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STUDIES ON LIPOXYGENASE FROM RICE BRAN

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ABSTRACT

Unfractionated rice bran extract showed on disc-gel electrophoresis three distinct bands with lipoxygenase activity. The major fraction was partially purified by repeated ammonium sulfate fractionation at pH 6.8. The partially purified lipoxygenase was optimally active at pH 8.5 and had a *Km* of 0.35 m*M* using linoleate as a substrate. It was stable for 15 days at 3° to 5° C. The enzyme activity was not affected by α,α'-dipyridyl and EDTA but partially by 1,10-phenanthroline. Ferrous and calcium ions were activators but copper ions were inhibitory. The enzyme may require tryptophan residues for activity.

Lipoxygenase (linoleate:oxygen oxidoreductase E.C. 1.13.1.13) is found in a wide variety of plants, particularly the legumes (1). It is present in alfalfa (2), groundnut (3), soya beans (4), peas (5,6), potato (7), and wheat (8). Lipoxygenase from soya beans (9–13) has been well characterized and crystallized. Under appropriate conditions, this enzyme leads to the deterioration of fat-soluble vitamins and of essential fatty acids of oils and fats. It also produces off-flavors and odors because of its action on unsaturated fatty acids in the lipids of food materials. Since little is known about this enzyme in rice bran (which is a good source for edible grade oil), work on this aspect was undertaken. This paper discusses the partial purification and some properties of the major lipoxygenase fraction.

MATERIALS AND METHODS

Materials

p-Chloromercuribenzoate (*p*-CMB); diisopropyl fluorophosphate (DFP); *N*-bromosuccinimide (NBS); 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB); *N*-ethylmaleimide (*N*EMI); iodoacetamide; bovine serum albumin; 2-hydroxy-5-nitrobenzyl bromide (HNB); 2-methoxy-5-nitrobenzyl bromide (MNB); and *O*-nitrophenylsulfenyl chloride (*p*-NSC) were purchased from the Sigma Chemical Company, St. Louis, Mo. Linoleic acid (technical grade), α,α'-dipyridyl, and EDTA were obtained from the British Drug House, England. Acrylamide, bisacrylamide, and other organic chemicals required for disc-gel electrophoresis were from Eastman Organic Chemicals. 1,10-Phenanthroline was from the

National Chemical Laboratory, Poona, India. Fresh rice bran was obtained from a rice mill in Mysore, India. All other chemicals were of analytical reagent grade. Double (glass) distilled water was used in all experiments and for preparing reagents, buffers, and other solutions.

Methods

Lipoxygenase activity was determined as described by Stevens *et al.* (14). Linoleic acid (0.1 ml) was dissolved in 60 ml of absolute alcohol, made up to 100 ml with water, and shaken thoroughly to disperse. Before use, 1.0 ml of this stock solution was diluted to 6 ml with 0.2*M* borate buffer (pH 8.5). The enzyme solution to be assayed was diluted 1:1 with 0.2*M* borate buffer (pH 8.5). Of the substrate, 2.9 ml was pipetted into a cuvet (10 mm light path) and oxygenated by bubbling oxygen through for a few minutes. Then 0.1 ml of the enzyme solution was added, mixed, and the change in absorbance at 234 nm measured at 1-min intervals as a function of time in a Beckman-DU spectrophotometer. One unit of enzyme activity produces a change in absorbance of 0.001 per min under the above conditions and the specific activity is the number of units per mg protein. Since activities were low, reactions had to be run for about 30 min with 5–10 mg protein.

For routine work, lipoxygenase activity was followed by measuring the oxygen uptake in a Warburg respirometer. Placed in the main compartment was 3 ml of the enzyme solution, 0.2 ml of 10% potassium hydroxide in the center well, and 0.5 ml substrate in the sidearm. Added to the main compartment was 0.3 ml water to make up the total volume to 4.0 ml. After equilibrating the flasks at 37° C for 15 to 20 min, the substrate and enzyme were mixed together and the oxygen uptake was measured at 15-min intervals for 2 hr. The specific activity was expressed as μl of oxygen consumed per min per mg protein.

Protein concentration was measured by the method of Lowry *et al.* (15) using bovine serum albumin as the standard. Polyacrylamide-gel electrophoresis was carried out essentially as described by Davis (16). For unfractionated rice bran extract, 400 μg total protein was applied per tube; for partially purified lipoxygenase preparation, 200 μg protein per tube. The method of Guss *et al.* (17) was employed to locate lipoxygenase activity on polyacrylamide gels.

Extraction

Fresh powdered bran (100 g) was stirred at 3°–5° C with 400 ml of 50 m*M* phosphate buffer (pH 7.0) for 30 min, filtered through cloth, and centrifuged at 3°–5° C at 10,000 × *g* for 30 min. The supernatant (about 280 ml) was used as a source of enzyme (total activity = 10 to 40 manometric units and specific activity = 0.01 to 0.04). Extraction with water was not satisfactory.

Purification

Partial purification of lipoxygenase was achieved by the following procedure conducted at 3°–5° C. The pH of the above extract was adjusted to 5.1 to 5.2 with 1*M* acetic acid and the turbid solution centrifuged at 10,000 × *g* for 30 min. The pH of the supernatant was adjusted to 6.8 with 1*M* NaOH. Finely powdered ammonium sulfate was added to the stirred solution to 50% saturation and immediately the pH was adjusted to 6.8 with 2*N* ammonia solution. It was then centrifuged and the precipitate collected, dissolved in water, and dialyzed against

water. The slightly turbid solution was centrifuged and the precipitate discarded. To the supernatant, ammonium sulfate was added as before to 30% saturation, pH adjusted to 6.8, centrifuged, and the precipitate discarded. The ammonium sulfate concentration of the supernatant was increased to 40% and the precipitate obtained was dissolved in water and dialyzed as before. The dialyzed enzyme was precipitated by ammonium sulfate (at 50% saturation), centrifuged, dissolved in water, and dialyzed against water. This solution was used as a source of the major lipoxygenase fraction to study some of its properties. By this procedure, a 10-fold purification with 8% recovery of total activity was achieved.

RESULTS AND DISCUSSION

Evidence for the Presence of Multiple Enzymes

The unfractionated rice bran extract and the partially purified lipoxygenase preparation were subjected to polyacrylamide disc-gel electrophoresis at pH 8.9, 3°–8° C, and 5 mA/tube for 2 hr. It was found that the gel containing the unfractionated rice bran extract showed three distinct lipoxygenase activity bands whereas the partially purified preparations had only one. The R_f values (mobility with respect to the tracking dye) of the bands showing lipoxygenase activity were 0.25–0.3, 0.7–0.75, and 0.85–0.90 for the unfractionated extract, and the band with R_f value of 0.85 was visually 3 to 5 times more intense than the other two. The partially purified fraction showed only one activity band with an R_f of 0.85–0.95, but it still contained five proteins as observed on disc-gel electrophoregrams. The purification procedure used therefore eliminated the two minor activity bands and it seemed probable that rice bran contained three lipoxygenase activities; this tentative conclusion, however, needs further evidence. Guss *et al.* (17) reported that under similar conditions a crude extract of soya bean had four lipoxygenase activity bands, mung bean one, and wheat four. Christopher *et al.* (18,19) obtained two isoenzymes from soya bean, the

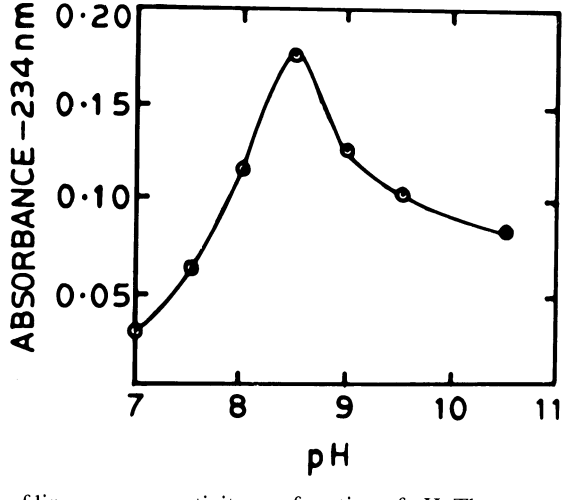

Fig. 1. Variation of lipoxygenase activity as a function of pH. The concentration of borate buffer was 50 m*M*. Activity was measured spectrophotometrically.

criteria being elution from DEAE-Sephadex, isoelectric point, pH activity profile, and their electrophoretic mobility.

Stability

When stored at pH 7 and 3° to 5°C, the partially purified preparation was stable for at least 15 days.

Optimum pH

Enzyme activity was tested at different pH values. The results are presented in Fig. 1. As seen from the figure, the optimum pH for activity was about 8.5; there was little detectable activity below pH 6.5, which could be due to the nonavailability of the substrate. Soya bean enzyme was reported to be optimally active at pH 9.4 (9) and the potato enzyme at pH 5.5 (7). Most workers found a bell-shaped curve with a maximum between pH 7 and 8 for the soya bean lipoxygenase (20).

Substrate Concentration and Activity

Enzyme activity was tested at different concentrations of the substrate. The results are graphically represented in Fig. 2 as a double reciprocal plot. The extrapolated apparent K_m value was 0.35 mM with linoleic acid as the substrate. This value is tentative since 1) a technical grade linoleate was used, and 2) the enzyme was not homogeneous and it is not known whether the other proteins present affect the activity of the enzyme itself. The corresponding values for the enzymes of soya bean (1,21) and wheat (7) are 1 and 5 mM, respectively. For the potato enzyme, K_m values of 0.3 and 0.8 mM have been reported (7).

Activators and Inhibitors

Enzyme activity was tested using different inhibitors and activators. The results are summarized in Table I. It was found that cupric ions (as copper sulfate) at 1 mM inhibited the activity completely. The lack of pronounced

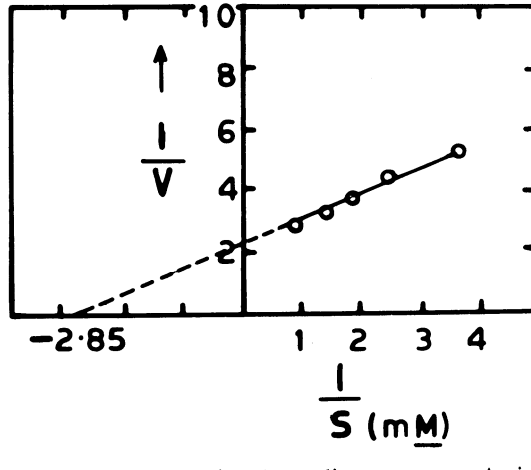

Fig. 2. Lineweaver-Burk plot of rice bran lipoxygenase. Activity was measured spectrophotometrically.

inhibition by 1,10-phenanthroline and α,α'-dipyridyl but a pronounced activation by ferrous ions is puzzling. EDTA also stimulated the activity about 20% which may be due to the removal of the inhibitory divalent metal ions present in the preparation.

Effect of Certain Group Specific Reagents

To ascertain in a preliminary fashion the active site amino acid composition, an experiment was carried out using group specific reagents. The results are summarized in Table II. Serine (threonine ?) hydroxyls were not necessary for enzyme activity since DFP and malathione had no effect on activity. The sulfhydryl reagents, iodoacetamide, *p*-CMB, and DTNB did not inhibit the enzyme activity and hence the enzyme sulfhydryl(s) were not essential for activity. The inhibition by Cu^{2+} was very strong (Table I), probably due to complex formation with the free amino and/or imidazole groups in the enzyme

TABLE I
Effect of Certain Metal Ions and Chelators on the Activity of Partially Purified Rice Bran Lipoxygenase

Inhibitor or Activator[a]	Activity %
None	100
1,10-Phenanthroline	76
α,α-Dipyridyl	94
Ferrous sulfate	150
Calcium chloride	118
EDTA	122
Copper sulfate	0
p-CMB	93
DTNB	100
Iodoacetamide	100

[a]The final concentration of each inhibitor was 1 m*M*. The enzyme was preincubated with each inhibitor or activator for 10 min and then mixed with the substrates containing the same amount of inhibitor or activator, and oxygen uptake measured.

TABLE II
Effect of Group Specific Reagents on the Activity of Partially Purified Rice Bran Lipoxygenase

Reagents[a]	Residual Activity %
Control	100
Diisopropyl fluorophosphate	96
Malathione	98
N-Ethylmaleimide	98
O-Iodosobenzoate	87
Dithioerythreitol	99
2-Hydroxy-5-nitrobenzyl bromide	95
2-Methoxy-5-nitrobenzyl bromide	95
O-Nitrophenylsulfenyl chloride	59
N-Bromosuccinimide	38

[a]The final concentration of each reagent was 1 m*M*. The enzyme was preincubated with the reagent for 15 min(1 hr preincubation for DFP and malathione)and the substrate containing the same amount of reagent was added to initiate the reaction. Oxygen uptake was measured.

protein. The lipoxygenases from soya bean, urd bean, mung bean, green peas, and wheat were not affected by 1 m*M* cyanide, azide, diethyldithiocarbamate, fluoride, pyrophosphate, EDTA, and quinacrine (20). The enzymes from urd bean and mung bean were reported to be sulfhydryl enzymes (20), but this needs to be confirmed. Considerable inhibition of the partially purified lipoxygenase of rice bran was observed only with tryptophan-specific reagents, *p*-NSC and NBS. NBS attacks other amino acid residues also, but *p*-NSC is supposed to be specific for tryptophan (22). Recently, the potato enzyme was shown to require tryptophan residues for its activity (7). Rice bran enzyme seems to be similar to the potato enzyme. Further studies are necessary to confirm these observations.

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