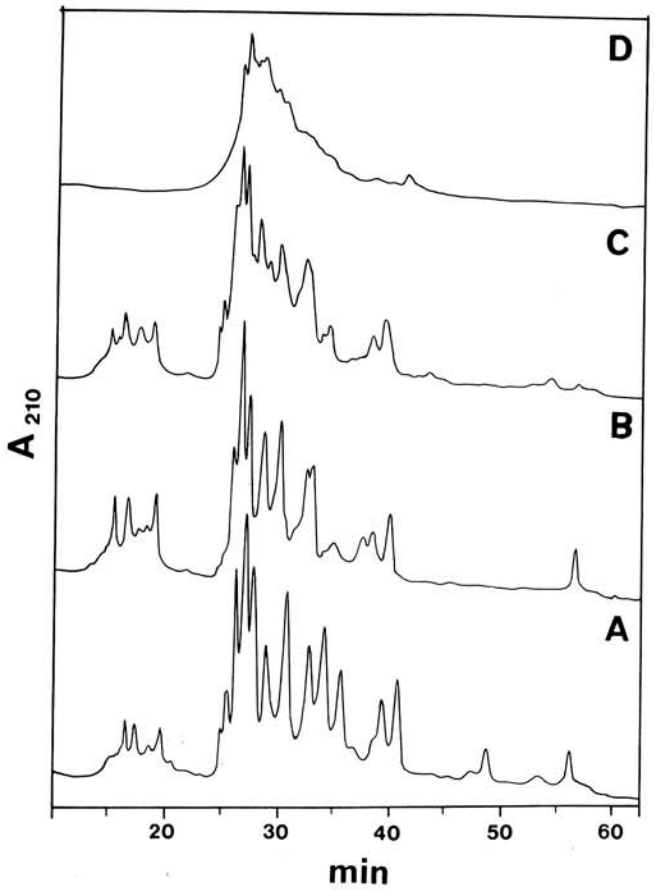

Fig. 3. Reversed-phase high-performance liquid chromatography analyses of laboratory-prepared and commercial zeins. Zeins were analyzed using a C_{18} column and a nonlinear 38–65% acetonitrile gradient for 52 min. A, B, and C represent zein from Popcorn, Golden cross bantam, and Koshu, respectively. D represents commercial zein.

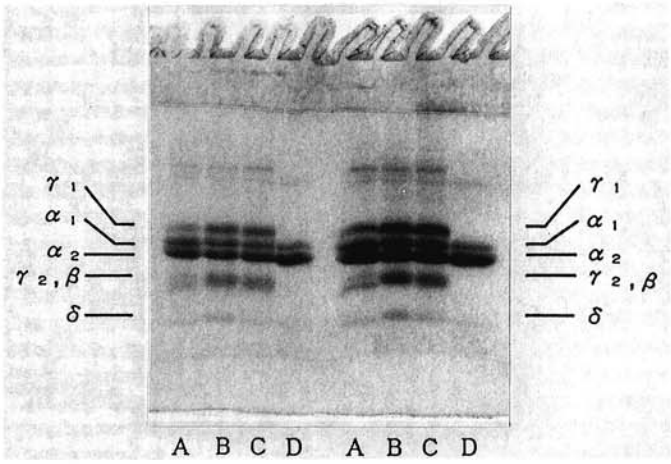

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analyses of laboratory-prepared and commercial zeins. Total amount of protein applied per lane was 10 μg in four lanes on left, and 20 μg in four lanes on right, respectively. Subunit names of the genetic classification are indicated on both sides. A, B, and C represent zein from Popcorn, Golden cross bantam, and Koshu, respectively. D represents commercial zein.

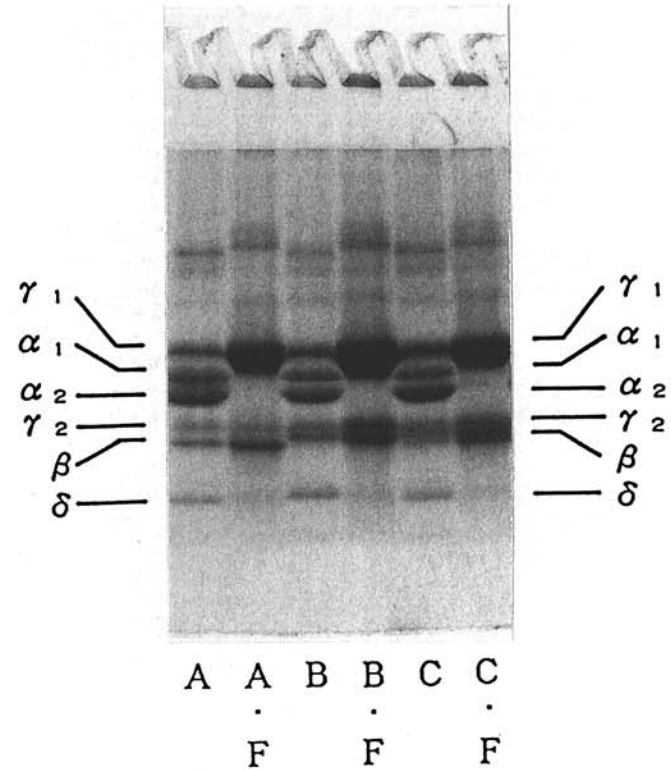

Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analyses (SDS-PAGE) of separated front fractions (F) of laboratory-prepared zeins. Front fractions (peaks eluting before 20 min) in reversed-phase high-performance liquid chromatography patterns (Fig. 3) were separated and analyzed by SDS-PAGE. Subunit names of the genetic classification are indicated on both sides. A, B, and C represent zein from Popcorn, Golden cross bantam, and Koshu, respectively. D represents commercial zein.

DISCUSSION

The first objective of this study was to examine the possibility that zein functions as an antioxidant against HUFA as well as linoleic acid. All of the commercial and the laboratory-prepared zeins were not capable of completely protecting DHE from the peroxidation. HUFA has more sites adjacent to double bonds and is more easily attacked by free radicals than is linoleic acid. Chain radical reaction, including the formation of conjugated dienes and endoperoxides, is also more likely to occur with HUFA. Although we expect the spatial protection of lipids from outer oxygen by zein protein membranes, a small amount of oxygen accessible to lipids may exist in the system, because contamination of oxygen during preparation of powder model systems cannot be avoided. Furthermore, diffusion of oxygen through zein membranes is also probable, although the diffusion constant of oxygen in zein membranes is fairly low. The amount of oxygen accessible to lipids in powder model systems should be very small, but even a trace amount of oxygen may be capable of causing oxidation of HUFA.

The results in the present study suggest that it should be difficult to fortify food systems with HUFA using zein as the antioxidant. Synergists such as chelating agents, radical scavengers, and terminators should be used together to improve or increase the antioxidant activity of the zein-oil powder model system.

There were significant differences ($P = 0.05$) in the antioxidant activity against DHE among the commercial and the laboratory-prepared zeins from different varieties, although no zein could stop the oxidation of DHE completely. Laboratory-prepared zeins, particularly the Golden cross bantam, showed improved antioxidant activity. This fact suggests the possibility of finding other zeins from varieties that have higher antioxidant activity. To make clear the properties relating to the antioxidant activity, the activity of physical encapsulation and the subunit compositions of the zeins were investigated. Comparison of results between Table I and Fig. 2 reveals some relation between the oil encapsulation activity and antioxidant activity of zeins. The higher absorbance of the commercial zein in the results of ATR-FT-IR-measurement (Fig. 2) is consistent with the fact that antioxidant activity of the commercial zein was inferior to those of the laboratory-prepared zeins. However, the antioxidant activity of the zein from Popcorn variety is inferior to those of other laboratory-prepared zeins, although results of ATR-FT-IR measurement suggest that this zein protected oil most effectively. Therefore, we can conclude that the degree of physical shielding of oil by zein is an important, but not the only, determinant for its antioxidant activity.

As the antioxidant activity of zein cannot be explained by physical mechanisms only, the relation between subunit compositions and the antioxidant activity was investigated. RP-HPLC and SDS-PAGE analyses pointed out that there are significant differences in the subunit compositions between the commercial zein and the laboratory-prepared zeins from the three varieties. The laboratory-prepared zeins have β- and γ-type subunits, while the commercial zein lacks these type subunits. Total amounts of β- and γ-type subunits present in the laboratory-prepared zein are 28.4, 45.5, and 43.1%, respectively, for Popcorn, Golden cross bantam, and Koshu (Table II). When results of Table II are compared with those of Table I, the correspondence between the subunit compositions and antioxidant activities of zeins can be acknowledged. The higher content of β and γ subunits (the lower content of α subunits) the zein has, the higher antioxidant activity it shows. For example, the zein from Golden cross bantam, which has the highest content of β and γ subunits (the lowest content of α subunits), exhibits the higher antioxidant effect on the peroxidation of DHE. In contrast, the commercial zein, which has no β and γ subunits, shows the lowest antioxidant activity. In the case of the zeins from Koshu and Popcorn, the antioxidant activity is proportional to the contents of β and γ subunits.

TABLE II
Relative Percentages of Subunit Bands in Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Patterns of Zein Samples Determined by Densitometrical Scanning[a]

	Subunit Name				
	τ_1	α_1	α_2	$\gamma_2 + \beta$	δ
Popcorn	16.2 b	28.3 a	40.5 b	12.2 c	2.8 a
Golden cross bantam	26.1 a	20.7 c	30.9 d	19.4 a	2.9 a
Koshu	26.5 a	19.4 c	35.8 c	16.6 b	1.7 b
Commercial	0 c	21.4 b	77.9 a	0 d	0.7 c

[a]Means followed by the same letters in columns are not significantly different at $P = 0.05$ according to Duncan's multiple range test. Values represent the means of three replicates.

We calculated correlation coefficients (r) between the values of Table I and Table II. Values of D/S ratio after three days storage were very well correlated ($r = 0.93$) to the contents of β and γ subunits of zeins. There was also a strong correlation ($r = 0.95$) between the values of D/S ratio after seven days of storage and the contents of β and γ subunits. Therefore, we can conclude that there is a correlation between the antioxidant activity against DHE and the presence of β- and γ-type subunits. Lower antioxidant activity of the commercial zein may originate from the absence of these subunits. Lending and Larkins (1989) indicated that β and γ zeins occur in the peripheral regions of protein bodies. Such location of the two subunits may be related to their antioxidant activities.

The reason for the higher antioxidant activity of β- and γ-type subunits is not yet understood. Chemical reactions of amino acid residues in these subunits against free radicals may be one reason. The β and γ subunits are richer in sulfur amino acids such as methionine and cysteine than are the α subunits (Pederson et al 1986, Prat et al 1987). The δ-type subunit, which is higher in the laboratory-prepared zeins than it is in the commercial zein (Table II), is also rich in methionine (Kurihara et al 1988). These amino acids are able to act as a trap for free radicals produced by the peroxidation of oil. Therefore, β-, γ-, and δ-type subunits could have a higher antioxidant activity, in terms of chemical reactivity, to peroxides than do α subunits. To confirm this, it should be checked whether methionine or cysteine residues in these subunits are damaged or not in the powder model system of zein and oil.

Not only amino acid composition, but also primary structure may be related to the antioxidant activity of zein subunits. Sequence analyses have revealed that α-type subunits, and β-, γ-, and δ-type subunits should belong to different groups genetically. The primary structures of the α-zein subunits are characterized by the presence of nine or ten tandem repeats of a 20 amino acid sequence (Geraghty et al 1981, Marks and Larkins 1982, Spena et al 1982). A structural model for these subunit proteins has been proposed (Argos et al 1982) that predicts folds into dense rod-like structures, perhaps allowing their close association in the protein bodies. Other type subunits exhibit no homology with α subunits. The C-terminal halves of the γ subunits contain regions with significant homology to the cysteine-rich prolamins of wheat and barley, such as α_1, β_1, γ gliadins, and β hordeins (Prat et al 1985, 1987; Pederson et al 1986). The β subunit has a low, but significant, degree of homology to γ-zein subunits. The δ subunit exhibits no homology with other zein subunits (Kurihara et al 1988). However, secondary structure predictions for the β- and δ-zein subunits possibly indicate a common three-dimensional structure with methionine-rich storage proteins from Brazil nut (Kurihara et al 1988). These findings suggest that α subunits belong to the subclass of zein fractions that evolved uniquely as zein components, while other zein subunits belong to another subclass, which has a close relationship to the family of the sulfur-rich storage proteins in cereals or legumes.

Our results in the present study suggest that zein subunits of the latter subclass, particularly γ-type subunits, have higher antioxidant activity than do α-type subunits. The feature in amino acid sequence of γ-type subunits (i.e., significant homology with

prolamins of wheat and barley) may be responsible for the improved antioxidant activity of this type subunit against DHE. Wang et al (1991a) suspected that other prolamins present in crops could offer similar antioxidant activity to zein. From the homology of primary structure, it is probable that α_1, β, γ-gliadins, β-hordein, and γ-zein have similar conformations. These conformations may be suitable for the spatial isolation of lipids from outer oxygen (encapsulation). It is also possible that such conformation increases the reactivity of methionine, cysteine, or other amino acid residues to peroxide radicals.

On the other hand, α-zein has a different conformation. It is expected that the efficiency and the mode of antioxidant activity of α zein is different from those of other prolamin subgroups. We did not exclude the possibility that α-zein subunits have significant antioxidant activity against unsaturated fatty acids. When Wang et al (1991a,b) demonstrated the antioxidant activity against methyl linoleate, they used the same commercial zein that we used in the present study. That zein should be composed mainly of α-zein subunits, though they did not analyze subunit compositions. We also found that commercial zein has the prominent antioxidant effect on peroxidation of methyl linoleate. Therefore, α zein is capable of protecting the peroxidation of uansaturated fatty acids whose double-bonding number is relatively low. However, it may be impossible for the α-type subunits to show the antioxidant effect on the HUFA such as EPA and DHA.

To make clear this point, the evaluation of antioxidant activity of zein should be performed at the subunit level. The next stage of the research on the antioxidant activity of zein is to separate various type of subunits by a preparative HPLC method and examine the antioxidant activities of separate subunits against several kinds of fatty acids. The permeability of oxygen through the protein membrane of each subunit also must be checked. From such findings, it should be clearer whether or not the antioxidant activity of α-zein subunits is inferior to other type of subunits. Furthermore, we should investigate the physical and chemical interaction of separated subunits with lipids by using several techniques, such as FT-IR, differential scanning calorimetry, etc., to understand the reason for the antioxidant activity of zein proteins.

Previous researchers (Wilson 1983, Wallace et al 1990) pointed out the presence of a mutant variety that has β- and γ-type subunits as the major components of the zein fraction. The selection of mutant types has been attempted to raise the level of sulfur amino acids in maize storage proteins. In the future, the varietal selection may be performed for the antioxidant activity of zein as well as for the nutritional value.

Not only the selection of the variety, but also the preparation method of zein is very important. Commercial zein is normally prepared according to the traditional method, by extraction with 70% (v/v) isopropanol. It is well known that the yields of zein by this method are low, and major extractable components are only α-type subunits. To extract β, γ, and δ zeins fully, in addition to α zein subunits, extraction by alcoholic solvent with reducing agent and sodium acetate is necessary (Paulis and Bietz 1986). We used 70% ethanol with 2-ME at 60°C for the extraction. The difference in subunit composition between the commercial and laboratory-prepared zeins found in the present study may be partially due to the different preparation or extraction methods of zein fractions. Therefore, the preparation method of zein should be carefully considered in application of zein as an antioxidant.

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