Biochemical Studies on Rice Bran Lipase

Part II. Chemical Properties

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Some chemical properties of the rice bran lipase were studied. The enzyme protein contained 14.98% nitrogen and consisted of 312 amino acid residues. It also contained a certain amount of lipid. The amino-terminal amino acids of the enzyme protein were shown to be glutamic acid and the carboxyl-terminal amino acids to be glycine and serine. The treatment of the enzyme protein with 8M urea containing 1×10^{-3} M EDTA (ethylenediaminetetraacetic acid) seemed to cause dissociation of the subunits of the enzyme protein. From this observation and the results of the terminal amino acids analysis, it was presumed that the enzyme protein was composed of at least two types of subunits.

As to the chemical properties of higher plant lipases, only a few papers have been published. For instance, Ory *et al.*¹⁻³⁾ reported that the lipase of castor bean consisted of three components, an apoenzyme, a cofactor and a proteinactivator. However, the chemical properties of other higher plant lipases remain obscure because of failure in their isolation.

In a preceding report,⁴⁾ the authors showed that a purified rice bran lipase was homogeneous in polyacrylamide gel disc electrophoresis and ultracentrifugation, and elucidated some physical properties of the enzyme protein. The isoelectric point of the enzyme was determined to be 8.56 by means of ampholine electrophoresis and the molecular

weight to be 40,000 by Archibald's method. The present paper deals with chemical characterization of the rice bran lipase and presents an assumption that the enzyme is composed of subunits.

MATERIALS AND METHODS

Materials. Rice bran lipase used throughout this study was purified as described in the preceding paper.⁴⁾ Anhydrous hydrazine was prepared from commercial 80% hydrazine after Kusama.⁵⁾ Benzaldehyde was refined before use. Benzoic acid in commercial benzaldehyde was extracted with 5% sodium bicarbonate solution and the benzaldehyde layer separated was washed with deionized water. After drying on calcium chloride, benzaldehyde was distilled. All the other reagents used in the present work were of analytical grade.

Determination of protein concentration. The concentration of enzyme protein was determined spectrophotometrically by using the value, $E_{280, \text{cm}}^{1\%} = 19.67$.

Determination of nitrogen content. The nitrogen

1) R. L. Ory, R. H. Barker and G. J. Boundreaux, *Biochemistry*, **3**, 2013 (1964).

2) R. L. Ory, H. W. Kircher and A. M. Altshul, *Biochem. Biophys. Acta*, **147**, 200 (1967).

3) R. L. Ory, A. J. St. Angelo, Ines V. deGruy and A. M. Altshul, *Can. J. Biochem.*, **45**, 1445 (1967).

4) M. Funatsu, Y. Aizono, K. Hayashi, M. Watanabe and M. Eto, *Agr. Biol. Chem.*, **35**, 734 (1971).

5) K. Kusama, *J. Biochem.*, **44**, 375 (1957).

content of enzyme protein was determined with a Yanagimoto C. H. N. Corder Model MT-1 at the Service Center of the Elementary Analysis of Organic Compounds in Kyushu University.

Analysis of amino acid. Into a cleaned test tube 2 ml of an enzyme solution (enzyme protein 1.498 mg/ml) was taken and allowed to stand at 50°C under air stream until the solution became dry. The dried enzyme protein was hydrolyzed with 1.25 ml of 6 N HCl in an evacuated sealed glass tube at 105°C for 22, 46 and 70 hr, respectively. The hydrolyzate was rapidly dried up *in vacuo* and then dissolved in 2.2 ml of 0.2 N citrate buffer, pH 2.2. One ml of this solution was analyzed for amino acids with a Hitachi Amino Acid Autoanalyzer Model KLA-2. On the other hand, the tryptophan content of the enzyme protein was determined spectrophotometrically by Goodwin-Morton's method.⁶⁾

Amino-terminal amino acid analysis. Dinitrophenylation of the enzyme protein of 9.45 mg (0.236 μ moles) was carried out for 5 hr under the conditions reported by Sanger.⁷⁾ DNP-protein was completely collected as follows. After centrifugation of the reaction mixture, the precipitated DNP-protein was suspended in 10 ml of 60% ethylalcohol (v/v) and then dialyzed against running tap water for 54 hr with a Visking tube #18/32. On the other hand, FDNB and DNP in the supernatant was separated with diethylether four times and discarded. The resulting solution was dialyzed in the same way as described above. Both the dialyzates were combined in a glass tube and dried up at 40°C under air stream. The DNP-enzyme protein was hydrolyzed with 2.5 ml of 6 N HCl at 105°C for 14 hr in a sealed glass tube. The diethylether-soluble fraction of the hydrolyzate was dried with warm air stream and then identified by means of two-dimensional chromatography on Toyo Roshi No. 51 paper in an upper layer of *n*-butanol-0.2% ammonia water (1:1) and 1.5 M phosphate buffer at pH 6.0. Since separation of DNP-glutamic acid from DNP-aspartic acid was often incomplete in this solvent system, both the DNP-amino acids were identified by high voltage paper electrophoresis on Toyo Roshi No. 51 (10×60 cm) in pyridine-acetic acid buffer of

pH 3.6.⁸⁾ Electrophoresis was carried out for 50 min at 5000 V with a current of 12 mA. Each spot on a chromatogram was cut out, and DNP-derivatives were extracted with 6 ml of 1% sodium bicarbonate for 15 min at 55°C. The optical densities of the extracts at 360 $m\mu$ and 390 $m\mu$ were measured, respectively. The water-soluble fraction of the hydrolyzate was applied to a talc column and DNP-amino acids adsorbed on the column were eluted with an ethylalcohol-1 N HCl (4 v/1 v) mixture.⁹⁾ The eluate free from amino acids was dried up at 40°C under air stream, dissolved in a small amount of acetone and then applied to a paper. One-way ascending chromatography on Toyo Roshi No. 51 paper was carried out in *n*-butanol-acetic acid-water (4 v:1 v:5 v). After development, DNP-arginine was detected on the paper with the Sakaguchi spray reagent.¹⁰⁾

Carboxyl-terminal amino acid analysis. The enzyme protein of 6~7 mg dried *in vacuo* at 80°C was taken into a cleaned test tube and to this was added 0.4 ml of anhydrous hydrazine. Hydrazinolysis was conducted in an evacuated sealed glass tube at 105°C for 8 or 11 hr. The removal of excess hydrazine on concentrated sulfuric acid in a vacuum-desicator followed with benzaldehyde treatment to remove the hydrazides was carried out, and then the free amino acids obtained were dinitrophenylated and isolated according to Nui and Fraenkel-Conrat.¹¹⁾ The identification of the DNP-amino acids was qualitatively accomplished with two-dimensional chromatography on paper as described in this report. On the other hand, the quantitative determination of free neutral and acidic amino acids was performed with a Hitachi Amino Acid Autoanalyzer Model KLA-3.

Thin-layer chromatography. Lipid components in the enzyme protein were extracted with chloroform-methanol (2:1) and analyzed by thin-layer chromatography. Silicagel G thin-layer plates (thickness 0.3 mm) activated at 110°C for 1 hr before use were employed and one-dimensional development was carried out in petroleum ether-diethylether-acetic acid (82:18:1) or chloroform-methanol-water (70:30:

8) A. P. Ryle, F. Sanger, L. F. Smith and R. Kitai, *Biochem. J.*, **60**, 541 (1955).

9) F. Sanger, *ibid.*, **45**, 563 (1949).

10) R. Archer and C. Crocker, *Biochem. Biophys. Acta*, **9**, 704 (1952).

11) C. I. Nui and H. Fraenkel-Konrat, *J. Am. Chem. Soc.*, **77**, 5882 (1955).

6) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

7) F. Sanger, *ibid.*, **39**, 507 (1945).

4). The lipids were detected on a thin-layer plate in iodine vapor or with ninhydrin and Zinzadze's spray reagent.

Polyacrylamide gel disc electrophoresis. A lyophilized enzyme preparation was dissolved in 8 M urea or 8 M urea containing 5×10^{-3} M EDTA and then allowed to stand for 12 hr. After the treatments, the sample solutions were subjected to disc electrophoresis in 7.5% polyacrylamide gel. In this experiment, the procedures of Reisfeld *et al.*¹²⁾ were used except adding urea at 8 M to all stock solutions for the preparation of the gels and to 0.35 M β -alanine-acetate buffer, pH 4.5. Protein bands were detected by staining them with Amido Black 10 B, followed by removal of unbound dye with several changes of 7% acetic acid. Absorbancies of stained bands in gel at 616 m μ were recorded on a Toyo Densitrol equipped with a recording device.

Ampholine electrophoresis. A lyophilized enzyme protein (7.0 mg) was subjected to an ampholine electrophoresis in 0.8% carrier ampholytes (pH 3~10) medium containing 8 M urea and 1×10^{-3} M EDTA. The other procedures employed in the present work were the same those described in a preceding report except measuring of pH value at 8°C.⁴⁾

RESULTS AND DISCUSSION

Nitrogen content and amino acid composition

The nitrogen content of a higher plant is generally known to be 15.5~17.5%, while that of a purified rice bran lipase was actually 14.98%. The data of the amino acid analysis of the enzyme protein are given in Table I. In the last column of this table, the averaged values for stable amino acids and the extrapolated values for unstable amino acids were shown. The measured values of threonine, serine and methionine were plotted against time of hydrolysis and extrapolated to zero time to give the corrected values, because these amino acids were progressively decomposed during hydrolysis. The content of proline was determined according to the value

TABLE I. AMINO ACID ANALYSIS OF RICE BRAN LIPASE

The data are presented as μ g of amino acid per 1.362 mg of anhydrous protein.

Amino acid	Time of hydrolysis			Average or extrapolated value
	22 hr	46 hr	70 hr	
Lysine	36.787	37.941	39.755	39.735
Histidine	19.338	19.163	19.475	19.325
Arginine	59.510	59.510	59.822	59.614
Aspartic acid	134.543	135.463	135.924	135.924
Glutamic acid	102.779	102.779	102.262	102.607
Glycine	87.293	87.863	87.749	87.635
Alanine	86.434	86.363	86.648	86.482
Valine	52.839	54.425	54.425	54.425
Leucine	47.189	46.962	47.981	47.377
Isoleucine	25.122	26.255	28.064	28.064
Proline	61.962	61.476	61.185	61.962
Serine	83.161	81.420	76.022	89.693
Threonine	59.654	59.350	58.339	60.867
Cystine	55.796	56.463	54.684	55.648
Methionine	18.368	16.925	16.531	19.155
Tyrosine	95.786	96.439	95.133	95.786
Phenylalanine	81.749	83.611	81.935	82.432
Tryptophan				61.079
			Total	1187.810
			Recovery	87.13%

of a 22 hr hydrolysate, as this amino acid, in this case, rather decreased with hydrolysis time. On the contrary, since the peptide bonds involving valine and isoleucine are recognized to be hydrolyzed completely after longer hydrolysis, the values of 70 hr hydrolyzates were adopted for these amino acids. Hence lysine and aspartic acid, in this experiment, increased during hydrolysis, the values of 70 hr hydrolyzates were also taken. Tryptophan was determined by spectrophotometrically. The average values of other amino acids were also listed. The amino acid recovery of the enzyme protein was estimated to be 87.13%. From this experimental result and the nitrogen content described above, it was inferred that the enzyme protein was composed of amino acids and other substances.

¹²⁾ R. A. Reisfeld, U. J. Lewis and D. E. Williams, *Nature*, **195**, 281 (1962).

TABLE II. AMINO ACID COMPOSITION AND MOLECULAR WEIGHT OF RICE BRAN LIPASE

	Corrected value (amino acids μg per 1.362 mg of protein)	Minimum molecular weight	Number of residues		Calculated molecular weight
			Calculated	Integral	
Lysine	39.735	4393.6	8.8	9	39541
Histidine	19.325	9665.9	4.0	4	38664
Arginine	59.614	3568.6	10.8	11	39255
Aspartic acid	135.925	1153.2	33.5	34	39209
Glutamic acid	102.607	1714.0	22.6	23	39422
Glycine	87.635	886.7	43.6	44	39015
Alanine	86.482	1119.5	34.5	35	39118
Valine	54.425	2415.2	16.0	16	38643
Leucine	47.377	3253.2	11.8	12	39038
Isoleucine	28.064	5492.0	7.0	7	38444
Proline	61.962	2134.8	18.1	18	38426
Serine	89.693	1322.3	29.2	29	38347
Threonine	60.867	2262.7	17.1	17	38466
Cystine	55.648	5440.7	7.1	7	38085
Methionine	19.155	9328.9	4.1	4	37316
Tyrosine	95.786	2320.3	16.7	17	39445
Phenylalanine	82.432	2431.8	15.9	16	38909
Tryptophan	61.079	4152.5	9.3	9	37370
			Total 312	Average 38706	

The significance of this finding will be discussed below. The amino acid composition and the molecular weight are presented in Table II. Since the calculated molecular weight of histidine, 38,664, approximated to the molecular weight of 40,000 determined according to Archibald's method, the content of histidine was assumed to be 4 moles per mole of the enzyme. The value of 38,664 was divided by the minimum molecular weight of each amino acid residue to give the residual numbers as shown in the third column. The total number of amino acid residues in the enzyme was estimated to be 312. For the purpose of calculating the molecular weight, each minimum molecular weight was multiplied by the integer value of corresponding amino acid residues, and then the molecular weight was averaged to be around 38,700.

Lipid composition

The qualitative analysis of the lipids contained in the enzyme protein was carried out by using silicagel G thin-layer chromatography. As seen in Fig. 1, triglycerides and fatty acids were identified on a thin-layer chromatogram as main components. On the contrary, no phospholipids were detected with iodine vapor or Zinzadze's reagent. Spot 4 and spot 5 indicated in Fig. 1 were slightly visualized by spraying ninhydrin reagent, but the identification of this ninhydrin sensitive compound was not made due to an insufficient amount of the sample. As to the significant role of the lipids, Okuda *et al.*¹³⁾ suggested that rat liver esterase is partly converted into liver lipase by forming a complex with lipid,

13) H. Okuda and S. Fujii, *J. Biochem.*, **64**, 377 (1968).

FIG. 1. Slicagel G Thin-layer Chromatograms of Lipids in Rice Bran Lipase.

Solvent system: (A) Petroleum ether-ether-acetic acid (82:18:1). (B) Chloroform-methanol-water (70:30:4).

Spots: 1. Hydrocarbones and others. 2. Triglycerides. 3. Fatty acids. 4. Polar lipids (ninhydrin positive, Zinzadze negative). 5. Triglycerides, fatty acids and others (ninhydrin positive, Zinzadze negative).

FA: fatty acid, TG: triglyceride, CEP: cephalin, LEC: lecithin. Intensity of spots: ○ medium, ○ weak.

and School *et al.*¹⁴⁾ also reported that the porcine pancreatic lipase made a complex with lipid, consequently being stabilized and prevented from losing activity. In this rice bran lipase, however, the significance of the triglycerides and fatty acids remains obscure.

The amino- and carboxyl-terminal amino acid residues

For the quantitative analysis of the amino-terminal amino acid residue the enzyme protein was dinitrophenylated. As the results of two dimensional paper chromatography, DNP-glutamic acid or DNP-aspartic acid and two minor DNP-amino acids were found as

FIG. 2. Paper Chromatograms of DNP-Amino Acids in Determination of Amino-terminal Amino Acid of Rice Bran Lipase.

Intensity of spots: ○ medium, ○ weak.

FIG. 3. High-voltage Paper Electrophoresis in Determination of Amino-terminal Amino Acid of Rice Bran Lipase.

(a) DNP-glutamic acid, Sample. (b) DNP-glutamic acid, NDP-aspartic acid. (c) Sample.

shown in Fig. 2(A). DNP-arginine, however, was not detected on a paper with the Sakaguchi spray reagent as seen in Fig. 2(B). The amino-terminal residue was finally determined by means of high-voltage paper electrophoresis. As shown in Fig. 3, only DNP-glutamic acid was identified. The results of amino-terminal analysis was summarized in Table III. From these experimental data glutamic acid was concluded to be the amino-terminal amino acid of the enzyme protein. In order to determine the carboxyl-terminal amino acid of the enzyme, hydrazinolysis method was employed. The carboxyl-terminal amino acids liberated were first dinitrophenylated and then identified with two-dimensional

14) W. P. School and P. Melius, *Biochem. Biophys. Acta*, **187**, 186 (1969).

paper chromatography. DNP-serine and DNP-glycine were dominant spots as shown in Fig. 4. Since dinitrophenylated basic amino acids were not evidently detected, the quantitative analysis of the carboxyl-terminal amino acids were performed with an amino acid autoanalyzer. On the basis of the analytical data summarized in Table IV, it was concluded that serine and glycine were the carboxyl-terminal amino acids of the enzyme protein.

The data of the end-group amino acid analysis indicate that there exist one mole of glutamic acid as amino-terminal amino acid

TABLE III. AMINO-TERMINAL AMINO ACIDS ANALYSIS OF RICE BRAN LIPASE

DNP-amino acid	Total O.D. ₃₆₀	μ moles ^{a)} per 0.236 μ moles ^{b)} of protein	Yield (%)
DNP-Glu	4.11	0.236	100.0
(minor 1)	0.09	0.005	2.2
(minor 2)	0.10	0.006	2.4

^{a)} The molecular extinction coefficient of DNP-amino acids at 360 m μ is assumed to be 17.4×10^3 .

^{b)} Calculated from molecular weight 40,000.

FIG. 4. Paper Chromatogram of DNP-Amino Acids in Determination of Carboxyl-terminal Amino Acid of Rice Bran Lipase.

Intensity of spots: ○ medium, ○ weak.

TABLE IV. CARBOXYL-TERMINAL AMINO ACIDS ANALYSIS OF RICE BRAN LIPASE

Amino acid	moles/mole of protein ^{a)}	
	Hidrazinolysis 8 hr	Hidrazinolysis 11 hr
Serine	0.573	0.433
Glycine	0.493	0.428
Alanine	0.154	—

^{a)} The value of 40,000 was adopted for the molecular weight of the enzyme protein.

and 0.50 mole of serine and 0.47 mole of glycine as carboxyl-terminal amino acid per mole of the protein. Although these values are represented without correction, on consideration of the usual recovery of amino-terminal analysis, it seems reasonable to presume that two moles of glutamic acid are present per mole of the enzyme protein. It is possible to conclude from the results given above that the rice bran lipase is a protein molecule which consists of two types of subunits.

Dissociation of enzyme protein to subunits and determination of their isoelectric point

After the treatment of the lyophilized enzyme protein with 8M urea or 8M urea containing 5×10^{-3} M EDTA, each sample solution was subjected to polyacrylamide gel disc electrophoresis in the same concentration of urea or EDTA. Nontreated enzyme protein migrated as a single defined band in gel as reported in a preceding paper, whereas the enzyme protein migrated in 8M urea solution as three major bands and one minor band as seen in Fig. 5(A). On the other hand, as seen in Fig. 5(B), by adding 5×10^{-3} M EDTA to 8M urea, band 2 increased evidently and band 1 decreased on the contrary. Although the significance of this phenomenon is not yet understood, it appears to be reasonable to assume that the dissociation of subunits is brought about by 8M urea or 8M urea containing 5×10^{-3} M EDTA. For the determination of the isoelectric point of the subunits,

FIG. 5. Analytical Disc Electrophoresis of Rice Bran Lipase in 7.5% Polyacrylamide Gel at pH 4.5 with a Current of 3.0 mA/tube (0.5×7 cm) for 5 hr.

(A) 8 M urea was contained in gel. (B) 8 M urea and 5×10^{-3} M EDTA were contained in gel.

a lyophilized enzyme protein was dissolved in 8 M urea containing 1×10^{-3} M EDTA and then applied to ampholine electrophoresis. Three peaks were obtained at pH 6.9, 7.2 and 8.8, respectively on a pH-slope drawn by a dotted line in Fig. 6. Being compared with the isoelectric point of a native protein, 8.56, reported in the preceding paper, the peak with isoelectric point, 8.8 was assumed to be a native one. Such a difference appeared to be caused by the temperature of measurement, as reported with respect to the isoelectric points of Myoglobin II₁ and Myoglobin II₂ by Vesterberg.¹⁵⁾ Therefore, the enzyme protein was assumed to be composed of two

FIG. 6. Ampholine Electrophoresis of Rice Bran Lipase in 0.8% Carrier Ampholite (pH 3~10) Containing 8 M Urea and 1×10^{-3} M EDTA at 8°C for 50 hr.

types of subunits with isoelectric points of 6.9 and 7.2, respectively. The results of the experiments described above suggest the possibility that the subunits are held together by two kinds of interaction; one is hydrogen and hydrophobic bonds which can be split out by 8 M urea, the other, covalent bonds involving a certain metal ion which is removed with EDTA.

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15) O. Vesterberg and H. Svenson, *Acta Chem. Scand.*, **20**, 820 (1966).