Biochemical Studies on Rice Bran Lipase

Part I. Purification and Physical Properties

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Lipase extracted from defatted rice bran with calcium chloride solution was purified by ammonium sulfate precipitation, followed by successive column chromatographies on DEAE-cellulose, Sephadex G-75, CM-Sephadex C-50 in the presence of calcium ion. The specific activity of the purified enzyme was 4.7 units/mg protein and 480 times that of starting crude extract. The homogeneity of the enzyme protein was criticized by polyacrylamide gel disc electrophoresis and ultracentrifugation. The enzyme protein also behaved homogeneously in ampholine electrophoresis, indicating the isoelectric point of 8.56. The sedimentation coefficient of the enzyme was determined to be 2.97 S, and the molecular weight to be 40,000 by Archibald's method. According to the measurement of optical rotatory dispersion of the enzyme, ORD constant, λ_e , Moffitt-Yang parameters, a_0 and b_0 , were evaluated to be 239 $m\mu$, -164 and -123, respectively.

Several lipases of animal origin, especially pancreas lipase, have been purified and their chemical and enzymatic properties were elucidated in detail.^{1,2)} As to lipase of microbial origin, Fukumoto *et al.*,³⁾ for instance, crystallized a preparation of lipase obtained from *Asp. niger* as a single protein. In contrast, studies on lipases in higher plant have advanced slowly, probably due to their solubilities and diversities. The existence of lipase in higher plant was first noticed by Green⁴⁾ in castor bean in 1890. Since then, the castor bean lipase has been investigated most intensively among the higher plant

lipases, because of its relatively high activity.^{5~8)} With respect to rice bran lipase, Kimura⁹⁾ studied the hydrolysis of lipids by crude enzyme preparation, and Noda and Kobayashi¹⁰⁾ examined the enzymatic properties of a partially purified enzyme preparation. Although these studies yielded appreciable informations on the enzymatic properties of the rice bran lipase, physical and chemical properties of the enzyme were not elucidated at all. The present paper, therefore, deals

5) E. Takamiya, *Nippon Nogeikagaku Kaishi*, **11**, 68, 216 (1935).

6) R. L. Ory, A. J. St. Angelo and A. M. Altschul, *J. Lipid Res.*, **1**, 208 (1960); *ibid.*, **3**, 99 (1962).

7) R. L. Ory, A. J. St. Angelo, I. V. deGruy and A. M. Altschul, *Can. J. Biochem.*, **45**, 1445 (1967).

8) R. L. Ory, J. Kiser and P. A. Pradel, *Lipids*, **4**, 261 (1969).

9) G. Kimura, *Yukagaku*, **9**, 154, 365, 486 (1960); *ibid.*, **10**, 136, 248 (1961).

10) M. Noda and K. Kobayashi, *Nippon Nogeikagaku Kaishi*, **42**, 574 (1968).

1) P. Desnuelle, "Advances in Enzymology," Vol. 23, ed. by F. F. Nord, Interscience Pub. Inc., New York, 1961, p. 129.

2) R. Verger, G. H. Haas, L. Sarda and P. Desnuelle, *Biochim. Biophys. Acta*, **188**, 272 (1969).

3) J. Fukumoto, M. Iwai and Y. Tsujisaka, *J. Gen. Appl. Microbiol.*, **9**, 353 (1963).

4) J. R. Green, *Pro. Roy. Soc. London*, **48**, 370 (1890).

mainly with the purification of the rice bran lipase for the purpose of investigating the physical properties of the enzyme.

MATERIALS AND METHODS

Materials. Rice bran used throughout this study was prepared from grains of "Hohyoku," one of *japonica* varieties, produced in Fukuoka prefecture in 1968.

All reagents used were of analytical grade except the followings. Tributyrin used as substrate in enzyme assay was purchased from Merk Co. Carrier ampholytes (pH 7~10) was obtained from LKB-Produkter AB, collodion membrane filter from Sartorius-Membranfilter GmbH, DEAE-cellulose (0.8~1.0 meq/g), from Brown Co., and CM-Sephadex C-50 (4~5 meq/g), Sephadex G-75 from Pharmacia Co.

Enzyme assay. The lipase activity was assayed by adding 0.2 ml of tributyrin to an enzyme solution consisted of 0.1 ml of 0.5 M KCl, 1.0 ml of 5×10^{-3} M CaCl_2 , and a given amount of the enzyme. The final volume of the reaction mixture was made up to 10 ml with deionized water. The enzyme reaction was carried out at pH 7.5 and 35°C with constant stirring under nitrogen gas stream, unless otherwise mentioned. The increase in free fatty acid produced in consequence of the enzyme reaction was followed by titration with either 0.05 or 0.1 N NaOH by using a pH-Stat (Radiometer Type TTT-1C).

Enzyme unit. The international unit was employed: one lipase unit was defined as the amount of enzyme that hydrolyzes one μ mole of ester bond in triglyceride per minute. The specific activity was expressed as the enzyme unit per mg of enzyme protein.

Determination of protein concentration. Protein concentration was determined spectrophotometrically with a Beckman DU spectrophotometer by using the value $E_{280, \text{cm}}^{1\%} = 19.67$.

Polyacrylamide gel disc electrophoresis. The pure enzyme preparation was subjected to disc electrophoresis in polyacrylamide gel prepared according to the method of Reisfeld *et al.*¹¹⁾ The electrophoresis was carried out for 3.5 hr at approximate 110 V

with a current of 2.5 mA/tube (7 cm $\times$ 0.5 cm) using 0.35 M β -alanine-acetate buffer (pH 4.5). Protein band in gel was detected by staining with Amide Black 10 B, followed by removing unbound dye with several changes of 7% acetic acid (v/v).

Ampholine electrophoresis. A lyophilized enzyme preparation (1.7 mg) was applied to an ampholine electrophoresis in 0.8% carrier ampholytes (pH 7~10) medium containing 1×10^{-3} M CaCl_2 . Electrophoresis was conducted using electrofocusing column, LKB 8101, for 50 hr at 2°C with about 1.1 Watt throughout the electrophoresis. After contents of the column were fractionated into 1.5 ml-portions, the amount of enzyme protein in each portion was determined spectrophotometrically at 280 m μ , and each pH value was measured by a Hitachi-Horiba pH-meter equipped with glass electrode 6028-10 T at 25°C.

Sedimentation analysis. Measurements of sedimentation velocity were made at five 8-min intervals by a Spinco Model E Ultracentrifuge equipped with a schlieren optical system and a synthetic boundary cell, operating at 56,100 rpm at 20.6°C. The solvent employed was a solution of 1×10^{-3} M CaCl_2 in phosphate buffer, pH 6.2.

Ultraviolet absorption spectrum. The ultraviolet absorption spectrum was determined with a Cary Model 14 Recording Spectrophotometer. The sample solution contained 0.441 mg of enzyme protein per 1 ml of 0.01 M phosphate buffer containing 1×10^{-3} M CaCl_2 .

Determination of molecular weight. The molecular weight of the enzyme protein was determined with Archibald's method^{12,13)} by using a Spinco Model E Ultracentrifuge equipped with a schlieren optical system. The enzyme solution at a concentration of 7% in phosphate buffer ($\mu=0.1$), pH 6.1, was ultracentrifuged by using a standard cell at 13,410 rpm at 20°C. The initial concentration was measured with synthetic boundary cell under the same conditions as described above. The molecular weight was calculated according to the following equation.

12) W. J. Archibald, *J. Phys. and Colloid Chem.*, **51**, 1204 (1947).

13) H. K. Schachman, "Methods in Enzymology," Vol. IV, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, 1957, p. 32.

11) R. A. Reisfeld, U. J. Lewis and D. E. Williams, *Nature*, **195**, 281 (1962).

$$M = \frac{RT}{(1-V\rho)\omega^2} \cdot \frac{(dc/dx)_m}{Xm \cdot Cm} = \frac{RT}{(1-V\rho)\omega^2} \cdot \frac{(dc/dx)_b}{Xb \cdot Cb}$$

where R is the gas constant, T the absolute temperature, V the partial specific volume of enzyme protein (assumed to be 0.75), ρ the density of enzyme solution, ω the angular velocity, Xm and Xb are the distance from the center of rotation to meniscus and bottom, Cm and Cb are the solute concentration at meniscus and bottom. Cm and Cb were determined according to the method reported by Klainer *et al.*¹⁴⁾

Optical rotatory dispersion measurement. Optical rotatory dispersion measurement was performed with a Jasco Model ORD/UV-5 Recording Spectropolarimeter at room temperature near 25°C. For the measurement in the visible and near ultraviolet region (600~300 m μ), 10 mm-cell was used with the enzyme solution at a concentration of 1.23%. The deionized water was used as solvent. The value of λ_e was calculated by Drude's equation (1) and the values of a_0 and b_0 by Moffitt-Yang's equation (2).¹⁵⁾

$$[\alpha]_i = \frac{K}{\lambda^2 - \lambda_e^2} \quad (1)$$

$$[M]_i = \frac{3}{n^2 + 3} \frac{M_0}{100} [\alpha]_i = \frac{a_0 \lambda_0^2}{(\lambda^2 - \lambda_0^2)} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad (2)$$

where M_0 is the average molecular weight per residue, λ_0 the absorption wavelength concerned with the rotation and n the solvent refractive index. In the present work, M_0 of 110, λ_0 of 210 m μ and n of 1.335 were used.

RESULTS AND DISCUSSION

A. Purification of enzyme

Extraction of lipase from rice bran (Step I). The rice bran was defatted five times with petroleum ether. The defatted rice bran was then suspended in 1×10^{-2} M calcium chloride and adjusted to pH 6.0 with 1 N NaOH. After mechanical stirring for 3 hr, the suspension was squeezed through cheese cloth and the turbid solution was centrifuged for 20 min

at 5000 rpm. A slightly turbid supernatant was obtained and designated as "crude extract".

Fractionation with ammonium sulfate (Step II). To the crude extract was added solid ammonium sulfate to 60% saturation under constant stirring. The resulting precipitate was separated by centrifugation and then suspended in a minimum amount of 1×10^{-3} M CaCl₂. The suspension was dialyzed against the same solution at 4°C for 2 days. Insoluble material was removed by centrifugation. The clear supernatant was designated as "crude enzyme solution"

DEAE-cellulose column chromatography (Step III). The crude enzyme solution was dialyzed against 0.005 M phosphate buffer containing 1×10^{-3} M CaCl₂, pH 7.0, at 4°C for 3 days. After removal of any insoluble material by centrifugation, 5 ml (106.9 mg enzyme) of the supernatant was applied onto a DEAE-cellulose column previously equilibrated with the same buffer solution as that used for dialysis. Elution was carried out stepwise with 0.005, 0.02, 0.06, 0.1 M phosphate buffer containing 1×10^{-3} M CaCl₂, pH 7.0 and finally with 0.1 M phosphate buffer (pH 7.0) containing 1 M NaCl additionally to 1×10^{-3} M CaCl₂. As shown in Fig. 1, protein was separated to six fractions. The fraction D-1, which exhibited the highest specific activity was collected and reprecipitated by saturation with ammonium sulfate. On the other hand, 5 ml (102.6 mg enzyme) of the crude enzyme solution, which was obtained by the similar purification procedures in the absence of calcium chloride, was chromatographed on DEAE-cellulose column in the same way as above using buffer solution without CaCl₂. As shown in Fig. 2, protein was separated similarly as above. However, each fraction exhibited more or less enzyme activity and the specific activity of the fraction D'-1 was about one half that of the fraction D-1. Thus, procedures with calcium chloride was proved to be of great advantage

14) S. M. Klainer and G. Kegels, *Arch. Biochem. Biophys.*, **63**, 247 (1956).

15) W. Moffitt and J. T. Yang, *Pro. Natl. Acad. Sci. U.S.*, **42**, 596 (1956).

FIG. 1. Column Chromatogram of Crude Enzyme on DEAE-cellulose in the Presence of CaCl_2 .

Column size; 2×20 cm, Content of each tube; 5.3 ml, Flow rate; 80 ml/hr.

Total recovery of protein: 97%.

Total recovery of activity: 96%.

Specific activities (mU/mg) of fractions: D-1: 290.8, D-2: 41.3, D-3: 53.2, D-4: 29.5, D-5: 26.6, D-6: 17.7.

FIG. 2. Column Chromatogram of Crude Enzyme on DEAE-cellulose in the Absence of CaCl_2 .

Column size; 2×20 cm, Content of each tube; 5.3 ml, Flow rate; 80 ml/hr.

Total recovery of protein: 92%.

Total recovery of activity: 95%.

Specific activities (mU/mg) of fractions: D'-1: 104.2, D'-2: 67.8, D'-3: 64.8, D'-4: 52.2, D'-5: 36.4, D'-6: 17.7.

to the purification of the enzyme.

Gel-filtration on Sephadex G-75 (Step IV). The precipitate obtained in Step III was dissolved in 0.01 M phosphate buffer (pH 6.2) containing 1×10^{-3} M CaCl_2 and dialyzed against the same buffer solution for 2 days. After removal of any insoluble material by centrifugation, 25 ml (679.7 mg enzyme) of the dialyzed solution was separated through a Sephadex G-75 column previously washed sufficiently with the same phosphate buffer. A typical gel filtration pattern is presented in Fig. 3. The fraction

FIG. 3. Gel-filtration Pattern of Fraction D-1 on Sephadex G-75.

Column size; 5×115 cm, Content of each tube; 10.5 ml, Flow rate; 90 ml/hr.

Total recovery of protein: 98%.

Total recovery of activity: 96%.

Specific activities (mU/mg) of fractions: DS-1: 66.8, DS-2: 237.8, DS-3: 407.2, DS-4: 306.7, DS-5: 31.4, DS-6: 0.

DS-3 with the highest specific activity was concentrated using collodion membrane filter under reduced pressure.

CM-Sephadex C-50 column chromatography (Step V). Six ml (309 mg enzyme) of the con-

FIG. 4. Column Chromatogram of Fraction DS-3 on CM-Sephadex C-50.

Column size; 2×19 cm, Content of each tube; 6.3 ml, Flow rate; 50.4 ml/hr.

Total recovery of protein: 98%.

Total recovery of activity: 96%.

Specific activities (mU/mg) of fractions: DSC-1: 561, DSC-2: 1908, DSC-3: 2949, DSC-4: 1402, DSC-5: 63.

concentrated enzyme solution in Step IV was applied onto a CM-Sephadex C-50 column previously equilibrated with 0.01 M phosphate buffer containing 1×10^{-3} M CaCl_2 , pH 6.2. The enzyme adsorbed on the column was eluted with a linear gradient change of the concentration of NaCl from 0 to 0.25 M by using a mixing chamber containing 300 ml of 0.01 M phosphate buffer (pH 6.2) and 1×10^{-3} M CaCl_2 and a reservoir containing 300 ml of the same buffer and 0.25 M NaCl. A typical column chromatogram is presented in Fig. 4. The fraction DSC-3 which showed the highest specific activity among five peaks was collected and concentrated using collodion membrane filter under reduced pressure.

Rechromatography on the CM-Sephadex C-50 column (Step VI). The concentrated enzyme solution in Step V was dialyzed against 0.01 M phosphate buffer containing 1×10^{-3} M CaCl_2 to remove NaCl. Four ml (146.9 mg enzyme) of the dialyzed enzyme solution was applied onto a CM-Sephadex C-50 column which had been equilibrated with the same buffer solution as used for dialysis. The column was developed by linear gradient elution with a mixing chamber which contained 450 ml of 0.01 M phosphate buffer and 1×10^{-3} M CaCl_2 and a reservoir which contained 450 ml of 0.15 M NaCl in the same buffer. As can be seen in Fig. 5, four peaks were obtained. The fraction RDSC-3 exhibited the highest specific activity. The lipase contained in the fraction RDSC-3 was behaved homogeneously on CM-Sephadex C-50 column chromatography as obviously seen in Fig. 6. The steps of the

FIG. 5. Column Chromatogram of Fraction DSC-3 on CM-Sephadex C-50.

Column size; 2.2×30 cm, Content of each tube; 8.6 ml, Flow rate; 68.8 ml/hr.

Total recovery of protein: 95%.

Total recovery of activity: 87%.

Specific activities (mU/mg) of fractions: RDSC-1: 2063, RDSC-2: 2055, RDSC-3: 4704, RDSC-4: 380.

TABLE I. A TYPICAL DATA IN THE PROCESS OF PREPARATION OF RICE BRAN LIPASE, STARTING WITH 1 kg OF DEFATTED RICE BRAN

Step of purification	Vol. (ml)	Activity (mU/ml)	Total activity (U)	Protein (mg/ml)	Specific activity (mU/mg)	Over-all yield (%)
Crude extract (Step I)	6100	127.4	777.1	13.0	9.8	100
Crude enzyme (Step II)	935	647.1	605.0	9.14	70.8	77.8
Column chromatography on DEAE-cellulose (Step III)	3160	98.0	309.7	0.337	290.8	39.9
Gel-filtration on Sephadex G-75 (Step IV)	160	900.0	144.0	2.21	407.2	18.5
Column chromatography on CM-Sephadex C-50 (Step V)	38	878.9	33.4	0.298	2949.3	4.3
Rechromatography on CM-Sephadex C-50 column (Step VI)	30	926.7	27.8	0.197	4704.1	3.6

FIG. 6. Column Chromatogram of Fraction RDSC-3 on CM-Sephadex C-50.

Column size; 2×26 cm, Content of each tube; 6.7 ml, Flow rate; 60.5 ml/hr.

purification procedure and yield of enzyme at each step are summarized in Table I. About 6 mg of purified enzyme was isolated from 1 kg of defatted rice bran. The sequence of the purification procedures effected a 480-fold increase in specific activity of the enzyme. The specific activity of the pure enzyme was 4.7 U/mg. The pure enzyme preparation was stored at -20°C in the presence of ammonium sulfate.

B. Homogeneity and physical properties of purified lipase

The homogeneity of the purified enzyme preparation, which appeared to the homogeneous by criterion of CM-Sephadex C-50 column chromatography, was further substantiated by the following physical tests. In addition, the physical properties of the purified enzyme were examined by means of several physical measurement.

Polyacrylamide gel disc electrophoresis. When the purified enzyme was subjected to disc electrophoresis in 7.5% polyacrylamide gel at pH 4.5, it migrated as a defined band, as shown in Fig. 7, indicating that the enzyme is electrophoretically homogeneous.

Ampholine electrophoresis. The purified enzyme was subjected to ampholine electrophoresis for the determination of isoelectric point. A single peak was obtained at pH 8.56 on a pH-slope drawn by dotted line in Fig. 8. The experimental result obtained indicates that the enzyme is a homogeneous and basic protein with isoelectric point of 8.56.

Sedimentation analysis. The purified enzyme solution was dialyzed against 0.01 M phosphate buffer containing 1×10^{-3} M CaCl_2 , pH 6.2 ($\mu = 0.02$) at 5°C for 3 days under constant stirring, and then concentrated to a final con-

centration of 0.94% using collodion membrane filter under reduced pressure. The resulting enzyme solution was ultracentrifuged with a

FIG. 7. Analytical Disc Electrophoresis of Rice Bran Lipase in 7.5% Polyacrylamide Gel at pH 4.5 with a Current of 2.5 mA/tube (0.5×7 cm) for 3.5 hr.

- (a) Purified enzyme preparation.
- (b) Purified and lyophilized enzyme preparation.

Spinco Model E Ultracentrifuge equipped with a synthetic boundary cell, operating at 56,100 rpm at 20.6°C . The sedimentation pattern is shown in Fig. 9. The enzyme preparation was homogeneous with a symmetric sedimenting boundary. The sedimentation coefficient, $s_{20,w}$ of the enzyme was calculated to be 2.97S according to the methods of Schachman¹⁵⁾ and Svedberg.¹⁶⁾

FIG. 8. Ampholine Electrophoresis of Rice Bran Lipase in 0.8% Carrier Ampholytes (pH 7~10) Containing 1×10^{-3} M CaCl_2 at 2°C for 50 hr.

FIG. 9. Sedimentaion Patterns of Rice Bran Lipase from a Run in the Synthetic Boundary Cell.

Sedimentaion is from right to left in photographs taken at indicated times after reaching 56,100 rpm. Protein concentration was 0.94% in 0.01 M phosphate buffer containing 1×10^{-3} M CaCl_2 , pH 6.2; $\mu=0.02$. The temperature was 20.6°C .

16) T. Svedberg and D. K. Pederson, "The Ultracentrifuge," Clearedon Press Inc., Oxford, 1940, p. 445.

Ultraviolet absorption spectrum. A typical absorption spectrum of the enzyme is shown in Fig. 10; the maximum and the minimum absorptions are found at 278 $m\mu$ and at 250 $m\mu$, respectively. The molecular extinction coefficient, ϵ_{280} of the enzyme was evaluated to be 7.868×10^4 from the value of $E_{280, cm}^{1\%} = 19.67$ and the molecular weight of 40,000 which was estimated with Archibald's method described later.

FIG. 10. Ultraviolet Absorption Spectrum of Rice Bran Lipase.

The concentration of the enzyme was 0.441 mg in 1 ml of 0.01 M phosphate buffer containing 1×10^{-3} M CaCl_2 , pH 6.2.

Molecular weight. The values of $(dc/dx)_m/Xm \cdot Cm$ and $(dc/dx)_b/Xb \cdot Cb$, which were estimated from photographs of boundary, were plotted against the time after reaching constant revolution and extrapolated to zero-time. From the intercept on ordinate, the values of $\{(dc/dx)_m/Xm \cdot Cm\}_{t=0}$ and $\{(dc/dx)_b/Xb \cdot Cb\}_{t=0}$ were found to be 0.800 and 0.835, respectively. The result is presented in Table II. The molecular weight of the enzyme protein was calculated to be 3.9×10^4 at the meniscus and 4.1×10^4 at the bottom of the cell with an

TABLE II. EVALUATION OF $\{(dc/dx)_m/Xm \cdot Cm\}_{t=0}$ AND $\{(dc/dx)_b/Xb \cdot Cb\}_{t=0}$ IN THE DETERMINATION OF MOLECULAR WEIGHT

Time (min)	$(dc/dx)_m/Xm \cdot Cm$	$(dc/dx)_b/Xb \cdot Cb$
24	0.767	0.854
32	0.772	0.859
40	0.763	0.849
48	0.758	0.861
56	0.744	0.862
Value of extrapolation to zero-time	0.800	0.835

Xm : 5.98 cm, Xb : 6.47 cm.

average value of 40,000.

Optical rotatory dispersion. ORD constant, λ_c , was estimated from the slope which was obtained from Yang's plot shown in Fig 11. A Moffitt-Yang's plot for calculating the Moffitt-Yang parameters, a_0 and b_0 , is shown in Fig. 12. In this plot, the value of a_0 is given from the intercept and the value of b_0 from the slope. The values of λ_c , a_0 and b_0 were 239 $m\mu$, -164 and -123, respectively. The values of a_0 and b_0 were similar to those of insulin at pH 8.5 or conalbumin at pH 5.8.¹⁷⁾

FIG. 11. Yang Plot of Rice Bran Lipase.

The protein concentration was 1.23% in de-ionized water. The temperature was 25°C.

17) "Handbook of Biochemistry," ed. by H. A. Sober, Chemical Rubber Co., Cleveland, Ohio, 1968, p. c-55.

FIG. 12. Moffitt-Yang Plot of Rice Bran Lipase.

The protein concentration was 1.23% in de-ionized water. The temperature was 25°C.

The α -helix content of the enzyme was calculated to be around 20% from the values of λ_e and b_0 . Discussion on these data will be made in the subsequent paper.

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