Biochimica et Biophysica Acta, 384 (1975) 127—137

BBA 67418

PURIFICATION AND PROPERTIES OF A LIPID ACYL-HYDROLASE FROM POTATO TUBERS

OSAMU HIRAYAMA, HIDEYUKI MATSUDA, HIDEHUMI TAKEDA, KUNIKO MAENAKA and HARUO TAKATSUKA

College of Agriculture, Shimane University, Matsue, 690 (Japan)

(Received September 9th, 1974)

Summary

1. A pure lipid acyl-hydrolase was prepared from potato tubers by acetone precipitation, Sephadex G-100 and DEAE-Sephadex A-50 column chromatography, and by electrofocusing.

2. The purified enzyme was an acidic protein of pI 5.0 and molecular weigth of about 70 000. K_m values were 0.38 mM for monogalactosyldiacylglycerol and 1.7 mM for phosphatidylcholine.

3. The hydrolytic activity of the enzyme on different substrates was determined. The relative rates were acylsterylglucoside > monogalactosyldiacylglycerol > monogalactosylmonoacylglycerol > digalactosyldiacylglycerol > diagalactosylmonoacylglycerol, while the rates for phospholipids were lysophosphatidylcholine > phosphatidylcholine > lysophosphatidylethanolamine > phosphatidylethanolamine.

4. Analyses of enzymatic hydrolysis products suggested that a single enzyme had both galactolipase and phospholipase activities, and for the phospholipids it showed activities similar to phospholipase B* and glycerylphosphorylcholine diesterase.

5. A competitive relation was found between monogalactosyldiacylglycerol and phosphatidylcholine as substrates of the enzyme, indicating that the active sites for both substrates may be the same.

6. It was suggested that histidine and probably serine residues were important to the enzymic activity, and that a tyrosine residue might be involved in the activity as an accessory component.

Introduction

Recently our knowledge of lipases and phospholipases of animal and

* The term "phospholipase B" used in the report refers to enzyme activity that removes both fatty acids from a diacylphospholipid to form a glycerylphosphoryl residue.

microbial origins has increased rapidly. In higher plants, however, little is known about the enzymic deacylation of glycolipids and phospholipids. Sastry and Kates [1] have reported the presence of galactolipase activity in leaves of some *Phaseolus* species, and Helmsing [2] has purified a galactolipase from leaves of *Phaseolus multiforus*. Galliard [3] has partially purified from potato tubers a lipid acyl-hydrolase that catalyses the deacylation of galactolipids, phospholipids and partial glycerides. He further suggests that a single enzyme is responsible for the deacylation of several classes of lipids. If this is true, it would be the first lipid acyl-hydrolase enzyme with a variety of activities, and the structure and nature of such an enzyme would be very interesting. The present work was carried out to further purify and characterize the acyl-hydrolase. In this paper, the enzyme is purified to homogeneity, and data for physical properties, substrate specificity, mode of action and structure of active site are provided and discussed.

Materials and Methods

Plant materials. Potato tubers (*Solanum tuberosum* L. cultivar Benimaru) were grown under standard agricultural conditions in the farm of Shimane University.

Substrates. Mono- and di-galactosyldiacylglycerol, sulfoquinovosyldiacylglycerol, phosphatidylglycerol, diphosphatidylglycerol and phosphatidic acid were prepared from spinach leaf lipids [4]. Acylsterylglucoside and monogalactosyldiacylglycerol were isolated from wheat flower lipids; phosphatidylcholine was purified from egg lipids [5], and phosphatidylethanolamine and phosphatidylinositol were prepared from soybean lipids. Mono- and di-galactosylmonoacylglycerol were prepared from the corresponding diacyl lipids with steapsin [6], while lysophosphatidylcholine, lysophosphatidylethanolamine and lysophosphatidylglycerol were obtained from the corresponding diacyl phospholipids with snake venom phopholipase A_2, according to the method of Hanahan et al. [7].

Reagents. *N*-Bromosuccinimide, *N*-ethylmaleimide and ethoxyformic anhydride were purchased from Wako Pure Chemical Industries Ltd., *N*-acetylimidazole and *p*-chloromercuribenzoate from Nakarai Chemicals Ltd., and diisopropyl fluorophosphate from Kishida Chemicals Ltd. Dimethyl (2-hydroxy-5-nitro-benzyl) sulfonium bromide was obtained from Dozin Chemicals Ltd., and cyanuric fluoride from Eastman Kodak Company.

Enzyme assay. In the routine procedure, monogalactosyldiacylglycerol and phosphatidylcholine were used as substrates for galactolipase and phospholipase activity, respectively. The lipid substrates (50 μmol) were dispersed by ultrasonic treatments in 10 ml of aqueous media containing 0.1 mmol of sodium deoxycholate. To small test tubers, 0.2 ml of 0.5 M buffer, 0.1 ml of the lipid substrates (0.5 μmol) containing 1 μmol of deoxycholate, 0.1 ml of enzyme preparation and 0.6 ml water were added (total volume, 1 ml). The buffers used were citrate buffer (pH 5.0, 0.1 mM $CaCl_2$) for galactolipids, and Tris · HCl buffer (pH 8.5, 1 mM $CaCl_2$) for phospholipids. The mixture was incubated with shaking at 35°C for 20 min. After incubation, 6 ml of a solvent mixture of *n*-hexane/ethanol/1.5 M H_2SO_4 (400 : 200 : 1, v/v) were added,

and the mixture was vigorously shaken, and centrifuged. Free fatty acids in the upper hexane layer were determined by the rhodamine method as described previously [8]. The enzyme activity was expressed as μmol of fatty acid released per 20 min, after correction for enzyme blanks.

Gel filtration. The gel was prepared by swelling of sephadex G-100 in 5 mM Tris · HCl (pH 7.0) containing 1 mM $CaCl_2$. The slurry was poured into a column with inner dimension of 5 cm × 35 cm. Elution was carried out with 5 mM Tris · HCl (pH 7.0) containing 1 mM $CaCl_2$, and fractions of 10 ml were collected.

Ion-exchange chromatography. DEAE-sephadex A-50 was equilibrated with 5 mM Tris · HCl (pH 7.0, 1 mM $CaCl_2$). The slurry was poured into a column with inner dimension of 2.5 cm × 32 cm. Elution was carried out at 4° C with 5 mM Tris · HCl (pH 7.0, 1 mM $CaCl_2$), linear salt gradient 0.0—0.6 M NaCl in the same buffer and finally 0.25 M NaOH. Fractions of 10 ml were collected.

Polyacrylamide gel disc electrophoresis. The method of Ornstein [9] and Davis [10] was followed. Polyacrylamide gel (5% or 10%, w/v) of 0.7 cm × 7.0 cm without an upper gel were used. Runs were made at 4 °C with 25 mM Tris/glycine buffer (pH 8.5) at 3 mA per gel. Protein was detected by staining the gel with 1% amide black 10 B solution, and esterase activity in the gel by incubating the gel with 2-naphthyl acetate and Fast blue salt B [11].

Electrofocusing. Electrofocusing was carried out in the 110 ml apparatus of LKB, using a carrier ampholite (pH range, 3—8) at a final concentration of 0.5% and a stepwise sucrose gradient (0—50%). Electrophoresis was performed at 900 V for 48 h at 4° C. After electrophoresis, the content of the column was cut into 3 ml fraction, followed by measuring pH, absorbance at 280 nm and enzymic activity.

Determination of molecular weight. A microcolumn of sephadex G-100 (inner dimension, 0.9 cm × 22 cm) was calibrated by applying a mixture of proteins of known molecular weights and developing with 5 mM Tris · HCl (pH 8.0, 1 mM NaCl). Eluates were fractionated into 0.5 ml, and analyzed.

Thin-layer chromatography. Thin-layer chromatography was performed on silica gel plates of 20 cm × 17 cm. The plates were developed with chloroform/methanol/acetic acid/water (70 : 20 : 2 : 2, v/v) as the first solvent, and with *n*-hexane/diethyl ether/acetic acid (90 : 10 : 1, v/v) as the second solvent, using a stepwise development technique [12,13]. The lipids separated were detected with color reagents, α-naphthol/H_2SO_4 (for glycolipids), molybdenum blue reagent (for phospholipids) and 50% H_2SO_4 (for all lipid components).

Photooxidation. The procedure was based on the method described by Ray and Koshland [14]. Reaction mixture (total volume, 1 ml) contained 50 mM Tris · HCl (pH 8.0), 0.01% methylene blue and the enzyme protein. The mixture was flushed with pure oxygen, and illuminated at 50 000 lux maintaining the mixture at 13° C. After illumination, the mixture was submitted to enzyme assay.

Analytical method. Organic and inorganic phosphorus were determined by the method of Allen [15] and by modification [16] of Allen's procedure, respectively. Total sugar was estimated by the method of Dubois et al. [17],

free reducing sugar by the method of Somogyi [18], and protein by the method of Lowry modified by Hartree [19]. Choline was determined by an improved method using cobalt sulfate and potassium ferrocyanide as color reagents (Hirayama, O. and Matsuda, H., unpublished).

Results

Enzyme isolation and purification

Enzyme extraction was carried out by a simplified modification of the method of Galliard [3]. Potato tubers were peeled, diced and homogenized with 2 mM sodium metabisulfite. The homogenate was filtered through nylon cloth, centrifuged at 15 000 × *g* for 60 min, and to the supernatant 4 vols of acetone at −25°C were added. The precipitate was collected by centrifugation, and washed with dry acetone and diethyl ether. The resulting acetone powder was then extracted with 5 mM Tris · HCl (pH 7.0), and the extracts were dialyzed against the same buffer to give a crude enzyme solution.

The crude enzyme solution was applied to a column (5 cm × 35 cm) of sephadex G-100, and elution with 5 mM Tris · HCl (pH 7.0, 1 mM $CaCl_2$) resulted in three peaks of protein (molecular weight about 80 000 or less), the first peak (the largest molecular weight) of which indicated enzymic activity. The active fractions were collected and concentrated, and then separated by a column (2.5 cm × 32 cm) of DEAE-sephadex A-50 as described in methods. In both the column chromatographies, galactolipase and phospholipase activities were eluted in one peak. The active fractions were dialyzed and concentrated, and then further purified by electrofocusing according to the procedure described in Methods. The electrofocusing pattern indicated one protein peak at pH 5.0 (pI 5.0) coinciding with both galactolipase and phospholipase activities. The active fractions were pooled, dialyzed, and used for subsequent analyses as a purified enzyme preparation.

The results in each step of purification are summarized in Table I. The ratios of both galactolipase and phospholipase activities in each enzyme preparation were similar through all steps of purification. The sequence of the purification procedures effected 380- and 350-fold increase in specific activity of galactolipase and phospholipase, respectively.

Homogeneity and properties of the purified enzyme

The homogeneity of the purified enzyme preparation, which appeared to

TABLE I

PURIFICATION OF LIPID ACYL-HYDROLASE FROM POTATO TUBERS

Stages	Total protein (mg)	Specific activity (μmol/20min/mg protein)	
		Galactolipase	Phospholipase
Acetone precipitate	2300	—	—
Crude extracts	432	0.45	0.46
Sephadex G-100 column	81	6.82	5.84
DEAE-sephadex A-50 column	13	102.00	97.20
Isoelectric focusing	5	170.00	162.00

be homogeneous by criterion of electrofocusing pattern, was substantiated by the following physical tests.

(1) Polyacrylamide gel disc electrophoresis. When the purified enzyme was subjected to disc electrophoresis in 10% polyacrylamide gel at pH 8.5, it migrated as one defined band, coinciding with the band stained with amide black and the band detected by 2-naphthyl acetate and Fast blue salt B.

(2) Sedimentation analysis. The purified enzyme solution (0.51% of protein) was ultracentrifuged with a Hitachi 282 analytical ultracentrifuge equipped with a Schlieren optical system, operating at 60 000 rev./min at 20°C. The enzyme preparation was homogeneous with a symmetric sedimenting boundary.

(3) pH dependence. The effects of pH on the rates of enzymatic hydrolysis of monogalactosyldiacylglycerol and phosphatidylcholine were studied. For monogalactosyldiacylglycerol the optimum pH was 5.0 in citrate buffer, whereas for phosphatidylcholine it was 8.5 in Tris · HCl buffer, although the pH curves showed differing maxima depending on the buffers used.

(4) Dependence of substrate concentration. The effects of substrate concentration on the rate of hydrolysis were investigated. The results are presented in Fig. 1. It was suggested that with monogalactosyldiacylglycerol a stepwise formation of molecular aggregates and micelles occurred with increase of its concentration, and the enzymic activity increased with progress of the substrate aggregation. A similar relationship was also observed with phosphatidylcholine. Extrapolation of the linear potions of the Lineweaver—Burk plots was made in the region where soluble monomeric substrate predominated. The K_m

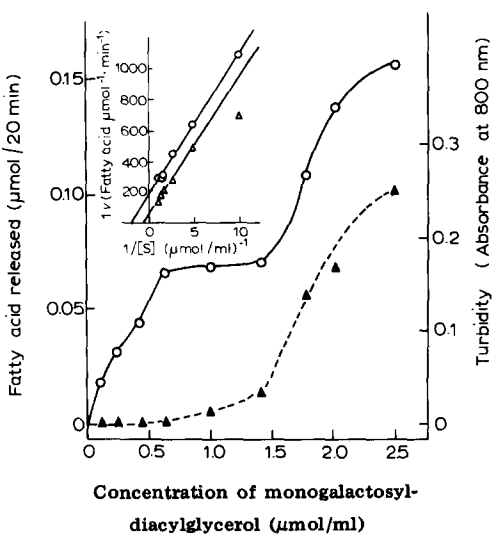

Fig. 1. Dependence of substrate concentration. Reaction mixtures (1 ml) contained 0.1—2.5 μmol of substrates, 0.2—5.0 μmol of deoxycholate, 0.1 ml of the enzyme preparation and 0.2 ml of buffer. The buffers used were 0.5 M citrate buffer (pH 5.0, 0.1 mM $CaCl_2$) for monogalactosyldiacylglycerol, and 0.5 M Tris · HCl buffer (pH 8.5, 1 ml $CaCl_2$) for phosphatidylcholine. Incubation at 35°C for 20 min. Lineweaver—Burk plots of these data 0.1—1.0 μmol/ml for [S] were presented in upper left corner. ○———○, monogalactosyldiacylglycerol; △———△, phosphatidylcholine; ▲- - - - -▲, turbidity (Absorbance at 800 nm) of monogalactosyldiacylglycerol solution in the reaction mixture without the enzyme preparation.

values obtained were 0.38 mM for monogalactosyldiacylglycerol and 1.7 mM for phosphatidylcholine.

(5) Molecular weight. The approximate molecular weight of the purified enzyme was determined by means of a microcolumn of Sephadex G-100 as described in Methods. A linear relationship was obtained between elution volume and log (molecular weight) for the following: cytochrome *C*, mol. wt. 12 500; chymotrypsinogen A, mol. wt. 25 000; bovine serum albumin, mol. wt. 67 000; aldolase from rabbit muscle, mol. wt. 158 000. The elution volume of the enzyme corresponded to that of 70 000.

Substrate specificity

The purified enzyme was tested for activity toward the various substrates shown in Table II. The enzyme exhibited a hydrolyzing activity for all lipid components of glyco- and phospholipids studied. The hydrolysis rates of monogalactolipids were higher than those of digalactolipids, and monoacylgalactosylglycerols were hydrolyzed at slower rate than the corresponding diacyl lipids. Acylsterylglucoside showed the highest hydrolysis rate of the lipids studied, and sulfolipid was also shown to be attacked. On the other hand, monoacylphospholipids such as lysophosphatidylcholine and lysophosphatidylethanolamine demonstrated much higher hydrolysis rates than those of the corresponding diacyl lipids. In general, the lysophospholipase reported [20,21] were markedly inhibited by sodium deoxycholate. However, the present lysophospholipase activity of the enzyme was not affected by sodium deoxycholate, indicating a different property of the activity from those previously reported.

Mode of action

Both monogalactosyldiacylglycerol and phosphatidylcholine were incu-

TABLE II

SUBSTRATE SPECIFICITY OF THE PURIFIED ENZYME

Incubation mixtures contained 0.5 μmol of substrate, 1 μmol of deoxycholate and 0.2 ml of buffer. The buffers used were 0.5 M citrate buffer (pH 5.0, 0.1 mM $CaCl_2$) for glycolipids, and 0.5 M Tris · HCl buffer (pH 8.5, 1 mM $CaCl_2$) for phospholipids. Total volume, 1 ml; incubation at 35°C for 20 min. Hydrolysis rates of glyco- and phospholipids were expressed as percentages of those of monogalactosyldiacylglycerol and phosphatidylcholine, respectively.

Substrates	Hydrolysis rates
Monogalactosyldiacylglycerol	100
Monogalactosylmonoacylglycerol	68
Digalactosyldiacylglycerol	62
Digalactosylmonoacylglycerol	26
Acylsterylglucoside	500
Sulfoquinovosyldiacylglycerol	52
Phosphatidylcholine	100
Lysophosphatidylcholine	304
Phosphatidylethanolamine	33
Lysophosphatidylethanolamine	71
Phosphatidylglycerol	151
Lysophosphatidylglycerol	20
Phosphatidic acid	16
Phosphatidylinositol	36

bated with the purified enzyme for 200 min, and the resulting products were followed. In monogalactosyldiacylglycerol, free fatty acids and water-soluble bound galactose were rapidly released with incubation time. The rate of release of fatty acids on a molar basis was about twice that of release of the water-soluble bound galactose, which was identified to be galactosylglycerol by paper chromatography. A very small amount of free galactose was released in the later stage of incubation. On the other hand, coline, water-soluble phosphorus and free fatty acids were released from phosphatidylcholine by the enzyme. The water-soluble phosphorous was identified as glycerophosphate by paper chromatography. No inorganic phosphorous was detected through the incubation.

For further investigation of hydrolysis products, the reaction products were extracted with chloroform, and applied to thin-layer chromatographic analysis. The chloroform-soluble products from galactolipids were isolated by preparative thin-layer chromatography, and identified according to the method by combination of thin-layer and gas-liquid chromatography [12]. The results indicated that monogalactolipid formed lyso-compound and an acylated monogalactolipid as well as free fatty acids while digalactolipid produced an acylated monogalactolipid and fatty acids. Gas-chromatographic analyses also showed that the fatty acids released from the wheat monogalactolipid by the enzyme were similar to those at the 1-position of the original lipid*, suggesting a tendency that the enzyme attacks the fatty acid esters at the 1-position preferentially to those at the 2-position of the monogalactolipid. On the other hand, phosphatidylcholine was found to release only free fatty acids as the chloroform-soluble reaction products, and lyso-compound could not be detected.

On the basis of the results, modes of action of the enzyme for galacto- and phospholipids were summarized as indicated in Fig. 2. It was assumed that for galactolipid the enzyme had a higher catalytic activity for deacylation and an additional activity for transformation of galactolipids [22]. And for phospholipids, the enzyme appeared to show actions similar to those of phospholipase B and glycerylphosphorylcholine diesterase.

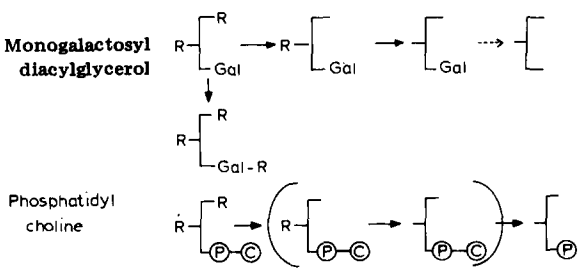

Fig. 2. Modes of action to monogalactosyldiacylglycerol and phosphatidylcholine, R, fatty acid; Gal, galactose; P, phosphate; C, choline. Reaction rate: →, higher; →, ordinary; →, lower.

* The positional distribution of fatty acids in the wheat monogalactosyldiacylglycerol was analyzed with steapsin according to the method of Noda and Fujiwara [6].

Examination of a competitive relation between galactolipids and phospholipids as substrates for the enzyme

As shown in the results, the lipid acyl-hydrolase had activity for both galactolipids and phospholipids. Thus, it is of interest whether the active sites for galactolipids and phospholipids are the same or not. For elucidation of the problem, monogalactosyldiacylglycerol and phosphatidylcholine were incubated with the purified enzyme, in separate solutions of each substrate and in a mixed solution containing both substrates, in concentrations as indicated in Fig. 3. The total amounts of fatty acids released from each substrate (0.75 μmol) were compared with those from the mixture of both substrates (0.75 μmol in total). The results were summarized in Fig. 3. It was found that the total fatty acids released from monogalactolipid and phosphatidylcholine (in separate incubations) were always much higher than those from their mixtures. The data suggest that there is a competitive relation between galactolipids and phospholipids as substrates for the enzyme, thus the active sites for these lipids may be the same.

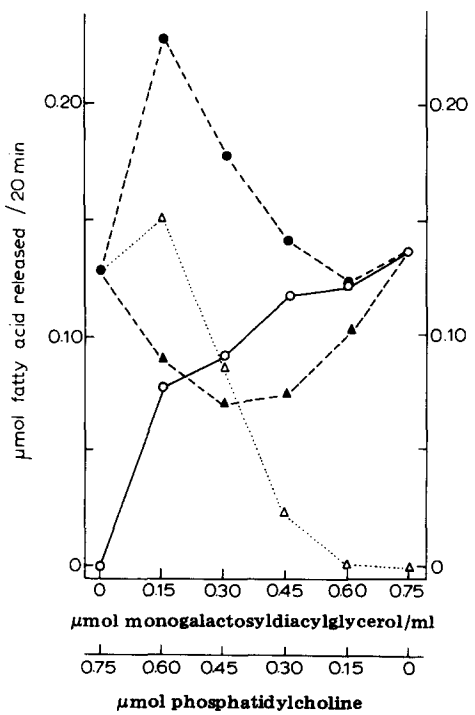

Fig. 3. Examination of a competitive relationship between monogalactosyldiacylglycerol and phosphatidylcholine as substrates for the purified enzyme. Reaction mixtures (1 ml) contained 0—0.75 μmol of monogalactosyldiacylglycerol, 0—0.75 μmol of phosphatidylcholine or mixture (0.75 μmol) of both the substrates with deoxycholate at twice the quantity of substrate in 0.1 M phosphate buffer (pH 6.0). Incubations were carried out at 35°C for 20 min. Fatty acids released were determined by the procedure as described in enzyme assay: ○———○, from monogalactosyldiacylglycerol; △· · · · · △, from phosphatidylcholine; ▲- - - - - ▲, from mixture of both the substrates; ●- - - - - ●, total from monogalactosyldiacylglycerol and phosphatidylcholine.

Examination of active site

The purified enzyme protein was treated with the reagents for chemical modification in conditions as indicated in Table III, according to methods described in references. The mixtures were passed through a Sephadex G-25 column (1.5 cm × 15 cm) to remove excess reagents, and subjected to enzyme assays. The results were summarized in Table III. There were no significant effects on the enzymic activity when treated with dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide, *N*-bromosuccinimide, *p*-chloromercuribenzoate and *N*-ethylmaleimide. These suggest that tryptophan residue and sulfohydroxyl group are not involved in the active site. A large molar excess of *N*-acetylimidazole did lead to a loss of enzymic activity, although subseqent treatment with hydroxylamine, which regenerate tyrosine, did not restore activity. Cyanuric fluoride also showed a marked inhibition for the activity. Thus, it may be assumed that tyrosine residue operates on catalysis with an accessory role. Diisopropyl fluorophosphate caused almost complete inactivation, suggesting that a serine residue is essential to activity. Similarly, ethoxyformic anhydride indicated a complete loss of activity, the subsequent treatment with hydroxylamine, which regenerates histidine, restoring activity to over 85% of the origi-

TABLE III

EFFECTS ON ENZYMATIC ACTIVITY OF CHEMICAL MODIFICATIONS

The purified enzyme protein was treated with the reagents for chemical modification in conditions as indicated in references. The mixtures were then passed through a Sephadex G-25 column to remove excess reagents, and subjected to enzyme assays. DM-HNBSB = dimethyl(2-hydroxy-5-nitrobenzyl)-sulfonium bromide.

Reagents	Reacting residues	Conditions		% activity remaining		Reference
		pH	Concentration	Galacto-lipase	Phospho-lipase	
Control				100	100	
DM-HNBSB	Trp	5.0	100-fold mol	97	104	23
		5.0	750-fold mol	99	93	
N-Bromosuccinimide	Trp > Tyr	5.0	5-fold mol	100	96	24
		5.0	10-fold mol	96	94	
p-Chloromercuribenzoate	-SH	5.0	50-fold mol	100	102	25
		5.0	135-fold mol	108	94	
N-Ethylmaleimide	-SH	7.0	30 mM	80	72	26
		7.0	60 mM	61	70	
N-Acetylimidazole	Tyr,	7.5	30-fold mol	70	92	24
	-SH > $-NH_2$	7.5	465-fold mol	52	92	
		7.5	180000-fold mol	0	1	
Cyanuric fluoride	Tyr	9.0	7 mM	84	89	27
		9.0	74 mM	7	2	
		9.0	741 mM	2	2	
Diisopropyl fluorophosphate	Ser, Tyr	7.5	30 mM	0	7	28
		7.5	60 mM	0	4	
Ethoxyformic anhydride	His > Lys, Ser	6.0	512-fold mol	91	87	24
		6.0	5120-fold mol	0	4	
		6.0	1000-fold mol*	0	0	

* Ethoxyformic anhydride was mixed in the substrates before incubation.

nal activity. These results suggest that a histidine residue is important to enzymic activity. Additional support for this suggestion was obtained when photooxidation of the enzyme was carried out at pH 8.7. With illumination, the activities decreased rapidly, although lipase and phospholipase from rice bran were not affected significantly under the same condition.

Discussion

A lipid acyl-hydrolase from potato tubers was extracted, and purified to a homogeneous protein by criteria of electrofocusing, disc electrophoresis and sedimentation analysis. It was found that the purified enzyme was an acidic protein of pI 5.0 and molecular weight about 70 000. The procedure of enzyme isolation and purification included an extraction with 2 mM sodium metabisulfite, acetone precipitation and chromatographic procedures. Tentatively, a simplified purification was carried out which included an extraction with 5 mM Tris · HCl (pH 7.0, 1 mM $CaCl_2$) instead of 2 mM sodium metabisulfite and omitted acetone precipitation. In this case an enzyme of characteristics similar to those of the enzyme prepared above was obtained. This suggests that lipid components did not have a significant effect on the nature of the enzyme, delipidation treatment giving only a slightly lower value of molecular weight. In previous paper [3] Galliard has reported 107 000 of molecular weight for lipid acyl-hydrolase partially purified from *Solannum tuberosum* var. Majestic. The difference might be interpreted as being due to the variety and the degree of purification.

As shown above, the purified acyl-hydrolase from potato tubers clearly exhibited a variety of activities for glyco- and phospholipids, indicating that a single enzyme protein was responsible for these activities. This was also supported by the fact that activity ratio of galactolipase to phospholipase was essentially constant through all purification steps. What enzyme structure is required to exhibit many sorts of activities? As one speculation, we can suppose the presence of more than two active sites in the enzyme protein molecule. However, Fig. 3 showed that there was a competitive relationship between monogalactolipid and phosphatidylcholine as substrates for the enzyme, suggesting the active site to be the same for both substrates. Additional evidence for this suggestion was also indicated in the results of chemical modifications as shown in Table III, in which enzymic activities for both galactolipid and phospholipid were changed at similar rates by many chemical modifications. Thus, a convincing interpretation for the data would be that the enzyme was an oligomeric enzyme containing one active site, and could behave as indicated by the induced fit theory. The presence of subunits in the enzyme protein has been confirmed, and the data will be reported.

Experimental data of chemical modifications and photooxidation showed that histidine and probably serine residues were essential to the enzymic activity, and a tyrosine residue might be involved in catalysis as an accessory residue. Studies are now under way to elucidate the structure of the active site. Detailed data will be available in the near future.

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