Isolation and Characterization of an Extracellular Lipase from the Conidia of *Neurospora crassa*

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A triacylglycerol lipase (EC 3.1.1.3) from the conidia of *Neurospora crassa* was purified and characterized. The enzyme was purified by Sephadex G-100 column chromatography. Homogeneity was checked by PAGE, and isoelectric focusing gave a single band corresponding to a pI of 6·4. The enzyme had an apparent M_r 54000 $\pm$ 1000 as determined by gel filtration. SDS-PAGE gave a single band of M_r 27000, suggesting the presence of two identical subunits. This lipase preferred triglycerides with C_{16}- and C_{18}-fatty acyl chains. It cleaved only the primary groups of triglycerides. The lipase also exhibited a marked preference for substrates containing endogenously occurring fatty acids and so may prove useful in detailed studies on the physiological relevance of fatty acyl specificity of lipases. The enzyme was not affected by detergents, or thiol-binding agents. Modification of free amino groups caused 90% inhibition, suggesting a role of these groups in the maintenance of lipase activity.

INTRODUCTION

Hydrolytic enzymes are essential for the utilization of carbon sources during germination of fungal conidia. In the conidia of *Neurospora crassa* three such enzymes, namely trehalase (Hanks & Sussman, 1969), invertase and β-arylglucosidase (Eberhart & Beck, 1970, 1973), are induced to facilitate germination. Triglycerides (TGs) which are considered to be storage forms of energy, are rapidly utilized during germination of fungal conidia and plant seeds, hence it is important to understand the nature and properties of the lipases responsible.

In spite of the increasing interest in microbial lipases, very few studies have been done on fungal lipases (Semeriva *et al.*, 1967; Tsujisaka *et al.*, 1973; Tomizuka *et al.*, 1966). We have previously reported the presence of phospholipases in the conidia of *N. crassa* (Chakravarti *et al.*, 1981). Phospholipase activities have also been detected in the late stationary phase of its mycelial growth (Chakravarti *et al.*, 1980). In the present paper we report the isolation and characterization of a triacylglycerol lipase from the same organism, the presence of which has been detected earlier (Guchhait *et al.*, 1983).

METHODS

Reagents. Glycerol tri[9,10-³H]oleate was obtained from Amersham; TGs, acrylamide, bisacrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED), Triton X-100, trinitrobenzenesulphonic acid, (TNBS), *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoic acid (PCMB) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were from Sigma; silica gel was from Merck; M_r markers, pI calibration kit and Sephadex G-100 were from Pharmacia and ampholines (pH 3·5–10·0) were from LKB Produkter. All other reagents were of analytical grade.

Purification of lipase. Stock culture of wild-type *N. crassa* ITCCF 1701, mating type A, was obtained from the Indian Agricultural Research Institute, New Delhi. The organism was grown at 30 °C for 6–7 d on Dox and

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Abbreviations: TEMED, *N,N,N',N'*-tetramethylethylenediamine; TNBS, trinitrobenzenesulphonic acid; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TG, triglyceride; MG, monoglyceride; FFA, free fatty acid.

Thom's modification of Czapek's medium as described previously (Chakravarti *et al.*, 1981). Bacto Agar (2%, w/v) was used to solidify the growth medium. Conidia were harvested, suspended in 0·9% NaCl, filtered through glasswool and kept in ice-cold 0·9% NaCl for 1 h to leach out the extracellular enzymes. The conidial suspension was centrifuged at 20000 g for 15 min at 4 °C and an orange supernatant was obtained. It was treated with activated charcoal (50 mg ml^{-1}) for 30 min at 4 °C to remove pigments and centrifuged at 20000 g for 10 min at 4 °C. The colourless supernatant was lyophilized and stored at -20 °C. Lyophilized material was dialysed against 0·1 M-sodium phosphate buffer (pH 7·0) and fractionated on Sephadex G-100 (1·6 × 70 cm). Proteins were eluted with 0·1 M-sodium phosphate buffer (pH 7·0) at a flow rate of 0·25 ml min^{-1} and 1·5 ml fractions collected at 4 °C. One unit of enzyme activity (U) was defined as the amount that liberated 1 μmol free fatty acid (FFA) min^{-1} at 30 °C. Fractions having lipase activity greater than 10 U ml^{-1} were pooled, lyophilized, dialysed against 50 mM-Tris/HCl (pH 7·0) and stored at -20 °C before use in subsequent studies. The protein content was measured by the Lowry method using BSA as standard.

Homogeneity was assessed by disc gel electrophoresis at pH 8·3 (Davis, 1964) and by SDS-PAGE (Weber & Osborn, 1969). Isoelectric focusing was performed on 5% polyacrylamide gels using ampholines (pH 3·5–10·0) and the broad pI calibration kit (Wrigley, 1976). The M_r of the lipase was determined by Sephadex G-100 column chromatography using BSA (67000), ovalbumin (43000) and chymotrypsinogen A (25000) as M_r markers.

Lipase assay. TG-hydrolase activity was measured using glycerol tri[9,10-^{3}H]oleate (specific activity 0·5 mCi mmol^{-1}; 7·4 TBq) dispersed in Triton X-100. The assay mixture, containing 10 mg Triton X-100 ml^{-1}, 3 mM-triolein, 20 mM-CaCl$_2$ and 50 mM-Tris/HCl (pH 7·0) in a final volume of 5 ml, was emulsified by sonication. The enzyme was added and the mixture was incubated for 30 min at 30 °C. The reaction was stopped by adding Dole's extraction mixture (Dole & Meinertz, 1960). The heptane phase was subjected to TLC using silica gel and n-hexane/diethyl ether/acetic acid (80 : 20 : 1, by vol.) as the developing solvent. The liberated [9,10-^{3}H]oleic acid was scraped off and measured by liquid scintillation counting (LKB Mini-Beta 1211). Triton X-100 at the above concentration did not affect lipase activity. Non-radioactive substrates were dispersed at the same concentration as above and the assay conditions were also the same. The FFA liberated was measured by titration with NaOH after dilution with ethanol (Brockerhoff & Jensen, 1974a). To obtain a good dispersion, tripalmitin and tristearin were first dissolved in a few drops of chloroform before emulsification in assay buffer containing Triton X-100. After emulsification, the chloroform was evaporated from the substrate emulsion in a stream of N$_2$ (Lin & Huang, 1983).

For qualitative determination of lipase activity, TGs obtained from lard were used as a substrate and the components of the reaction mixture were separated by TLC as described above. The fatty acid composition of monoglycerides and FFAs liberated by the hydrolysis of lard TGs with the *N. crassa* lipase was determined according to the method of Luddy *et al.* (1960). Methyl esters were analysed by GLC in a Pye Unicam, 104 series gas chromatograph fitted with a dual flame ionization detector on a 10% polyethylene glycol adipate column (100–120 mesh) at 170 °C.

All results are mean values of five separate determinations (SE ± 5%).

RESULTS AND DISCUSSION

Purification of lipase

The triacylglycerol lipase activity was purified by Sephadex G-100 column chromatography as shown in Fig. 1. The fractions having lipase activity greater than 10 U ml^{-1} (fractions 51–53) were pooled. By this method the lipase was purified 26-fold with a specific activity of 44 U (mg protein)$^{-1}$ (Table 1). The lipase thus purified showed a single band after PAGE (Fig. 2a) and isoelectric focusing (Fig. 2d) with a pI of 6·4, thereby confirming the homogeneity of the protein. Gel filtration on Sephadex G-100 gave an apparent M_r 54000 ± 1000. SDS-gel electrophoresis in the absence as well as in the presence of 2-mercaptoethanol showed a single band corresponding to an M_r 27000, suggesting that the lipase contains two subunits of M_r 27000 each, which are not associated by disulphide bonds (Fig. 2c).

Properties of the enzyme

The *N. crassa* lipase showed optimum activity at 30 °C which was reduced by some 60% at 45 °C. The optimum pH for activity was about 7, and activity was barely detectable at pH 5·5 and 8·5. The *N. crassa* lipase-mediated hydrolysis of triolein was stimulated by 20 mM-Ca^{2+}, but it was unaffected by Mg^{2+} under the same conditions. Ca^{2+} might function by precipitating the liberated fatty acids as calcium salts.

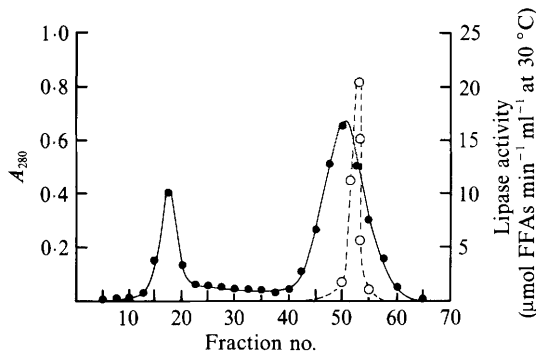Effects of various detergents and group-specific modifying agents on the lipase activity were

Fig. 1. Purification of lipase by gel filtration using Sephadex G-100. The fractions with lipase activity greater than 10 U ml^{-1} (nos 51–53) were pooled. ●, A_{280}; ○, lipase activity.
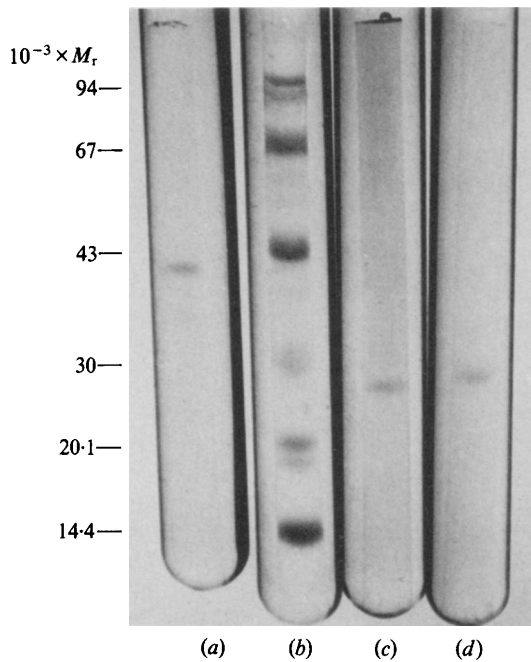

Fig. 2. (a) PAGE (7·5% polyacrylamide) of native lipase; (b) SDS-PAGE of marker proteins (from top): phosphorylase, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin; (c) SDS-PAGE of purified lipase and (d) isoelectric focusing of purified lipase.

Table 1. *Purification of N. crassa lipase*

Step	Total protein (mg)	Yield of protein (%)	Total activity (U)	Specific activity [U(mg protein)$^{-1}$]	Purification (-fold)
Saline extract	50	100	85	1·7	–
Charcoal treatment and dialysis	35	70	75	2·14	1·25
Sephadex G-100 filtration	1	2	44	44	25·88

Table 2. *Positional specificity of lipase*

Fatty acid content (% of total wt)

Fatty acid	Lard TG	Lard TG after treatment with *N. crassa* lipase	
		MG	FFA
14:0	3·3	5·1	ND
16:0	19·2	64·4	ND
18:0	12·5	25·5	ND
16:1	2·8	0·5	1·0
18:1	52·8	0·8	96·8
18:2	8·6	2·6	2·0

ND, Not detected.

studied. Many lipases have been reported to be affected by detergents, but in this case DOC (0·1% and 1%, w/v) and SDS (2 and 20 mM) did not affect lipase activity. Activity was not inhibited by thiol-binding reagents like PCMB (0·1 mM), NEM (10 mM) and DTNB (1 mM), suggesting the absence of thiol groups at the active site of the lipase. Similar results have been reported for the lipase from *Staphylococcus aureus* (Muraoka *et al.*, 1982). TNBS (1 mM) inhibited 90% of the lipase activity. Besides amino groups, TNBS is known to react with thiol groups also, but under alkaline conditions (pH 9·5) the thiol derivative is unstable (Glazer *et al.*, 1975). Modification with TNBS was done at pH 9·5 (50 mM-borate buffer) followed by dialysis against assay buffer (50 mM-Tris/HCl, pH 7·0). The results suggested that free amino groups are required for the maintenance of enzyme activity.

Influence of the nature of fatty acids on the rate of hydrolysis

The effect of chain length as well as unsaturation of fatty acids on lipase activity was studied using different triacylglycerols. Five different sets of assays were done in each case. The *N. crassa* lipase preferred tripalmitin, tristearin, tripalmitolein, triolein and trilinolein and hydrolysed these at almost the same rates (40–44 µmol mg^{-1} min^{-1}). TGs with short-chain fatty acids (C_4, C_6, C_8, C_{10}) were hydrolysed at a very slow rate (10–16 µmol mg^{-1} min^{-1}). Preference towards TGs with long-chain fatty acids (C_{16}, C_{18}) appears to be metabolically significant since these fatty acids are the most abundant in fungal lipids, palmitic acid (28·4 %) and linoleic acid (42·5%) being the major fatty acids in TGs of *N. crassa* (Kushwaha & Kates, 1976). Such preference towards substrates containing endogenously occurring fatty acids has also been reported in the case of several seed lipases (Lin *et al.*, 1986). Substrate specificity has been demonstrated in the case of some other microbial lipases (Mudherwa *et al.*, 1985). Triolein is more rapidly hydrolysed than tristearin by the lipases of *Candida paralipolytica*, *Corynebacterium acnes* and *Candida deformans* (Mudherwa *et al.*, 1985), and the lipase from *Propionibacterium acnes* hydrolyses trilaurin, triolein, trimyristin and tripalmitin at decreasing rates (Ingham *et al.*, 1981). The greater solubility and physical characteristics of the TGs of shorter fatty acids, such as C_{10} and C_{12}, often make them more suitable substrates for lipases such as pancreatic lipase (Lin *et al.*, 1986). However, in this case tripalmitin and tristearin were better substrates than tricaprin or trilaurin in spite of their lower solubility.

Positional specificiy of the lipase

Positional specificity of the lipase was determined using lard TG as substrate. It contains long-chain saturated fatty acids (16:0 and 18:0) in position 2 and mainly oleic acid (18:1) in positions 1 and 3. The GLC analysis (Table 2) revealed that MGs produced by the *N. crassa* lipase-mediated hydrolysis contained mainly saturated fatty acids, while the FFA fraction was only oleic acid. This result suggests that the *N. crassa* lipase preferentially cleaves the primary ester group of triacylglycerols in the same way as pancreatic lipase (Brockerhoff & Jensen,

1974*b*) and the lipases from *Rhizopus arrhizus* (Benzonana, 1974), *Candida deformans* (Mudherwa *et al.*, 1985) and different *Alcaligenes* strains (Kokusho *et al.*, 1982).

Microbial lipases have received much attention in recent years because of their application in industry and medicine, but their physiological relevance has not been studied in detail. Using mixed TGs, lipases from several seed species have been shown to be relatively specific for substrates containing the major fatty acid components of the storage triacylglycerols of the same species (Lin *et al.*, 1986). Such studies on the significance of substrate specificity of microbial lipases are scant. The preference of the *N. crassa* lipase for substrates containing endogenously occurring fatty acids suggests the need to extend these studies using mixed TGs to determine whether microbial lipases are relatively specific for the characteristic TGs of the same species.

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