Isolation and Characterization of an Extracellular Heat-Stable Lipase Produced by *Pseudomonas fluorescens* MC50

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The extracellular, heat-stable lipase produced by *Pseudomonas fluorescens* MC50 was purified by successive gel chromatography on Sephacryl S-500 and Sephadex G-200. The purified enzyme was homogeneous as judged by gel electrophoretic analyses and exhibited characteristics of a true lipase. Substrates required emulsification to obtain activity; trioctanoin appeared to be best synthetic triacylglycerol and coconut oil was the best natural substrate. The activity showed an alkaline pH optimum and was inhibited by treatment with EDTA and partially inhibited by reagents specific for reaction with sulfhydryl groups. Comparison of the amino acid composition with that of pancreatic lipase indicated a relatedness ($S\Delta Q = 90$) of the two enzymes, suggesting possible similar functioning. A subunit weight of 55 000 was estimated by gel electrophoresis in sodium dodecyl sulfate. The hydrodynamic molecular radius obtained by gel chromatography and gel electrophoresis in Triton X-100 was 40 Å.

Thermostable proteases and lipases that are produced by psychrotrophic bacteria are a major concern during storage of heat-processed, commercially sterile foods (Speck and Adams, 1976; Cogan, 1977; Adams and Brawley 1981a,b; Morttar, 1981). Since *Pseudomonas fluorescens* is the most common psychrotroph isolated from milk (Law, 1979), enzymes produced by this organism could have significant effects on milk and derived products. Thus, the presence of lipase activity associated with this microorganism has been correlated with development of rancid flavor (Andersson et al., 1981). The heat stability of the lipase activity is such that it can survive heat treatments commonly used for commercial sterilization (Driessen and Stadhouders, 1974; Andersson et al., 1979).

P. fluorescens MC50 has also been shown to produce a heat-stable extracellular lipase (Adams and Brawley, 1981a). However, neither this enzyme nor any of the other extracellular lipases have been purified or characterized in detail. This paper describes the purification and some of the kinetic and molecular properties of the extracellular lipase, triacylglycerol acylhydrolase (EC 3.1.1.3), produced by MC50.

EXPERIMENTAL SECTION

Production and Purification of MC50 Lipase. *P. fluorescens* MC50 was isolated originally from raw milk (Adams and Brawley, 1981a). Freeze-dried MC50 was initially subcultured in 10 mL of reconstituted 10% nonfat dry milk in screw-cap tubes by incubation for 24 h at room temperature. Lipase was produced by inoculation of 1.0 mL of this culture into a 250-mL Erlenmeyer flask containing 100 mL of autoclaved (121 °C for 15 min) reconstituted nonfat dry milk and incubated statically at room temperature for 4 days. Insoluble milk solids and cells were then removed by centrifugation at 12000*g* for 20 min. The supernatant fraction was concentrated 10-fold by ultrafiltration using a membrane with a nominal exclusion limit of 100 000 (Amicon Corp., Danvers, MA) and diafiltered against 5 volumes of 10 mM sodium citrate buffer (pH 8.5) by using the same ultrafiltration unit (Amicon, Model LP-1) and a membrane with a nominal exclusion limit of 10 000. The diafiltered concentrate was diluted 10-fold with the citrate buffer, filter sterilized by passage through a 0.45-μm filter (Millipore Corp., Bedford, MA), and lyophilized.

Crude lyophilized lipase was dissolved in the citrate buffer to give approximately 10 units of activity/mL (~30 mg/mL) and chromatographed on Sepharose 6B (Pharmacia, Piscataway, NJ). The column (2.5 cm × 40 cm) was maintained at 4 °C and eluted at a flow rate of 6.1 mL h^{-1} cm^{-2}. Alternatively, the crude lipase was chromatographed on Sephacryl S-500 (Pharmacia) by using a 0.9 cm × 30 cm column and a flow rate of 31.4 mL h^{-1} cm^{-2}. Final purification was accomplished by chromatography of the pooled active fraction from Sephacryl S-500 on Sephadex G-200 (Pharmacia) using a 2.6 cm × 40 cm column and a flow rate of 3.8 mL h^{-1} cm^{-2}.

Enzyme Assay. Lipolytic activity was measured by a pH-stat method (Parry et al., 1966) using an automatic titration system (Sargent-Welch, Skokie, IL). The titrant used was 0.025 N NaOH. A standard emulsion was prepared by mixing 38 mL of H_2O, 2.5 mL of corn oil, 9 mL of 10% gum arabic (Fisher Scientific), 1 mL of 2% sodium deoxycholate, and 0.1 mL of 20% $CaCl_2$ in a Waring blender for 3 min, followed by five passages through a hand homogenizer (C. W. Logeman Co., American Scientific Products). Emulsions of other substrate lipids (triacylglycerols from Eastman, Rochester, NY; coconut oil from Fisher Scientific; olive oil and milk fat from Land O'Lakes, Minneapolis, MN; monolaurin, monocaprylic, and linoleic acids from Sigma Chemical Co., St. Louis, MO) were prepared in a similar manner by substitution for the corn oil. In the standard assay, 1 mL of enzyme solution was added to 5 mL of this emulsion and the mixture maintained at 40.5 °C and pH 8.5. To evaluate the effects of pH, temperature, and substrate, the unchanging variables remained at the standard values. The initial slope was taken as a measure of activity. Lipase activity (units) was defined as the milliequivalents of alkali added per minute per milliliter of enzyme solution. All assays were performed in duplicate.

Determination of Stokes Radius. The Stokes radius was determined by gel chromatography using a column (0.9 cm × 25 cm) of Sephadex G-200 equilibrated with 10 mM citrate buffer (pH 8.5) containing 0.1% Triton X-100. Gel characteristics were calibrated according to Ackers (1967) by using as protein standards bovine serum albumin (35.5 Å), hen egg ovalbumin (30.5 Å), and rabbit muscle aldolase (48.1 Å). Fractions containing 0.5 mL of volume were collected, and the elution position was determined from the protein content and activity by weighing the contents of previously tared tubes.

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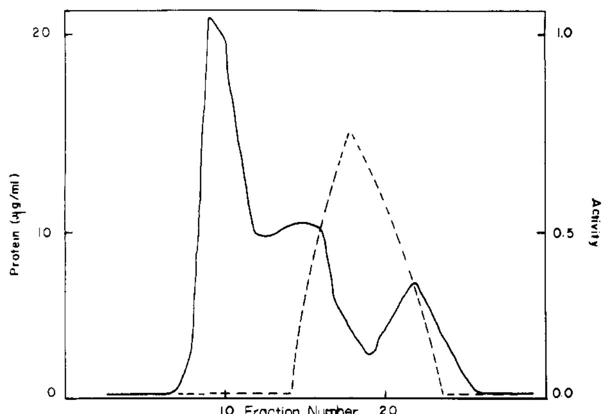

Figure 1. Gel chromatography of crude *P. fluorescens* MC50 lipase on Sepharose 6B. A 2.5 cm × 40 cm column maintained at 4 °C was eluted with a flow rate of 30 mL/h. 4-mL fractions were collected. (—) Protein concentration determined by the dye-binding assay; (---) activity units, mmol min⁻ mL^{-1}.

The molecular size was also estimated by using a Ferguson plot analysis (Ferguson, 1964; Chrambach et al., 1976) of gel electrophoretic data. Disc gels for protein resolution were prepared from acrylamide, *N,N'*-methylenebis(acrylamide), and *N,N,N',N'*-tetramethylenediamine (Bio-Rad Laboratories, Richmond, CA) in Tris (Trizma, Sigma Chemical Co., St. Louis, MO) buffer at pH 8.5. The method of gel preparation and the discontinuous buffer system were those described by Davis (1964). Also the method of polymerization and the gel apparatus have been previously described (Davis, 1964). Lyophilized enzyme was dissolved and equilibrated in 0.1% Triton X-100 for 1 h prior to electrophoresis. Lipase activity in the gel was located by incubation of 0.3-cm gel slices in tributyrin agar overnight at 35 °C.

Subunit Weight Estimation. The subunit molecular weight was estimated by gel electrophoresis in 0.1% sodium dodecyl sulfate as described by Weber et al. (1972).

Amino Acid Analysis. The enzyme preparation was dried and weighed directly in Pyrex hydrolysis vials. Following addition of 6 N HCl, the contents were evacuated, sealed, and hydrolyzed by heating in a forced-air oven at 145 °C for 2 h (Young, 1978). The analyses were performed using a Durrum Model D-500 analyzer (Dionix Corp., Sunnyvale, CA).

Protein Concentration. Protein was estimated spectrophotometrically by using the dye-binding assay described by Bradford (1976). A standard kit obtained from Bio-Rad Laboratories was used for these determinations.

RESULTS

Isolation of the Enzyme. The highest production of lipase was observed when *P. fluorescens* MC50 was grown in 10% nonfat dry milk. Concentration and diafiltration of the cell-free preparation followed by chromatography on Sepharose 6B resulted in a 500-fold purification of the lipase with a 78% yield (Figure 1). This preparation was used for studies of the activity characteristics. A similar elution profile and purification were obtained by chromatography on Sephacryl S-500, and these preparations were used for further purification. Fractions having the highest enzymic activity were pooled, concentrated by ultrafiltration, and chromatographed on Sephadex G-200, resulting in complete resolution of active and inactive proteins (Figure 2). Gel electrophoresis of the enzyme obtained gave a single protein-staining band with lipase activity. These preparations were used for studies of the molecular characteristics.

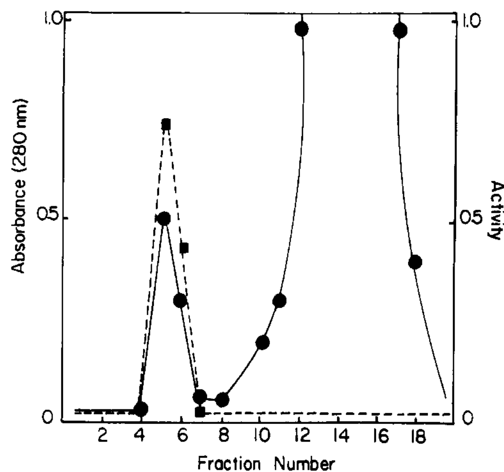

Figure 2. Gel chromatography using Sephadex G-200 of *P. fluorencens* MC50 lipase partially purified by elution from Sephacryl S-500. A 2.6 cm × 40 cm column of Sephadex G-200 maintained at room temperature was eluted with a flow rate of 20 mL/h. 2-mL fractions were collected. Absorbance at 280 nm (●); lipase activity, mmol min^{-1} mL^{-1} (■).

Table I. Hydrolysis of Various Substrates by MC50 Lipase[a]

substrate	% relative activity[b]
butter oil	108
olive oil	113
coconut oil	116
corn oil	100
trilaurin	61
monolaurin	17
Tween 20	26
Tween 80	11
monocaprylic acid	24
linoleic acid methyl ester (emulsified)	28
linoleic acid methyl ester (in solution)	0

[a] The temperature and the pH of the assay were 41 °C and 8.5, respectively. [b] Activity for corn oil was taken as 100%.

Characteristics of the Catalytic Activity. The ability of the enzyme to hydrolyze a variety of lipid substrates, triacylglycerols, monacylglycerols, fatty acid esters, and natural lipids, was examined and the results are listed in Table I. Comparison of hydrolysis of trilaurin to that of monolaurin indicated that activity on monoacylglycerols is substantially less. Thus, as characteristic of true lipases, the activity was greater for triacylglycerols. Also, the ester of linoleic acid was hydrolyzed only in the emulsified form. No activity could be detected for the soluble form. Comparison of the rates of hydrolysis of synthetic triacylglycerols of varying acyl chain length from C_6 to C_{16} indicated that trioctanoin was hydrolyzed most rapidly (Figure 3). This observation is consistent with the data in Table I showing that coconut oil, which has the highest content of saturated C_8–C_{10} fatty acids, was hydrolyzed more rapidly than the other natural lipids.

The optimum pH for activity was between 8.0 and 9.0 for all the triacylglycerols tested (Figure 4). More than 40% of the optimal activity was observed throughout the pH range from 7.0 to 9.0. MC50 lipase also exhibited complete stability in the pH range between 6.0 and 9.0; however, in the extreme pH regions irreversible loss of activity occurred. The optimum temperature was examined for these synthetic triacylglycerols and found to be

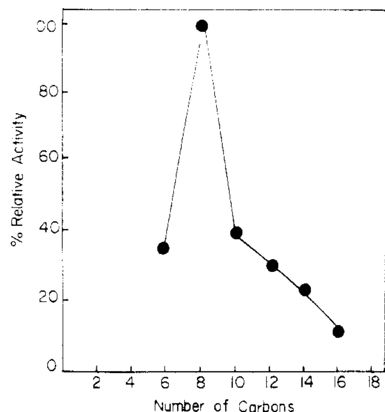

Figure 3. Hydrolysis of synthetic triacylglycerols catalyzed by MC50 lipase. Activity was measured on emulsions maintained at 41 °C and pH 8.5.

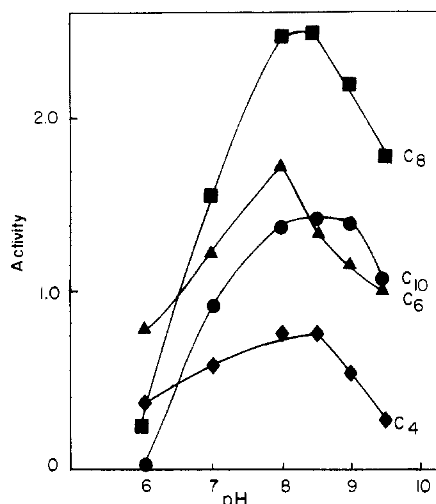

Figure 4. Effect of pH on the activity of MC50 lipase by using various synthetic triacylglycerol substrates. Activity (mmol min^{-1} mL^{-1}) was measured on emulsions maintained at 41 °C. (♦) Tributyrin; (▲) trihexanoin; (■) trioctanoin; (●) tridecanoin.

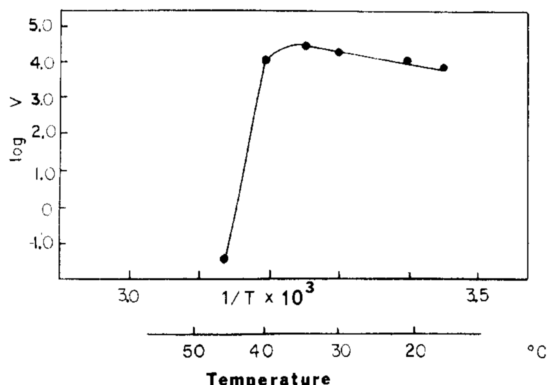

Figure 5. Arrhenius plot of the data for MC50 lipolytic activity by using tributyrin as the substrate between 15 and 50 °C. Activity was assayed at pH 8.5.

between 30 and 40 °C. An Arrhenius plot of the data for tributyrin showed that inactivation occurred above 40 °C (Figure 5). Therefore, it appears that, although the enzyme is stable to irreversible thermal denaturation at high temperatures (Swaisgood and Bozoğlu, 1984), the activity is reversibly lost above 40–50 °C. The activation energy for tributyrin hydrolysis was 1175 cal/mol.

Previous investigations [e.g., see Finkelstein et al. (1970)] have used chemical inhibitors and activators to distinguish the activities of various lipases, lipoprotein lipases, and esterases. The effect of various inhibitors and alkylating reagents on the enzymic activity is listed in Table II. The enzyme was not inhibited by NaCl or heparin as is lipoprotein lipase; nor was the enzyme completely inhibited by eserine or NaF, which are potent inhibitors of esterases at low concentrations [see Finkelstein et al. (1970)]. In addition to these treatments, loss of activity was observed upon exposure to EDTA (treatment with 0.1 M EDTA caused complete loss of activity). Although more than 50% of the activity was restored by addition of Ca^{2+}, nearly complete reactivation was observed following addition of skim milk (Table III). Treatment with *o*-phenanthroline, which specifically chelates Zn^{2+}, however, had no effect on the activity even at concentrations of 0.1 M. Moreover, enzyme partially inactivated by removal of Ca^{2+} was further inactivated by addition of Zn^{2+} (Table III).

Table II. Effect of Various Inhibitors on MC50 Lipase Activity[a]

compound	final concentration	% inhibition
protamine	100 μg/mL	28
	2000 μg/mL	100
heparin	100 μg/mL	0
eserine	1 mM	34
	5 mM	51
NaF	1 mM	35
	5 mM	50
NaCl	1 M	0
sodium taurocholate	1 mM	0
	10 mM	34
p-(hydroxymercuri)-benzoate[b]	10 mM	21
iodoacetate[b]	10 mM	8
2-mercaptoethanol[b]	10 mM	15
Hg^{2+}	1 mM	65

[a] Activity was assayed at 41 °C and at pH 8.5 by using corn oil as the substrate. [b] Incubated for 30 min at room temperature prior to assay.

Table III. Metal Ion Reactivation of Apoenzyme[a]

cation[b] (10 mM)	% of original activity
none	22.4
Ca^{2+}	54.0
Zn^{2+}	7.5
Zn^{2+} (5 mM)/Ca^{2+} (5 mM)	10.0
Fe^{2+}	17.0
milk	92.0

[a] Apoenzyme prepared by treatment with 10 mM EDTA for 20 min at room temperature followed by exhaustive dialysis against water. [b] Enzyme was incubated with the cation for 20 min at room temperature prior to assay using corn oil as the substrate. The gum arabic was treated with EDTA to minimize interference from extraneous cations. Diluted 1/1 with deionized water.

Molecular Characteristics. The Stokes radius of purified MC50 lipase was determined by gel chromatography in 0.1% (v/v) Triton X-100. Use of the nonionic detergent was necessary to effect solubilization and dissociation of the aggregates. A plot of R_s vs. $\mathrm{erfc}^{-1}\sigma$, according to the relationship $R_s = a_0 + b_0\,\mathrm{erfc}^{-1}\sigma$, should yield a straight line (Ackers, 1967), where a_0 and b_0 are calibration constants for a given gel. The Stokes radius determined for the enzyme was 39.7 Å. Under these experimental conditions, a_0 (intercept) was 15.62 and b_0 (slope) was 29.88 (correlation coefficient 0.99) for Sephadex G-200 (Figure 6).

The molecular size of purified MC50 lipase was also determined from a Ferguson (1964) plot analysis of gel

Table IV. Amino Acid Composition of *P. fluorescens* MC50 Lipase

amino acid	mmol/ 100 g	residues/ 100 residues	porcine pancreatic lipase residues/ 100 residues[a]	Δ[b]	Δ^2
Asx	37.06	12.44	12.92	−0.48	0.23
Thr	25.86	8.69	6.03	2.66	7.07
Ser	29.08	9.73	6.53	3.2	10.24
Glx	40.02	13.48	8.70	4.78	22.85
Pro	24.94	8.37	5.70	2.67	7.13
Gly	18.98	6.38	8.93	−2.55	6.50
Ala	15.39	5.18	4.67	0.51	0.26
$^1/_2$-Cys	9.36	3.11	3.08		
Val	17.84	5.98	7.85	−1.87	3.50
Met	3.31	1.12	0.95	0.17	0.03
Ile	11.83	3.99	6.30	−2.31	5.34
Leu	23.26	7.81	6.60	1.21	1.46
Tyr	5.18	1.75	3.53	−1.78	3.17
Phe	7.52	2.55	5.06	−2.51	6.30
Lys	14.37	4.86	4.90	−0.04	0
His	11.21	3.75	2.28	1.47	2.16
Arg	2.31	0.80	4.49	−3.69	13.62
					$S\Delta Q$ = 89.86

[a] Given by Desnuelle (1972) for porcine lipase L_A. [b] The difference between residues per 100 residue values for MC50 lipase and porcine lipase.

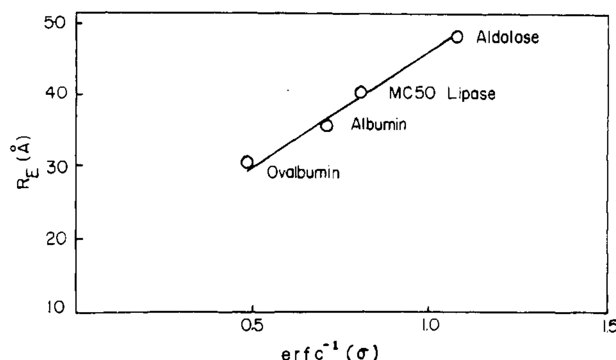

Figure 6. Calibration of the Sephadex G-200 gel and determination of the Stokes radius of *P. fluorescens* MC50 lipase in 0.1% Triton X-100.

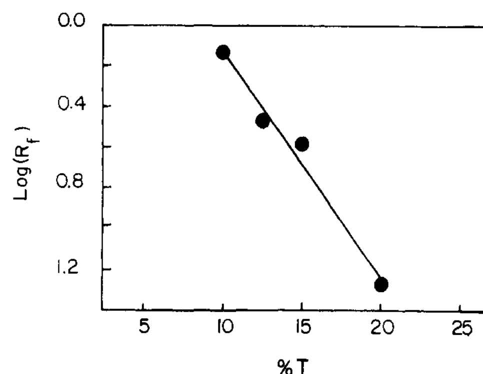

Figure 7. Ferguson plot of gel electrophoretic data for *P. fluorescens* MC50 lipase in 0.1% Triton X-100. The slope of this pilot allows calculation of the molecular radius.

electrophoretic data obtained in the presence of 0.1% (v/v) nonionic detergent. A plot of log R_f vs. gel concentration (% T) should give a linear relationship (Ferguson, 1964). The slope of this plot (K_R) is a measure of molecular sieving in the gel and is related to the molecular radius (Rodbard, 1976). The relationship between the slope and molecular radii used for these calculations is given by Newby et al. (1978). The molecular hydrodynamic radius determined for MC50 lipase is 41.0 Å (Figure 7).

An approximate subunit molecular weight for the purified enzyme was estimated by electrophoresis in 0.1% sodium dodecyl sulfate using a 7.5% polyacrylamide gel. The value obtained was 55 000.

Results for amino acid analyses of purified MC50 lipase are shown in Table IV together with that for porcine pancreatic lipase as a comparison. Tryptophan was not determined because of a limited amount of sample. Although there are significant differences, their compositions are similar enough to conclude that they are part of the same family of enzymes. Quantitatively, their relatedness is indicated by an $S\Delta Q$ (Marchalonis and Weltman, 1971) of 90, which is rather good for a comparison between a procaryotic and a eucaryotic protein.

DISCUSSION

Although the heat stability of lipase produced by *P. fluorescens* has been investigated (Andersson et al., 1979, 1981; Driessen and Stadhouders, 1974), the enzyme has not been purified nor have the molecular characteristics been determined. Characteristics of the MC50 lipase, such as the requirement for an interface for catalysis, allow it to be classified as a true lipase (Desnuelle, 1972; Lawrence, 1967a; Brockman, 1981). Although the influence of fatty acid chain length is difficult to assess since the degree of emulsification of different triacylglycerols may vary, it appears that MC50 lipase is more active with trioctanoin (C_8). Similar apparent specificity was reported for *Pseudomonas fragi* lipase (Nashif and Nelson, 1953) although some microbial lipases are more active with tributyrin, e.g., *Staphylococcus aureus* (Shah and Wilson, 1965).

The pH for optimal activity of most bacterial lipases is in the alkaline region (Lawrence, 1967b) similar to that for MC50 lipase. For example, staphylococcal lipases have an optimum between pH 7.5 and pH 8.5 (Vadehra, 1974; Shah and Wilson, 1965), that for *P. fragi* is between pH 8 and pH 9 (Lawrence et al., 1967; Lu and Liska, 1969), and that for *Pseudomonas aeruginosa* is pH 8.9 (Finkelstein et al., 1970). In contrast, fungal or yeast lipases often exhibit acid pH optima (Lawrence, 1967b), e.g., an optimum of pH 5.6 for lipases from *Rhizopus delemar* and *Aspergillus niger* (Fukumoto et al., 1964).

The effect of potential inhibitors on MC50 lipase activity is qualitatively similar to that reported for a lipase from *P. aeruginosa* (Finkelstein et al., 1970). Chemicals that inhibit esterases more strongly than lipases, such as eserine and NaF (Finkelstein et al., 1970; Cohen et al., 1953;

Renshaw and San Clemente, 1966), did not appreciably affect MC50 lipase activity except at relatively high concentrations. Partial inhibition resulting from treatment with Hg^{2+}, iodoacetate, and pHMB suggest that sulfhydryl groups, although not part of the catalytic site, are close to the active site or participate in maintenance of the active structure. Likewise, partial inhibition by mercaptoethanol suggests that disulfide bonds contribute to the structural stability. Similar observations have been made for pancreatic lipase (Wills, 1960), which contains two sulfhydryl groups and six disulfides per mole (Desnuelle, 1972). These similarities in substrate requirements, inhibition patterns, and amino acid composition relatedness ($S\Delta Q$) suggest that the pancreatic enzyme and MC50 lipase may function in a like manner. However, the microbial enzyme apparently does not require a colipase for binding to the substrate interface in the presence of bile salts as does the pancreatic enzyme (Brockman, 1981; Chapus et al., 1978). Consequently, the interface binding function must be a part of the covalent structure of MC50 lipase since low concentrations of taurocholate did not inhibit this enzyme.

Associated forms of lipase are commonly observed, presumably resulting from association with small amounts of lipid (Gelotte, 1964; Kimura et al., 1972; Renshaw and San Clemente, 1966; Desnuelle, 1972). Thus, the active form of MC50 lipase observed in 0.1% nonionic detergent was substantially smaller than that present in buffer alone. The molecular radius of the smallest active species observed in nonionic detergent (~40 Å) does not necessarily correspond to the subunit existing in sodium dodecyl sulfate. An estimate of the molecular weight can be obtained from the relationship

$$M_2 = \frac{4\Pi N_A R_0^3}{3(\bar{\nu}_2 + \delta_1\bar{\nu}_1 + \delta_D\bar{\nu}_D{}^0)(f/f_0)^3} \quad (1)$$

where R_0 is the Stokes radius, $\bar{\nu}_2$ is the partial specific volume of the protein, δ_1 is the hydration in grams of H_2O per gram, δ_D is the amount of detergent bound in grams per gram, and $\bar{\nu}_1{}^0$ and $\bar{\nu}_D{}^0$ are their respective specific volumes. Assuming that the protein–detergent complex is spherical, that $\bar{\nu}_2 = 0.74$, and an average hydration of 0.53 g of H_2O/g (Squire and Himmel, 1979) gives molecular weights ranging from 60 000 to 90 000 by using the range of Triton X-100 binding that has been observed for proteins (Hackenberg and Klingenberg, 1980). If one allows for asymmetry of the complex, it appears very likely that the 55 000-dalton species observed by SDS gel electrophoresis is the active species in the nonionic detergent–enzyme complex. The estimated molecular weight and Stokes radius for MC50 lipase are slightly larger than those for the pancreatic enzyme, 45 000–52 000 and 30.3 Å (Brockman, 1981), and considerably larger than some values previously reported for microbial lipases, e.g., 27 000 (Sugiura and Isobe, 1974) and 25 000 (Lawrence et al., 1967).

Registry No. Lipase, 9001-62-1; trioctanoin, 538-23-8.

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