Chemical Studies on Novel Rice Hull Antioxidants. 2. Identification of Isovitexin, A *C*-Glycosyl Flavonoid

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Methanol extract of rice hull from long-life rice seeds, having strong antioxidant activity, was fractionated by column chromatography and HPLC techniques, and the active components were characterized by FAB-MS, ^1H NMR, and ^{13}C NMR techniques. The active components were found to be flavonoid substances, and one of them exhibiting antioxidant property as strong as α-tocopherol was identified as isovitexin, a *C*-glycosyl flavonoid.

Rice (*Oryza sativa* Linn.) is the principal cereal food in Asia and the staple food of nearly half of the world's population. The quality of rice seeds selected for breeding and cultivation is of great agricultural importance, especially in the context of increasing the agricultural output and improvement in the nutritional quality of harvested grain. The conditions for postharvest storage of rough rice (paddy) vary from place to place depending on the technological advancement and cultural needs. This leads to variations in changes in physiological properties of rice seeds, biochemical properties of rice constituents, and also the quality of cooked rice kernels.

It has long been known that germination of rice seeds is influenced by components present in the husk (Mikkelsen and Sinah, 1961; Roberts, 1961). Kato et al. (1977) investigated the growth and germination inhibition factors in rice husk of short-life seeds and reported the presence of ineketone, *S*-(+)-dehydrovomifoliol, momilactone C, and *p*-coumaric acid in addition to the presence of known momilactones A and B. Moreover, it has been observed that there exists a distinctive difference in the storability of rice seeds among the two main geoecographical races *indica* and *japonica* and that a relationship between the longevity of rice seeds and the antioxidative activity of the seed coat or the husk was also established (Osawa et al., 1985; Ramarathnam et al., 1986). In our previous paper we reported the partial characterization of the antioxidants present in long-life and short-life rice seeds (Ramarathnam et al., 1988). In a continuation of the same investigation, we report the isolation, characterization, and identification of novel flavonoid glycosides in the rice husk of long-life rice seeds.

MATERIALS AND METHODS

Rice Hull. Rice hull from Katakutara (*indica*) was prepared according to the method as described in our previous paper (Ramarathnam et al., 1988).

Extraction of Rice Hull. A 500-g portion of Katakutara rice hull was extracted with 3 L of methanol overnight, followed by filtration and evaporation of the filtrate to dryness in vacuo on a rotary evaporator, below 40 °C. The crude sample thus obtained was fractionated on an Amberlite XAD-2 (Organo Co., Ltd.) column (80 mm × 100 cm). The column was eluted stepwise with glass-distilled water, MeOH–H_2O (50:50), MeOH–H_2O (75:25), methanol, and acetone.

High-Pressure Liquid Chromatography (HPLC). The active MeOH–H_2O (50:50) fraction of Katakutara rice hull (Ramarathnam et al., 1988) was further subjected to preparative HPLC on a Jasco Twincle HPLC (Japan Spectroscopic Co., Ltd.) using reversed-phase Develosil ODS-10 (20 mm (i.d.) × 250 mm) column (Nomura Chemical Co., Ltd.). The elution solvent was a linear gradient ranging from H_2O to MeOH–H_2O (80:20) over 90 min with a solvent flow rate of 5 mL/min. The UV detector Jasco UVIDEC-100-III (Japan Spectroscopic) at 280 nm was used as the monitor.

Antioxidative Activity. Antioxidative activity of the HPLC-separated fractions was carried out by 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/0.2M 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 reaction 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 for comparison of antioxidative activity.

Purification of the Active Subfractions. The active subfractions separated by HPLC were further purified by preparative HPLC, and the antioxidative activity of the component fractions was monitored by the thiocyanate method (Osawa and Namiki, 1981).

Instrumental Analysis of the Active Fractions. (*a*) *UV Spectrometry.* UV absorption spectra of the purified active fractions were recorded on a spectrophotometer (Hitachi 200-10) with dilute solutions in methanol. Shifts in UV absorption were determined with use of shift reagents, NaOMe/MeOH, $AlCl_3$/MeOH, $AlCl_3$/HCl, NaOAc, and NaOAc/H_3BO_3. All recordings were carried out at room temperature (ca. 25 °C).

(*b*) *Fast Atom Bombardment Mass Spectrometry (FAB-MS).* FAB-MS spectra of the purified active components were recorded on a JMS DX-300 mass spectrometer (Japan Electron and Optics Laboratory Co. Ltd., Tokyo, Japan) with glycerol as the mounting matrix.

(*c*) *^1H NMR and ^{13}C NMR Spectrometry.* ^1H NMR (200 MHz) and ^{13}C NMR (50 MHz) were measured on a JNM-FX 200 (Japan Electron and Optics) with tetramethylsilane (TMS) as the internal standard, and the chemical shifts were given in δ values.

RESULTS AND DISCUSSION

High-Pressure Liquid Chromatography. The HPLC chromatogram of the XAD-2 column separated MeOH–H_2O (50:50) fraction of Katakutara rice hull is shown in Figure 1. It was found that this fraction could be separated into 16 subfractions. Of the various fractions separated, fraction 4 (retention time 53.3 min), fraction 11 (retention time 67.0 min), and fraction 13 (retention time 70.9 min) were found to be present as major peaks.

Antioxidative Assay. Antioxidative activity of 1 mg each of the separated subfractions, determined by thiocyanate method, is illustrated in Figure 2. It can be seen

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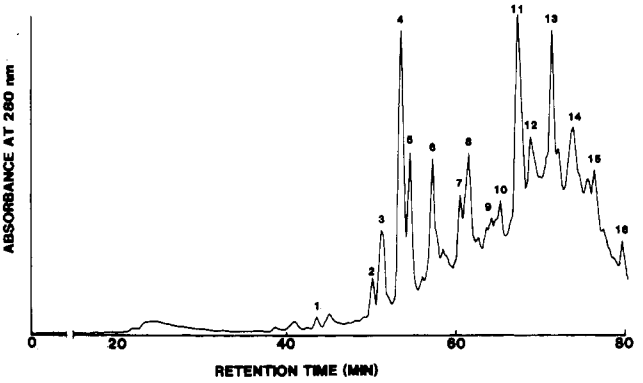

Figure 1. HPLC chromatogram of the MeOH–H_2O (50:50) fraction of Katakutara rice hull. Conditions: column, Develosil ODS-10; eluent, H_2O (90 min) MeOH–H_2O (80:20); flow rate, 5 mL/min; detector, UV 280 nm.

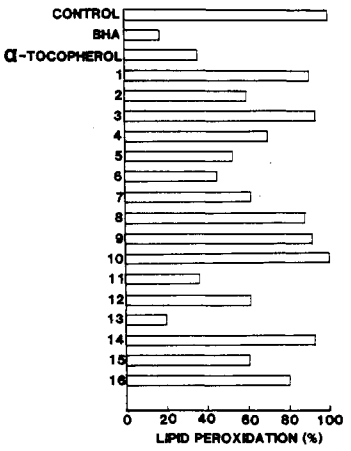

Figure 2. Antioxidative activities of HPLC-separated subfractions of Katakutara rice hull MeOH–H_2O (50:50) fraction (thiocyanate method).

from this figure that the major fraction, fraction 13 (retention time 70.9 min), possessed strong antioxidative activity, stronger than that of α-tocopherol and BHA, while the activity of fraction 11 (retention time 67.0 min) was only as strong as that of α-tocopherol. Fraction 4 (retention time 53.3 min), which was also present as a major component, however, showed only weak antioxidative activity.

Characterization of the Active Subfractions 11 and 13. The active subfractions 11 and 13 were further purified on HPLC by a reversed phase Develosil ODS-10 (20 μm × 250 cm) column and MeOH–H_2O (45:55) and MeOH–H_2O (50:50) as the respective eluents. Flow rates in both the cases were maintained at 6 mL/min. Samples were repeatedly injected, and fractions were collected with a UV detector at 280 nm as the monitor. The HPLC chromatogram during purification of subfraction 11 is shown in Figure 3. It was observed that this fraction was a mixture of four fractions, with peak 11-(iii) being the major component.

Antioxidative activity of the HPLC-separated components of subfraction 11 is given in Figure 4. It was observed that the antioxidative activity decreased slightly after separation. However, the activity of component 11-(iii) was still found to be as strong as that of α-tocopherol. The minor components 11-(i), 11-(ii), and 11-(iv) showed relatively weaker antioxidative activities.

The HPLC chromatogram on further separation of subfraction 13 is shown in Figure 5. It was found that this fraction could be separated into five smaller components, with peak 13-(iv) appearing to be the main component.

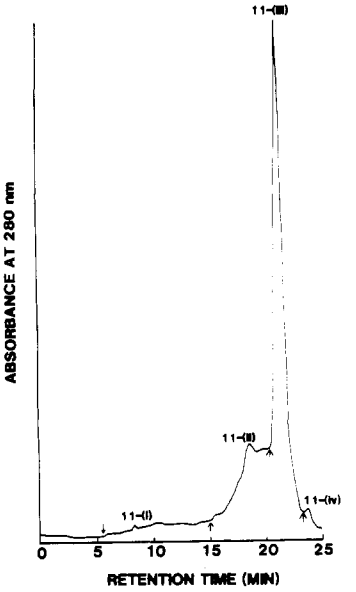

Figure 3. HPLC chromatogram of subfraction 11 of the MeOH–H_2O (50:50) fraction of Katakutara rice hull sample. Conditions: column, Develosil ODS-10; eluent, MeOH–H_2O (45:55); flow rate, 6 mL/min; detector, UV 280 nm.

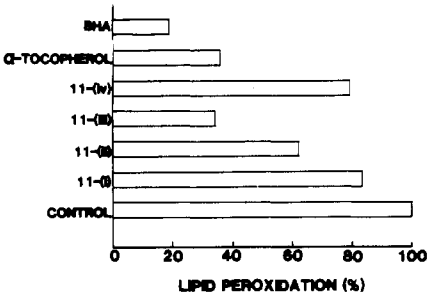

Figure 4. Antioxidative activities of separated constituents of subfraction 11 (thiocyanate method).

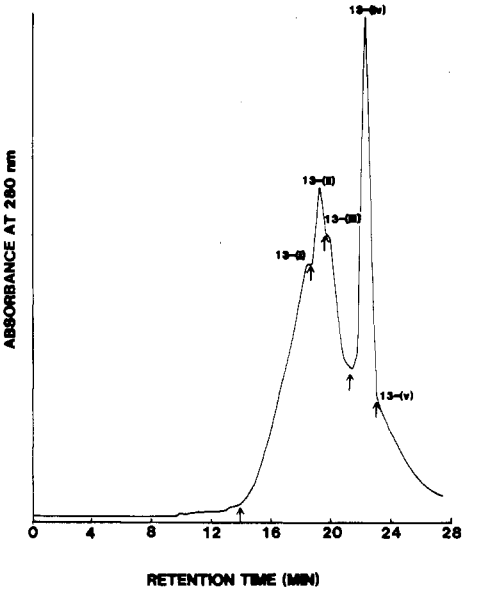

Figure 5. HPLC chromatogram of subfraction 13 of the MeOH–H_2O (50:50) fraction of Katakutara rice hull sample. Conditions: column, Develosil ODS-10; eluent, MeOH–H_2O (50:50); flow rate, 6 mL/min; detector, UV 280 nm.

The result of the antioxidative activity assay of the HPLC-separated components of subfraction 13, determined by thiocyanate method, is shown in Figure 6. It was observed that the peaks 13-(ii), 13-(iii), and 13-(iv)

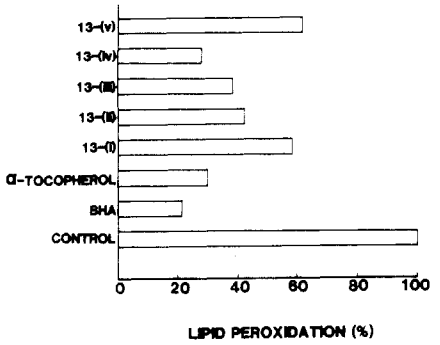

Figure 6. Antioxidative activities of separated constituents of subfraction 13 (thiocyanate method).

Table I. Assignment of Chemical Shifts in the ^{13}C NMR Spectrum of Component 11-(iii)

δ	assgnt	δ	assgnt	δ	assgnt
183.9	C-4	123.0	C-1′	82.5	C-5″
165.9	C-2	116.9 (d)	C-3′, C-5′	80.1	C-3″
164.6	C-7	109.1	C-6	75.3 (d)	C-1″
162.5	C-4′	105.1	C-10	72.7	C-2″
161.5	C-5	103.8 (d)	C-3	71.8	C-4″
158.4	C-9	95.3 (d)	C-8	62.9	C-6″
129.3 (d)	C-2′, C-6				

exhibited strong antioxidative activity. However, the antioxidative activity of the separated constituents was found to become considerably weaker after separation into minor component fractions.

Structural Analysis of 11-(iii). UV absorption spectra of HPLC-purified component 11-(iii), before and after addition of shift reagents, indicated that the spectra were characteristic of flavonoid components showing bathochromic shifts with the reagents due to the presence of phenolic OH groups in their A and B rings. The spectral λ_{max} values in MeOH are 271 and 335; +NaOMe 278, 329, and 398; +$AlCl_3$/MeOH 262 (sh), 278, 304, 349, and 382; +$AlCl_3$/HCl 260 (sh), 280, 302, 344, and 380 nm; +NaOAc 279, 303, and 385; and +NaOAc/H_3BO_3 274, 346, and 408 (sh). The FAB-MS of component 11-(iii) gave an (M + 1) ion peak at 433, and hence a molecular ion peak (M^+) of 432 was suggested. On the basis of mass spectral data and the characteristic nature of the UV spectrum, component 11-(iii) was thought to represent a flavonoid glycoside.

The ^1H NMR spectrum of component 11-(iii), in MeOH-d_4, showed that the aromatic protons of the B ring appeared at δ 6.95 (H-3′ and H-5′, d, J = 9 Hz) and 7.78 (H-2′ and H-6′, d, J = 9 Hz), while those of A ring appeared at δ 6.49 (H-3, s) and 6.56 (H-8, s), respectively. The glycosyl H-1″ protons appeared at δ 4.21 while the remaining glucosyl protons appeared in the range of δ 3.78–3.93. C–C glycosidic linkage was recommended on the basis of appearance of H-1″ proton of the glucose moiety at δ 4.21 as in the case of O-glycosidic linkage the same proton should have appeared at a lower region of δ 5.1.

^{13}C NMR of component 11-(iii), in MeOH-d_4, gave the signals for 21 carbon atoms, and on the basis of FAB-MS and ^{13}C NMR spectral data, a molecular formula of $C_{21}H_{20}O_{10}$ was suggested, it representing a flavonoid glycoside, the glycosidic linkage being a C–C linkage. The chemical shifts of all the 21 carbon atoms were interpreted by comparing the standard values of a known flavonoid C-glycoside, vitexin (Mabry et al., 1982). The assignments are shown in Table I.

On the basis of the UV spectral data, FAB-MS, and ^1H NMR and ^{13}C NMR spectral data, component 11-(iii) was identified as isovitexin (Figure 7), which has been reported to be present widely in plants along with its isomer vitexin (Briggs and Cambie, 1958; Horowitz and Gentili, 1964). Instrumental analysis of 13-(iv) was carried out with FAB-MS and ^1H NMR. However, the data obtained did not give any conclusive information as the sample still seemed to contain minor components that need to be eliminated before further structural characterization could be carried out. As the yield of component 13-(iv) at this stage is very low (2.2 mg/500 g of hull), the chemical characterization remains incomplete as yet. However, preliminary UV data indicated this component to be of flavonoid type. Further screening and characterization of other active antioxidant(s) in addition to 13-(iv) in Katakutara rice hull and similar cultivars having greater longevity are in progress.

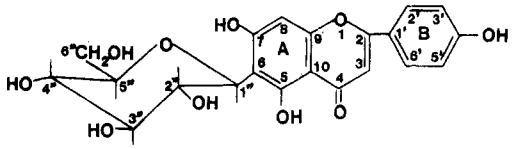

Figure 7. Structure of subfraction 11-(iii) identified by instrumental analyses as isovitexin.

CONCLUSION

Flavonoid substances have been reported to be widely distributed in plants. The function of flavonoids in plants seems to vary extensively, though mainly as attractants of agents for pollination (Harborne, 1972). They are also regarded as regulators of growth, the activity being attributed to interaction of flavonoids with IAA-oxidase (Thimann, 1969). The metal-chelating capability together with their radical scavenging property has enabled flavonoids to be accounted as natural plant antioxidants (Simpson and Uri, 1956; Mehta and Seshadri, 1959; Letan, 1966; Bors and Saran, 1987). As the flavonoid substances are found to be accumulating in the rice hull during the ripening phase of the grain development (20 days after flowering), it may well be assumed that the main function of flavonoids in rice hull is to provide chemical defense for the fully developed edible rice caryopsis constituting the rice germ, from oxidative deterioration during postharvest storage. Besides this, what specific functions they have and to what extent they differ in their functions in the main ecogeographical races, *indica* and *japonica*, are still subjects for further research. Isovitexin, the novel C-glycosyl flavonoid identified in Katakutara rice hull, is found to be absent in the short-life rice seeds. The demonstration of the presence of flavonoid components in the rice hull and their antioxidant property in this report not only is a totally new contribution to the studies on rice chemistry and physiology but also will certainly lead to new avenues for investigating the disease-resistance properties of *indica* and *japonica* rice seeds and serve as a marker for predicting the longevity of the newly developed rice seeds. In this context, it will be worth exploring into identification of the other minor flavonoid components of the rice hull that have also demonstrated antioxidant property.

Registry No. Isovitexin, 38953-85-4.

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Thermally Induced Gelation of Succinylated Canola Protein Isolate

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Thermally induced gelation of unmodified and succinylated canola protein isolate (54% and 84% modification of free amino groups) was examined over a wide range of sodium chloride concentrations (0.0–0.7 M) and pH (3.5–11.0). Protein dispersions were heated at 72 °C for 30 min to simulate cooking conditions for a comminuted meat product. Succinylation improved gelation. For unmodified isolate, gels formed at only 4 of 18 combinations of pH and NaCl, while 12 gels formed from each level of succinylation under the same conditions. Gels from unmodified isolate formed only at high pH (≥9.5) whereas those from succinylated isolate formed from pH 5.0 to 11.0. Above pH 6.5, succinylated protein formed gels only in the presence of NaCl. In general, the firmest gels were obtained with moderate succinylation. All gels from unmodified isolate and those at pH 5.0 from succinylated isolate formed opaque gels due to the presence of insoluble particulates; all others were translucent. Translucent and opaque gels responded differently to rheological tests and were related in different ways to physicochemical and rheological properties of protein dispersions. Bonds involved in gel formation and stability were tentatively identified as hydrophobic interactions and hydrogen bonds.

Food gels consist of a continuous phase of interconnected particles and/or macromolecules intermingled with a continuous liquid phase such as water (Powrie and Tung, 1976). Gelling agents are generally present at levels of 10% or less and form a three-dimensional matrix such that the system behaves as a soft solid yet retains many properties characteristic of the fluid component and are thus termed "viscoelastic". As a rule, to obtain gels from globular proteins requires protein concentrations 1 order of magnitude higher than is required for gelation of polysaccharide or gelatin dispersions.

The mechanisms of gelation of globular proteins are not yet completely understood. The most generally accepted hypothesis was proposed by Ferry (1948) who suggested a two-step mechanism beginning with an initiation step involving unfolding or dissociation of the protein molecules, followed by aggregation and association and, under appropriate thermodynamic conditions, formation of a gel. Hermansson (1978, 1979a,b) described a globular protein gel as a state intermediate between a protein sol and precipitate, where a gel may form if a proper balance between protein–protein and protein–solvent interactions is achieved. Tombs (1970, 1974) suggested that gels are formed from globular proteins as a result of aggregation of protein molecules into strands followed by interaction of the strands to form a gel network. Bonds in protein gels vary quantitatively and qualitatively with type of protein and gelation environment and may include hydrophobic interactions, ionic attractions, hydrogen bonds, or disulfide linkages.

Although much work has been done on examination of gelation behavior of soy protein, little has been reported on gelation of canola protein. Sosulski et al. (1976) reported that rapeseed flours, concentrates, and isolate had poor gelation properties. Similarly, Thompson et al. (1982) also reported poor gelation of rapeseed protein concentrate. In contrast, Gill and Tung (1976, 1978) examined gelation behavior of the 12S glycoprotein fraction of rapeseed by both rheological and microscopical techniques and reported gelation at protein concentrations as low as 4.5%, with measurable thickening at 1% protein. Although gelation mechanism and the bonds involved in gel formation and stability were not fully elucidated, the authors concluded

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