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.

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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

be due to the presence of a protective system in the leaf waxes; they also succeeded in isolation of novel β-diketone-types of antioxidants (Osawa and Namiki, 1985). Further, Osawa et al. (1985) investigated the germination potentials of rice seeds during long storage and showed that the difference in viability of Japonica and Indica rice seeds, effected by aging, could be due to the difference in the effectiveness of defense systems in the rice hull that has been believed until now to offer only physical protection to rice grain.

Later studies indicated that the contents of natural antioxidants in rice, α-tocopherol and oryzanol, did not differ appreciably in the rice seeds that maintain viability even after storage at room temperature for 1 year (long life) and those that lost viability on storage for 1 year at the same temperature (short life) (Ramarathnam et al., 1986). However, the hull fraction of long-life rice seeds were shown to have stronger antioxidative activity and were richer in phenolic constituents than the short-life rice cultivars. Such phenolic components were believed to function either as natural antioxidants or as lipoxygenase enzyme inhibitors (Ramarathnam et al., 1986). Model studies to demonstrate the presence of defense mechanism in the rice hull also reveled that rice seeds having relatively longer viability were given better protection by their hull against γ irradiation or heat-induced oxidative damages (Ramarathnam et al., 1987a,b).

Though extensive work has been done on the growth- and germination-inhibiting factors in rice hull (Mikkelsen and Sinah, 1961; Roberts, 1961; Datta, 1972; Kato et al., 1977), no attempt has been made yet to undertake chemical investigation on the antioxidtive defense system in rice hull. This paper reports for the first time chemical studies involving isolation, fractionation, and partial characterization of natural antioxidants of rice hull.

MATERIALS AND METHODS

Rice Hull. Two rice cultivars (*Oryza* Sativa Linn.), Katakutara (Indica; long life) and Kusabue (Japonica; short life), were cultivated under controlled conditions in the experimental farm of Nagoya University. Immediately after harvest (120 days after sowing), the seeds were sundried for about 2 weeks, to a moisture level of about 10%. The seeds were subsequently dehulled on a manual rubber–rolled-type dehuller, taking care not to cause mechanical damage to the intact kernels and the bran layers. The hull samples from the respective rice seeds were ground in a laboratory mill and made to pass through a 60-mesh sieve to get a uniform sample for extraction. Preparation of the hull samples for extraction was achieved within 1 month after harvest.

Extraction and Fractionation of Crude Antioxidants. The scheme for preparation of rice hull antioxidants is shown in Figure 1. Hull samples (50 g) of the respective rice seeds were extracted twice with 300 mL of methanol overnight, followed by filtration and evaporation of the filtrate to dryness in vacuo. The crude samples thus obtained were partly used (1 mg) for antioxidative assay, by the thiocyanate method (Osawa and Namiki, 1981), while the remaining portion was used for the fractionation of an Amberlite XAD-2 (Organo Co., Ltd.) column (25 mm × 300 mm). The column was eluted stepwise with glass-distilled water, 50:50 MeOH–H_2O, 75:25 MeOH–H_2O, methanol, and acetone. The separated fractions were evaporated to dryness in vacuo and weighed to determine the respective yields of soluble constituents in the two cultivars.

Antioxidative Activity Determination. Antioxidative activity of these fractions was carried out by the thiocyanate method using 1 mg of each fraction for the assay (Osawa and Namiki, 1981). Each sample was added to a solution mixture of linoleic acid–99.0% ethanol–0.2 M phosphate buffer (pH 7.0). The mixed solution in a conical flask was incubated at 40 °C, and the peroxide value was determined by reading the absorbance at 500 nm after coloring with $FeCl_2$ and thiocyanate at intervals during incubation. BHA (butylated hydroxyanisole; Tokyo Kasei Kogyo Co., Ltd.) and α-tocopherol (Wako Pure Chemical Industries, Ltd.) (200 μg) were used as standards for comparison of antioxidative activity.

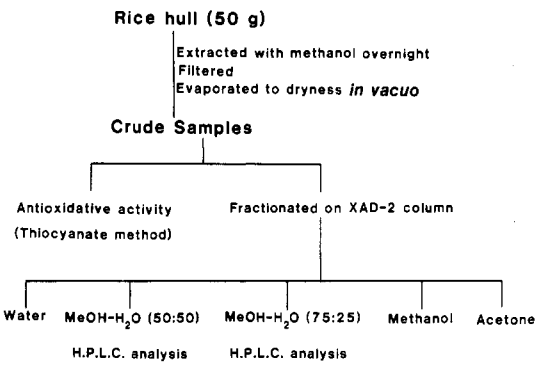

Figure 1. Scheme for extraction, fractionation, separation, and analysis of antioxidants from rice hull.

High-Pressure Liquid Chromatography (HPLC). Analyses of 50:50 MeOH–H_2O and 75:25 MeOH–H_2O fractions were carried out on a Jasco Twincle HPLC (Japan Spectroscopic Co., Ltd.) using a Develosil ODS-7 (8 mm (i.d.) × 250 mm) column (Nomura Chemical Co., Ltd.) with a UV spectrophotometer detector (Jasco UVIDEC-100-III, Japan Spectroscopic) at 280 nm as the monitor. The 50:50 MeOH–H_2O fractions were eluted with a linear gradient ranging from H_2O to 80:20 MeOH–H_2O over 30 min at a flow rate of 3 mL/min while the 75:25 MeOH–H_2O fractions were eluted with a linear gradient ranging from 50:50 MeOH–H_2O to MeOH over 30 min at the same flow rate. The HPLC-separated subfractions of Kusabue and Katakutara rice hull MeOH–H_2O (50:50 and 75:25) fractions were assayed for antioxidative activity by the thiocyanate method (Osawa and Namiki, 1981).

A mixture of standard phenolic acids consisting of caffeic acid, *p*-coumaric acid, ferulic acid, syringic acid, vanillic acid (Tokyo Kasei Kogyo Co., Ltd.), and gallic acid (Wako) was also analyzed under the above HPLC conditions to investigate the contribution of these constituents toward the antioxidative activity of the respective hull fractions.

Determination of Total Phenolic Contents. Quantification of total phenolic contents in the separated hull fractions was done spectrophotometrically, in triplicate, according to the method prescribed by the Association of Official Analytical Chemists (1984). The hull fractions in methanol (0.5 mL), in a test tube, were diluted to 5 mL with glass-distilled water. Folin-Denis reagent (5 mL) was added, and the contents of the test tube were mixed thoroughly. After 3 min, Na_2CO_3 solution (10%) was added and the mixture was allowed to stand for 1 h with intermitent shaking. The blue color was measured in a Hitachi Model 200-10 spectrophotometer, and on comparison with the optical density values of different concentrations of standard catechin, the concentrations of total phenolic contents in the separated fractions were determined.

RESULTS AND DISCUSSION

Antioxidative Activity of the Hull Extracts. Antioxidative activities of the methanol extracts of rice hull from Kusabue and Katakutara seeds are shown in Figure

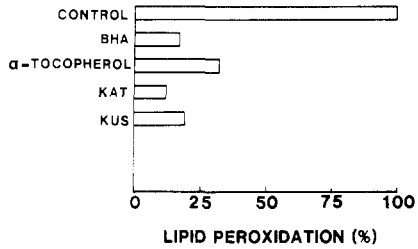

Figure 2. Antioxidative activities of crude methanol extracts of Kusabue (Kus) and Katakutara (Kat) rice hull, determined by the thiocyanate method.

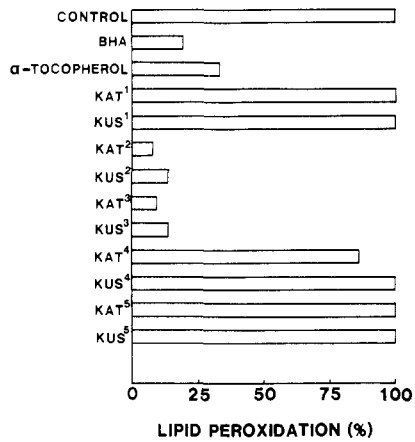

Figure 3. Antioxidative activities, by the thiocyanate method, of different fractions of Kusabue (Kus) and Katakutara (Kat) rice hull samples, separated on an XAD-2 column: 1, water; 2, 50:50 MeOH–H_2O; 3, 75:25 MeOH–H_2O; 4, methanol, 5, acetone.

Table I. Yield of Different Fractions of Methanol Extracts of Kusabue and Katakutara Rice Hull, Separated on an XAD-2 Column (mg/50-g Hull)

fraction	cultivar	
	Kusabue	Katakutara
water	58.8	40.5
50:50 MeOH–H_2O	44.1	64.2
75:25 MeOH–H_2O	45.7	80.6
methanol	61.2	69.9
acetone	57.6	31.9

2. As can be seen in this figure, Katakutara rice hull extract exhibited strong antioxidative activity, inhibiting lipid peroxidation to the extent of 88% in comparison with Kusabue rice hull extract which inhibited lipid peroxidation to the extent of 81%. However, both varieties showed antioxidative activities stronger than those of BHA and α-tocopherol, which showed inhibition of lipid peroxidation to the extent of 83% and 68%, respectively. This observation supports the earlier assumption for the presence of antioxidative defense system in rice hull by Osawa et al. (1985).

Fractionation of the Crude Extracts. Yields of different fractions separated on the XAD-2 column are given in Table I. As can be observed in this table, Katakutara rice hull had higher amounts of soluble constituents in MeOH–H_2O (50:50, 75:25) and methanol extracts than Kusabue rice hull. Also, Kusabue rice hull was found to have higher levels of acetone- and water-soluble constituents than Katakutara rice hull.

Antioxidative Activity of the XAD-2 Fractions. Antioxidative activities of the different XAD-2 fractions are shown in Figure 3. Of the various fractions separated, water, methanol, and acetone fractions of both rice hull samples did not show appreciable activity. Both 50:50 and 75:25 MeOH–H_2O fractions showed strong antioxidative activities, stronger than the α-tocopherol and BHA standards. Katakutara rice hull extract showed a stronger inhibiting effect on lipid peroxidation than Kusabue rice hull extract in both fractions. The 50:50 MeOH–H_2O fraction of Katakutara rice hull extract showed relatively stronger antioxidative activity (93% inhibition) than the 75:25 MeOH–H_2O fraction (91% inhibition).

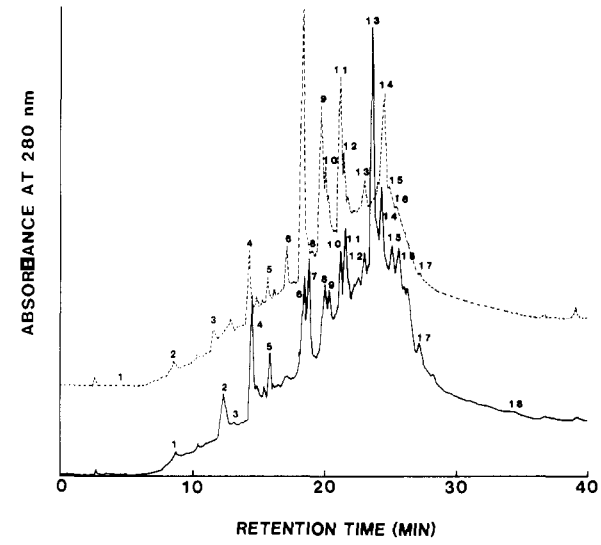

Figure 4. HPLC chromatograms of the 50:50 MeOH–H_2O fraction of Kusabue (---) and Katakutara (—) rice hull extracts. Conditions: column, Develosil ODS-7 (8 mm (i.d.) × 250 mm); eluent, linear gradient from H_2O to 80:20 MeOH–H_2O over 30 min; flow rate, 3 mL/min; detector, UV 280 nm.

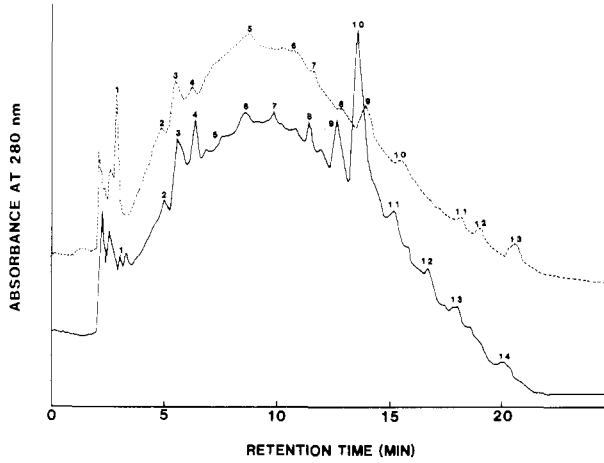

Figure 5. HPLC chromatograms of the 75:25 MeOH–H_2O fraction of Kusabue (---) and Katakutara (—) rice hull extracts. Conditions: eluent, linear gradient from 50:50 MeOH–H_2O to 100% MeOH over 30 min; other conditions, same as in Figure 4.

High-Pressure Liquid Chromatography (HPLC). HPLC chromtograms of the MeOH–H_2O (50:50) fractions of Kusabue and Katakutara rice hull samples, separated an ODS-7 (8 mm (i.d.) × 250 mm) column, are shown in Figure 4. While the pattern of the chromatograms was almost identical in the retention time region 15–30 min, Katakutara showed a major peak at retention time 23.6 min, while Kusabue had a major peak at 18.2 min. Thus, the strong antioxidative activity of Katakutara rice hull could be due to the major component at 23.6 min that is either absent or present as a minor component in Kusabue rice hull sample.

The HPLC chromatograms of 75:25 MeOH–H_2O fractions of Kusabue and Katakutara rice hull samples are illustrated in Figure 5. Both varieties showed identical

chromatograms, with major components at 5.6, 6.4, 12.8, and 13.6 min in Katakutara and at 5.6 and 13.8 min in Kusabue rice hull samples. As both varieties exhibited strong antioxidative activities in this fraction, it is believed that the difference in their degree of antioxidative activities could be due to quantitative differences in their constituents. However, it appears from the two chromatograms that the component at retention time 13.6 min must be mainly responsible for the strong antioxidative activity of 75:25 MeOH–H_2O fractions.

The hull constituents of seeds, especially phenolic acids, are well-known for their role as regulators for germination (Wurzburger et al., 1974; Chen et al., 1982). The HPLC analysis of the standard mixture of phenolic acids separated under the conditions identical with that of separation of 50:50 MeOH–H_2O fractions indicated that though most of the known phenolic acids reported to be present in husks of other cereal grains (Van Sumere et al., 1958; Smart and O'Brien, 1979; Chen et al., 1982; Nordkvist et al., 1984) could be separated within 15 min, under the same conditions, no similar major peaks could be detected in the rice hull samples (data not shown). Moreover, in our previous report, we also reported the absence of the natural rice antioxidants α-tocopherol and oryzanol in the rice hull samples (Ramarathnam et al., 1986). Thus, the component(s) responsible for the antioxidative activity must be something other than the known rice antioxidants or standard phenolic acids used in the analysis, namely gallic acid (3.4 min), vanillic acid (9.9 min), caffeic acid (11.4 min), syringic acid (11.9 min), *p*-coumaric acid (15.1 min), and ferulic acid (16.3 min).

Antioxidative Activity of HPLC-Separated Components of 50:50 and 75:25 MeOH–H_2O Fractions of Kusabue and Katakutara Rice Hull. The antioxidative activity of individual subfractions of the 50:50 MeOH–H_2O fraction of Kusabue rice hull sample separated on HPLC is illustrated in Figure 6a. It is very clear from this figure that fractions 14 and 13, which are at retention times 24.4 and 23.2 min (Figure 4), exhibited antioxidative activities stronger than that of α-tocopherol. However, these components were present only as minor components in the case of Kusabue rice hull. Around the same retention time, Katakutara rice hull fraction exhibited very strong antioxidative activity and was found to have a major peak in this region (Figure 4). The other component in Kusabue rice hull exhibiting strong antioxidative activity, comparable to that of α-tocopherol, was fraction 7, which was present as a major peak at retention time 18.2 min. Thus, the antioxidative activity of the crude 50:50 MeOH–H_2O fraction of Kusabue rice hull could be due to a combined effect of the activities of the above-mentioned subfractions.

The antioxidative activities of the individual subfractions of the 50:50 MeOH–H_2O fraction of Katakutara rice hull are illustrated in Figure 6b. This figure clearly demonstrates that strong antioxidative activity was exhibited by fraction 13, which is found to be present as a major component (retention time 23.6 min; Figure 4) in this fraction of Katakutara rice hull. The activity was found to be as strong, as that of BHA, a synthetic antioxidant, but certainly twice as strong as that of the natural food antioxidant α-tocopherol. The other fraction that exhibited antioxidative activity comparable to that of α-tocopherol is subfraction 6, which was found to be present only as a minor component in this fraction. Thus, the strong antioxidative activity of Katakutara rice hull could be mainly due to subfraction 13. Isolation, purification, and structural characterization of this active component from a large-scale batch of Katakutara rice hull have been completed and will be soon reported elsewhere.

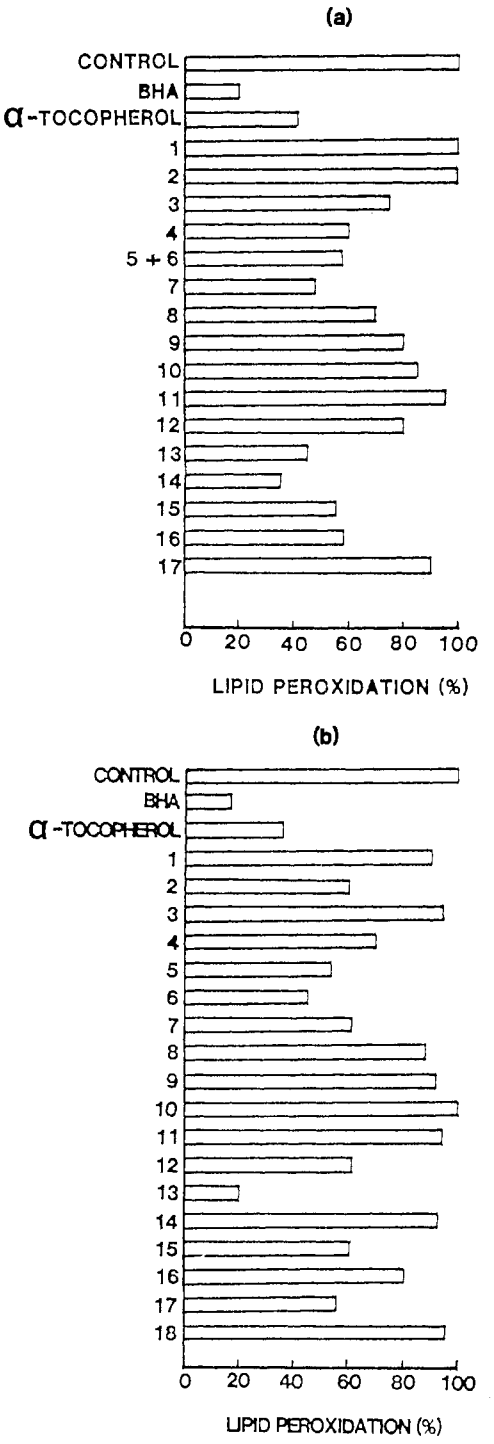

Figure 6. Antioxidative activities of different fractions of the 50:50 MeOH–H_2O fraction of (a) Kusabue and (b) Katakutara rice hull (by the thiocyanate method).

Antioxidative activities of the different subfractions of 75:25 MeOH–H_2O fractions of Kusabue and Katakutara rice hull separated on HPLC are illustrated in parts a and b of Figure 7, respectively. It was found that both rice hull fractions exhibited strong antioxidative activity in the retention time region 10–20 min. However, the activity of the separated subfractions was considerably weaker after separation of the crude mixture into individual subfractions.

Determination of Total Phenolic Content. The contents of total phenolic constituents as estimated by the Folin–Denis method in the different fractions separated on the XAD-2 column are given in Table II. As can be

Table II. Content of Total Phenolics in Different Fractions of Kusabue and Katakutara Rice Hull, Separated on an XAD-2 Column (mg/50-g Hull)[a]

fraction	cultivar	
	Kusabue	Katakutara
water	1.885 ± 0.041	1.499 ± 0.012
50:50 MeOH–H_2O	5.865 ± 0.011	9.309 ± 0.010
75:25 MeOH–H_2O	5.621 ± 0.025	11.687 ± 0.014
methanol	3.182 ± 0.011	3.355 ± 0.011
acetone	2.131 ± 0.023	1.276 ± 0.014

[a] Reported values are mean ± SD, n = 3.

seen from the results, the more active MeOH–H_2O fractions contained higher amounts of phenolic constituents. Katakutara had higher levels of phenolic constituents [9.309 ± 0.010 mg/50-g hull (MeOH–H_2O, 50:50) and 11.687 ± 0.014 mg/50-g hull (MeOH–H_2O, 75:25)] than Kusabue [5.865 ± 0.011 mg/50-g hull (MeOH–H_2O, 50:50) and 5.621 ± 0.025 mg/50-g hull (MeOH–H_2O, 75:25)]. These results indicated that phenolic components, other than those investigated in the present work, could be the major contributing factor for the antioxidative activities of these rice hull fractions. The difference in the antioxidative activities of Kusabue and Katakutara rice hull extracts could also be due to differences in their qualitative and quantitative compositions of such phenolic compounds.

All the foregoing observations indicated the presence of a novel antioxidative defense system in rice hull that is entirely different from the known rice antioxidants α-tocopherol and oryzanol or the known phenolic acids reported as natural antioxidants in other plant systems. Our previous studies (Ramarathnam et al., 1986, 1987a,b) undertaken with the two cultivars used in the present investigation have suggested the presence of effective antioxidative defense system in the long-life (Katakutara) rice hull, which offered better protection against oxidative damages induced by γ irradiation or heat. Moreover, as the antioxidative activities of the rice hull constituents are stronger than any of the aforementioned constituents, there is probability that the active rice hull constituents could be playing a vital role during long-term preservation of rice seeds. Further detailed biochemical investigations are in progress to understand the mechanism of action of this defense system in the rice seeds.

CONCLUSION

Seeds have a wide range of defense systems such as tocopherols, carotenoids, ascorbic acid, and many other phenolic components that inhibit lipid peroxidation and protect the membrane functions against damage by oxygen radicals. Research on rice antioxidants, α-tocopherol and oryaznol, have received only commercial attention to explain the storage stability of rice bran oil. Hence, much attention in the past has been paid to investigations on rice bran rich in oil. However, physiological studies on the role of natural defense systems in rice seeds, to explain the difference in viability of long-life and short-life rice seeds, during long-term storage, have been topics of research interest only in recent years. The present investigation reports chemical aspects of the novel antioxidative defense system in rice hull that was established recently. Despite the presence of α-tocopherol and oryzanol in long-life and short-life rice seeds, the antioxidative constituents of rice hull were shown to be relatively stronger in their activity, and hence they may play a vital role in prevention of aging of rice seeds. The long-life and short-life rice seeds were shown to differ in the qualitative and quantitative composition of the active fractions of their hull extracts.

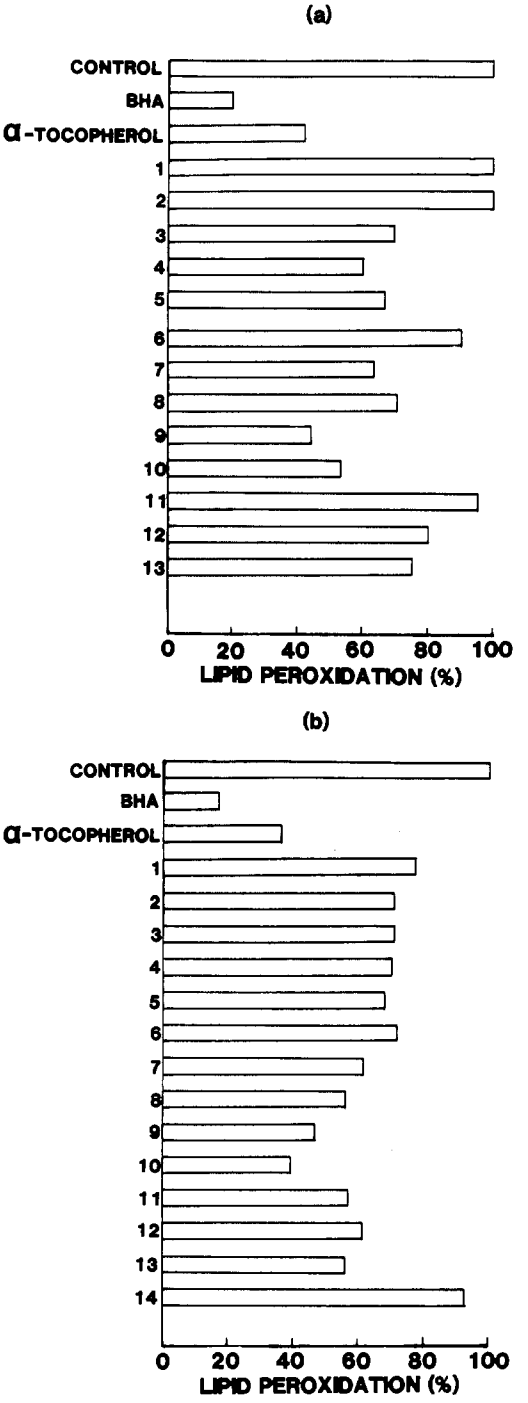

Figure 7. Antioxidative activities of different fractions of the 75:25 MeOH–H_2O fraction of (a) Kusabue and (b) Katakutara rice hull (by the thiocyanate method).

However, in both active hull extracts, phenolic constituents seemed to be more responsible for the antioxidative activity.

ACKNOWLEDGMENT

This work was supported in part by research funds from the Foundation for Promotion of Food Science and Technology, Japan, for which we are deeply indebted. We thank Dr. Toru Tashiro for providing this valuable assistance and cooperation in the cultivation of rice samples.

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Received for review February 11, 1987. Revised manuscript received January 26, 1988. Accepted March 11, 1988. This work was presented at the annual meeting of the Agricultural and Chemical Society of Japan, Tokyo, April 1–4, 1987.

Structural and Conformational Basis of the Resistance of β-Lactoglobulin to Peptic and Chymotryptic Digestion

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The structural basis accounting for the resistance of β-lactoglobulin (β-Lg) to peptic and chymotryptic digestion was studied. Native β-Lg was resistant to peptic and chymotryptic digestibility because of its stable conformation. Heating at 50, 60, and 70 °C for 15 min did not affect resistance of β-Lg, while heating at 80 and 90 °C significantly decreased its resistance to proteolysis. Surface polarity measurements and fluorescence spectra revealed that very little change in conformation of β-Lg occurred upon heating at 50, 60, or 70 °C. Measurable changes in the conformation of β-Lg occurred at 80 and 90 °C, increasing its susceptibility to hydrolysis. Cleavage of S–S bonds caused extensive changes in conformation and significantly decreased the resistance of β-Lg to peptic and chymotryptic hydrolysis. The results suggest that disruption of native conformation exposes susceptible peptide bonds and decreases the resistance of β-Lg to proteolytic digestion.

The nutritive value of a protein is related to its amino acid composition and the bioavailability of these amino acids. Milk proteins in general and whey proteins, β-lactoglobulin (β-Lg) in particular, have a high content of essential amino acids (Hambraeus, 1982; McKenzie, 1971). However, in experimental animals, native β-Lg is resistant to gastric digestion and apparently remains intact after it passes through the stomach/abomasum (Miranda and Pelissier, 1983; Yvon et al., 1984), and thus its component amino acids may be nutritionally unavailable. Recently, Jakobsson et al. (1985) reported the presence of immunoreactive bovine β-Lg in human milk and correlated its presence to the development of colic in breast-fed babies.

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Since β-Lg is thermolabile, heat processing may alter its digestibility characteristics and render it biologically available. Milk undergoes heat processing such as preheating, pasteurization, sterilization, concentration, dehydration, etc., which affects the structure and properties of milk/whey proteins, either reversibly or irreversibly. The effect of heat on the denaturation of β-Lg has been reviewed by McKenzie (1971). Dupont (1965a,b) and Dewit and Swinkels (1980) showed that reversible conformational change/denaturation of β-Lg occurred below 70 °C, but above this temperature, denaturation resulted in irreversible polymerization. There is limited information concerning the effects of heat-induced conformational changes of β-Lg on its resistance to proteolytic digestion especially at pH 1–2 where pepsin digestion occurs.

β-Lg contains four S–S groups per dimer of molecular weight 36000 (McKenzie, 1971). Intramolecular S–S bonds

0021-8561/88/1436-0737$01.50/0