Note

Partial Purification and Study of Some Properties of Rice Germ Lipoxxygenase

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Development of stale flavor in rice grain is one of unfavorable changes occurring during storage, and thus is barring institution of an increasing plan for the national stock holdings. Hexanal is generally accepted as one of the major components responsible for objectional off-flavors developing in many plant foods. In long-term-stored rice kernels, hexanal is also an important flavor contributor, arising presumably from decomposition of hydroperoxides of unsaturated fatty acids¹⁾. Lipoxxygenase (EC 1.13.11.12) is implicated with heme proteins in the hydroperoxide production in many plant tissues. Yet, presence of lipoxxygenase in rice grain has not been established unequivocally, though Shastry *et al.* attributed the hydroperoxidizing activity present in rice bran to a lipoxxygenase reaction.²⁾ The present investigation was undertaken to give more detailed characterization of this activity so that we can understand more precisely the mechanism by which the stale flavor components arise in stored rice. In this paper, we describe the partial purification and characterization of lipoxxygenase from rice germ.

Lipoxxygenase activity was determined polarographically and spectrophotometrically as described previously.³⁾ The standard assay conditions were, unless otherwise stated, 0.01 M Na-linoleate, 0.1 M phosphate buffer (pH 7.0) and 240 μ M O₂ (in equilibrium with air at 25°C). The enzyme activity was expressed in katal.⁴⁾

A relatively high lipoxxygenase activity was detected in the unfractionated fresh rice bran which was successively removed from brown rice of different *japonica* varieties in a laboratory test mill (an abrasive type). The unfractionated bran, corresponding to the out-

side layers of brown rice, is composed of the pericarp, the seed coat, most of aleurone layer, broken kernels, and the whole of the germ removed together with these bran components. However, the enzyme activity was found not distributing uniformly among the bran components but localized mostly, if not entirely, in the germ fraction (Table I). This localization, hitherto not fully realized, conduced to the purification of lipoxxygenase.

TABLE I. LIPOXYGENASE ACTIVITY IN UNFRACTIONATED RICE BRAN

	Total activity (μ kat/kg material)	Specific activity (μ kat/kg protein)
Unfractionated rice bran	5.80	71.5
Bran fraction	4.22	62.5
Germ fraction	12.0	160

Unfractionated rice bran from a *japonica* variety (Nipponbare, 1976) was passed through 20 and 32 mesh sieves to separate into the bran and germ fractions. The two fractions were extracted with 4 volume of acetate buffer (pH 4.5) and the resultant extracts were assayed for the enzyme activity under the standard assay conditions.

The enzyme purification procedure involved extraction of powdered rice germ with 0.1 M acetate buffer (pH 4.5), and fractionation of the protein with ammonium sulfate precipitation, heat treatment, and column chromatography on a DEAE-Sephadex column. Table II summarizes the results of a typical purification. Disc electrophoresis on 7% polyacrylamide gel⁵⁾ of the peak fraction obtained in the gel filtration on a Sephadex G-100 column gave one major protein band bearing lipoxxygenase activity and two trace protein bands both being negative in activity staining reaction of Guss *et al.*⁶⁾ The result indicates homogeneity of the final preparation with respect to lipoxxygenase. The molecular weight of the enzyme estimated by the gel filtration method was 95,000.

Properties of the isolated rice germ enzyme were typical of lipoxxygenase, standing comparison with those of soybean lipoxxygenase-a.^{8,7,9)} Initial velocity of the reaction was proportional to the enzyme concentration in both polarographic and spectrophotometric assays. This linear relationship is characteristics of the lipoxxygenase-catalyzed reaction and distinct from those nonlinear relationships usually observed for the reactions catalyzed nonspecifically by many heme proteins. The rice germ enzyme reaction was highly specific for linoleic and linolenic acids, with a *K_m* value of 0.10 mM for Na-linoleate. Trilinolein and methyl linoleate were the less effective substrate and oleic acid was inactive. The reaction was optimal

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TABLE II. SUMMARY OF THE PURIFICATION PROCEDURE FOR RICE GERM LIPOXYGENASE

Fraction	Total protein (g)	Total activity (μ kat)	Recovery of activity (%)	Specific activity (mkat/kg)	Purification (fold)
Crude extract	255	73.2	100	0.287	1.00
(NH ₄) ₂ SO ₄ , 30~70% saturation	133	100	137	0.753	2.62
Heat treatment, 63°C, 5 min	43.8	63.1	86.1	1.44	5.02
(NH ₄) ₂ SO ₄ , 0~70% saturation	11.2	46.6	63.7	4.16	14.5
DEAE-Sephadex:					
Pooled fractions	0.365	27.2	37.1	74.5	260
Peak fraction	0.0176	2.45	—	139	484

Rice germ from a *japonica* brown rice (Gohyakumangoku, 1976) was ground in a Centri-mill and 5 kg of the resultant powder was homogenized with 25 l of 0.1 M acetate buffer (pH 4.5). Purification was compared in relative to the crude extract.

FIG. 1. pH-Activity Profile for Isolated Rice Germ Lipoygenase.

The enzyme activity was determined under the standard assay conditions except that pH of the reaction medium was varied using the following buffers: acetate (■—■) for pH 2.0 to 6.0, potassium phosphate (○—○) for pH 6.0 to 8.0, and borate (▲—▲) for pH 8.0 to 9.0.

at around pH 6.5 to 7.0 as shown in Fig. 1. The enzyme proved stable at temperature up to 60°C and could be stored over a month in a refrigerator without any significant loss of activity.

The rice germ enzyme lost more than 60% of the original activity on incubation with 0.1 mM H₂O₂, which was discovered previously as a specific inactivator of lipoygenase.⁶⁾ This finding provides an unequivocal evidence supporting our identification that the isolated enzyme is a member of true lipoygenase. Among those divalent cations which affected

little the enzyme activity at a concentration up to 10 mM were included Mg²⁺, Sr²⁺, Ba²⁺, Mn²⁺, Ni²⁺, Cu²⁺, and Cd²⁺. Calcium ion also neither activated nor inhibited the enzyme in the crude extracts and in a highly purified preparation both at pH 7 and 9. This immunity to added Ca²⁺ is not parallel with the susceptibility of soybean enzymes.⁷⁾ The rice germ enzyme activity was not modified to any detectable extent by incubation at 25°C for 50 hr with 10 mM NaN₃, NaCN, and NaF respectively.

The rice germ enzyme was inhibited about 40% by 10 mM EDTA, while not by other chelators such as 8-hydroxyquinoline, tiron, 2,2'-dipyridyl, *o*-phenanthroline, dithizone and pyrophosphate. The specific action of added EDTA remains to be accounted for precisely.

Some of the experimental results we obtained for the rice germ enzyme are apparently inconsistent with those reported by Shastry *et al.*,²⁾ who showed that partially purified lipoygenase from unfractionated rice bran of an *indica* variety is optimal at around pH 8.5, inhibited by Ca²⁺ and activated by both Fe²⁺ and EDTA. Although further work is required to explain fully those discrepancies, possible explanation might involve variety and tissue variations of rice lipoygenase: *japonica* versus *indica* varieties and germ versus bran portions.

A preliminary study on the positional specificity of the reaction product indicated that the rice germ enzyme gave predominantly 9-hydroperoxide from linoleic acid in contrast to soybean lipoygenase-a which yielded exclusively the 13-hydroperoxide. The physiological implication of rice germ enzyme will be discussed elsewhere pursuant to its positional- and stereo-specificity.

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REFERENCES

- 1) K. Yasumatsu, S. Moritaka and S. Wada, *Agric. Biol. Chem.*, **30**, 483 (1964).
 - 2) B. S. Shastry and M. R. Raghavendra Rao, *Cereal Chem.*, **52**, 597 (1975).
 - 3) H. Mitsuda, K. Yasumoto, A. Yamamoto and T. Kusano, *Agric. Biol. Chem.*, **31**, 115 (1967).
 - 4) IUPAC-IUB Commission on Biochemical Nomenclature. Enzyme Nomenclature Recommendations (1972).
 - 5) L. Ornstein and B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 321 (1964).
 - 6) P. L. Guss, T. Richardson and M. A. Stahmann, *Cereal Chem.*, **44**, 607 (1967).
 - 7) A. Yamamoto, K. Yasumoto and H. Mitsuda, *Agric. Biol. Chem.*, **34**, 1169 (1970).
 - 8) H. Mitsuda, K. Yasumoto, and A. Yamamoto, *Agric. Biol. Chem.*, **31**, 853 (1967).
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