Purification of a Galactolipase from Rice Bran by Affinity Chromatography on Palmitoylated Gauze

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Galactolipase (galactolipid acyl hydrolase, EC 3.1.1.26) was purified 147-fold in good yield (91%) from rice bran by affinity chromatography, in which the enzyme was adsorbed on a palmitoylated gauze column at pH 5.5 and then was eluted with a buffer solution containing a detergent such as sodium deoxycholate or Triton X-100 at pH 8.0. The preparation obtained was further purified by gel filtration on a Sephadex G-100 column and isoelectric focusing. After electrophoresis, the enzyme separated into four components with different isoelectric points. It seems that galactolipase in rice bran exists in multiple forms. The major component (G-2) with isoelectric point of 7.3, one of them, was purified 268-fold and electrophoretically homogeneous. The enzyme (G-2) hydrolyzed rapidly galactolipid and also slowly phospholipid, but hardly triglyceride.

Galactolipase (EC 3.1.1.26) catalyzes the hydrolysis of fatty acid ester bonds of galactolipids. Since the enzyme was first detected in the extract of *Phaseolus multifloris* primary leaves by Sastry and Kates,¹⁾ the enzymes in several higher plants such as spinach leaves,²⁾ bean primary leaves,^{3,4)} and potato tubers^{5,6)} have been purified and characterized. Especially, the galactolipase of potato tubers has been examined in detail. For example, the authors reported that the enzyme which was purified from potato tubers to homogeneity clearly exhibited the hydrolyzing activities for both galacto- and phospholipids.⁶⁾ Galliard has proposed that these lipolytic acyl-hydrolases occurring in plants should be classified as non-specific acyl-hydrolase.⁷⁾

The authors also reported that galactolipases exist in rice bran and the enzymes together with lipase play significant roles in the lipid degradation during the storage of rice grains.^{8,9)} During the investigation, it was presumed that the rice bran galactolipase hydrolyzes not only galactolipids but also phospholipids as the potato enzyme mentioned above does.

On the other hand, rapid and effective methods for purification of galactolipase have been desired to investigate properties and physiological roles of the enzyme. Rock *et al.*,¹⁰⁾

Verine *et al.*,¹¹⁾ and Lairon *et al.*¹²⁾ reported affinity chromatographies on agarose derivatives of phospholipase, adipose tissue lipase and pancreatic lipase, respectively. Recently, Horiuti *et al.*¹³⁾ described affinity chromatography on palmitoylated gauze of microbial lipase. However, with respect to galactolipase, no such report has apparently been published up to date.

The present paper describes the effective purification of a galactolipase from rice bran by means of affinity chromatography on palmitoylated gauze, gel filtration, and isoelectric focusing. The final preparation appeared to be homogeneous electrophoretically and had an isoelectric point of 7.3.

MATERIALS AND METHODS

Plant material. Rice bran used throughout this study was prepared from grains of "Koshihikari," one of Japonica varieties, produced in a Central Agricultural Experiment Station of Shimane Prefecture in 1976.

Substrates. Monogalactosyldiacylglycerol was prepared from spinach leaf lipids.¹⁴⁾ Phosphatidylcholine was purified from egg lipids.¹⁵⁾ Triacylglycerol was prepared from olive oil.¹⁶⁾

Reagents. Carrier ampholyte was obtained from LKB-Produktor AB, Sweden. Sephadex G-100 was

purchased from Pharmacia Fine Chemicals AB, Sweden. All other reagents used were of analytical grade.

Enzyme assay. Enzyme was assayed by determining free fatty acid released from a substrate, spinach monogalactosyldiacylglycerol, according to the method reported previously.^{9,17)} The assay system contained the following components in a total volume of 1.0 ml: 0.1 ml of the micellar substrate solution (5 mM substrate, 10 mM sodium deoxycholate) which was dispersed by ultrasonic treatment, 0.2 ml of 0.5 M Tris-HCl buffer (pH 7.5), 0.1 ml of enzyme preparation and 0.6 ml of water. The enzyme activity was expressed as enzyme unit, where one unit of enzyme is defined as the amount of the enzyme that releases one μmol of free fatty acid from the substrate per minute. The specific activity was expressed as the enzyme unit per mg of enzyme protein. Protein was determined by the Lowry method modified by Hartree.¹⁸⁾

Preparation of palmitoylated gauze. Palmitoylated gauze was prepared from well-dried and -defatted gauze by the method of Horiuti *et al.*¹³⁾ The amount of palmitoyl groups in the prepared palmitoylated gauze was determined to be about 2.4 mmol/g by using the rhodamine method as described previously.¹⁷⁾

Gel filtration. A Sephadex G-100 column (3 $\times$ 40 cm) equilibrated with 5 mM phosphate buffer (pH 8.0) was used, and applied proteins were eluted with the same buffer as described previously.⁹⁾

Isoelectric focusing. Isoelectric focusing was carried out in the 110 ml-apparatus of LKB, using a carrier ampholyte (the pH range of 3.5~10) at a final concentration of 0.5% and a stepwise sucrose gradient (0~50%).⁶⁾ Electrophoresis was performed at 900 V for 48 hr at 4°C. After electrophoresis, the contents of the column were fractionated into 3 ml portions. Each portion was subjected to the measurements of pH, optical density at 280 nm, and the enzymic activity.

Polyacrylamide gel disc electrophoresis. Disc gel electrophoresis was carried out on polyacrylamide gels (7.0% acrylamide, pH 9.0) based on the method described by Ornstein¹⁹⁾ and Davis.²⁰⁾ Protein was detected by staining the gel with 1% amido black 10 B in 7% acetic acid.

RESULTS

Preliminary experiments for the purification of rice bran galactolipase with palmitoylated gauze

Effects of pH on the adsorption of galactolipase to palmitoylated gauze. It has been

reported that lipase and phospholipase are well adsorbed on palmitoylated gauze at pH 7.0 probably owing to hydrophobic interactions.¹⁸⁾ In this connection, the effect of pH on the adsorption of galactolipase on palmitoylated gauze was examined. The results are shown in Fig. 1. The amount of the galactolipase adsorbed was markedly influenced by pH. The maximum adsorption was attained at about pH 5.5, where 95% or more of the galactolipase was adsorbed on palmitoylated gauze.

FIG. 1. Effects of pH on the Adsorption of Rice Bran Galactolipase to Palmitoylated Gauze.

One ml of rice bran crude extract (20 mg of protein) was loaded on a palmitoylated gauze column (1 $\times$ 7 cm) previously equilibrated with 5 mM phosphate buffer of various pH. The charged column was washed with 10 ml of the same buffer, and the remaining enzyme proteins on the washed column were eluted with 10 ml of 50 mM phosphate buffer (pH 8.0) containing 1 mM sodium deoxycholate. The eluted fractions were subjected to enzyme assay. Relative adsorption was expressed as a percentage of total activity of the enzyme adsorbed to palmitoylated gauze at pH 5.5.

Effects of reagents on adsorption. As Fig. 2 shows, the adsorption of the enzyme was strongly inhibited by detergents such as sodium deoxycholate and Triton X-100, but it was not affected by 0.1 M NaCl and CaCl_2 .

Effects of pH and detergents on elution. It was possible to elute the enzyme readily from palmitoylated gauze with 50 mM phosphate buffer containing sodium deoxycholate or Triton X-100 at pH 8.0 rather than at pH 5.5, as shown in Fig. 3. Triton X-100, however,

FIG. 2. Effects of Reagents on the Adsorption of Galactolipase.

Palmitoylated gauze columns equilibrated previously with 5 mM phosphate buffer (pH 5.5) containing the reagents of various concentrations were used. The charged column was washed with 10 ml of the same buffer used for equilibration, and then the remaining enzyme proteins were eluted with 10 ml of 50 mM phosphate buffer (pH 8.0) containing 1 mM sodium deoxycholate. The eluted fractions were dialyzed to remove the excess reagents and were subjected to enzyme assay. The other conditions were similar to those described in Fig. 1.

Relative adsorptions were expressed as a percentage of total activity of the enzyme adsorbed at the same condition without the reagents.

△—△, sodium deoxycholate; □—□, Triton X-100; ○—○, CaCl_2 ; ●—●, NaCl.

inhibited the enzyme activity, while sodium deoxycholate stimulated the activity (Table II). It is known that the substrate solution dispersed with sodium deoxycholate has the negative charges on the surface of the substrate micelles, whereas it dispersed with Triton X-100 does not have such charges. It seems likely that the negative charges on the surface of the micelles stimulate the enzymic activity as the activation of *Penicillium natatum* phospholipase B demonstrated by Dawson.²¹ Therefore, sodium deoxycholate was well suited both for emulsifying the substrate and for the elution of the enzyme from the column. In the present work, the enzyme was eluted with 50 mM phosphate buffer (pH 8.0) containing 1 mM sodium

FIG. 3. Effects of pH and Detergents on Elution of Rice Bran Galactolipase from Palmitoylated Gauze.

The enzyme protein adsorbed at pH 5.5 on palmitoylated gauze column was eluted with 10 ml of 50 mM phosphate buffer (pH 5.5 and 8.0) containing the detergents of various concentrations. The eluted fractions were dialyzed to remove the detergents and then subjected to enzyme assay. The other conditions were similar to those described in Fig. 1.

○—○, pH 8.0; sodium deoxycholate; ●—●, pH 8.0; Triton X-100; △—△, pH 5.5; sodium deoxycholate; ▲—▲, pH 5.5; Triton X-100.

deoxycholate, thereby the enzyme activity retained could be released completely.

Purification of rice bran galactolipase

Unless otherwise described, all purification steps were performed at about 4°C.

Extraction. The enzyme was extracted by the method described previously.⁹ Fresh rice bran prepared from brown rice was immediately defatted five times with petroleum ether at -20°C. The resulting defatted rice bran was homogenized with 5 mM Tris-HCl buffer (pH 8.0) in a waring blender. The homogenate was filtered through nylon cloth, and the turbid filtrate was centrifuged at 15,000 × *g* for 30 min. The enzyme was further extracted from the pellets twice by the same method. The combined supernatant was concentrated by freeze-drying and the preparation obtained was designated as "a crude extract."

Affinity chromatography on palmitoylated gauze. The crude extract was dialyzed against

5 mM phosphate buffer (pH 5.5) for 24 hr, and the dialyzate was applied to a palmitoylated gauze column (3×20 cm) equilibrated previously with the same buffer. The charged column was washed with the same buffer, and then the adsorbed enzyme was eluted with 50 mM phosphate buffer (pH 8.0) containing 1 mM sodium deoxycholate. As Fig. 4 shows, galactolipase was found in the adsorbed fractions, and could be well separated from other proteins. The active fractions were collected, dialyzed for 24 hr against 5 mM phosphate buffer (pH 8.0), and then concentrated by freeze-drying. The enzyme obtained was purified about 150-fold over the crude extract with a yield of 91%. The ratio of the specific activity of galactolipase to that of lipase in the crude extract (about 6:10) was changed to about 100:0.1 by the gauze column.

FIG. 4. Affinity Chromatography of Rice Bran Crude Extract on a Palmitoylated Gauze Column.

Rice bran crude extract (220 mg of protein) was loaded on a palmitoylated gauze column (3×20 cm) previously equilibrated with 5 mM phosphate buffer (pH 5.5), and then the remaining enzyme proteins on the washed column were eluted with 50 mM phosphate buffer (pH 8.0) containing 1 mM sodium deoxycholate. The fractions of 5 ml were collected and subjected to enzyme assay.

○—○, galactolipase activity; ×---×, optical density at 280 nm.

Gel filtration on a Sephadex G-100 column.

The enzyme preparation obtained by affinity chromatography was loaded on a Sephadex G-100 column (3×40 cm) equilibrated with

5 mM phosphate buffer (pH 8.0) and was eluted with the same buffer. The elution pattern of the galactolipase activity gave a single peak, as shown in Fig. 5. The active fractions (fraction Nos. 32~40) were combined, concentrated by freeze-drying, and dialyzed against 5 mM phosphate buffer (pH 8.0) for 12 hr.

FIG. 5. Gel Filtration of Rice Bran Galactolipase on a Sephadex G-100 Column.

The enzyme fraction (170 mg of protein) obtained by affinity chromatography was loaded on a Sephadex G-100 column (3×40 cm) equilibrated with 5 mM phosphate buffer (pH 8.0). The fractions were collected and subjected to enzyme assay.

○—○, galactolipase activity; ×---×, optical density at 280 nm.

Isoelectric focusing. The resulting dialyzate was subjected to isoelectric focusing. As Fig. 6 shows, the enzyme activity was separated into four peaks (G-1, 2, 3, and 4), their isoelectric points being pH 9.6, 7.3, 4.9, and 3.5, respectively. The combined fractions of Nos. 3~7 (peak G-1), Nos. 9~14 (peak G-2), Nos. 19~25 (peak G-3), and Nos. 27~34 (peak G-4) gave the yields of the enzyme activity of 19, 57, 9, and 7%, respectively, of the total activity applied to the column. In this experiment, the major peak (G-2) with pI 7.3, one of them, was investigated as "a purified galactolipase." The steps of the purification procedure and the yield of enzyme at each step are summarized in Table I. About 45 mg of the purified enzyme was isolated from 800 g of the fresh defatted rice bran. The enzyme was purified 268-fold in a yield of about 40%.

TABLE I. PURIFICATION OF GALACTOLIPASE FROM RICE BRAN

Stages	Total protein (mg)	Total activity (U)	Specific activity (mU/mg)	Purification (-fold)	Over-all yield (%)
Crude extract	30,160	412	14	1	100
Palmitoylated gauze column	181	372	2055	147	91
Sephadex G-100	98	308	3156	225	75
Isoelectric focusing (G-2 fraction)	44.8	168	3750	268	41

FIG. 6. Isoelectric Focusing of Rice Bran Galactolipase.

The enzyme fraction (41 mg of protein) obtained by gel filtration was subjected to isoelectric focusing. Isoelectric focusing was carried out for 48 hr at 900 V and 4°C in a LKB 8101 column using ampholyte for the pH range of 3.5~10 with the cathode at the bottom. After electrophoresis, the content of the column was fractionated into 3 ml portions. Each fraction was assayed for pH, optical density at 280 nm, and the enzymic activity.

-----, pH; x---x, optical density at 280 nm; O—O, galactolipase activity.

Homogeneity of enzyme protein. The homogeneity of the purified enzyme preparation was substantiated by polyacrylamide gel disc electrophoresis, as well as by isoelectric focusing and gel filtration on Sephadex G-100. As Fig. 7 shows, the purified enzyme gave a single protein band on polyacrylamide gel disc electrophoresis. Furthermore, both isoelectric focusing and gel filtration of the purified enzyme preparation (G-2) also gave a single protein peak coinciding with the enzymic activity.

The purified enzyme hydrolyzed rapidly

FIG. 7. Disc Electrophoresis of the Purified Rice Bran Galactolipase.

The purified rice bran galactolipase was subjected to disc electrophoresis in 7% polyacrylamide gel. Electrophoresis was carried out at a constant current of 3 mA per gel for 3 hr.

TABLE II. HYDROLYSIS RATES FOR LIPIDS

The incubation mixtures contained 0.5 μ mol of a substrate and 1 μ mol of sodium deoxycholate (for monogalactolipid and phosphatidylcholine), or 10 μ mol of long-chain triacylglycerol and 2 μ mol of sodium deoxycholate (for triacylglycerol) and 0.2ml of 0.5 M Tris-HCl buffer (pH 7.5, 8.0 and 9.0 for galactolipid, phospholipid and triacylglycerol, respectively). Total volume, 1 ml; incubation at 35°C for 20 min. Hydrolysis rates were expressed as a percentage of the rate of monogalactosyldiacylglycerol.

Substrates	Hydrolysis rates(%)
Monogalactosyldiacylglycerol	100
Phosphatidylcholine	27
Triacylglycerol	trace

monogalactosyldiacylglycerol, and also slowly phosphatidylcholine, but hardly long chain triacylglycerol, as Table II shows.

DISCUSSION

Previously, the authors partially purified a galactolipase from rice bran and examined its properties.⁹⁾ However, further purification of

the enzyme by means of DEAE- and CM-Sephadex ion exchange chromatographies was unsuccessful, because of the lability of the enzyme, and the enzyme preparation was not completely separated from lipase activity.⁹⁾ In this work, galactolipase was separated from other (non-enzymatic) proteins by means of affinity chromatography on palmitoylated gauze, and was further purified about 270-fold in a yield of 40% by gel filtration on a Sephadex G-100 column and isoelectric focusing. The purified enzyme (G-2) was shown to be homogeneous electrophoretically and rarely to involve lipase activity.

The adsorption of galactolipase on palmitoylated gauze was inhibited by detergents such as sodium deoxycholate and Triton X-100 but was not affected by NaCl and CaCl₂ (Fig. 2). These results suggest that the adsorption is due to hydrophobic interactions rather than electrostatic forces between the substrate and the enzyme as pointed out by Horiuti *et al.*¹³⁾ and Lairon *et al.*¹²⁾ However, the adsorption of the enzyme was markedly influenced by pH (Fig. 1). Such an effect was also found in the affinity chromatography of other galactolipases in higher plants such as *Phaseolus vulgaris* leaves²²⁾ and potato leaves.²³⁾ It seems that the adsorption of these galactolipases on palmitoylated gauze is also dependent on the conformation of the enzyme proteins. Both galactolipase and lipase were adsorbed on the column, but only the former was eluted under the condition used in this experiment. Therefore, palmitoylated gauze is useful for the purification of galactolipases from other sources. Similar results with respect to affinity chromatography have been reported with agarose derivatives^{24~29)} and with *p*-cellulose.³⁰⁾

The purified enzyme rarely catalyze the hydrolysis of long-chain triacylglycerol, so that the galactolipase was well separated from lipase activity. However, it clearly exhibited phospholipase activity, too (Table II). The enzyme was found to be a single protein electrophoretically (Fig. 7). In addition, galactolipase and phospholipase activities were not separated from each other throughout all purification

procedures examined. These results suggest that a single enzyme exhibits not only galactolipase but also phospholipase activities as the purified lipolytic acyl-hydrolase from potato tubers does.⁶⁾ Consequently, the purified rice bran galactolipase may belong to a non-specific acyl-hydrolase. The structure and nature of such an enzyme are very interesting and will be reported in near future.

After isoelectric focusing, galactolipase was separated into four peaks with different isoelectric points (Fig. 6). Since the four galactolipases were present even in the crude extract from brown rice,⁹⁾ they would not be so-called artifacts. The enzymatic relationship among these enzymes is yet not clear. However, it seems that galactolipase in rice bran exists in multiple forms similar to those of rice bran lipase previously described.^{9,31)}

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