Prominent Antioxidant Effect of Wheat Gliadin on Linoleate Peroxidation in Powder Model Systems at High Water Activity

Kimikazu Iwami,* Masahiko Hattori, and Fumio Ibuki

Various proteins were examined for their antioxidant effects in powder model systems. Wheat gliadin that was prepared from gluten by extraction with 70% ethanol, but not gluten and its component "glutenin", was the most effective in antioxidation against linoleate peroxidation under the experimental conditions (40 °C, A_w = 0.6). Maize zein, ovalbumin, and soy protein isolate followed it to a lesser extent. The antioxidant effect of gliadin was found closely correlated with the moisture in the model system. At a high A_w, the peroxide value was maintained at a marginal level during storage. A quite similar tendency was observed for residual linoleic acid determined by gas chromatography; the ratio of linoleic acid to palmitic acid (an internal standard) remained unchangeable throughout the experimental period.

Polyunsaturated fatty acids (PUFA) such as linoleic, linolenic, and arachidonic acids are essential nutrients for mammals, and their beneficial effects in counteracting cholesterolemia and/or atherosclerosis have become of much interest in recent years (Spritz and Mishkel, 1969; Dyerberg et al., 1978; Ramesha et al., 1980; Toussant et al., 1981). Nevertheless, supplementation of processed foods by PUFA should be carefully considered, because PUFA are extremely susceptible to peroxidation under aerobic conditions and their excess ingestion often causes injury to health (Dianzani and Ugazio, 1978; Slater, 1979). Various antioxidants including phenolic compounds such as (butylhydroxy)anisole and (butylhydroxy)toluene have been used to protect oxidation-sensitive foods against their oxidative deterioration. Free-radical terminators, reducing agents, and chelating agents are involved in antioxidants for foods (Dziezak, 1986). Certain proteins are likewise expected to function as potent antioxidants under suitable conditions (Karel et al., 1975; Kawashima et al., 1979; Yamaguchi et al., 1980; Taylor and Richardson, 1980a; Funnes et al., 1982; Laakso, 1984; Houlihan and Ho, 1985; Pratt, 1985). The proteins of amphipathic properties have the ability to emulsify, bind, and stabilize PUFA (Lin et al., 1974; Wang and Kinsella, 1976; Pearce and Kinsella, 1978). Thus, entrapment of PUFA by protein may be more effective in retarding autoxidation due to spatial isolation from the outer oxygen besides reactions with peroxy radicals (Rosenberg et al., 1985). If food proteins avail as antioxidants, their application to food preservation will be most favorable in view of safety and nutrition.

For the purpose of obtaining information on this possibility, a variety of proteins were examined for their antioxidant activities in powder model systems. The present paper deals with the effectiveness of food proteins, especially of glaidin, at different water activities.

MATERIALS AND METHODS

Materials. Linoleic and palmitic acids of analytical grade were obtained from Nakarai Chemicals Ltd., Kyoto, Japan. Gliadin and glutenin were prpared from wheat gluten (a product of Nakarai Chemicals Ltd.) in our laboratory by extraction with 70% ethanol and 0.01 N acetic acid (Osborne, 1907), respectively, followed by lyophilization after evaporation. Soy protein isolate (SPI) was a gift of Fuji Oil Co., Osaka, Japan. Peptic digests were prepared from SPI, casein, gluten, and ovalbumin commercially available in the following manner: each food protein (5 g) was suspended in 20 volumes of distilled water, adjusted to pH 2 with HCl, incubated at 37 °C together with 50 mg of porcine pepsin (a product of Sigma Chemical Co.) overnight, and lyophilized after adjustment to neutral pH with NaOH. Other reagents also were commercially obtained and used without further purification.

Powder Model System for Antioxidant Activity. Linoleic acid or a 7:3 mixture of linoleic and palmitic acids was added to various proteins (or peptides) in a ratio of 1:9 by weight and adequately blended in an electric mixer. In addition, starch, cellulose, β-cyclodextrin, and quartz sand were used as reference instead of proteins and peptides. Samples in powder states were subdivided in small portions and stored in a humidity-controlled plastic vessel at 40 °C. Water activity (A_w) was arbitrarily adjusted with different concentrations of sulfuric acid (Troller and Christian, 1978): H_2SO_4 (%, w/w) = 55, 44, 38, 33, and 22 for A_w = 0.3, 0.5, 0.6, 0.7, and 0.9, respectively. Their corresponding samples were taken out of the vessel at stated intervals, of which the inner parts were packed every time with oxygen.

Measurement of Peroxide Value (POV). The POV was determined by the ferric thiocyanate method as described previously (Mitsuda et al., 1966). A 20-mg portion of each sample was extracted with 4 mL of chloroform–methanol (2:1, v/v) and centrifuged at 3000 rpm for 10 min. The supernatant (0.25 mL) was diluted with 4.55 mL of the same solvent, to which were added 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of 0.02 M ferrous sulfate and barium chloride before use. The absorbance at 500 nm was measured exactly 3 min after the start of reaction, from which the amount of hydroperoxide accumulation was obtained by comparison with standards of ferric ions.

Gas Chromatography. Samples of 20 mg were three times extracted with 1 mL of hexane, and the supernatants after centrifugation were combined and filtered through a Whatman 1PS filter to remove insoluble materials. The clarified solution was evaporated to dryness under a stream of nitrogen gas and dissolved in 100 μL of hexane. Its aliquot (4 μL) was directly injected into the column inlet of Shimadzu GC-4B apparatus equipped with hydrogen flame ionization detector. Analytical conditions are as follows: column size, 0.26 (o.d.) × 200 cm glass capillary; packing for separation, diethylene glycol succinate + phosphoric acid (5 + 1%) on Choromosorb W (60–80 mesh); column temperature, 195 °C (not programmed); injection temperature, 220 °C; carrier gas, N_2 at a flow rate of 40 mL/min; N_2 pressure, 6 kg/cm^2; H_2 pressure, 0.55 kg/cm^2; air pressure, 0.82 kg/cm^2.

Radical-Trapping Ability. The assay was based on a modification of the method described by Blois (1958) using a stable radical diphenylpicrylhydrazyl (DPPH, a

Department of Agricultural Chemistry, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606, Japan.

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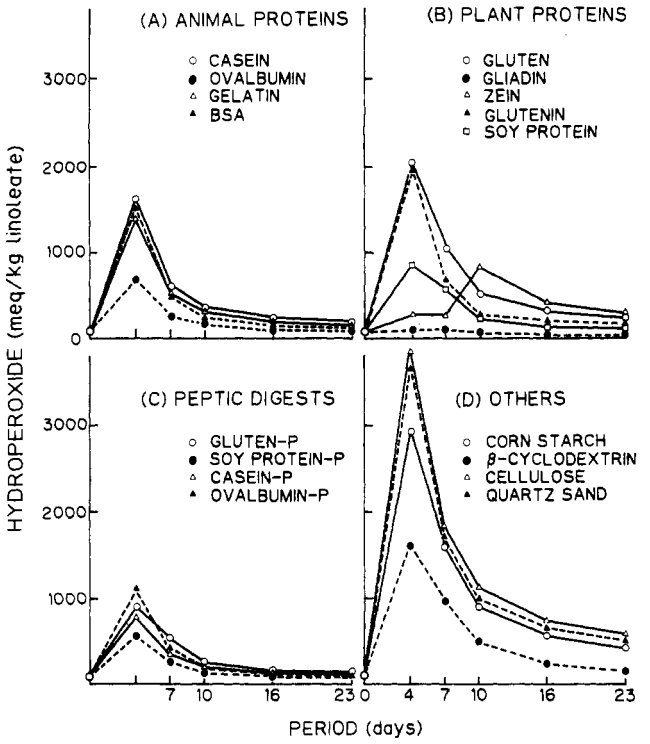

Figure 1. POV changes in powder model systems during storage. Powder model systems composed of linoleic acid and (A) animal protein, (B) plant protein, (C) peptic digest, or (D) other in the 1:9 ratio by weight were stored at 40 °C and A_w = 0.6 in a closed vessel packed with oxygen and assayed for their POV at stated intervals. The procedures for POV measurement are described in Materials and Methods.

product of Nakarai Chemicals Ltd.). A 50-mg portion of protein was suspended in 4 mL of 0.1 M, pH 8, Tris-HCl buffer containing 8 M urea and 2.5 mM ethylenediaminetetraacetic acid. An aliquot of the suspension, 0.5 mL, was added to 2 mL of 0.1 M, pH 6.5, phosphate buffer, followed by addition of 2 mL of 0.25 mM DPPH in ethanol. After incubation of 60 min, the mixture was filtered through a 0.45-μm membrane filter (Millipore Co.) and assayed for the absorbance at 525 nm.

Other Assays. Total sulfhydryl content in protein was measured with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, a product of Sigma Chemical Co.) according to a modification of the method described by Taylor and Richardson (1980b). Oil absorbability was determined in a small scale according to the procedure used by Lin et al. (1974).

RESULTS AND DISCUSSION

Figure 1 shows the time course of linoleate peroxidation in powder model systems at A_w = 0.6 that was monitored by POV in relation to antioxidant activities of various foodstuffs and others. More or less, increases in the POV were observed 4 days after the beginning of experiment, with the exception of only a few proteins. The exceptional typical protein was gliadin, which gave a marginal level in the POV throughout the experimental period. Simiarly zein was effective, but a moderate increase in the POV was observed a several days later. For SPI, ovalbumin, and peptic digests of food proteins, POV values on the fourth day were at relatively low levels. On the other hand, cellulose, α-corn starch, and quartz sand gave POV values near or more than 3000 mequiv/kg of linoleate and β-cyclodextrin to a small extent. From the viewpoint of antioxidant activity, food proteins and their digestive products were more effective than dextrans. The effectiveness of gliadin was conspicuous among them. In order to substantiate this phenomenon, the amount of linoleic acid

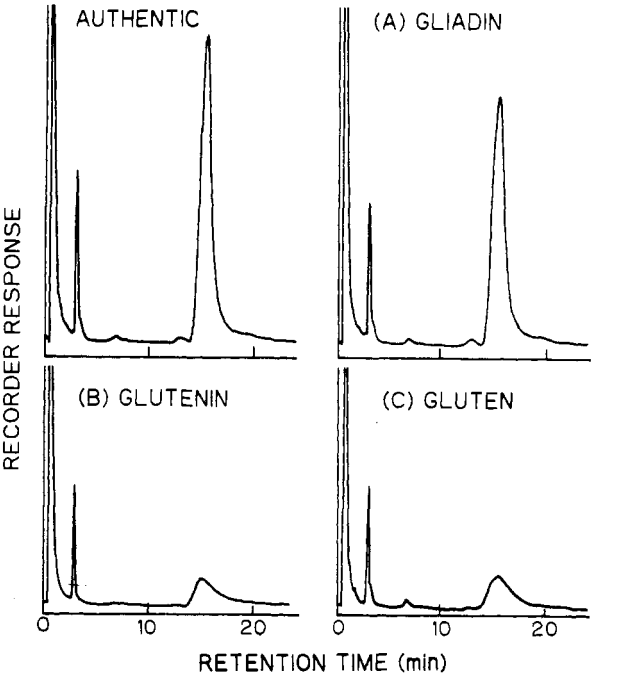

Figure 2. Typical gas chromatographic patterns of linoleic acid remaining in powder model systems after 1 month of storage. The storage conditions are the same as in Figure 1. Hexane extracts from powder systems (5 mL/g sample), in each 4 μL, were directly injected into the gas chromatograph and analyzed for residual linoleic acid to be recorded in a chart.

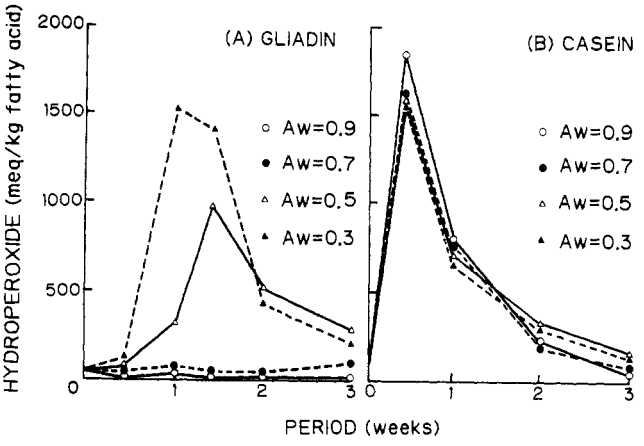

Figure 3. POV changes in gliadin- or casein-based powder systems as functions of time and A_w. The experimental conditions are the same as in Figure 1, except that protein (gliadin or casein)–linoleic acid–palmitic acid (90:7:3) mixtures were stored at varied A_w.

remaining in the above model system was analyzed by gas chromatography after storage for 1 month. Apparently, as shown in Figure 2, linoleate in gliadin was found mostly recovered in the "intact" form without suffering oxidative deterioration, whereas such was not the case with gluten and glutenin. A remarkable diminution or disappearance of peak corresponding to linoleic acid was seen for other samples that had given high POV values (data not shown).

The water content in foods is genrally accepted to affect their resistance to lipid peroxidation. For this reason, the function of gliadin as an antioxidant in the powder model systems was again investigated at various A_w. Figure 3 shows the results of POV measurements of protein–linoleate–palmitate (90:7:3) mixtures stored under the circumstances of a fixed temperature (40 °C) and various A_w. The POV reached a maximum on the third day at a low A_w and later at a middle A_w. At a high A_w, however,

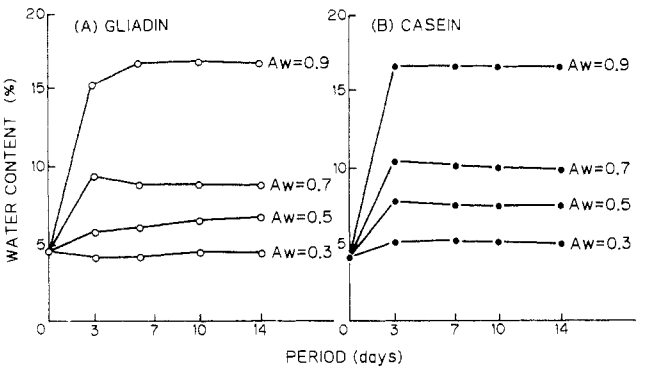

Figure 4. Water contents in gliadin and casein stored at various A_w (40 °C). The experimental conditions are the same as in Figure 3.

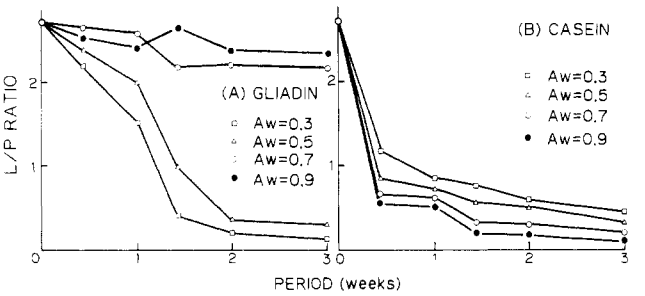

Figure 5. Ratio of linoleic acid to palmitic acid remaining in the gliadin- or casein-based powder systems during storage at various A_w (40 °C). Samples are the same as in Figure 3. The ratio between both fatty acids was determined by gas chromatography.

linoleate peroxidation settled down throughout the experimental period. When the protein (gliadin) in the mixture was replaced by casein, its POV became greater at a similar level 3 days after the start of experiment irrespective of variation in A_w, implying that casein would have little ability to protect linoleate against its autoxidation. Changes in the water content of test samples at various A_w are depicted in Figure 4. The samples just before storage contained approximately 5% water by weight. When these samples were allowed to stand at A_w = 0.3, there was no increase in their water content. The water content increased with increasing A_w levels but attained equilibrium within a few days. Those in equilibrium state were 6–8%, 9–10%, and 16–17% by weight at A_w = 0.5, 0.7, and 0.9, respectively, the values being scarcely different between the gliadin- and casein-based samples.

Although fatty acids in the mixtures were mostly extractable with either hexane, ether, or chloroform–methanol (2:1), a survival of unsaturated fatty acid during storage was evaluated by the ratio of linoleic acid to an internal standard "palmitic acid", by which a possible error due to adsorption to protein was rectified. The ratio between the peak areas corresponding to both unsaturated and saturated fatty acids on the gas chromatogram was obtained as a function of time at various A_w. The relationship is shown plotted in Figure 5. The L:P ratio at time zero was the same with gliadin as with casein. A considerable decrease in the ratio was observed for casein over the range of low to high A_w, and to a lesser extent at low and middle A_w for gliadin. Noteworthy, the L:P ratio in the gliadin-based model systems was kept constant at high A_w throughout the experimental period. This was well reflected in a marginal level in the POV for periods exceeding 3 weeks.

When antioxidant effects of various food proteins were investigated by measuring changes in the POV and by gas chromatographic determination of fatty acids, gliadin has proven to be the most effective among samples tested. Its effectiveness continued longer at high A_w than at low A_w. Gliadin was prepared as a 70% ethanol-soluble fraction from gluten in our laboratory according to the classical definition by Osborne (1907) and, after lyophilization, used without further purification. It thus would be better to say that the final preparation is heterogeneous in purity. If active components responsible for antioxidant activity either were left or were concentrated in preparing gliadin, the original source "gluten" must have a somewhat inhibitory effect on lipid peroxidation as well. Actually gluten did not exhibit such an effect. Similarly, gluten was not quite different from gliadin in radical-trapping ability, which was far less than for other food proteins such as zein, ovalbumin, and SPI. Although the sulfhydryl contents in these proteins were also measured, the difference between gliadin and the other proteins was never to such an extent able to account for that in the antioxidant effect. It was described long ago that the sulfhydryl groups of native proteins became "reactive" on denaturation (Larson and Jennes, 1952), but Taylor and Richardson (1980b) mentioned later that the antioxidant activity of ovalbumin is not improved by its reduction with sodium borohydride in 8 M urea and that β-lactoglobulin has still negligible antioxidant activity after $NaBH_4$ treatment is spite of its high sulfhydryl content.

If prooxidants were removed in preparaing gliadin, nonconstant effects of gliadin at different A_w may partly be interpreted in terms of influences due to the hydration of residual prooxidants in trace amounts and/or of temporarily produced hydroperoxides. In contrast, an increase in the water content has been described to raise not only the mobility of prooxidants such as metal catalyst but also the oxygen absorption from the atmosphere and to accelerate the propagation of autoxidation thereby (Heiderbaugh et al., 1971; Labuza et al., 1972; Chou and Labuza, 1974). As far as gliadin is concerned, its antioxidant activity tended to be rather enhanced at high A_w.

Gliadin is not very soluble in aqueous solutions, where it varies in state of resinous aggregation. This property is mainly due to the presence of nonpolar amino acid residues such as glutamine, proline, and leucine at high concentrations in the protein molecule. It seems more likely that the amide group of glutamine plays an important role in intra- or intermolecular association via hydrogen bond, because the insolubility of gliadin is improved by deamidation (Holme and Briggs, 1959). Addition of a small amount of fatty acid to protein, even though by simple mixing, may cause such infiltration into the hydrophobic regions as to affect the rate of lipid peroxidation. For this reason, the oil absorbability of gliadin was compared with those of other food proteins. Gliadin showing the most excellent antioxidant activity was not as good in oil absorbability as would be expected from its feature containing nonpolar amino acids at high concentrations.

Since linoleic acid was used as a model of unsaturated lipid in the autooxidation system, it was conceivably possible that the antioxidant activity of gliadin was an artifact due to the use of free fatty "acid". However, the usefulness of gliadin as an antioxidant has been verified with a gliadin–safflower (9:1) mixture (Taguchi et al., 1986). In any case, the fact that the antioxidant effect of gliadin increases at the higher A_w suggests the significance of water participation in protection against lipid peroxidation. Water in gliadin may retard autoxidation not only by hydration of "trace" prooxidants but also by direct action on hydroperoxides or free radicals in cooperation with functional groups of the protein.

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