

Cloning, expression and characterization of a cDNA encoding a lipase from *Rhizopus delemar*

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SUMMARY

A λ gt11 cDNA library was constructed in *Escherichia coli* using poly(A)-selected mRNA from the fungus, *Rhizopus (Rp.) delemar*. Lipase-producing members of the library were identified by means of a phenotypic score wherein the release of fatty acids by lipase causes a characteristic color change in the growth medium. One such isolate contained a 1287-bp insert (*LIP* cDNA) which hybridizes to 1.25- to 1.35-kb mRNA species from *Rp. delemar*. The lipase produced in *E. coli* containing the *LIP* cDNA exhibits the same substrate selectivity as the authentic fungal enzyme, hydrolyzing ester bonds at the stereospecific numbering (*sn*) *sn*-1 and *sn*-3, but not the *sn*-2, positions of triglycerides. The complete nucleotide sequence of the *LIP* cDNA was determined. By reference to the N-terminal sequence of authentic *Rp. delemar* lipase, the lipase-encoding region was identified within this fragment. The *LIP* cDNA encodes a putative preprolipase consisting of a 26-amino-acid(aa) signal sequence, a 97-aa propeptide, and a 269-aa mature enzyme. The predicted mature lipase has the same molecular weight and aa composition as that of *Rp. delemar*, is highly homologous to that produced by the fungus *Rhizomucor miehei*, and contains the consensus pentapeptide (Gly-Xaa-Ser-Yaa-Gly) which is conserved among lipolytic enzymes. It is concluded that the *LIP* cDNA is an essentially full-length analogue of the lipase-encoding gene of *Rp. delemar*. The lipase encoded by the *LIP* cDNA occupies a cytoplasmic location when synthesized in *E. coli*. Unprocessed forms of the lipase accumulate in *E. coli*.

INTRODUCTION

Triglycerides represent a rich source of reduced carbon and energy to living organisms. Their metabolism is initiated by enzymatic hydrolysis of their ester bonds. These reactions are catalyzed by lipases (acylglycerol acyl-

hydrolases, EC 3.1.1.3) (Brockerhoff and Jensen, 1974; Borgström and Brockman, 1984).

Due to their pivotal roles in fat metabolism, there is considerable interest in the biochemistry and molecular biology of lipases (Mickel et al., 1989; Kirchgessner et al., 1989). There is also substantial interest in the biotechnological applications of these enzymes. Lipases catalyze the synthesis and hydrolysis not only of glycerides but also of a variety of ester and ester-like bonds (Margolin and Klibanov, 1987; Langrand et al., 1988; Posorske et al., 1988; Sztajer and Zboinska, 1988; Nishio et al., 1989).

Fungal and bacterial lipases are usually employed in these applications. The extracellular lipases of the fungus *Rp. delemar*, a member of the Class Zygomycetes, have been shown to catalyze a variety of reactions involving the synthesis and hydrolysis of esters (Okumura et al., 1979;

Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); IPTG, isopropyl- β -D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; *LIP*, gene (DNA) encoding lipase; nt, nucleotide(s); ORF, open reading frame; PAGE, polyacrylamide-gel electrophoresis; *Rm.*, *Rhizomucor*; *Rp.*, *Rhizopus*; SDS, sodium dodecyl sulfate; *sn*, stereospecific numbering; Xaa, any aa; [], denotes plasmid-carrier state.

Macrae, 1983; Yagi et al., 1990). Several factors, such as limitations in supply, stability and specificity, hinder the fuller utilization of lipases such as this one in applied catalysis. To characterize the lipase gene from this organism, and to facilitate its high level expression and further modification, we have isolated and characterized an expressed *Rp. delemar* lipase cDNA.

RESULTS AND DISCUSSION

(a) cDNA library construction and recovery of lipase-producing clones

Rp. delemar ATCC34612 (American Type Culture Collection, Rockville, MD) was grown at 30°C from a spore inoculum in minimal salts medium (Westergaard and Mitchell, 1947) fortified with 0.5% casamino acids (Difco)/30 mM glycerol/5 ng biotin per ml. Total RNA was isolated from 5 g of 40-h mycelia using a guanidinium/CsCl protocol (Maniatis et al., 1982). Poly(A)⁺ RNA isolated by affinity chromatography on oligo(dT)-cellulose (Pharmacia, Piscataway, NJ) (Maniatis et al., 1982) served as the template for cDNA synthesis, using an oligo(dT) primer and reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). Internal *Eco*RI sites in the product were blocked with M·*Eco*RI methyltransferase, and *Eco*RI linkers (New England Biolabs, Beverly, MA) were added by ligation. The product was cut with *Eco*RI (Bethesda Research Laboratories, Gaithersburg, MD), ligated to λ gt11 arms, and packaged in vitro using Packagene extracts (Promega, Madison, WI). *E. coli* Y1090r⁻ (Promega, Madison, WI) was infected with the resulting phage and plated in top agar containing a lipase indicator medium consisting of an emulsion of rhodamine B and olive oil (Kouter and Jaeger, 1987). Phage replication was temperature-induced in the presence of 10 mM IPTG. Following overnight incubation at 37°C, lipase-positive plaques (bright pink under ultraviolet light) were identified at a frequency of 10⁻⁵. *E. coli* 1089r⁻ (Promega, Madison, WI) was lysogenized with R45A4, a lipase-encoding phage identified in this manner.

Phage R45A4 contains a single *Eco*RI insert, estimated by electrophoresis to be 1.3 kb in size. The expression plasmid, pUC8-2.14, was derived by cloning this fragment into the *Eco*RI site of pUC8-2, a derivative of pUC8 (Vieira and Messing, 1982) in which inserts are under the control of the *lacZ* promoter, and expressed in the same frame as fragments inserted in the *Eco*RI site of λ gt11 (Hanna et al., 1984). Plasmid pUC8-2.14 was introduced into *E. coli* JM101 by transformation (Hanahan, 1983) and Lip⁺ transformants were identified on lipase indicator media containing Ap. Lipase activity was detectable only in cells harboring pUC8-2.14, and only upon induction with IPTG.

Thus, the *LIP* cDNA is necessary and sufficient for the synthesis of lipase by *E. coli* JM101[pUC8-2.14], and this synthesis is regulated by IPTG.

(b) Northern-blot analysis of *LIP* RNA

Poly(A)⁺ RNA from *Rp. delemar* was electrophoresed on agarose gels, transferred to a nitrocellulose membrane, and hybridized with radiolabeled *LIP* cDNA (Fig. 1) (Davis et al., 1986). The probe hybridized primarily to fungal RNAs approx. 1.25–1.55 kb in size (Fig. 1). This establishes that the *LIP* cDNA has homology to *Rp. delemar* poly(A)⁺ RNA, and is large enough to be a full-length analogue of the fungal lipase mRNA.

(c) Nucleotide sequence determination

Three contiguous, nonoverlapping, restriction fragments which comprise the entire *LIP* cDNA (*Eco*RI-*Kpn*I, *Kpn*I-

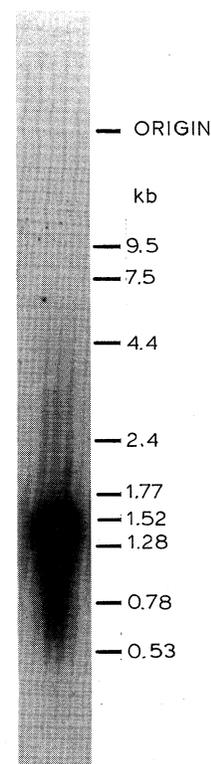


Fig. 1. Northern-blot analysis of *LIP* RNA. Poly(A)⁺ RNA from *Rp. delemar* was fractionated on 1% agarose-0.66 M formaldehyde gels (Davis et al., 1986), along with marker preparations of 0.16–1.77 kb and 0.24–9.5 kb RNA ladders (Bethesda Research Laboratories, Gaithersburg, MD). After staining with ethidium bromide to visualize bands, the nucleic acids were transferred to nitrocellulose (Davis et al., 1986). Filters were prehybridized overnight at 42°C in 50% formamide/5 × Denhardt's solution (per liter: 1 g polyvinylpyrrolidone/1 g bovine serum albumin/1 g Ficoll 400)/5 × SSPE (0.75 M NaCl/0.05 M NaH₂PO₄/0.005 M EDTA-Na₂ pH 7.4)/0.1% SDS/100 μg per ml sheared, heat-denatured herring sperm DNA. Radiolabeled probe was then added and hybridizations were conducted for 18 h at 42°C. The membranes were washed twice in 0.2 × SSPE/0.01% SDS, followed by autoradiography. The numbers indicate the sizes of the marker fragments in kb.

HindIII, and *HindIII-EcoRI*), were isolated by electrophoresis-electroelution and subcloned in both orientations in phage M13mp18 and 19 (Yanisch-Perron et al., 1985). The *Sau3AI* sub-fragments of the *EcoRI-KpnI* region, and the *RsaI* sub-fragments of the *HindIII-EcoRI* region were similarly subcloned. Nucleotide sequences were determined by the dideoxy method employing either Klenow enzyme (Sanger et al., 1977) or T7 polymerase (Sequenase, USB, Cleveland, OH, nt 145–243, 452–566, and the final 200

3'-proximal nt), using synthetic oligodeoxynucleotide primers. Sequence analysis was performed with PC/GENE software (IntelliGenetics, Mountain View, CA).

The sequence of the *LIP* cDNA is shown in Fig. 2. It is 1287 bp in length and has a G + C content of 45%. A polyadenylation consensus sequence lies at nt 1253–1258 (Birnstiel, 1985). Only one reading frame has an ORF sufficient in length to encode the *Rp. delemar* lipase, which is composed of a single polypeptide with a mass of 30.3 kDa

GAATTCCTTTTCTTCTTACCCCTTCCAGTTCTTTACTATCAATC	ATG GTT TCA TTC ATT TCC ATT TCT	69
	met val ser phe ile ser ile ser	-116
CAA GGT GTT AGT CTT TGT CTT CTT GTC TCT TCC ATG ATG CTC GGT TCA TCT GCT GTT CCT		129
gln gly val ser leu cys leu leu val ser ser met met leu gly ser ser ala val pro		-96
GTT TCT GGT AAA TCT GGA TCT TCC AAC ACC GCC GTC TCT GCA TCT GAC AAT GCT GCC CTC		189
val ser gly lys ser gly ser ser asn thr ala val ser ala ser asp asn ala ala leu		-76
CCT CCT CTC ATC TCC AGC CGT TGT GCT CCT CCT TCT AAC AAG GGA AGT AAA AGC GAT CTC		249
pro pro leu ile ser ser arg cys ala pro pro ser asn lys gly ser lys ser asp leu		-56
CAA GGT GAA CCT TAC AAC ATG CAA AAG AAT ACA GAA TGG TAT GAG TCC CAT GGT GGC AAC		309
gln ala glu pro tyr asn met gln lys asn thr glu trp tyr glu ser his gly gly asn		-36
CTG ACA TCC ATC GGA AAG CGT GAT GAC AAC TTG GTT GGT GGC ATG ACT TTG GAC TTA CCC		369
leu thr ser ile gly lys arg asp asp asn leu val gly gly met thr leu asp leu pro		-16
AGC GAT GCT CCT CCT ATC AGC CTC TCT AGC TCT ACC AAC AGC GCC TCT GAT GGT GGT AAG		429
ser asp ala pro pro ile ser leu ser ser ser thr asn ser ala <u>SER</u> ASP GLY GLY LYS		5
GTT GTT GCT GCT ACT ACT GCT CAG ATC CAA GAG TTC ACC AAG TAT GCT GGT ATC GCT GCC		489
VAL VAL ALA ALA THR THR ALA GLN ILE GLN GLU PHE THR LYS TYR ALA GLY ILE ALA ALA		25
ACT GCC TAC TGT CGT TCT GTT GTC CCT GGT AAC AAG TGG GAT TGT GTC CAA TGT CAA AAG		549
THR ALA TYR CYS ARG SER VAL VAL PRO GLY ASN LYS TRP ASP CYS VAL GLN CYS GLN LYS		45
TGG GTT CCT GAT GGC AAG ATC ATC ACT ACC TTT ACC TCC TTG CTT TCC GAT ACA AAT GGT		609
TRP VAL PRO ASP GLY LYS ILE ILE THR THR PHE THR SER LEU LEU SER ASP THR ASN GLY		65
TAC GTC TTG AGA AGT GAT AAA CAA AAG ACC ATT TAT CTT GTT TTC CGT GGT ACC AAC TCC		669
TYR VAL LEU ARG SER ASP LYS GLN LYS THR ILE TYR LEU VAL PHE ARG GLY THR ASN SER		85
TTC AGA AGT GCC ATC ACT GAT ATC GTC TTC AAC TTT TCT GAC TAC AAG CCT GTC AAG GGC		729
PHE ARG SER ALA ILE THR ASP ILE VAL PHE ASN PHE SER ASP TYR LYS PRO VAL LYS GLY		105
GCC AAA GTT CAT GCT GGT TTC CTT TCC TCT TAT GAG CAA GTT GTC AAT GAC TAT TTC CCT		789
ALA LYS VAL HIS ALA GLY PHE LEU SER SER TYR GLU GLN VAL VAL ASN ASP TYR PHE PRO		125
GTC GTC CAA GAA CAA TTG ACC GCC CAC CCT ACT TAT AAG GTC ATC GTT ACC GGT CAC TCA		849
VAL VAL GLN GLU GLN LEU THR ALA HIS PRO THR TYR LYS VAL ILE VAL THR <u>GLY HIS SER</u>		145
CTC GGT GGT GCA CAA GCT TTG CTT GCC GGT ATG GAT CTC TAC CAA CGT GAA CCA AGA TTG		909
<u>LEU GLY</u> GLY ALA GLN ALA LEU LEU ALA GLY MET ASP LEU TYR GLN ARG GLU PRO ARG LEU		165
TCT CCC AAG AAT TTG AGC ATC TTC ACT GTC GGT GGT CCT CGT GTT GGT AAC CCC ACC TTT		969
SER PRO LYS ASN LEU SER ILE PHE THR VAL GLY GLY PRO ARG VAL GLY ASN PRO THR PHE		185
GCT TAC TAT GTT GAA TCC ACC GGT ATC CCT TTC CAA CGT ACC GTT CAC AAG AGA GAT ATC		1029
ALA TYR TYR VAL GLU SER THR GLY ILE PRO PHE GLN ARG THR VAL HIS LYS ARG ASP ILE		205
GTT CCT CAC GTT CCT CCT CAA TCC TTC GGA TTC CTT CAT CCC GGT GTT GAA TCT TGG ATC		1089
VAL PRO HIS VAL PRO PRO GLN SER PHE GLY PHE LEU HIS PRO GLY VAL GLU SER TRP ILE		225
AAG TCT GGT ACT TCC AAC GTT CAA ATC TGT ACT TCT GAA ATT GAA ACC AAG GAT TGC AGT		1149
LYS SER GLY THR SER ASN VAL GLN ILE CYS THR SER GLU ILE GLU THR LYS ASP CYS SER		245
AAC TCT ATC GTT CCT TTC ACC TCT ATC CTT GAC CAC TTG AGT TAC TTT GAT ATC AAC GAA		1209
ASN SER ILE VAL PRO PHE THR SER ILE LEU ASP HIS LEU SER TYR PHE ASP ILE ASN GLU		265
GGA AGC TGT TTG TAA AACACTTGACGTGTTACTCTAATTTTATA <u>ATAAA</u> ATTAAGTTTTTATACAATAAAAGGA		1283
GLY SER CYS LEU STOP		

ATTC

Fig. 2. Nucleotide sequence of the mRNA-like strand of *LIP* cDNA, and the deduced aa sequence from the most probable start codon in the only ORF sufficiently long to encode lipase. The proposed sites of proteolytic removal of the signal sequence, between Ala⁻⁹⁸ and Val⁻⁹⁷, and of the propeptide, between Ala⁻¹ and Ser⁺¹ are indicated by blackened arrowheads. The consensus lipase pentapeptide at aa 143–147 is underlined. A consensus sequence signalling poly(A)-addition, at nt 1253–1258, is double underlined. GenBank accession No. M38352.

(M.J.H. and D. Cichowicz, in preparation). Within the first 110 bp of the sequence there are three Met codons. The one at nt 46–48 is most likely the start codon (Kozak, 1984). A translation terminator lies at nt 1222–1224 and is followed by 63 untranslated nt.

The protein encoded between the probable start and stop codons contains 392 aa and has a mass of 42.1 kDa. The N terminus of this putative protein contains a typical signal peptide. Proteolytic cleavage to remove this signal is most likely to occur between Ala²⁶ and Val²⁷ (von Heijne, 1986).

Removal of the signal sequence would leave a protein with 366 aa and a mass of 39.5 kDa. This is substantially larger than the *Rp. delemar* lipase, implying that the enzyme is initially synthesized as a larger precursor. To identify the position of the mature lipase within this precursor, the aa sequence predicted from the *LIP* cDNA was compared with the N-terminal sequence of authentic *Rp. delemar* lipase (M.J.H. and D. Cichowicz, in preparation). A sequence identical to the N-terminal 28 aa residues of the fungal lipase is encoded downstream from nt 415 in the *LIP* cDNA (Fig. 2). Proteolytic cleavage of a proenzyme just before the Ser residue (encoded by nt 415–417) would generate a mature lipase with a mass of 29.6 kDa, in agreement with that of authentic lipase. The aa composition of the predicted mature lipase agrees with that of the authentic fungal enzyme (Table I). These results indicate that the lipase is synthesized as a proenzyme containing a 26-aa signal peptide and a 97-aa propeptide.

One highly conserved region, with the consensus sequence Gly-Xaa-Ser-Yaa-Gly where Xaa is often His, has been identified in all lipases examined to date (Brenner, 1988; Antonian, 1988). This region has been variously implicated in the binding of lipases to micellar substrates (Guidoni et al., 1981) or as the location of an active site Ser (Brady et al., 1990). A sequence (Gly-His-Ser-Leu-Gly) homologous to this consensus site is encoded by the *LIP* cDNA between nt 841–855. With the exception of the lipase of the fungus *Rm. miehei* (below), the enzyme shows no significant homology to other lipases whose sequences are known.

The 6102 entries of the SWISS-PROT protein data base (release 6.0) were scanned for homology with the predicted aa sequence of the mature lipase. Other than homologies with the consensus sequences of other lipases, the *Rp. delemar* enzyme exhibits no homologies greater than a pentapeptide.

The predicted lipase is substantially homologous to the lipase of *Rm. miehei*, another member of the Class Zygomycetes (Boel et al., 1988). The *Rm. miehei* enzyme exhibits the same positional selectivity toward triglyceride substrates as the *Rp. delemar* enzyme: both enzymes hydrolyze esters at the *sn*-1 and *sn*-3, but not the *sn*-2, positions of glycerides. The organizational and sequence homologies

TABLE I

The aa compositions of mature *Rhizopus delemar* lipase^a

aa	Predicted ^b	Determined ^c
Ala	15	19
Arg	9	10
Asn	10	28
Asp	13	
Cys	6	8
Gln	13	24
Glu	9	
Gly	21	21
His	7	8
Ile	17	14
Leu	16	17
Lys	15	18
Met	1	2
Phe	15	14
Pro	15	16
Ser	24	22
Thr	22	21
Trp	3	N.D.
Tyr	12	11
Val	26	19
Total	269	

^a Expressed as number of aa residues per lipase molecule.

^b From nt sequence of the *LIP* cDNA (see Fig. 2). For aa + 1– + 269.

^c Samples of pure lipase were incubated at 110°C for 24 h in 5.7 N HCl. Amino acid contents of the resulting hydrolysates were determined on a Beckman 119CL amino acid analyzer according to the manufacturer's instructions (M.J.H. and D. Cichowicz, unpublished). N.D., not determined.

between the cDNAs encoding these enzymes are considerable. Their coding portions are 56% identical overall. The degrees of identity within the regions encoding the signal, propeptide and mature lipase regions are 47%, 53% and 59%, respectively, at the DNA level and, considering both identical and functionally identical aa, the predicted aa sequences are 29% and 68% homologous in the pre/pro and mature domains.

The predicted mature lipases from both organisms contain 269 aa. Maximum alignment is obtained by the introduction of only one gap, a single aa long, in each (Fig. 3). In the C-terminal halves of the enzymes, beginning at Pro¹³⁷ in the *Rp. delemar* lipase and including the lipase consensus sequence, the sequence conservation is substantially greater. Sixty-seven percent of the aa in this region are identical, and an additional 9% are functionally similar. The mature *Rp. delemar* lipase contains two potential Asn-glycosylation sites, at Asn⁹⁶ and Asn¹⁶⁹. Only the latter is also found in the *Rm. miehei* enzyme.

The three dimensional structure of the *Rm. miehei* lipase has recently been reported (Brady et al., 1990). Key features of that structure appear to be conserved in the *Rp. delemar*

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Rd- SDGGKVAATTAQIQEFTKYAGIAATAYCRSVVPGNKWDCVQCQKVVDPDG 50
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Rm- SIDGGIRAATSQEIINELTYTFLSANSYCRTVIPGATWDCIHCDA-TEDL 49
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Rd- KIITTFSTLLSDTNGYVLRSDKQKTIYLVFRGNTSFRSAITDIVFNFSY 100
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Rm- KIKTWSTLIYDTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPSY 99
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Rd- KPVKAKVHAGFLSSYEQVVDYFPVQEQQLTAHPTYKVIIVTGHSLGGAQ 150
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Rm- PPVSGTKVHKGFLLDSYGEVQNELVATVLDQFKQYPSYKVAVTGHSLGGAT 149
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Rd- ALLAGMDLYQREPRLSPKNLSIFTVGGFRVGNPTFAYYVESTGIPFQRTV 200
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Rm- ALLCALGLYQREGLSSNLFYLTQQQPRVGDPAFANYVVSTGIPYRRTV 199
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Rd- HKRDIVPHVPPQSFGLHFGVESMIK-SGTSNVQICTSEIETKDCSNSIV 249
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Rm- NERDIVPHLPPAFAFGFLHAGEEYWITDINSPEYVQVCTSDLETSDCSNSIV 249
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Rd- PFTSILDHLSYFDINEGSCL 269
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Rm- PFTSVLDHLSYFGINTGLCT 269
    : : : : : : : : : : : : : : : : : : : : : : : : : : : :

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Identity : 151 (56.1%)
Similarity: 33 (12.3%)
Number of gaps inserted in Rd: 1
Number of gaps inserted in Rm: 1

Fig. 3. Alignment of the aa sequences of the mature lipases of *Rp. delemar* (Rd), determined from the *LIP* cDNA sequence, and of *Rm. miehei* (Rm) (Boel et al., 1988). Alignment was performed by the method of Myers and Miller (1988) with open gap and unit gap costs of 50. Identical aa are marked by colons; similar aa are connected by dots. The groups of similar aa are A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W.

enzyme. These include the catalytic triad, S144, D203 and H257, the extended loop (85–97) and the pattern of disulfide bonds (C29/268, C40/43, C239/249).

(d) Characterization of the lipase encoded by the *LIP* cDNA

Lipolytic activity was quantitated with a continuous titrating pH-stat method (Junge, 1984). A unit of activity is that amount which releases one μ mole of free fatty acid from emulsified olive oil per min at 26°C, pH 7.5.

The subcellular location of the *Rp. delemar* lipase when synthesized by *E. coli* was determined by fractionating the cells and determining the level of lipase in each fraction. *E. coli* JM101[pUC8-2.14] was grown to late logarithmic

phase in the presence of IPTG. The cells were harvested and subjected to osmotic shock to release periplasmic constituents. The cells were recovered and broken by sonication, releasing soluble cytoplasmic contents. Particulate material, containing membrane-bound and insoluble cell components, was recovered by centrifugation. Lipase activities were determined in the resulting fractions, and in the spent growth medium. To monitor the efficiency of fractionation of the cells, the levels of alkaline phosphatase, a periplasmic marker (Heppel, 1971), and lactate dehydrogenase, a cytoplasmic marker (Mahler and Cordes, 1971) in these fractions were also determined. A high degree of separation of marker enzymes was obtained, indicating that the subcellular fractions were well separated (Table II). Of the recovered lipase activity, 68% was in the cytoplasmic fraction, with an additional 32% in the particulate fraction (Table II). This latter activity may be membrane bound or in the form of inclusion bodies. No detectable lipase was present in the periplasmic fraction or in spent growth medium (Table II). Therefore, the lipase occupies a cytoplasmic location in *E. coli*.

Induction of *LIP* gene expression in *E. coli* JM101[pUC8-2.14] did not cause a detectable change in the protein complement of the cells, as monitored by SDS-PAGE (Fig. 4A). The appearance of lipase cross-reacting material upon induction was detected, however, by immunoblotting with polyclonal antibodies raised against purified *Rp. delemar* lipase (Fig. 4B). The cross-reacting species were of 45.0, 41.2, 39.3 and 34.4 kDa. The first of these sizes corresponds to that predicted for the unprocessed protein resulting from initiation at a vector Met codon. The second corresponds to initiation at a Met codon within the cDNA (Fig. 2, nt 46–48). The third corresponds to the size predicted for prolipase. No material with a mass of 30 kDa, the size of mature lipase, was detected. Thus, *E. coli* is unable to fully process the initial *LIP* gene product

TABLE II

Activities of selected enzymes in subcellular fractions of *E. coli* JM101[pUC8-2.14]

Preparation ^a	Total enzyme activity units and % distribution (in parentheses, below)		
	Lipase ^b	Alkaline phosphatase ^c	Lactate dehydrogenase ^d
Supernatant, post-osmotic shock	0	3.8×10^{-1} (93)	0
Sonicated, post-shock cells	28.6 (68)	2.5×10^{-2} (6)	1.05 (89)
Debris following sonication and shock	13.3 (32)	3.2×10^{-3} (1)	1.25×10^{-1} (11)

^a Cells grown to mid-log phase in 500 ml of low phosphate media (Inouye et al., 1981) containing 1 mM IPTG and 100 μ g Ap/ml were subjected to osmotic shock (Haas and Dowding, 1975). The shocked cells were resuspended in 4 ml of 10 mM Tris · Cl, pH 8.0, and sonicated. Cellular debris was removed by centrifugation at 38000 × g for 15 min. The debris pellet was resuspended in 4 ml 10 mM Tris · Cl, pH 8.0.

^b Determined as described in section d.

^c Determined as described by Garen and Levinthal (1960).

^d Determined according to the 1982 Worthington Enzyme Catalog (diaphorase assay) and the instructions accompanying kit LDH 228-UV (Sigma Chemical Co., St. Louis, MO).

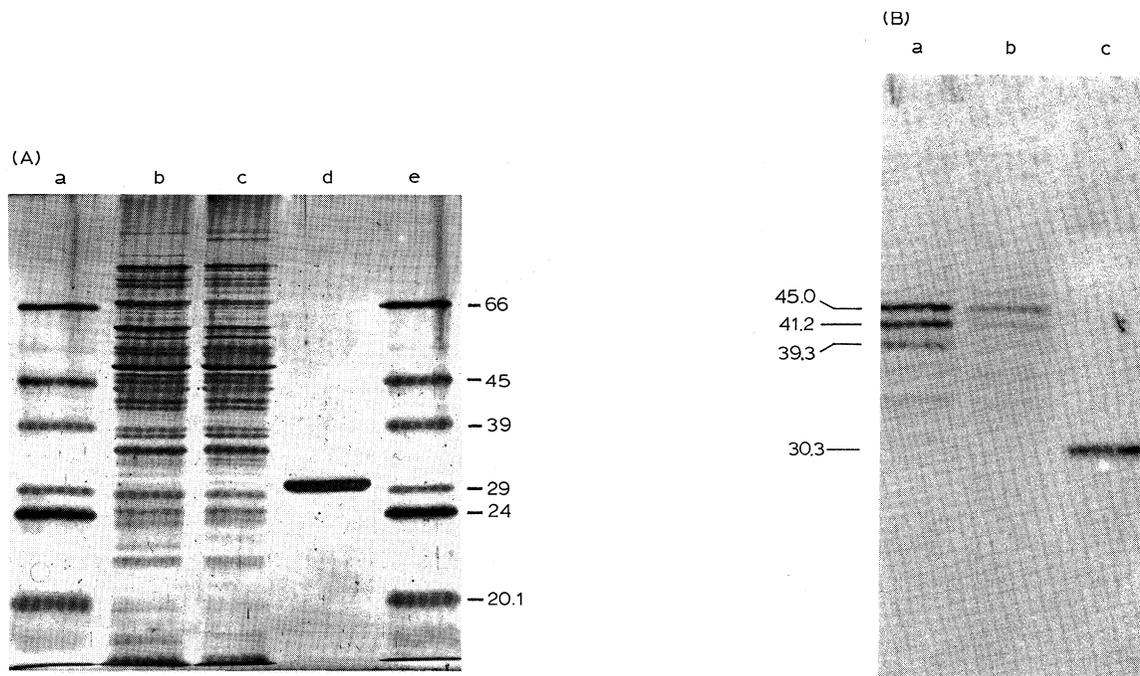


Fig. 4. Electrophoretic and immunological characterization of the products of *lip* gene expression in *E. coli* JM101[pUC8-2.14]. Extracts of mid-log phase cells, prepared by sonication, were resolved by 0.1% SDS-12% PAGE. The method of Winston et al. (1987) was used to detect lipase immunologically. Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose sheets, and treated with polyclonal rabbit antibodies raised against purified *Rp. delemar* lipase. The membranes were then treated with alkaline phosphatase-conjugated *Staphylococcus aureus* Protein A (Sigma Chemical Co., St. Louis, MO). Sites of immunological cross-reaction were visualized by incubating the membranes with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO). (Panel A) Silver stained gel (Morrissey, 1981); lanes: a, 1.5 μ g mixed M_r standards (Sigma, St. Louis, MO), b, 5.6 μ g protein from induced *E. coli* JM101[pUC8-2.14], c, 3.8 μ g protein from uninduced *E. coli* JM101[pUC8-2.14], d, 1 μ g pure *Rp. delemar* lipase, e, 2.2 μ g mixed M_r standards. (Panel B) Immunoblots; lanes: a, 5.6 μ g protein from induced *E. coli* JM101[pUC8-2.14], b, 5.6 μ g protein from uninduced *E. coli* JM101[pUC8-2.14], c, 60 ng pure *Rp. delemar* lipase.

to its final mature form. Small amounts of cross reacting material with these same masses were detected in uninduced cells (Fig. 4B).

To determine the positional selectivity of the lipase, sonicates of *E. coli* harboring and expressing the *LIP* cDNA were incubated at room temperature with triolein. Before hydrolysis had reached 10% of maximum, the glyceride composition of the reaction products was determined by thin layer chromatography on silica gel plates (Bilyk et al., 1991). The diglyceride population contained 1,2 (2,3) and no 1,3 diglycerides. Thus, the product of the *LIP* cDNA specifically hydrolyzes primary, and not secondary fatty acyl esters. This is the same specificity displayed by authentic *Rp. delemar* lipase, consistent with the conclusion that the *LIP* cDNA was derived from an *Rp. delemar* *LIP* gene.

(e) Conclusions

(1) cDNA encoding a lipase produced by *Rp. delemar* has been cloned and the product expressed intracellularly in *E. coli*.

(2) The *LIP* cDNA encodes a predicted 392-aa preproenzyme consisting of a 26-aa signal sequence, a 97-aa propeptide and a 269-aa mature lipase. The mature

enzyme contains the highly conserved lipase consensus pentapeptide and exhibits 56% sequence identity with the lipase produced by *Rm. miehei*, providing evidence for lipase gene families in the lower eukaryotes. No homology to other lipases could be detected.

(3) Forms of lipase corresponding to the prepro- and pro-enzymes, to the product of initiation at a vector methionine codon, and to a species intermediate in size to those of preprolipase and the mature enzyme, are found in *E. coli* JM101[pUC8-2.14]. The mature form of the lipase does not accumulate.

ACKNOWLEDGEMENTS

We thank Karen Kolaetis and Deborah Woolf for expert technical assistance, Alex Bilyk, George Piazza and David Cichowicz for assisting in the determination of the activity and selectivity of the lipase and R. Brousseau for generously providing pUC8-2.

Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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