Esterase Activity and Free Fatty Acid Accumulation in the Bran of Selected Rice Cultivars

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ABSTRACT

Cereal Chem. 71(2):162–165

To increase our understanding of lipid degradation in rice bran, the esterase activities of crude enzyme solutions extracted from rice bran were compared for 10 rice cultivars. The activities of rice cultivars Kinuhikari, Saikai191, and Hishiyutaka were higher than those of other rice cultivars we examined. Changes in the free fatty acid content of the polished bran from four rice cultivars were studied using by fluorometric high-performance liquid chromatography using 9-bromomethylacridine. Free fatty acids in the polished bran of Saikai191 and Hoshiyutaka accumulated more rapidly than the those of Tomochi396 and Koshihikari, which supports the observation that the esterase activity in polished bran is cultivar-dependent. Furthermore, the species of free fatty acids that accumulated in the polished rice bran differed depending upon the storage conditions. This suggests that the storage temperature and relative humidity affected the activities of several enzymes that participate in the lipid hydrolysis.

The accumulation of free fatty acids (FFA) in rice seed and the associated rancidity is a problem during storage. Rapid degradation of the polished rice bran oil into FFA through lipolytic hydrolysis prevents rice bran from becoming a major source of edible oil. Several kinds of lipases are involved in lipid degradation. The biochemical aspects of purifying lipases in rice seed have been studied by many investigators (Funatsu et al 1971; Aizono et al 1973, 1976; Heltved 1984; Saunders and Heltved 1985; Galliard 1986a; Takano et al 1987a,b). Information from those studies is important in understanding the physiological action of lipases during seed storage and their effective utilization in the food industry. The storage conditions of rice seed have also been discussed (Matsuda and Hirayama 1973, Hirayama and Matsuda 1975, Lin et al 1982, Huang et al 1988, (Chrastil 1990). Methods to depress lipase activity have also been developed (Heltved 1984; Saunders and Heltved 1985; Galliard 1986a,b). However, information on the comparison of lipolytic hydrolysis among different rice cultivars is limited.

The FFA content in cereals has been measured by titration methods, but those methods lack sensitivity; detecting any change requires large samples and long storage times. Although gas chromatography and O_2 electrodes are highly sensitive methods for determining FFA content, they require special apparatus. Recently, several fluorescent-labeling reagents for fatty acids have been developed. 9-Bromomethylacridine (9-BMA), one of the fluorescent-labeling reagents of carboxyl groups, has been used to determine the fatty acid composition of buckwheat seed lipids and butter (Akasaka et al 1987, Tsuzuki et al 1991). The 9-BMA method has a high sensitivity for FFA.

In this report, crude enzyme solution with measurable esterase activity was extracted from the bran of 10 rice cultivars, and their activities were compared. Four cultivars that had notable differences in esterase activities were stored under various conditions and tested by fluorometric high-performance liquid chromatography (HPLC) to detect FFA in the bran. The FFA accumulation in the four rice cultivars we studied indicated that the lipid degradation by lipolytic hydrolysis is characteristic of rice cultivars.

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MATERIALS AND METHODS

Materials and Reagents

The 10 samples of unhulled rice grain used in this experiment were harvested in autumn of 1990 in Japan. (Samples harvested in 1991 were also used as specified.) As soon as they were harvested, the grains were threshed and sent to our laboratory. They were stored at 4°C and 60% rh until used. Ammonium sulfate and calcium chloride ($CaCl_2$) for the extraction of crude enzymes were obtained from Wako Pure Chemical Co. (Osaka, Japan). Analytical grade chloroform and methanol used for the FFA extraction were also purchased from Wako Pure Chemical Co. 4-Methylumbelliferyl oleate, used as a substrate of the esterase assay, was obtained from Sigma Chemical Co. (St. Louis, MO). All solvents for HPLC were of analytical or superpure grade (Wako Pure Chemical Co. and Kanto Chemicals, Tokyo, Japan) and used without further purification. Lauric acid, a standard for the determining FFA content, and standard fatty acids for HPLC analysis were obtained from Sigma Chemical Co. 9-BMA and tetraethylammonium carbonate, a catalyst for the labeling reaction, were prepared according to a previously published method (Akasaka et al 1987).

Preparation of Crude Enzyme

The crude enzyme solution was prepared according to the method of Funatsu et al (1971). Rice bran was polished from 50 g of unhulled rice seed with a commercial polisher (Toshiba, Tokyo, Japan) and defatted five times with petroleum ether; it was then suspended in 100 ml of 10m*M* $CaCl_2$. The suspension was stirred vigorously for 3 hr and centrifuged after filtration through paper. Solid ammonium sulfate was added to the supernatant to 60% saturation. The resulting precipitate was separated by centrifugation and then suspended in 10 ml of 1m*M* $CaCl_2$. The suspension was dialyzed against the 1m*M* $CaCl_2$ at 4°C for two days, followed by dialysis against deionized water for two days. After centrifugation to remove insoluble materials, the supernatant was lyophilized overnight. The resulting powder was stored at 4°C until use.

Determination of Esterase Activity

Esterase activities were measured using the synthetic fluorescent substrate 4-methylumbelliferyl oleate (Shimura et al 1991). The enzyme powder (1.25 mg) was suspended in 1 ml of Tris-HCl buffer (50m*M*, pH 7.5). After centrifugation, the supernatant was designated as *crude enzyme solution*. The protein content was determined using a protein assay kit (Bio-Rad Laboratories). For esterase assay, a 100 μl aliquot of 10m*M* 4-methylumbelliferyl oleate dissolved in 50m*M*, Tris-HCl (pH 7.5), and 200 μl of the

crude enzyme solution were incubated at 37°C for 20 min. A 1-ml portion of 0.1*N* HCl was added to the reaction mixture to terminate the enzyme reaction. After the reaction mixture was adjusted to pH 4.3 by the addition of 2 ml of 0.1*M* sodium citrate, the amount of 4-methylumbelliferone released by the enzymes was measured fluorometrically at 450 nm with an excitation at 320 nm. Using a standard solution, changes in the fluorescent intensity induced during the assay were converted into picomoles of 4-methylumbelliferone generated per minute.

FFA Extraction and Fluorometric-HPLC Determination

After rice seeds were polished, about 10% was removed as rice bran. FFA was extracted from about 150 mg of ground rice bran with 10 ml of chloroform and methanol (2:1, v/v), containing a known amount of lauric acid as a standard for calibration of FFA content. The combined extraction solvent was added to 6 ml of 0.8% $CaCl_2$ solution and stirred vigorously. After centrifugation, the aqueous phase ($CaCl_2$ solution and methanol) was removed. An aliquot of 0.5 ml of chloroform was transferred to the test tube. The solvent was completely evaporated under a nitrogen gas stream. The residue was dissolved in a mixture of 0.1 ml of 10m*M* 9-BMA-dimethylformamide and 0.1 ml of 5m*M* tetraethylammonium carbonate-dimethylformamide. The labeling reaction was performed at room temperature for 2 hr. A portion of the sample (20 μl) containing 1.1 mg of rice bran fraction was injected during HPLC. The fluorescent-labeled fatty acids were analyzed according to the method reported by Tsuzuki et al (1991).

RESULTS

Esterase Activity of Rice Bran

The protein content and esterase activities of the crude enzyme solutions extracted from 10 rice cultivars are summarized in Table I, which shows differences in the esterase activities depending on the cultivar. Among the 10 rice cultivars examined, Kinuhikari, Saikai191, and Hoshiyutaka exhibited the highest esterase activity; Tomochi396, Habataki, and Hokuriku147 had the lowest. The total esterase activity of Saikai191 was about three times higher than that of Tomochi396. To see whether the differences in esterase activity were really cultivar characteristics, Saikai191 and Tomochi396 samples harvested in 1991 were also tested (Table I). The total activities of both cultivars were in good accordance with the samples harvested in 1990, suggesting that the difference in the esterase activities of rice bran might be attributable to the rice cultivar.

TABLE I
Protein Content and Esterase Activities of Crude Enzyme Solution Extracted from Bran of 10 Rice Cultivars

Rice Cultivar	Protein Content[a] (mg)	Specific Activity[b] (μmol)	Total Activity (mmol)
Oochikara	21.3	306	6.52
Kinuhikari	23.4	431	10.08
Koshihikari	20.4	318	6.49
Saikai191	31.7	458	14.52
Saikai191(1991)	30.4	438	13.32
Chugokumochi124	27.2	307	8.36
Tomochi396	18.8	230	4.33
Tomochi396(1991)	23.2	222	5.15
Touhokumochi149	25.8	243	6.27
Habataki	24.0	217	5.21
Hokuriku147	20.0	242	4.84
Hoshiyutaka	31.1	343	10.74

[a] Protein content of crude enzyme powder extracted from 50 g of unhulled rice seed.
[b] Expressed as μmol of the amount of 4-methylumbelliferone released per milligram of protein per minute.

Determination of FFA by HPLC

The HPLC separation pattern of the typical FFA derivatives extracted from the bran of unhulled seed is shown in Figure 1. Because rice bran lipids contain an extremely small amount of lauric acid (0.15%), it was used as a standard for determining the rice bran FFA. Each peak of FFA from rice bran could be identified by comparison with the peaks of standard fatty acids. Under the present experimental conditions, the retention times

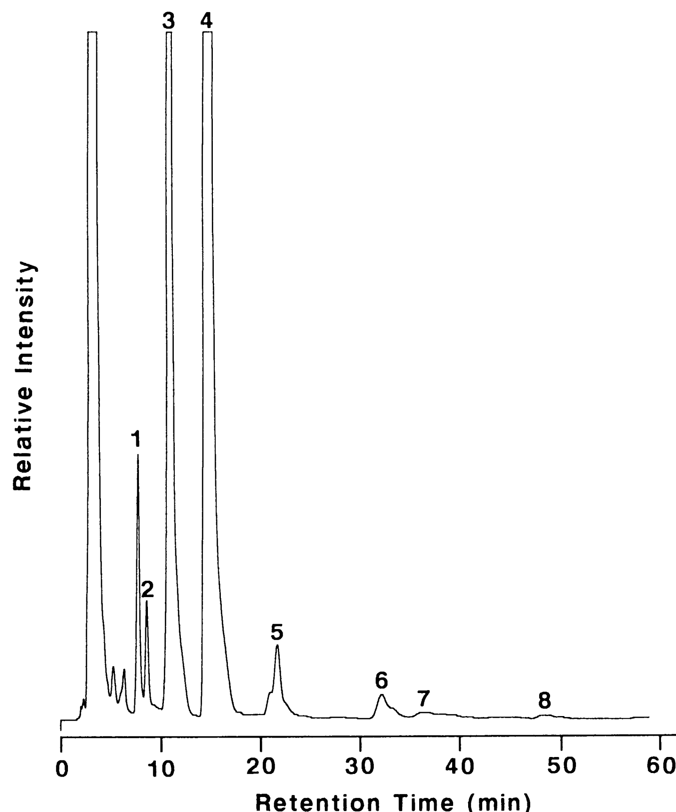

Fig. 1. High-performance liquid chromatogram of the 9-bromomethylacridine derivatives of fatty acids in the bran of unhulled seed of the rice cultivar Hoshiyutaka. 1 = Lauric acid for standard, 2 = C18:3, 3 = C14:0 + C16:1 + C18:2, 4 = C16:0 + C18:1, 5 = C18:0 + C20:1, 6 = C20:0, 7 = C22:0, 8 = C24:0.

TABLE II
Free Fatty Acid Composition (nmol)[a]

Rice Cultivar	C18:3	C14:0 C16:1 C18:2	C16:0 C18:1	C20:1	C20:0	C22:0	C24:0	Total
Tomochi396	0.21	4.29	6.33	0.31	0.11	0.01	0.02	11.28
	(1.9)	(38.0)	(56.1)	(2.8)	(1.0)	(0.0)	(0.0)	(100)
Koshihikari	0.41	9.42	13.16	0.55	0.25	0.04	0.07	23.90
	(1.7)	(39.4)	(55.1)	(2.3)	(1.0)	(0.0)	(0.0)	(100)
Hoshiyutaka	0.17	4.05	5.98	0.22	0.12	0.04	0.07	10.65
	(1.6)	(38.0)	(56.2)	(2.1)	(1.1)	(0.0)	(0.1)	(100)
Saikai191	0.17	3.76	5.46	0.22	0.10	0.05	0.07	9.83
	(1.7)	(38.3)	(55.5)	(2.2)	(1.0)	(0.1)	(0.1)	(100)

[a] For 1.1 mg of bran from unhulled seed. Percentages are given in parentheses. Average of three replicates.

TABLE III
Change in Free Fatty Acid Content[a] in Polished Rice Bran Stored for Seven Days

Rice Cultivar	Storage Conditions °C	Storage Conditions % rh	C18:3	C14:0 C16:1 C18:2	C16:0 C18:1	C18:0 C20:1	C20:0	C22:0	C24:0	Total
Tomochi396	4	60	97	98	109	123	108	106	102	105
	4	80	157	120	117	136	128	113	110	122
	37	80	171	136	154	133	143	113	130	147
Koshihikari	4	60	98	100	111	122	111	128	120	107
	4	80	171	122	138	144	138	125	129	133
	37	80	184	131	148	143	148	150	157	143
Hoshiyutaka	4	60	128	117	118	131	124	125	129	118
	4	80	120	135	141	121	115	120	129	138
	37	80	163	152	166	157	131	120	143	160
Saikai191	4	60	118	115	120	150	120	114	111	116
	4	80	167	138	151	163	120	125	133	146
	37	80	175	152	165	147	120	150	156	159

[a] FFA and total FFA content expressed as a percentage of those in unpolished rice bran before storage (100%). Average of three replicates; standard deviation is between 2 and 5.

of three fatty acid derivatives (C14:0, C16:1, and C18:2) were similar, and their peaks could not be separated. The derivatives of two fatty acids (C16:0 and C18:1) were eluted at the almost same time, as were those of C18:0 and C20:1. As the rice bran oil contains very little (<1%) C14:0, C16:1, and C20:1 fatty acids (Nasirullah et al 1989), the FFA released from the bran oil would consist of a very small amount of the corresponding FFA.

FFA Content in the Bran of Unhulled Rice Seed

The FFA content in the bran of four cultivars of unhulled rice seed were determined by the fluorometric-HPLC method. The values of FFA (nmole) included in 1.1 mg of the bran are shown in Table II. Total FFA content in rice bran varied among rice cultivars, but it was not clear whether these variations resulted from cultivar characteristics or from the handling after harvest. However, no differences could be detected in the FFA composition among the four rice cultivars. More than 80% of the FFA content consisted of the two main peaks corresponding to the FFA mixture of C14:0 + C16:1 + C18:2, and C16:0 + C18:1. The FFA composition in the rice bran was in good agreement with the fatty acid composition of the rice bran oil reported previously by Nasirullah et al (1989).

Changes in the FFA content in the bran of unhulled rice seed were investigated using four rice cultivars stored at 4°C and 80% rh for 42–56 days. As shown in Figure 2, no increase in FFA content could be detected under these conditions, although there are notable cultivar differences in FFA content.

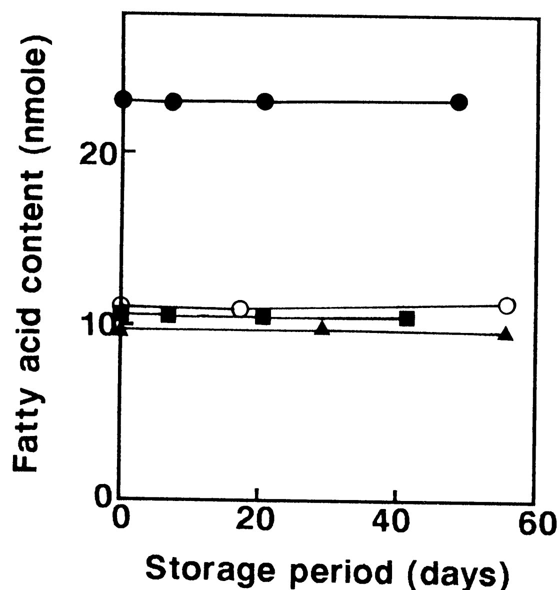

Fig. 2. Change in free fatty acid content of 1.1 mg of the bran of unhulled rice seed during storage at 4°C, 80% rh. ● = Koshihikari, ○ = Tomochi396, ■ = Hoshiyutaka, ▲ = Saikai191.

Accumulation of FFA in Polished Rice Bran

Change in the FFA in the polished rice bran was compared among four cultivars. The rice seeds were polished to separate the white-polished rice. The rice bran powder fraction was stored seven days at 4°C, 60% rh; 4°C, 80% rh; or 37°C, 80% rh.

At 4°C and 60% rh, FFA increased about 1.05–1.18 fold, most of which was in C18:0 + C20:1. In storage at 4°C and 80% rh, total FFA content increased by 1.22–1.46 fold, with the largest increase in C18:3 and C16:0 + C18:1. The FFA increase in polished rice bran was much larger than that in the bran of unhulled seed stored under the same conditions (Table III). Thus, our results confirmed the findings of Saunders and Heltved (1985).

When the storage temperature was set at 37°C, total FFA content in the polished rice bran increased about 50% over that of the starting materials. Under these conditions, C18:3, C16:0 + C18:1, and C18:0 + C20:1 accumulated more rapidly than did the other components (Table III).

DISCUSSION

This report is the first to show that there are differences in the esterase activities of rice cultivars. The diversity of the esterase activity was supported by the study on the accumulation of FFA in the rice bran. Cultivars that were high in esterase activity had higher rates of lipid degradation. The results also suggest that the activities of lipolytic hydrolysis in the polished rice bran may be cultivar-dependent. These findings may be important not only for quality control in rice bran during food processing, but also for the studies on esterase and lipase in rice bran.

The report that polished rice bran lipid rapidly breaks down into FFA through lipolytic hydrolysis (Saunders and Heltved 1985) was confirmed in our study. Rapid degradation of polished rice bran may be attributed to the easier availability of water, a substrate of the hydrolysis reaction. It could also be attributed to the easier interaction between the lipolytic enzymes and the substrate in the homogenized powder sample.

Most of the studies on FFA in rice bran have not included the FFA composition. The fluorometric-HPLC method used in this work could determine the content of 12 FFA species simultaneously in one analysis. This procedure can provide much information on the lipolytic properties of rice bran. The measurement of FFA content using the fluorometric-HPLC method with 9-BMA is more sensitive and uses smaller samples than do other current methods.

Comparison of the changes of FFA in polished rice bran stored under various conditions demonstrated that the accumulated FFA species differed depending on the storage conditions. Thus, different storage conditions determine which lipid classes would be hydrolyzed and which lipases might be active. A previous study

on lipases purified from rice bran demonstrated that lipase with high activity in rice bran stored at low temperature was not the same as lipase activated during germination (Hirayama and Matsuda 1975).

ACKNOWLEDGMENTS

We thank H. Meguro of Tohoku University for his helpful suggestions. Parts of this investigation were performed under the Super Rice project of the Ministry of Agriculture, Forestry, and Fisheries.

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[Received November 12, 1992. Accepted October 21, 1993.]