Cloning and Characterization of Two Lipases and a Lysophospholipase from *Aspergillus niger*

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Abstract

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a Lysophospholipase from *Aspergillus niger*

Shu-Sen Zhu

We identified from a collection of partially sequenced *Aspergillus niger* cDNA clones four cDNAs that were predicted to encode secreted lipases or phospholipases B/lysophospholipases. The deduced amino acid sequence of two of the four genes, *lipA* and *lipB*, exhibited approximately 50% identity with each other and with a lipase from the thermophilic fungus *Thermomyces lanuginosus*, The other two cDNAs, *plbA* and *plbB*, share amino acid sequence 63% identity with each other and about 65% identity with *Aspergillus fumigatus* PlbAp. We functionally expressed the four genes in *Pichia pastoris* and *A. niger*, and purified and characterized the recombinant proteins. LipA showed optimal activity at 40°C and pH 4.5-5.0. LipA was stable at a pH range of 2.2-10.6 and up to 70°C. The pH optimum for LipB was pH 3.5-4.0, and it was stable between pH 3.0 and pH 9.6. The temperature optimum for LipB was 15°C and it retained 70% of its peak activity at 0°C. LipB has the lowest temperature activity profile when compared with reported cold-adapted enzymes. Substrate specificity determination with triacylglycerols and *p*-nitrophenyl esters showed that both lipases preferred esters with middle- and long-chain fatty acids.

The pH optimum for the lysophospholipase activity encoded by PlbA was 3.0 and the activity was stable during incubation from pH 2 to 8.6. The temperature optimum of PlbA was 50°C and it retained at least 80% activity when incubated at 50°C for 3 h.

PlbA exhibited a rather broad specificity towards lysophospholipids with maximal activity on lysophosphatidylcholine and lysophosphatidylserine and preferred the shorter C12 substrate relative to substrates with 14 to18 carbons. PlbA did not exhibit phospholipase B, A1 or A2, lipase or general esterase activity and appeared to be lysophospholipid specific. PlbA lysophospholipase activity displayed cooperative kinetics with a Hill coefficient of 2.036. Recombinant PlbB could only be expressed at an extremely low level in both *Pichia pastoris* and *Aspergillus niger*. We were unable to purify and characterize this enzyme.

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List of Abreviations

BSA, bovine serum albumin

cDNA, DNA complementary to RNA

EDTA, ethylendiaminetetraacetic acid

ESTs, expressed sequence tags

LPL, Lysophospholipase

lyso-PC, Lysophosphatidylcholine

lyso-PE, lysophosphatidyllethanolamine

lyso-PG, lysophosphatidylglycerol

lyso-PL, lysophospholipid

lyso-PS, lysophosphatidylserine

MALDI-TOF, Matrix-assisted laser desorption ionization/time of flight

M_r, relative molecular mass

PAGE, polyacrylamide-gel electrophoresis

PCR, polymerase chain reaction

PL, phospholipid

PLA, phospholipase A

PLB, phospholipase B

PMSF, phenylmethylsulfonyl fluoride

*p*NPCx, *p*-nitrophenyl carboxylate with x-carbon chain

PtdCho, phosphatidylcholine

PtdEtn, phosphatidylethanolamine

PtdIns, phosphatidylinositol

PtdSer, phosphatidylserine

SDS, sodium dodecyl sulphate

SGNH, motif consensus amino acid sequence of Ser, Gly, Asn, and His.

Chapter 1: Literature review

At present, there is no satisfying definition for lipase (triacylglycerol acylhydrolase, EC 3.1.1.3). Lipolytic reactions occur at the lipid-water interface where lipolytic substrates usually form an equilibrium between monomeric, micellar, and emulsified states. Until recently, two criteria have been used to classify a lipolytic enzyme as a "true" lipase: (*a*) It should be activated by the presence of an interface, that is, its activity should sharply increase as soon as the triglyceride substrate forms an emulsion. This phenomenon was termed "interfacial activation". (*b*) It should contain a "lid", which is a surface loop of the protein covering the active site of the enzyme and moving away on contact with the interface (Brzozowski et al. 1991, Derewenda et al. 1992a, Van Tilbeurgh et al. 1993). However, these obviously suggestive criteria proved to be unsuitable for classification, mainly because a number of exceptions were described of enzymes having a lid but not exhibiting interfacial activation (Verger, 1997). Therefore, lipases are simply defined as carboxylesterases catalyzing the hydrolysis (and synthesis) of long-chain acylglycerols (Ferrato, et al. 1997). There is no strict definition available for the term "long-chain," but glycerolesters with an acyl chain length of $\geq$10 carbon atoms can be regarded as lipase substrates, with trioleoylglycerol being the standard substrate. Hydrolysis of glycerolesters with an acyl chain length of <10 carbon atoms with tributyrylglycerol (tributyrin) as the standard substrate usually indicates the presence of an esterase (Jensen, 1983). It should be emphasized that most lipases are perfectly capable of hydrolyzing these esterase substrates.

Lipases catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol, and fatty acids, and under certain conditions they also catalyze the reverse

reaction, esterification, forming glycerides from glycerol and fatty acids. Some lipases are able to catalyze transesterification and enantioselective hydrolysis reactions. Lipases are ubiquitous enzymes playing a pivotal role in all aspects of fat metabolism. In human and other vertebrates a variety of lipases control the digestion, absorption, and reconstitution of fat, as well as lipoprotein metabolism (Desnuelle, 1961). In plants, for example, lipases are abundant in energy reserve tissues (Hassanien and Mukherjee, 1986). Microorganisms are also known to produce a wide spectrum of extracellular lipid-degrading enzymes (Lie *et al.*, 1991). These lipases represent a large array of enzymes that have been classified into 16 superfamilies which display no global sequence similarity between each other (Fischer and Pleiss, 2003).

1.1 General features of lipase structure

Many attempts have been made to identify sequence motifs conserved in lipolytic enzymes originating from a broad variety of organisms, including higher and lower vertebrates, invertebrates, fungi and bacteria, and to relate them to three-dimensional (3D) structural elements involved in substrate recognition and catalysis, and therefore being essential for the enzyme's function. In recent years through the elucidation of many gene sequences and the resolution of numerous crystal structures, our knowledge of the structure of lipases has increased considerably (Cygler and Schrag, 1997; Schrag and Cygler, 1997). Determination of the three-dimensional structures of different lipases has confirmed their classification as 'serine hydrolases'. Their active site is composed of three residues: a serine residue hydrogen-bonded to a histidine residue, and a carboxylate-residue hydrogen bonded to this same histidine residue. The carboxylate may be either an

aspartate or a glutamate residue. The architecture of the catalytic triad of lipases is very similar to the one found in serine proteases (Winkler et al. 1990, Brady et al. 1990). The active-site Ser residue is contained in the consensus sequence GxSxG (where x is any residue), known as the substrate-binding site from many esterases. During the reaction, a tetrahedral intermediate is formed which decomposes into an acyl-enzyme complex. The free lipase is regenerated by a hydrolytic reaction mediated by a water molecule.

All the lipases investigated so far vary considerably in size and in their amino acid sequences. They all have a general common structural motif, known as the α/β-hydrolase fold (Brady et al. 1990; Winkler et al. 1990; Schrag et al. 1991; Grochulski et al. 1993; Derewenda et al. 1994; Ollis et al, 1992). The structural superfamily of α/β-hydrolases defined by Ollis et al. (1992) comprises a wide variety of enzymes whose activities rely mainly on a catalytic triad usually formed by Ser, His and Asp residues. This triad is functionally (but not structurally) identical with that of serine proteases. In the amino acid sequences of α/β-hydrolases the three residues follow the order Ser-Asp- His. The serine residue usually appears in the conserved pentapeptide G-X-S-X-G.

In addition, another common feature shared by many lipases is the presence of a helical structure, referred to as the 'lid', that covers the active site of the enzyme, rendering it inaccessible to the substrate. This helical lid presents non-polar sidechains over the catalytic group, and polar sidechains to the enzyme surface. Although the 'lids' are very similar in construction in all lipases, they belong to very different parts of the polypeptide chain. They may consist of a single helix (Brzozowski et al. 1991; Derewenda et al. 1992a), or two helices (Kim et al. 1997; Schrag et al. 1997), or a loop region (Grochulski et al. 1994a). The catalytic activity of lipases with soluble substrates

is very low, and it increases dramatically when the substrate is aggregated forming a lipid/water interface [Sarda and Desnuelle 1958]. The most favored hypothesis to explain interfacial activation involves a conformational change in the enzyme on binding to the interface, essentially a displacement of the lid by rotating around its hinge regions to expose the active site (Desnuelle et al. 1960). This structural rearrangement is supported by the X-ray structures of lipases complexed with inhibitors (Brzozowski et al. 1991; Lawson et al. 1994; van Tilbeurgh et al. 1993; Grochulski et al. 1994b; Hermoso et al. 1997). The large hydrophobic patch created by this movement will likely be stabilized by interaction with a non-polar interface, such as micelles or bilayers, where lipase will adopt the catalytically active or 'open' conformation (van Tilbeurgh et al. 1992; Cygler and Schrag 1997). Theoretical studies on lid opening indicate that the open conformation of the lid is more favorable at low dielectric constant, as found in lipids and organic solvents, and unfavorable at high dielectric constants, such as in water solution (Norin et al. 1994). The X-ray structure of lipases from *Rhizomucor miehei* and *Thermomyces lanuginosa* (Lawson, et al, 1994) shows that the catalytic sites are clearly buried beneath the amphipathic helical lid which arises from a long external loop between two β strands. This is known as the 'closed' structure of the enzyme, and it is believed to be the structure adopted in aqueous solution in the absence of an interface, where lipases display very low or no catalytic activity.

1.2 The three-dimensional structure of fungal lipases.

At present, nine X-ray structures of fungal lipases have been elucidated, *R. miehei* (Derewenda et al. 1992b), *Penicillium camembertii* (Derewenda et al. 1994), *T.*

lanuginosa (Brzozowski et al. 2000), *Rhizopus niveus*(Kohno et al. 1996), *Geotrichum candidum* (Schrag et al. 1991), *Candida antarctica* (Uppenberg et al. 1994) and three from *Candida rugosa* (Grochulski et al. 1993). These lipases can be divided into three groups based on their sequence homology and structure similarity. *R. miehei*, *P. camembertii*, *T. lanuginosa* and *R. niveu* lipases are the first group, all sharing a similar structure. The second group include *G. candidum* and three *Candida rugosa* lipases. The structures of these lipases are very similar since the lipases share a high level of sequence homology. *Candida antarctica* lipase shows little sequence homology with all the other lipases and has a different structure.

Three-Dimensional Structure of a Lipase from a Filamentous Fungus *Rhizomucor miehei*

Rhizomucor miehei lipase (Derewenda, et al, 1992b) is structurally closely related to human pancreatic lipase (Winkler, et al, 1990) although protein sequence alignment fails to indicate any close relationship. This extracellular enzyme is a relatively small (269 amino acids) single polypeptide chain with one domain. All but the first five residues of this lipase have been positioned in the 1.9-Å electron density map (Derewenda et al. 1992b). The molecule adopts an uncommon fold that consists of a sequential, predominantly parallel, singly wound nine-stranded β sheet, with all connecting fragments showing the classic right-handed twist, and six main α helices. One face of the sheet is adjacent to a single N-terminal helix and the other face makes numerous interactions with most of the loops, turns, and interstrand connections. This results in unique asymmetry of the molecule. Three disulfide bridges stablilize the fold and link the following pairs of cysteines: 29-268, 40-43, and 235-344. Four cis peptide

bonds are present in the *R. miehei* lipase and they all precede prolines. A number of hydrophilic internal cavities containing ordered solvent molecules were found. This is an interesting observation, particularly in the light of the *G. candidum* lipase structure (see below), in which Schrag *et al.* (1991) also note the presence of a number of internal water molecules.

The catalytic site in the *R. miehei* lipase was identified originally from the location of the known lipase/esterase consensus sequence G-X-S-X-G (Brenner, 1988) containing the nucleophilic serine (Ser-144), This amino acid was found to be involved in a hydrogen-bounded constellation also including His-257 and Asp-203. Overall this hydrogen-bonding network is reminiscent of the catalytic triad of serine proteinases. Howerver, in contrast to proteinases, the triad is concealed under a short helix, the "lid," and is therefore inaccessible to solvent.

***Geotrichum candidum* Lipase**

Geotrichum candidum Lipase contains 544 amino acids in a single chain folded into one domain, making it one of the largest structural domains observed to date in a protein (Schrag *et al.* 1991). Like *R. miehei* lipase, *G. candidum* lipase is an α/β structure with a central, predominantly parallel β sheet. There are 11 strands in the central sheet, 3 more in a small additional sheet, and 17 α helices. The catalytic Ser-217, part of the G-X-S-X-G pentapeptide, is located at a tight turn between the C terminus of a β strand and the N terminus of an α helix, exactly as observed in *R. miehei* lipase. The hydroxyl of Ser-217 is hydrogen bonded to the imidazole of His-463, which in turn donates a hydrogen bond to Glu-354. Thus, GcL constitutes the first known example of a serine hydrolase in which the acid residue of the triad is glutamate and not aspartate.

The putative active site is covered by two nearly parallel α helices (residues 66-76 and 294-310) coming from different parts of the polypeptide chain. Schrag *et al.* (1991) note that both of these helices can be easily displaced following relatively minor adjustments in the main-chain conformation.

***Candida antarctica* lipase**

The primary sequence of the *Candida antarctica* lipase has no significant homology to any other known lipase and deviates from the consensus sequence around the active site serine that is found in other lipases. The crystal structure determination of the *C. antarctica* lipase was determined by Uppenberg et al (1994). The *C. antarctica* lipase is an α/β type protein that has many features in common with previously determined lipase structures and other related enzymes. It is built up from a subset of the α/β-hydrolase fold and contains a Ser-His-Asp active site triad. The catalytic triad is made up of Ser105, Asp187 and His224 and, therefore, shares the sequential order of catalytic residues of all lipases and α/β- hydrolases for which structures have been determined. The active site serine is surrounded by many polar residues. A rather narrow and deep channel leads into an open active site that contains an oxyanion hole. The shape of the channel probably accounts for the enzyme's stereospecificity. The lipase crystal structures that have appeared in recent years indicate that activation at the interface may be caused by a conformational change that exposes the active site of the enzyme. A putative lid has been identified based on the observed mobility of a short α–helix. The long carboxy-terminal helix may also play an important role since it has no hydrogen bonds to other parts of the structure and interacts mainly through hydrophobic side chains. The relatively low activity of the enzyme on large triglyceride substrates and the easy adoption of an open

conformation suggests that the *C. antarctica* lipase may be an intermediate between an esterase and a true lipase.

1.3 Interfacial activation and kinetics

The physical properties of lipids in general have caused many difficulties in studying the properties of lipolytic enzymes. Desnuelle (1961) demonstrated a fundamental difference between esterase and lipase activity based on their ability to be activated by interfaces. Esterase activity is a function of substrate concentration as described by Michaelis- Menten kinetics with the maximal reaction rate being reached long before the solution becomes substrate-saturated; the formation of a substrate/water emulsion does not change the reaction rate. In contrast, lipases show almost no activity with the same substrate as long as it is in its monomeric state. However, when the solubility limit of the substrate is exceeded, there is a sharp increase in enzyme activity as the substrate forms an emulsion.

Experiments demonstrate that lipase activity depends on the presence of an interface. This led to the definition of lipases as carboxylesterases acting on emulsified substrates. This property found an elegant explanation when the first three-dimensional structures of lipases had been elucidated. This increase in enzymatic activity is triggered by structural rearrangements of the lipase active-site region, as witnessed from crystal structures of lipases complexed by small transition state analogs (Brzozowski et al. 1991; Derewenda et al. 1992a; van Tilbeurgh et al. 1993). It was found that the active site of lipase was covered by a lid-like polypeptide chain which rendered the active site inaccessible to substrate molecules, thereby causing the enzyme to be inactive on

monomeric substrate molecules (Winkler et al. 1990; Brady et al. 1990). However, when a lipase was bound to a lipid interface, a conformational change took place causing the lid to move away whereby the active site of the lipase became fully accessible. As a result, the hydrophobic side of the lid became exposed to the lipid phase, thus enhancing hydrophobic interactions between the enzyme and the lipid surface (Brzozowski et al.1991; Van Tilbeurgh et al. 1993). This observation explains the interfacial activation phenomenon with the lid causing inactivation if no lipid interface is present and has been used to discriminate between 'true' lipases and esterases by defining a lipase as an enzyme which shows interfacial activation in the presence of long-chain triacylglycerols as substrates. If an enzyme hydrolyzing these substrates does not show interfacial activation it should be called an esterase. However, this definition should be used with care for several reasons: (1) the detection of interfacial activation requires pure lipase enzyme to avoid potential effects of other carboxyl hydrolases; (2) the same lipase may show a distinctly different behaviour depending on the 'quality' of the interface. An example is *S. hyicus* lipase which is able to degrade acylglycerols as well as phospholipids. It is activated in the presence of a tributyrin interface, but not in the presence of an interface composed of diheptanoyl-phosphocholine (Van Oort et al. 1989). (3) Lipases from *P. aeruginosa* (Jaeger et al. 1993), *B. subtilis* (Lesuisse et al. 1993) and guinea pig pancreatic lipase (Hjort et al. 1993) do not show activation in the presence of emulsified substrates; instead, their activity continuously increases indicating that these enzymes are able to degrade both emulsions and monomeric substrates, whereas true esterases degrade only monomeric substrates. Therefore, a lipase should not be defined

solely according to its interfacial activation behaviour, but also according to its capability to hydrolyse emulsions of long-chain acylglycerols.

Kinetics of lipases cannot be described with the Michaelis-Menten model since this model is valid only in the case of one homogenous phase, i.e. for soluble enzymes and substrates. Therefore, an alternative model has been proposed to describe the kinetics of catalysis by lipolytic enzymes (Verger and de Haas 1976) which consists of two steps: (1) the physical adsorption of the enzyme at the lipid interface may include an activation of the enzyme (opening of the lid which blocks the active site) (Brzozowski et al. 1991; Van Tilbeurgh et al. 1993); (2) the formation of the enzyme/substrate complex which can be hydrolysed to give the product and regenerate the adsorbed enzyme. This second step may be described by an 'interfacial' Michaelis-Menten model with the substrate concentrations expressed in [mol/surface] instead of [mol/volume]. Accumulation of products at the interface may cause problems, such as induced changes of the physico-chemical properties of the interface and products inhibition. The kinetic model takes into account a lipase inactivition step caused by the interface. This inactivation depends upon the surface pressure, with low pressure (corresponding to high surface energy) being the most strongly denaturing. The production rate depends therefore both on the adsorption and the inactivation, and becomes constant in time when the adsorption is fast compared with the inactivation, or when the two processes have equal fluxes. The reversibility of the adsorption step seems to depend on the surface pressure. Equations have been reported which perfectly describe the experimental results (Verger and de Haas 1976). Additionally, models to describe the kinetics of competitive inhibition of lipases in the

presence and absence of detergents as well as for interfacial inactivation have been proposed (Ransac et al. 1990, 1991).

1.4 lipase inhibitors

Lipase inhibitors have been used in the study of their structural and mechanistic properties. Further, the search for lipase inhibitors is also of pharmacological interest. Lipase inhibitors are used for designing drugs for the treatment of obesity and the problem of acne. Following is an account of general lipase inhibitors. Broadly, inhibitors of enzymes are classified as reversible or irreversible. The reversible inhibitors can be further classified as non-specific and specific.

Reversible non-specific inhibitors

Compounds that do not act directly at the active site, but inhibit lipase activity by changing their conformation or the interfacial properties are defined as non-specific inhibitors. Surfactants (Iizumi et al. 1990; Patkar and Bjorkling 1994), bile salts (Borgstrom and Donner 1976; Wang et al. 1999), some proteins (Gargouri et al. 1984; Bezborodov et al. 1985) and some metal ions, such as calcium (Liu et al. 1973), iron (Iwai et al. 1970) and mercury (Fredrikson et al. 1981), belong to this group of inhibitors. However, surfactants, bile salts and calcium can activate the enzyme in some cases.

Specific inhibitors

Specific inhibitors are those compounds, which directly interact with the active site of the enzyme. Such inhibitors can be either reversible or irreversible. Specific reversible inhibitors include: (1) boronic acid derivatives, which form reversible but long-lived complexes with the active-site serine of lipases (Lolis and Petsko 1990) and (2) substrate

analogues including triacylglyceride ether, which is also a competitive inhibitor of pancreatic lipase (Hadvary et al. 1988). However, the affinity of the enzyme for this compound is not high enough, compared with the substrate, and hence it is difficult to obtain useful information from these analogues. Specific irreversible inhibitors generally react with the amino acids at or near the active site and thus inhibit the catalytic activity.

Lipases belong to the class of serine hydrolases with the catalytic triad Ser-His-Asp/Glu. Therefore, serine inhibitors are potential irreversible active-site lipase inhibitors, e.g. phenylmethylsulfonyl fluoride (PMSF), phenylboronic acid, diethyl *p*-nitrophenyl phosphate. However, the lipase from *Acinetobacter calcoaceticus* LP009 was not inhibited by PMSF (Dharmsthiti et al. 1998). Generally, lipases are not sulphydryl proteins; and thus in most lipases neither free –SH nor S–S bridges are important for their catalytic activity. This is substantiated by the use of 2-mercaptoethanol, *p*-chloromercuric benzoate and iodoacetate, which have no detectable effect on the lipase from *Chromobacterium viscosum* (Sugiura et al. 1974), *Staphylococcus aureus* 226 (Muraoka et al. 1982) and *A. calcoaceticus* LP009 (Dharmsthiti et al. 1998). Further, EDTA does not affect the activity of most lipases (Gilbert et al. 1991; Sugihara et al. 1991; Kojima et al. 1994). However, it is inhibitory to lipases from *Pseudomonas aeruginosa* 10145 (Finkelstein et al. 1970), *Pseudomonas* sp. nov. 109 (Ihara et al. 1991), *Bacillus* sp. THL027 (Dharmsthiti and Luchai 1999) and *A. calcoaceticus* LP009 (Dharmsthiti and Kuhasuntisuk 1998). Tryptophan residues play an important role in maintaining the conformation of lipases (Patkar and Bjorkling 1994). Modification of tryptophan residues in lipases from *Pseudomonas fragi* CRDA 037 (Schuepp et al. 1997) and *Pseudomonas*

fluorescens (Sugiura et al. 1977) by *N*-bromosuccinimide leads to decreased lipase activity.

1.5 Substrate specificity

The glycerol molecule as the basic building block of the lipase substrate triacylglycerol contains two primary and one secondary hydroxyl groups. Although the molecule has plane symmetry, the two primary groups are sterically distinct. Substitution of these hydroxyl groups with two different substituents will lead to optically active derivatives. In a generally adopted nomenclature (IUPAC-IUB Commission on Biochemical Nomenclature, 1967), glycerol is written in a Fisher projection with the secondary hydroxyl group to the left, and the carbon atoms numbered 1, 2, and 3 from top to bottom (*sn-*, i.e. stereospecifically numbered glycerol), thereby allowing the unambiguous description of isomeric glycerides.

In general, specificity of lipases is low and in many cases the hydrolytic potential extends even to phospholipids and other organic compounds with an ester bond (Deckelbaum et al. 1992). This is a logical consequence of the primary function of most lipases, the hydrolytic degradation of acylglycerols in general. Even so, lipases may be divided into three categories: namely nonspecific, regiospecific and fatty acid-specific, based on their substrate specificity. Nonspecific lipases act at random on the triacylglyceride molecule and result in the complete breakdown of triacylglyceride to fatty acid and glycerol. Examples of this group of lipases include those from *G. candidum, Penicillium cyclopium* (Okumura et al. 1976), *S. aureus* (Vadehra and Harmon, 1967), *Staphylococcus hyicus*

(Van Oort et al. 1989), *Corynebacterium acnes* (Hassing 1971) and *C. viscosum* (Jaeger et al. 1994).

In contrast, regiospecific lipases are 1,3-specific lipases which hydrolyze only primary ester bonds (i.e. ester bonds at atoms C1 and C3 of glycerol) and thus hydrolyze triacylglyceride to give free fatty acids, 1,2(or 2,3)-diacylglyceride and 2-monoacylglyceride. Pancreatic lipase has been known for its positional specificity toward the 1(3) chains of triglycerides (Yang et al. 1990). Gastric lipase (Barrowman & Darnton 1970), hepatic lipase (Waite and Sisson 1974) and lipases from fungi *Rhizopus arrhizus, A. niger* and *Rhizopus delemar* (Benzonana 1974) were also reported to exhibit a positional specificity similar to that of pancreatic lipase. Bacterial lipases of this group are those from *Bacillus* sp. (Lanser et al. 2002), *B. subtilis* 168 (Lesuisse et al. 1993), *Bacillus* sp. THL027 (Dharmsthiti and Luchai 1999), *Pseudomonas* sp. f-B-24 (Yamamoto and Fujiwara 1995), *P. aeruginosa* EF2 (Gilbert et al. 1991) and *Pseudomonas alcaligenes* 24 (Misset et al. 1994).

The third group comprises fatty acid-specific lipases, which exhibit a pronounced fatty acid preference. Gastric lipase (Gargouri et al. 1986) and lipases from *G. candidum* (Iwai and Tsujisaka 1984), *Bacillus* sp. (Wang et al. 1995), *P. alcaligenes* EF2 (Gilbert et al.1991) and *P. alcaligenes* 24 (Misset et al. 1994) show specificity for triacylglycerides with long-chain fatty acids, while lipases from *P. cyclopium, R. delemar* (Iwai and Tsujisaka 1984), *B. subtilis* 168 (Lesuisse et al. 1993), *Bacillus* sp. THL027 (Dharmsthiti and Luchai 1999), *P. aeruginosa* 10145 (Finkelstein et al. 1970), *P. fluorescens* (Sugiura et al. 1977), *Pseudomonas* sp. ATCC 21808 (Kordel et al. 1991), *C. viscosum* (Horiuti and Imamura 1977) and *Aeromonas hydrophila* (Angultra et al. 1993)

prefer small- or medium-chain fatty acids. Lipase from *S. aureus* 226 shows a preference for unsaturated fatty acids (Muraoka et al. 1982).

Another important property of lipases is their enantio-/stereoselective nature, wherein they possess the ability to discriminate between the enantiomers of a racemic pair. Such enantiomerically pure or enriched organic compounds are steadily gaining importance in the chemistry of pharmaceutical, agricultural, synthetic organic and natural products (Reetz 2001). Mostly lipases from *Pseudomonas* family fall in this category (Reetz and Jaeger 1998). The stereospecificity of a lipase depends largely on the structure of the substrate, interactions at the active site and the reaction conditions (Cambou and Klibanov 1984; Muralidhar et al. 2002).

1.6 Applications of lipases

Lipases are versatile biocatalysts. In addition to their hydrolytic activity on triglycerides, they can catalyze other reactions such as esterification, interesterification, acidolysis, alcoholysis, and aminolysis (Fig. 1). As hydrolases, lipases do not require cofactors. Most regioselective lipases act preferentially on ester bonds at the *sn*-1 and *sn*-3 position of the triglyceride structure, whereas few lipases are active at the *sn*-2 position. Lipases can be found with optimum activities over a wide range of temperatures. Several three-dimensional structures of these enzymes have been resolved allowing the design of rational engineering strategies. The commercial use of lipases is a billion-dollar business that comprises a wide variety of different applications, such as in the detergent, food, leather, textile, oil and fat, cosmetic, paper, and pharmaceutical industries (Kademi et al. 2004). Many lipases are currently commercially available; because of their high

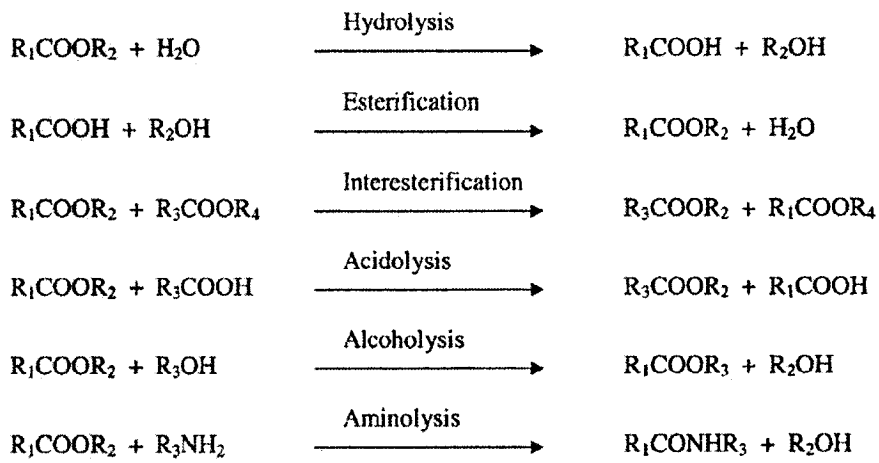

Figure 1. Different reactions catalyzed by lipase

performance, some lipases are commercialized for one specific application whereas others can be used in different industrial fields. However, despite the relatively high number of commercial lipases available, industrial applications remain limited owing to the high cost of some lipases, the low number of available lipases in industrial amounts, and the low performance of some lipase-mediated processes. Nevertheless, lipases are currently used mainly in the food, detergent, and pharmaceutical industries.

Food Industry

Fats are generally divided into three categories with respect to the degree of saturation of their fatty acids as: saturated, monounsaturated, and polyunsaturated. Monounsaturated and polyunsaturated fats are liquid at room temperature and are considered healthier than saturated fats owing to their role in decreasing serum cholesterol and therefore the risk of coronary disease as well as tumor development (Balcao and Malcata 1998).

Structured lipids are modified fats or oils modeled to be more nutritious and with specific functional and physical properties that make them more suitable for food applications. These modifications include changes in fatty acid content, changes in the position of the fatty acid on the glycerol backbone, and changes in the length of the fatty acid as well as its degree of saturation. Structured lipids may be the most effective approach to include the desired fatty acid in the diet for nutritive and therapeutic purposes. Lipids possessing these higher-added-value attributes can also be produced through lipase-catalyzed interesterification reactions and/or by acidolysis of a less desirable and cheaper lipid. As structured lipids, plastic fats intended for food applications, such as the production of margarine, shortening, and modified butter, are solid in appearance and possess a low

resistance to small stresses, thereby making them easy to spread and rapidly melt in the mouth.

Econa® oil or diacylglycerol (DAG) oil was produced enzymatically from natural oil and contains 80% or more DAG. DAG oil was introduced to the market by Novozymes and Kao and possesses virtually the same energy value as triacylglycerol oil but is not transformed into body neutral fat (Pszczola 2001). This oil represents the first industrial application to obtain a product using an immobilized lipase process. In Japan, the total sale of Econa oil and derived products reached annual sales exceeding $150 million in 2002 (Hosoya and Hirahara 2002).

Infant formula offers a good alternative to breast milk and ideally tends to mimic human milk as much as possible. Milk fat represents the main source of energy in human milk and provides the lipids required to build the structure of cell membranes. The fat present in most infant formulas comes from vegetable sources and has an unsaturated fatty acid usually located at the *sn*-2 position of the triglyceride. Modification of the triglyceride by lipase to increase the proportion of palmitic acid at the *sn*-2 position leads to a fat with an improved absorption capability in infants (Osborn and Akoh 2002). Betapol™ (Loders Croklaan) was the first commercial product made by the 1,3-specific lipase treatment of tripalmitin with unsaturated fatty acids that resulted in 1,3-diunsaturated-2- saturated triglycerides intended for infant formula (Osborn and Akoh 2002, Yang et al. 2003).

Although fats possess a high-calorie intake, their good taste and smoothness make them difficult to be circumvented. Reduced calorie and substituted fats are now available. Akoh and Yee (1997) produced a low-calorie lipid by interesterification of tristearin

(C18:0) with either tricaprin (C10:0) or tricaprylin (C8:0) using an immobilized lipase. Kanjilal et al. (Kanjilal et al. 1999) performed an interesterification of sunflower oil with a lipase and incorporated behenic acid at the *sn*-1 and *sn*-3 positions. The resulting oil, Bohenin, produced by Fuji Oil, is a commercial triglyceride containing behenic acid at the *sn*-1 and *sn*-3 positions with oleic acid at the *sn*-2 position and has 5.36 cal/g. Bohenin has a taste very close to sunflower oil (Osborn and Akoh 2002).

Cocoa butter is a mixture of oil and fat composed of triglycerides possessing palmitic acid, stearic acid, and oleic acid as the major components. The high price of cocoa butter is the result of its low availability. Consequently, interesterification of abundant and less expensive fats, including illipe fat, shea butter, sal fat, and kokum butter, offers a good alternative for the production of cheaper cocoa butter substitutes. The introduction of palmitic or stearic acids at the *sn*-1 and *sn*-3 positions by a selective lipase produces cocoa butter substitutes with a cooling, melting sensation characteristic of chocolate and similar physical properties at a lower cost (Osborn and Akoh 2002). Newlase, an immobilized lipase from *Rhizopus niveus*, specifically incorporates stearic acid at the *sn*-1 and *sn*-3 positions of triglycerides in safflower oil or sunflower oil. Fuji Oil has exploited this process since 1993 to produce a cocoa butter substitute.

Cheese ripening is composed of a complex sequence of events and is the result of many transformation processes such as proteolysis and lipolysis in milk by indigenous microflora. Cheese texture is related to the fat content, and aroma is generated by fat degradation. In a regular cheese-making process, milk fat is hydrolyzed during lipolysis to liberate free fatty acids, which contribute directly to the aroma and also act as precursors for methyl ketones, secondary alcohols, and aliphatic and aromatic esters. The

addition of exogenous lipase accelerates the ripening process. However, the addition of free lipases to the process can lead to excessive lipolysis resulting in texture and flavor defects, whereas lipase encapsulation regulates the enzyme/substrate ratio and overcomes this problem (Kheadr et al. 2002). As potential industrial application, the use of an encapsulated enzyme cocktail containing a lipase (Palatase M from Novozymes) and a protease results in a full-flavored cheese without a bitter taste even after 90 days of ripening (Kheadr et al. 2003).

High-intensity cheeselike products find numerous applications in the food industry, where they can add cheese flavor to salad dressings, dips, soups, sauces, snacks, frozen foods, and so on. They include enzyme-modified cheeses (EMCs), cheese powders, and cheese flavors. The flavor intensity of EMCs can be increased up to 15–30 times that found in natural cheese. EMCs do not increase the fat content in the intended application but provide rich mellow tones, a pleasant flavor-enhancing effect, a fatty feel in the mouth, reduced production costs, and enhanced product stability. A wide variety of lipases are available and their contribution to the resulting EMC depends on the type or source of enzyme. Thus, knowledge of the metabolic pathways involved in flavor production and the specific activity of the enzyme are both essential for a reproducible cheese flavor.

Detergent Industry

In the past, ground porcine or bovine pancreases, rich in lipases, were used in the fine chemical industry as detergent additives. Actually, the use of lipases in the detergent industry represents the main significant application of these enzymes. In 1995, detergent enzymes represented 30% of the total enzyme market, estimated at US $30 million

(Godfrey and West 1996). In 2000, this market reached US $1.5 billion (Kirk et al. 2002). Novozymes, formerly Novo Nordisk, launched Lipolase® in 1988, the first commercial lipase developed for the detergent industry. Lipolase has an optimal pH of 10.5–11.0; is active over a broad range of temperatures, with an optimum at 40°C; is stable in proteolytic wash solutions; shows oxidation stability; and is stable toward several other detergent ingredients including surfactants. This enzyme is widely used in detergent formulations to remove fat-containing stains including fried fat, salad oils, butter, fat-based sauces, soups, human sebum, and lipstick. Lipolase also has a broad range of substrate specificity. This lipase is currently added to a significant number of major detergent brands throughout the world. Novozymes later introduced three variants of Lipolase: Lipolase® Ultra, LipoPrime™, and Lipex®.

Oleochemical Industry

The scope for the application of lipases in the oleochemical industry is enornous. Fats and oils are produced world-wide at a level of more than 60 million tons per annum and a very substantial part of this (more than 2 million tons p. a.) is utilised in high-energy-consuming processes such as hydrolysis, glycerolysis and alcoholysis (Arbige and Pitcher 1989, Falch 1991). The conditions for steam fat-splitting and conventional glycerolysis of oils involve temperatures of 240-260°C and high pressures (methanolysis is currently performed under slightly milder conditions). The resulting products are often unusable as obtained and require re-distillation to remove impurities and products of degradation. In addition to this, highly unsaturated heat-sensitive oils cannot be used in this process without prior hydrogenation.

The saving of energy and minimisation of thermal degradation are probably the major attractions in replacing the current chemical technologies with biological ones. A wide range of fatty acid esters can be synthesised by esterification and transesterification reactions catalysed by lipases in non-aqueous systems. Lipases have been shown to have broad substrate specificities and can thus catalyse conversions with many other non-glycerol based compounds. Many steps used in conventional chemical methods can be avoided by a selective enzymatic ester production, where only esterification is catalysed. The avoidance of the formation of by-products in the low temperature synthesis means that purification procedures are no longer necessary. In particular, as there is no bleaching, no bleaching residues or remains of bleaching agents contaminate the products. Thus cleaner products with better colour and odour, which are important criteria for cosmetic products, and with narrower specifications due to the more efficient synthesis can be obtained (Hills 2003).Miyoshi Oil & Fat Co., Japan, reported the commercial use of *Candida cylindracea* lipase in the productin of soaps (Hoq et al. 1985). The company claimed that the enzymic method yielded a superior product and was cheaper overall than the conventional Colgate-Emery process.

Pharmaceutical Industry

In the pharmaceutical industry, biocatalysis offers numerous advantages over chemical synthesis, thereby justifying the growing demands for enzymes. These advantages include enantio- and regioselectivity; mild conditions that avoid isomerization, racemization, epimerization, and rearrangement reactions; overexpression of the enzymes; reuse of the immobilized biocatalysts; economy of the process; and mutagenesis of the enzymes for specific functions. The ability of lipases to resolve racemic mixtures by the

synthesis of a single enantiomer is currently exploited for drug production by the pharmaceutical industry. In fact, only one enantiomer of a drug is responsible for the desired therapeutic effect, and milder or fewer side effects are observed when using optically pure drug products compared with those found with the use of racemic mixtures.

Some lipases are suitable to be used in the synthesis of various enantiopure molecules such as alcohols, amides, carboxylic acids, and esters. These molecules are used in antiinflammatory drugs (ibuprofen, naproxen) (Kademi et al. 2004), anticancer drugs (Taxol®, spergualin), an antiviral drug (lobucavir), an antihypertensive drug (captopril), anticholesterol drugs (squalene synthase inhibitor), an anti-Alzheimer disease drug ([*S*]-2-pentanol), and vitamin A (Bonrath et al. 2002).

The anticancer drug Taxol® (paclitaxel) is an antimitotic agent that inhibits the depolymerization process of microtubulin during mitosis. This drug is used in the treatment of ovarian cancer and metastatic breast cancer. Taxol® generates annually about US $1 billion in sales (Anonymous 1998). Paclitaxel was originally extracted and purified from the bark of the yew *Taxus brevifolia* in a very low yield. Paclitaxel can also be obtained by a semisynthetic process by coupling baccatin III (paclitaxel without the C-13 side chain) or 10-deacetylbaccatin II ((10-DAB), paclitaxel without the C-13 side chain and the C-10 acetate) to C-13 paclitaxel side chains. Baccatin III and 10-DAB can be extracted from renewable sources such as the extract of needles, shoots, and young *Taxus* plants, therefore eliminating the cutting of yew trees. The C-13 paclitaxel side chain was obtained from the enantioselective hydrolysis of racemic acetate-*cis*-3-(acetoxy)-4-phenyl-2- azetidione to the corresponding (3*S*)-alcohol and the intact desired (3*R*)- acetate. Hydrolysis was catalyzed by the lipase PS-30 from *Pseudomonas cepacia*

or Bristol-Myers Squibb (BMS) lipase from *Pseudomonas* sp. SC 13856. Both lipases were immobilized on Accurel Polypropylene before use. An enantiomeric excess of >99.5% and reaction yields of >48% (maximum theoretical yield: 50%) were obtained for (3*R*)-acetate. The process was scaled up to 75 and 150 L using immobilized BMS lipase and Lipase PS-30, respectively. The (3*R*)-acetate was then converted chemically into (3*R*)-alcohol (C-13 paclitaxel side chain) (Patel 1998).

Ibuprofen, a nonsteroidal antiinflammatory drug commercialized as Advil or Motrin with sales estimated to be about $290 million in the United States for Motrin alone (www.thehindubusinessline.com/bline/2003/01/04/stories/2003010401600200.htm), represents another example of the applications of lipases. Ibuprofen, 2-(4-isobutylphenyl) propionic acid, inhibits the binding of arachidonic acid and prevents the synthesis of prostaglandins acting on the inflammatory response. Ibuprofen is a racemic mixture containing two enantiomers. The (*S*)-ibuprofen molecule is 160 times more potent in inhibiting the prostaglandin synthesis than the (*R*)-ibuprofen one (Sharma et al. 2001). As a potential application, resolution of racemic ibuprofen by esterification of (*S*)-ibuprofen with methanol or butanol in organic media using a specific lipase leads to synthesis of the corresponding (*S*)-ester. This ester is then completely separated from the (*R*)-ibuprofen and chemically transformed to (*S*)-ibuprofen (Kademi et al. 2004).

With an annual market of about US $85 million in 1996 and 22.5 billion yen in 2002, diltiazem, a calcium channel blocker, constitutes another economical, important industrial lipase application. Tanabe manufactures 50 t of diltiazem annually. Resolution of racemic epoxyesters represents a key step in the production of an important intermediate essential for the synthesis of diltiazem. This enantiospecific hydrolysis is

catalyzed by a lipase from *Serratia marcescens*. The product of the reaction, 2(*R*), 3(*S*)-methyl-*p*-methoxyphenylglycidate, is found in an ee of >98% and is later converted into diltiazem (Jaeger et al. 1999). Sepracor has successfully operated a multiphase membrane bioreactor at a multikilogram scale to produce the key diltiazem intermediate 2(*S*), 3(*R*)-methoxyphenyl glycidate (www. au-kbc. org).

Synthesis of Ingredients for Personal-care products

Although the cost of lipase-catalysed esterification typically remains very high for the manufacturing of many bulk products, the synthesis of several speciality esters has found its way into the market-place. Unichem International has recently launched the production of isopropyl myristate, isopropyl palmitate and 2-ethylhexyl palmitate (Macrae et al. 1990) for use as an emollient in personal-care products such as skin and sun-tan creams, bath oils etc. Immobilised *Mucor miehei* lipase was used as a biocatalyst in the solvent-free esterification, which was driven to completion by vacuum distillation of the water produced during the reaction. The company claims that the use of the enzyme in place of the conventional acid catalyst gives products of much higher quality, requiring minimum downstream refining.

Wax esters (esters of fatty acids and fatty alcohols) have similar application in personal care products and are also being manufactured enzymically (Croda Universal Ltd.). *Candida cylindracea* lipase is used in a batch bioreactor. According to the manufacturer, the overall production cost is slightly higher than that of the conventional method, but the cost is justified by the improved quality of the final product.

Polymer synthesis

Optically active polymers have found several applications: as asymmetric reagents, as absorbents and in the field of liquid crystals. Excellent stereoselectivity has been demonstrated in the lipase-mediated polytransesterification of racemic diesters and a diol (Wallace and Morrow 1989, Margolin et al. 1987).

For the enzymatic synthesis of alkyds, unsaturated diesters are combined with aliphatic or aromatic diols in a polytransesterification reaction using a *Pseudomonas* lipase. No isomerization of the double bond was observed under the mild conditions of the lipase-catalyzed reaction, in contrast to the extensive isomerization found during chemical polycondensation (Geresh and Gilboa 1990, 1991). In a subsequent cross-linking reaction, alkyds can be polymerized to industrially applicable 'general purpose polyesters'. Several chemoenzymatic processes have been described for the preparation of various polyacrylates. After a stereoselective reaction of a racemic alcohol with a (meth) acrylate ester as acylating agent using a *Pseudomonas* lipase, (meth)acrylate polymers of higher molecular mass could be obtained employing an additional chemical polymerization step (Margolin et al. 1991). P. *cepacia* lipase (Martin et al. 1992) catalyzed the transesterification of various monosaccharides with vinylacrylates, whereupon the resulting sugar-acrylate esters were chemically polymerized. The use of the resulting polymers for biomedical applications and membranes was suggested (Martin et al. 1992).

Pulp and paper industry

The enzymatic pitch control method using lipase was put into practice in a large scale paper-making process as a routine operation in the early 1990s and was the first case in the world in which an enzyme was successfully applied in the actual papermaking

process. Pitch is composed of fatty acids, resin acids, sterols, glycerol esters of fatty acids, other fats, and waxes and is usually defined empirically as the wood component that is soluble in methylene. It is less than 10% of the total weight of wood but causes major problems (Farrell et al. 1997). Pitch reduction with enzymes is a very efficient biotechnological method (Fischer and Messner, 1992; Fischer et al., 1993; Fujita et al., 1992). Different lipases have been used for removal of pitch. Few commercial preparations of lipases for pitch removal are available (Fujita et al., 1992). Enzymatic pitch control helps to reduce pitch-related problems to a satisfactory level. It reduces defects on paper web as well as the frequency of cleaning pitch deposits in the paper machine. At the same time, it also offers other advantages, such as ecofriendly and nontoxic technology, improved pulp and paper quality, reduction in bleaching chemical consumption, reduction of effluent load, and space and cost saving in a mill wood yard by using unseasoned logs. By reducing the outside storage time of logs, this method reduces wood discoloration, wood yield loss, and the natural wood degradation which occurs over longer storage time. With chemical (sulfite) pulps, the applications of lipase improves the properties of resins by lowering their effectiveness. Since 1990, this method has been used commercially. Nippon Paper Industries in Japan developed a pitchcontrol method that uses a fungal lipase from *C. rugosa* to hydrolyse up to 90% of the triglycerides.

1.7 Lysophospholipases

Phospholipases constitute a diverse group of enzymes that attack phospholipids; this group can be divided into two broad categories, acylhydrolases and

phosphodiesterases. The nomenclature of phospholipases follows their functional specificity, as indicated in Figure 2. Lipolytic enzymes are characterised by their ability to hydrolyse aggregated (phospho)lipids with a much higher velocity that the same (phospho)lipid in its monomolecular form. This rate enhancement at lipid-water interfaces is the central theme in the study of lipolysis and distinguishes (phospho)lipases from all other kinds of water-soluble enzymes, which invariably act on monomolecularly dispersed substrates.

The acyl ester bond at position 1 of 3-*sn*-phosphoglycerides is attacked by phospholipase A1(EC 3.1.1.32) and the 2-*sn* acyl ester bond by phospholipase A2 (EC 3.1.1.4). Phospholipase B (EC 3.1.1.5) displays both activities of type A1 and A2, removing both acyl chains from phospholipids, and it also hydrolyses lysophospholipids (lyso-PLs). However, the terms phospholipase B and lysophospholipase have sometimes been employed synonymously without any conscious difference between their meanings. Transacylase combines two molecules of lyso-PL to form one molecule of phospholipid and one molecule of a glycerolphosphate derivative. Acyltransferase reacylates the lyso-PL to form phospholipid using a fatty acyl group derived either from a fatty acyl-CoA or from another phospholipid. Two phospholipase species attack the phosphodiester linkage, and this results either in the release of diacylglycerol by the action of phospholipase C (EC 3.1.4.3) or phosphatidic acid by phospholipase D (3.1.4.4) (Fig 2, 3).

Lysophospholipases (LPLs, EC 3.1.1.5, Fig 2) catalyze the hydrolysis of the ester bonds of lyso-PLs to free fatty acids and glycerolphosphate derivatives, such as glycerophosphocholines and glycerophosphoethanolamines. LPLs are widely distributed both in prokaryotic and eukaryotic cells. They have been isolated from a number of

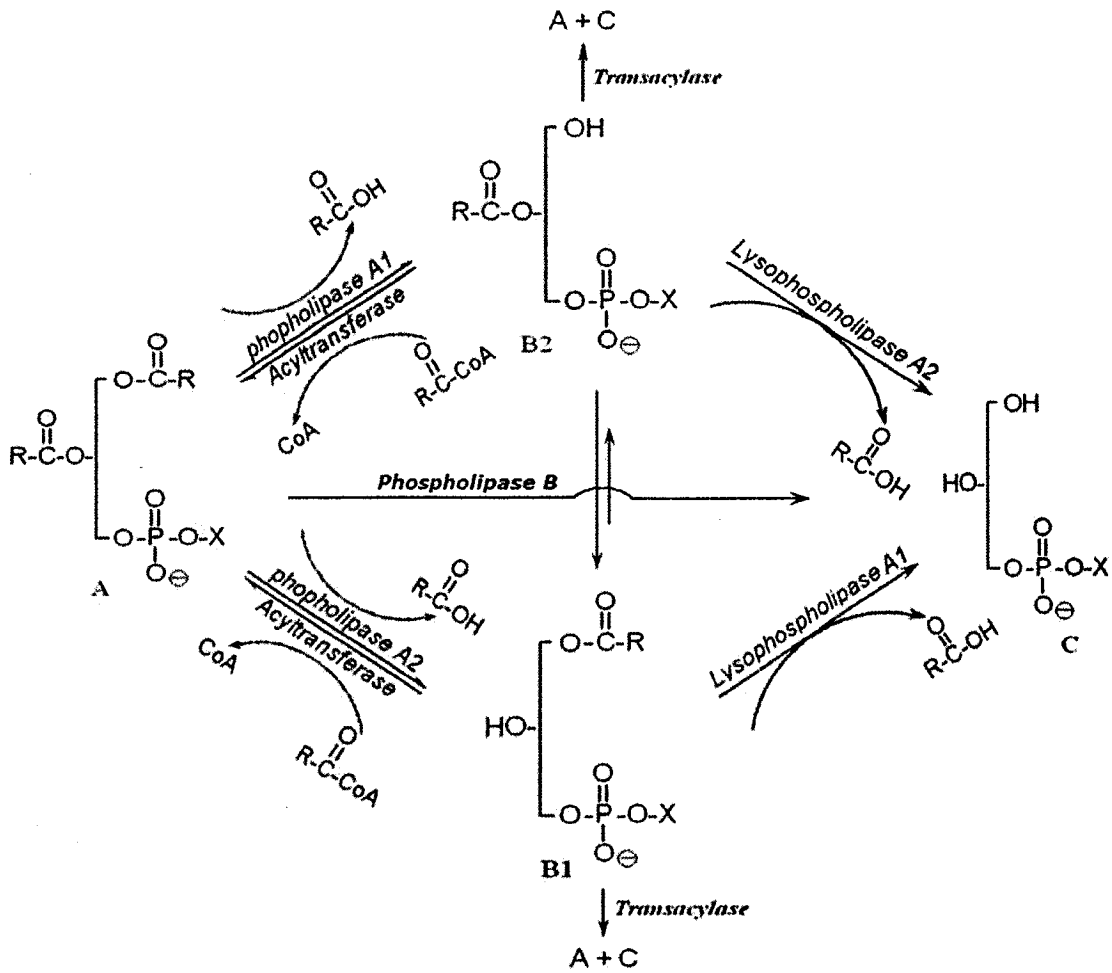

Figure 2. Possible metabolic interconversions of phospholipids (A), l-acyl-lysophospholipids (B1), 2-acyl- lysophospholipids (B2) , and glycerolphosphate derivative (C). (Modified from (Wang and Dennis 1999))

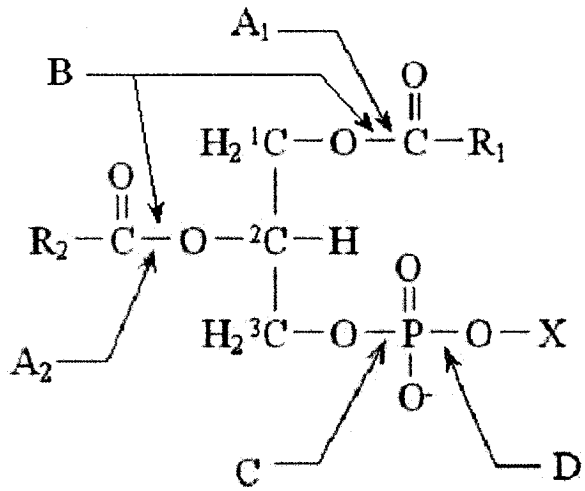

Figure 3. Cleavage sites of different phospholipases on phospholipids. A_1, phospholipase A_1; A_2, phospholipase A_2; B, phospholipase B; C, phospholipase C; D, phospholipase D.

sources (Kawasaki and Saito 1973, Van den Bosch et al. 1973, Victoria and Korn 1975, Weller 1980, Fujikura and Baisted 1985, Karasawa et al. 1985, Garsetti et al. 1992, Merkel et al. 1999, Chen et al. 2000). These enzymes are remarkably variable as to their size, specificity, and pH and temperature optima. Most studies focus on mammalian lysophospholipases. Mammalian lysophospholipases can be classified into two large groups according to their molecular masses (Waite 1991). Both groups of lysophospholipase exist as monomers and no divalent metal requirement is known. The small form enzymes (16.5–28 kDa) only catalyze deacylation whereas the large form enzymes (50-63 kDa) were shown to catalyze not only deacylation, but transacylation as well (Brumley and van den Bosch 1977, Gross et al 1983). The relationship between the reported lysophospholipases has not been well studied.

The knowledge of lysophospholipase structure is very limited. The protein sequence of mammalian lysophospholipase A I (LPLA I) contains the GXSXG consensus sequence that is conserved in the active site. The Ser-119 in the GXSXG motif was demonstrated to be at the active site of the LPLA I by site-directed mutagenesis (Wang 1997a). The other residues in the catalytic triad of the enzyme, namely Asp-174 and His-208, were identified by protein sequence analysis and subsequently confirmed by site-directed mutagenesis (Wang 1997b). The predicted secondary structure of the LPLA I resembles that of the α/β hydrolase fold, with the Ser-119, Asp-174 and His-208 occupying the conserved topological location of the catalytic triad in the α/β hydrolases (Wang 1997b). Sequence comparison of LPLA II with LPLA I revealed that the catalytic triad identified in LPLA I was conserved in LPLA II, i.e. Ser- 122, Asp-176, His-210.Therefore, the catalytic mechanism of both LPLA I and II resembles the classical serine hydrolase, with

the active site composed of the catalytic triad Ser-Asp-His. The active site of three Phospholipases B/Lysophospholipases from fungus *Saccharomyces cerevisiae*, Plb1p, Plb2p and Plb3p, were predicted to consist of similar catalytic triad Ser-Arg-His, and the Ser is located in the conseved GXSXG consensus sequence (Pickard *et al.* 1996, Merkel *et al* 1999). Conparison of the protein sequences of the LPLs from the fungi *A. fumigatus, P. chrysogenum* and *N. crassa* revealed that all the LPLs contain the conseved GXSXG sequence and catalytic triad Ser-Arg-His.

Little is known about the molecular structures of LPLs except for *Escherichia coli* Lysophospholipase L1. *E. coli* Lysophospholipase L1 is a multifunctional enzyme with thioesterase, esterase, arylesterase, protease and LPL activities. So it has been documented with three different names, including thioesterase I, protease I and lysophospholipase L1. Now it is usually named *E. coli* Thioesterase I/Protease I/Lysophospholipase L1 (TAP). Crystal structure of native TAP, the first solved structure of a multifunctional LPL, was determined at 1.9Å, revealing a minimal SGNH-hydrolase fold (Lo 2003). An asymmetric unit of the TAP crystal contains one monomer of TAP, folded into a single α/β domain. The domain comprises a core of predominantly five parallel β strands flanked by seven α helices. Of the seven helices, four helices are located at the concave surface of the five aligned β-sheet, while the remaining three helices reside at the convex surface of the β-sheet. The arrangement of the five β strands resembles the central β-sheet of a minimal α/β hydrolase fold. The size (182 residues) of TAP is much smaller than that of most lipases, phospholipases, carboxylesterases and thioesterases with an α/β hydrolase fold. The structure of TAP displays a single compact domain without a lid, an important domain of most lipases, which have been extensively

applied in industry. The catalytic triad of TAP is composed of Ser10, Asp154, and His157, a sequence which obeys the Nuc-Acid-His rule of α/β hydrolases and SGNH-hydrolases (Cygler 1993, Schrag and Cygler 1997, Nardini and Dijkstra 1999). An oxyanion hole consists of three residues, Ser10-Gly44-Asn73, each separated from the other by more than 3.5Å, implying that all of them are highly polarized when substrate bound. As TAP belongs to the SGNH-hydrolase family, and sequence analysis shows low sequence homology with other LPLs, the crystal structure of TAP may not represent the three-dimensional structure of most LPLs.

LPLs are important enzymes that hydrolyze lyso-PLs. Lyso-PLs are detergent-like intermediates in phospholipids metabolism and play essential roles in many physiological processes. Lysophosphatidylcholine (lyso-PC), a normal constituent of cell membranes, may act as a lipid messenger, transducing signals initiated from membrane receptors (Flavahan 1993, Oishi et al. 1988, Fang et al. 1997). The primary function of LPLs may be removal of cytoxic Lyso-PLs, produced as a result of phospholipase A activity (Chopra and Khuller 1983). Higher concentrations of lyso-PLs have been shown to disturb membrane conformation, affect the activities of many membrane-bound enzymes, distort cell membrane integrity and may even lead to cell lysis (Welthzien 1979, Silverman et al. 1984). Aside from direct lytic effects, Lyso-PLs have been associated with a wide range of phenomena, such as induction of lethal dysrhythmias in myocardial infarction (Gross et al. 1982), segmental demyelination of peripheral nerves (Low et al, 1983), and stimulation or inhibition of several important enzymes (Kelly et al. 1986; Tamura et al. 1987; Shier et al. 1976; Anttinen 1976). Lysophosphatidic acid (LPA), the simplest naturally occurring Lyso-PL, is also a multifunctional phospholipid messenger

that evokes a variety of biological responses, including platelet aggregation, smooth muscle contraction, cell proliferation and differentiation, growth cone collapse and neurite retraction, focal adhesion assembly and stress fiber formation (Moolenaar 1995a,b; Moolenaar et al. 1997). Such diversified biological responses to PLA appear to the activation of a specific G-protein coupled receptors, which in turn couples to multiple independent effector pathways including the small GTP-binding proteins Ras and Rho. Indeed, LPA-specific receptors were cloned and were identified as members of the G-protein coupled receptors (An et al. 1998; Fukushima et al. 1998; Hecht et al. 1996). Accumulation of lyso-PLs in cells is therefore strictly regulated, and at least five different types of enzymes are directly involved in producing or removing them as shown in Fig 2. Included among these enzymes are LPLs and phospholipase As.

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Chapter 2: Characterization of two *Aspergillus niger* genes encoding lipases with distinct properties

Keywords: triacylglycerol acylhydrolase; *Pichia pastoris*; heterologous expression; psychrophilic enzyme; secreted protein

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Abstract

We identified from a collection of partially sequenced *Aspergillus niger* cDNA clones two genes that were predicted to encode secreted lipases. We completed the sequencing of these two genes. The deduced amino acid sequences of the two genes, *lipA* and *lipB*, exhibited approximately 50% identity with each other and with a lipase from the thermophilic fungus *Thermomyces lanuginosus*. We functionally expressed *lipA* and *lipB* in *Pichia pastoris*, and purified and characterized biochemically the recombinant proteins. Lipase A showed optimal activity at 40°C and pH 4.5-5.0. This enzyme was stable in a pH range of 2.2-10.6 and up to 70°C. The pH optimum for LipB was pH 3.5-4.0, and it was stable between pH 3.0 and pH 9.6. The temperature optimum for LipB was 15°C and it retained 70% of its peak activity at 0°C. Comparing to reported cold-adapted enzymes, LipB has the lowest temperature activity profile. Substrate specificity determination with triacylglycerols and *p*-nitrophenyl esters showed that both lipases preferred esters of the middle- and long-chain fatty acids.

Introduction

Lipases (triacylglycerol acylhydrolase; EC 3.1.1.3) catalyze the hydrolysis of fats and oils into diglycerides, monoglycerides, glycerol, and fatty acids in an aqueous environment; and in organic medium they catalyze the reverse esterification reaction, forming glycerides from glycerol and fatty acids. Some lipases are able to catalyze trans-esterification and enantioselective hydrolysis reactions. Lipases are widely distributed in animals, plants and microorganisms. They represent a large array of enzymes that have been classified into 16 superfamilies which do not display global sequence similarity (Fischer and Pleiss 2003). Biochemical characterization of lipases has revealed a wide heterogeneity in catalytic properties and specificities. Lipases have therefore found many applications in the food, dairy, pharmaceutical, agrochemical, oils and fats, cosmetic, tanning, detergent, textile, and oleochemical industries (Jaeger and Eggert 2002).

Owing to their ready availability in large quantities and their low cost of production, secreted lipases from fungi are widely used for characterization and industrial applications. The secreted lipases from *Candida rugosa* are some of the best characterized lipolytic enzymes. Commercial lipase preparations of *C. rugosa* are mixtures of at least 7 distinct enzymes with distinct biochemical properties. The characteristics of *C. rugosa* lipase systems vary significantly with preparations and suppliers (Dominguez de Maria et al. 2006). On the other hand, recombinant lipases provide reliable sources for pure *C. rugosa* isozymes (Tang et al. 2001). The filamentous ascomycete *Aspergillus niger* is used extensively for the production of organic acids and secreted enzymes. This organism has a long history of use in the food industry (Mattey 1992) and many *A. niger* enzymes have been afforded the GRAS (Generally Regarded As

Safe) status by the U.S. Food and Drug Administration. Commercial preparations of *A. niger* lipases can be obtained readily. Like *C. rugosa* lipase systems, the commercial lipase preparations of *A. niger* appear to contain a mixture of isozymes ranging from 19 kDa to 65 kDa (Tombs and Blake 1982; Höfelmann et al. 1985; Sugihara et al.1988; Torossian and Bell 1991; Carrea 2000; Fernandez-Lorente et al. 2005). In addition, a few other lipase isozymes have been identified in culture filtrates of *A. niger* (Hatzinikolaou et al. 1986; Pokorny et al. 1997; Namboodiri and Chattopadhyaya 2000; Mahadik et al. 2002). Besides molecular sizes, the lipases of *A. niger* are reported to possess different catalytic properties. Hence extracting lipase from wild-type strains would result in a mixture of isozymes whose proportions cannot be controlled easily. A mixture of isozymes complicates analysis and limits the potential uses of the enzymes. Expressing recombinant *A. niger* lipases in conditions where a single isozyme is produced would provide pure isozymes for analysis and facilitate the development of industrial applications. Furthermore, identifying the genes encoding secreted lipases and characterizing their gene products would reveal the number of distinct lipases that are encoded by the *A. niger* genome. To date, the genes encoding secreted lipases of *A. niger* have not been reported in journal publications. Here, we describe the cloning, production, purification, and characterization of two secreted *A. niger* lipases. The relationships of the recombinant lipases with previously characterized *A. niger* lipases is also described.

Materials and Methods

Strains and culture conditions

Pichia pastoris strain KM71 was purchased from Invitrogen (Burlington, Ontario). Maintenance, culturing, and transformation by electroporation of *P. pastoris* were conducted according to the procedures provided by the manufacturer. Strain DH5-α of *E. coli* was used as the host for the construction and preparation of recombinant plasmids. The procedures for manipulating bacterial cultures and recombinant DNA were conducted essentially as described by Sambrook et al (1989).

Sequence analysis

The presence of translation start codons in the collection of *A. niger* ESTs (Semova et al. 2006) and tentative functional identity of the cDNA clones were predicted using the tool TargetIdentifier (Min et al., 2005a). Signal peptides in the amino acid sequences were predicted using SignalP v3 (Bendtsen et al. 2004). Two cDNA clones, Asn_07937 and Asn_02385, were predicted to encode intact ORFs for two different secreted lipases. The partial sequence of these two clones is available in Genbank with the following accession numbers, ***DR707255*** for Asn_07937 and ***DR702752*** for Asn_02385. The two clones were completely sequenced using an ABI 3730xl automated analyzer at the Génome Québec Innovation Centre.

Homology search was performed using the BLAST algorithms (Altschul et al. 1990) against the non-redundant database of NCBI. The search for protein families, domains, and active sites was performed using InterProScan (http://www.ebi.ac.uk/InterProScan). Multiple sequence alignment and analysis of relatedness were performed using CLUSTALW (Thompson et al. 1994) at the server provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw).

Expression of lipase genes

For expression in *P. pastoris* we used pPID3.5, a derivative of pPIC3.5K (Invitrogen) where the multiple cloning site (*Bam*HI, *Sna*BI, *Eco*RI, *Avr*II, *Not*I) was replaced by with recognition sites for restriction endonucleases *Bam*HI, *Pac*I, *Asc*I, *Sna*BI, *Fse*I, *Avr*II and *Not*I. The protein-coding regions of *lipA* and *lipB* were PCR amplified from Asn_07937 and Asn_02385 respectively using gene-specific primers. The forward primers contain the *Asc*I recognition sequence and the reverse primers possess the *Fse*I recognition sequence. For *lipA* the sequence for the forward primer is 5'GGCGCGCCATGTTCT CTGGACGGTTTGGA 3' and for the reverse primer is 5'AGCCTAGGCCGGCCCTATAGCAGACACTCTGAAATTGC 3'. The sequences for the forward and reverse primers for *lipB* amplification are respectively 5'GGCGCGCCATGTTTTTCCGCAGGGAATTTGG 3' and 5'AGCCTAGGCCGGCCCACAGCACCAGCAATGCTCTAT 3'. Following amplification, the PCR products were digested with *Fse*I and *Asc*I and cloned into the corresponding sites of pPID3.5. The recombinant plasmids were propagated in *E. coli* DH5-α cells, column purified (Qiagen), and transformed into *P. pastoris*.

The transformed *P. pastoris* cells were spread on MD plates (1.34% yeast nitrogen base, 4×10^{-5} % biotin, 2% dextrose, 1.5% agar) and incubated at 30°C for 3-4 days. His^+ transformants were cultured in liquid GCM (1% Yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5} % biotin and 1% glycerol) with shaking at 200 rpm and 30°C. When the culture reached an OD_{600} of 2 to 6, cells were collected and transferred to MCM (same as GCM except that 1% glycerol was replaced by 0.5% methanol) to induce the expression of recombinant proteins (Minning et al. 2001).

Protein purification

The enzymes in the culture filtrates, concentrated by centrifugation through Centricon membranes (YM-10), were purified by column chromatography using a Waters 625 LC system. One milliliter of protein in 25 mM Tris-HCl, pH 7.5, was resolved in a Superdex®75 column at a flow rate of 0.4 ml/min^{-1}. The fractions containing lipase activity were pooled and subjected to anion exchange chromatography using a Mono-Q column. The proteins were eluted with a linear gradient of 0-0.5 M NaCl in Tris-HCl, pH 7.5. Partially purified proteins were analyzed by SDS-PAGE gels and stained with Coomassie Blue R250.

Determination of peptide mass

The major protein bands from the purified lipases were excised from the Coomassie Blue-stained SDS-PAGE and subjected to in-gel digestion with trypsin according to the method of Shevchenko et al. (Shevchenko et al. 1996) The trypsin-digested peptides diffused from the gel fragments were mixed with the matrices and prepared for MALDI-TOF analysis (Laugesen and Roepstorff 2003) using a MALDI-LR mass spectrometer (Waters-Micromass). The data were acquired in a positive-ion reflectron mode.

Enzyme assays

Lipase activity was detected qualitatively using the agar plate assays of Kouker and Jaeger (Kouker and Jaeger 1987). Aliquots of culture filtrates were added to punched holes on 0.75% agar impregnated with rhodamine B, 0.002% (w/v), and 1% (v/v) of either tricaprin, triolein, olive oil, or soy oil in 0.1 M sodium acetate buffer (pH 4.0). Lipase from *Candida rugosa* (Sigma-Aldrich, Toronto, Canada) was used as a positive control. The plates were incubated at room temperature for up to 4 days. Relative enzyme

activity is proportional to the diameter of the dark purple halo formed around the punched hole.

Lipase activity was quantified spectrophotometrically using *p*-nitrophenyl fatty acid esters as substrates. The procedure for detection of activity was modified from that described previously (Yang et al. 2002; Bourne et al. 2004). The reactions were performed as follows. The substrates were dissolved in acetone at 100-fold the working concentrations. Prior to the addition of the enzyme, the substrate was mixed by vigorous vortexing with the buffer until the solution became clear. Unless specified otherwise, the reactions were carried out in 150 µl of 50 mM potassium acetate buffer, pH 4.5, containing 1 mM of *p*-nitrophenyl caprate (*p*NPC10) or *p*-nitrophenyl laurate (pNPC12) and 0.67% Triton X-100. Following incubation with the enzyme at 30°C for 30 min, the reaction was stopped by the addition of 50 µl of 1 M Na_2CO_3. The absorbance was measured at 410 nm with a microplate reader (Power Wave HT, Bio-Tek Instruments, Winooski, Vermont). The standard curve was prepared with *p*-nitrophenol. One unit of lipase activity is defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per min under the assay conditions while 1 milli-unit (mU) is the amount of enzyme needed to release 1 nmole of *p*-nitrophenol from the substrate. Protein concentration was determined using a Protein Assay kit (Bio-Rad) with bovine serum albumin as a standard.

Biochemical characterization of the enzymes

The pH optimum of the lipases was determined using the assay conditions described above except in different buffer systems. Buffers used at a final concentration of 20 mM were glycine-HCl for pH 2.2 to 3.6; sodium acetate, pH 3.6 to 5.6; Tris-malate,

pH 5.6 to 8.6; Tris-HCl, pH 7.4 to 9.0; and glycine-NaOH, pH 8.6 to 10.6. For determination of pH stability the enzymes were incubated at 22°C for 3 h in various pH, and the activity was measured at optimal pH and temperature (pH 5.0 and 40°C for LipA, and pH 4.0 and 15°C for LipB). Temperature optima were assayed at temperatures ranging from 20°C to 80°C for LipA and 0°C to 60°C for LipB. For determination of temperature stability, the enzymes were incubated at temperatures ranging from 20°C to 80°C for 60 min at optimal pH, and then the activities were determined at optimal pH and temperature.

Purified lipases were used to determine substrate specificity and kinetic properties at optimal pH and temperature for the two enzymes. Substrate specificity was determined with *p*-nitrophenyl esters of fatty acid with chain lengths varying from C2 to C18. The substrates *p*-nitrophenyl acetate (*p*NPC2), butyrate (*p*NPC4), caprylate (*p*NPC8), caprate (*p*NPC10), laurate (*p*NPC12), myristate (*p*NPC14), palmitate (*p*NPC16), and stearate (*p*NPC18) were purchased from Sigma-Aldrich (Toronto, Canada).

Sequence submission

The determined nucleotide sequence and predicted amino acid sequence of *lipA* (Asn_07937) and *lipB* (Asn_02385) have been submitted to Genbank and can be retreived under the accession numbers ***DQ680030*** and ***DQ680031***, respectively.

Results

The cDNA clones Asn_07937 and Asn_02385 encode two putative lipases

Analysis of ESTs of two *A. niger* cDNA clones Asn_07937 and Asn_02385 (Semova et al. 2006) revealed sequence similarity with known lipases. These cDNA

clones were completely sequenced (Fig. 4). Using the OrfPredictor tool (Min et al., 2005b), we showed that Asn_07937 contained an open-reading frame of 891 bp, which encoded a polypeptide of 297 amino acid residues with a calculated mass of 31718.37. Similar analysis showed that Asn_02385 harboured an open-reading frame of 894 bp which encoded a polypeptide of 298 residues with a calculated mass of 31953.36. The deduced amino acid sequences of Asn_07937 and Asn_02385 share 53% identity. Both polypeptides were predicted by the SignalP algorithm (Bendtsen et al., 2004) to possess a 19-residue signal peptide that is characteristic for proteins which are exported to the extracellular region through the ER/Golgi pathway.

A lipase_3 domain (NCBI Conserved Domain cd00519) was detected in the Asn_07937 and Asn_02385 polypeptides, suggesting that they encode enzymes which hydrolyze long-chain acyl-triglycerides. Sequence comparison showed that Asn_07937 and Asn_02385 were 50% identical to a well characterized triacylglycerol acylhydrolase of the thermophilic ascomycete *Thermomyces lanuginosus* (Accession no. *AAC08588*). The active site of lipases comprises three residues: a nucleophilic serine located in a highly conserved Gly-X-Ser-X-Gly pentapeptide, an aspartate or glutamate, and a histidine (Brady et al., 1990). Alignment with the *T. lanuginosus* lipase showed that the positions for the catalytic triad for LipA were Ser173, Asp228, and His285; and for LipB were Ser175, Asp230, and His287. The serine in the active site of both enzymes resides in the consensus pentapeptide Gly-X-Ser-X-Gly. Based on the presence of a signal peptide, a conserved lipase domain, a lipase active site motif, and similarity to the *T. lanuginosus* lipase, we assigned Asn_07937 and Asn_02385 as putative secreted lipases

and termed them *lipA* and *lipB* respectively. The proteins encoded by these two genes are referred to as LipA and LipB.

A search of Genbank Release 153 showed that *lipA* and *lipB* of *A. niger* exhibit 51-69% amino acid identity to four proteins predicted from the genome sequences of *A. fumigatus*, *A. nidulans*, and *A. oryzae* (Accession nos. *EAL86100*, *EAA59668*, *BAE56692*, *BAE58376*). Since the genome sequences of these three species are known, these identified sequences may represent the closest homologues of *lipA* and *lipB*. Considering the sequence identity between *lipA* and *lipB* and their homologues is over 50%, it is possible that they all encode the same catalytic activity but with distinct functionalities.

Genomic organization of lipA and lipB

The genome sequence of *A. niger* was released in April 2006 by the Joint Genome Institute, and the information can be accessed via the portal http://genome.jgi-psf.org. We compared the sequences of *lipA* and *lipB* (Fig. 4) with the genome sequence using BLASTP and BLASTN independently. The *lipA* sequence mapped to the protein model ASN210730, while *lipB* aligned to ASN212664.The nucleotide sequence of *lipA* deviates from the coding region of ASN210730 by 2 basepairs, but its amino acid sequence is identical that of ASN210730. Since the cDNA and genomic sequences are from different strains, the minor deviations may represent nucleotide polymorphisms. Both the nucleotide and amino acid sequences of *lipB* are identical to that of ASN212664. The perfect mapping of the coding regions of *lipA* and *lipB* to ASN210730 and ASN212664 implies that these two protein models were correctly predicted.

The alignments of the cDNAs to the genome sequence showed that both gene models contain three introns. With respect to the coding sequences, the locations of the

introns for the two genes are identical or near identical. This suggests that the introns were present in the ancestral gene before the duplication event that gave rise to *lipA* and *lipB*. In the current version of the genome sequence, ASN210730 (*lipA*) is located 430 kb from the end of the 1.8-Mb Scaffold 7 while ASN212664 (*lipB*) is located 450 kb from the end of 1.68-Mb Scaffold 11. This datum indicates that the two genes are not closely linked.

Assigning molecular function to lipA and lipB

The open-reading frames of *lipA* and *lipB* were cloned independently into the expression vector pPID3.5, and transformed into *P. pastoris*. In agar plate assays, culture filtrates of *P. pastoris* carrying the *lipA* or *lipB* genes reacted positively with tricaprin, triolein, olive oil and soy oil as substrates, whereas culture filtrates from the parental strain did not show lipase activity. On agar impregnated with triglycerides and rhodamine B, LipB formed haloes with diameters much wider than that of LipA and *C. rugosa* lipase. Figure 5 shows the diameter of the halo formed by 17 mU of LipB on soy oil agar was wider than that formed by 125 mU of LipA or 2,500 mU of *C. rugosa* lipase.

The lipase activities from the culture filtrates of the recombinant strains were purified by column chromatography. Table 1 summarizes the results from the purification steps and Figure 6 shows the SDS-PAGE of protein samples obtained following column chromatography. Both *lipA* and *lipB* directed the production of recombinant proteins with M_r of 37 kDa as determined by SDS-PAGE (Fig 6). These two protein bands on SDS-PAGE were analyzed by MALDI-TOF mass spectrometry. Table 2 shows the peptides identified, and they covered 49.49% and 28.29% of the predicted amino acid sequences

for LipA and LipB respectively. These results confirm that the lipase activities were derived from the recombinant LipA and LipB.

Biochemical properties of LipA and LipB

The effects of pH on the lipase activity and stability of LipA and LipB were investigated. Figure 7A shows that the pH optimum for LipA was 4.5-5.0 and for LipB was 3.5-4.0. Figure 7B shows that LipA was stable from pH 2.2 to pH 10.6 whereas LipB was stable from pH 2.2 to pH 9.6. These results indicate that the two *A. niger* lipases prefer acidic conditions for activity, but they are stable in a broad pH range.

Determination of optimal temperature for activity revealed that LipA was most active at 40°C and this enzyme was stable up to 70°C (Fig 8). The temperature optimum for LipB was 15°C. Lipase B is a particularly cold-active enzyme, retaining 70% of its peak activity at 0°C (Fig. 8A). However LipB was thermolabile, rapidly losing its activity above 40°C (Fig. 8B).

The recombinant lipases were reacted with *p*-nitrophenyl esters of various fatty acids. Figure 9 shows that both LipA and LipB exhibited significant activities against *p*NPC4 – *p*NPC18, but preferred medium- to long-chain (C8 to C18) substrates with the highest activity toward *p*NPC12.

Comparison of LipA and LipB with other secreted lipases identified from A. niger

Several publications describe the characterization of secreted lipases from *A. niger*. It is therefore probable that LipA and LipB have been characterized in one or more of the previous studies. Table 3 summarizes the physical and biochemical properties of several secreted lipases of *A. niger* identified in most of the studies to date. Enzymes with molecular weights ranging from 19 kDa to 65 kDa have been reported. Based on the

molecular size, pH optimum, temperature optimum, and substrate specificity, LipA is most likely the same enzyme previously characterized by two groups independently (Sugihara et al.1988; Torossian and Bell 1991). This is further confirmed by comparing the amino acid sequence of LipA (Fig. 4) and the N-terminal sequence of the reported enzymes (Sugihara et al. 1988; Torossian and Bell 1991).

The lipase identified by Namboodiri and Chattopadhyaya (Namboodiri and Chattopadhyaya 2000) has a molecular size similar to LipA and LipB, but the N-terminal sequence is different. Fernandez-Lorente *et al.* (Fernandez-Lorente et al. 2005) purified three lipases with molecular sizes of 31 kDa, 43kDa, and 65 kDa. Based on the molecular sizes, they may be the same enzymes reported by other groups (Höfelmann et al. 1985; Pokorny et al. 1997).Two reports identified a lipase that has the characteristics of a cold-adapted enzyme, temperature optimum at 25°C and inactivation above 40-45°C (Fukumoto et al. 1964; Hatzinikolaou et al. 1986). With the reported properties it is not clear if these two preparations are the same enzyme. These two enzymes have temperature and pH optima higher than those of LipB. Based on the above comparisons, we concluded that LipB is a secreted lipase which has not been characterized previously.

A

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1     gtgcattgcagcagtccgttggtctcacgtctctggttgcctcgattgtatatatactgc
61    aggatgttctctggacggtttggagtgcttttgacggcgcacgctgcgctgagtgctgcg
         M  F  S  G  R  F  G  V  L  L  T  A  H  A  A  L  S  A  A
121   gcaccgacaccacttgatgtgcggagtgtctcgacttccacgttggatgagctgcaattg
       A  P  T  P  L  D  V  R  S  V  S  T  S  T  L  D  E  L  Q  L
181   ttctcgcaatggtctgccgcagcttattgctcgaacaatatcgactcggacgactctaac
       F  S  Q  W  S  A  A  A  Y  C  S  N  N  I  D  S  D  D  S  N
241   gtgacatgcacggccgacgcctgtccatcagtcgaggaggcgagcaccaagatgctgctg
       V  T  C  T  A  D  A  C  P  S  V  E  E  A  S  T  K  M  L  L
301   gagtttgacctgacaaataactttggaggcacagccggtttcctggccgcggacaacacc
       E  F  D  L  T  N  N  F  G  G  T  A  G  F  L  A  A  D  N  T
361   aacaagcggctcgtggtcgccttccgaggcagtagcaccatcaagaactggattgctgat
       N  K  R  L  V  V  A  F  R  G  S  S  T  I  K  N  W  I  A  D
421   ctcgacttcatcctgcaagataacgatgacctctgtactggctgcaaggttcacactgga
       L  D  F  I  L  Q  D  N  D  D  L  C  T  G  C  K  V  H  T  G
481   ttctggaaggcatgggaagccgctgcagacaatctgacgagcaagatcaagtccgcgatg
       F  W  K  A  W  E  A  A  A  D  N  L  T  S  K  I  K  S  A  M
541   agcacgtattcgggctataccctctacttcaccgggcacagcttgggcggcgcattggct
       S  T  Y  S  G  Y  T  L  Y  F  T  G  H  S  L  G  G  A  L  A
601   acactgggagcaacggtcttgcgaaatgacggttatagcgttgaactgtacacctatgga
       T  L  G  A  T  V  L  R  N  D  G  Y  S  V  E  L  Y  T  Y  G
661   tgtcctcgagtcggaaactatgcgctggccgagcacatcaccagccagggatctggagcg
       C  P  R  V  G  N  Y  A  L  A  E  H  I  T  S  Q  G  S  G  A
721   aacttccgcgttacacacttgaacgacatcgtcccccggttgccacccatggactttgga
       N  F  R  V  T  H  L  N  D  I  V  P  R  L  P  P  M  D  F  G
781   ttcagccagccaagtccagaatactggatcaccagtggcaccggagccagtgtcacggcg
       F  S  Q  P  S  P  E  Y  W  I  T  S  G  T  G  A  S  V  T  A
841   tcggatattgaactcatcgagggaatcaattcgacggcggggaatgcaggcgaagcaacg
       S  D  I  E  L  I  E  G  I  N  S  T  A  G  N  A  G  E  A  T
901   gtggacgttttggctcacttgtggtacttttttcgcaatttcagagtgtctgctatagctg
       V  D  V  L  A  H  L  W  Y  F  F  A  I  S  E  C  L  L  -
961   gacagtccgatgaaataagtgcggagagaaagtgtaaatagtaattagtataaaagcagg
1021  cagagaagcagtggtggtcagagaaaaaaaaaaaaaaaaaaa
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B

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1     tgccctcaagagtgtcctgcattgtattgcctggctcccaagatgtttttccgcagggaa
                                                    M  F  F  R  R  E
61    tttggggctgttgcagccctatctgtgctggcccatgctgctcccgcacctgctccgatg
       F  G  A  V  A  A  L  S  V  L  A  H  A  A  P  A  P  A  P  M
121   cagcgtagagacatctcctctaccgtcttggacaatatcgacctcttcgcccaatacagt
       Q  R  R  D  I  S  S  T  V  L  D  N  I  D  L  F  A  Q  Y  S
181   gcagcagcttactgctcctcgaacatcgagtccaccggcacgactctgacctgcgacgta
       A  A  A  Y  C  S  S  N  I  E  S  T  G  T  T  L  T  C  D  V
241   ggcaattgccctctcgtcgaggcagccggtgccacgaccatcgatgagtttgacgacacc
       G  N  C  P  L  V  E  A  A  G  A  T  T  I  D  E  F  D  D  T
301   agcagctacggcgacccgactgggttcatcgccgttgacccaacgaacgagttaattgtt
       S  S  Y  G  D  P  T  G  F  I  A  V  D  P  T  N  E  L  I  V
361   ctgtctttccggggtagttccgacctctcgaactggattgccgacctagacttcggcctc
       L  S  F  R  G  S  S  D  L  S  N  W  I  A  D  L  D  F  G  L
421   acctccgtaagcagcatctgtgatggctgtgagatgcacaagggcttctacgaggcctgg
       T  S  V  S  S  I  C  D  G  C  E  M  H  K  G  F  Y  E  A  W
481   gaagtcatcgcggacaccatcactagcaaggtggaggctgctgtctccagctatccggac
       E  V  I  A  D  T  I  T  S  K  V  E  A  A  V  S  S  Y  P  D
541   tacaccctcgtgttcactggacacagctacggcgctgcattggcggctgtcgcggccacc
       Y  T  L  V  F  T  G  H  S  Y  G  A  A  L  A  A  V  A  A  T
601   gtgctccgcaacgccggatacactcttgacctgtacaacttcggccagccccgtattggc
       V  L  R  N  A  G  Y  T  L  D  L  Y  N  F  G  Q  P  R  I  G
661   aacctcgccttagccgactatatcaccggccaaaatatgggcagcaactaccgcgtcacg
       N  L  A  L  A  D  Y  I  T  G  Q  N  M  G  S  N  Y  R  V  T
721   cacaccgatgacatcgtgcctaagctgcctccggagctgctgggctaccaccacttcagc
       H  T  D  D  I  V  P  K  L  P  P  E  L  L  G  Y  H  H  F  S
781   ccggagtactggatcaccagcggtaatgatgtgacggtgactacgtcggacgtgaccgag
       P  E  Y  W  I  T  S  G  N  D  V  T  V  T  T  S  D  V  T  E
841   gtcgtgggggtggattcgacggctgggaatgacggcacgctgcttgacagtacgactgcc
       V  V  G  V  D  S  T  A  G  N  D  G  T  L  L  D  S  T  T  A
901   catcggtggtacacgatctacattagtgaatgctcatagagcattgctggtgctgtgctg
       H  R  W  Y  T  I  Y  I  S  E  C  S  -
961   tgattatgtgggttggatatagtcaagatggaatgaatctgtatatagttttagtaataa
1021  caggcgaagtaaataggctagtctgatcatgaaaccgttcgttcgaggcaaaaaaaaaaa
1081  aaaaaaaaaaa
```

Figure 4. Nucleotide and deduced amino acid sequences of *lipA* and *lipB*.

The cDNA clones Asn_07937 and Asn_02385 were completely sequenced. The nucleotide sequence is shown in lowercase and the deduced amino acid is shown in uppercase. The three amino acid residues of the active site are shown in boldface and underlined. The shadowed areas mark the conserved pentapeptides Gly-X-Ser-X-Gly encompassing the serine residue in the active site. Panel A, sequence of *lipA* (Asn_07937); and Panel B, sequence of *lipB* (Asn_02385).

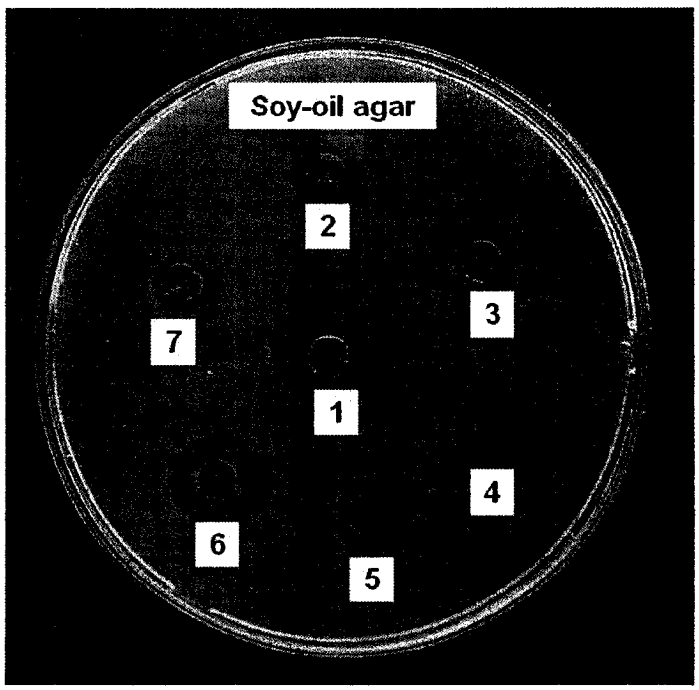


Figure 5. Enzyme activity on agar containing soy oil and rhodamine B. Culture filtrates or control enzymes were added to holes punched on agar as described in methods. (1) culture filtrate from *P. pastoris* carrying the pPID3.5 vector; (2) 2,500 mU of lipase from *C. rugosa* lipase; (3) 250 mU of lipase from *C. rugosa*; (4) 250 mU of LipA; (5) 125 mU of LipA; (6) 17 mU of LipB; and (7) 3 mU of LipB.

Table 1. Summary of the purification of recombinant lipases

Enzyme	Steps	Total protein (μg)	Total Activity (mU)	Specific Activity (U/mg)	Purification (fold)	Activity Yield (%)
LipA	Crude	229.5	2563	11.2	1	100
	Superdex	21.7	1762	81.3	7.3	68.7
	Mono-Q	3.3	794	241.6	21.6	31.0
LipB	Crude	281	429	1.5	1	100
	Superdex	54	317	5.8	3.8	73.9
	Mono-Q	14	97	6.8	4.5	22.7

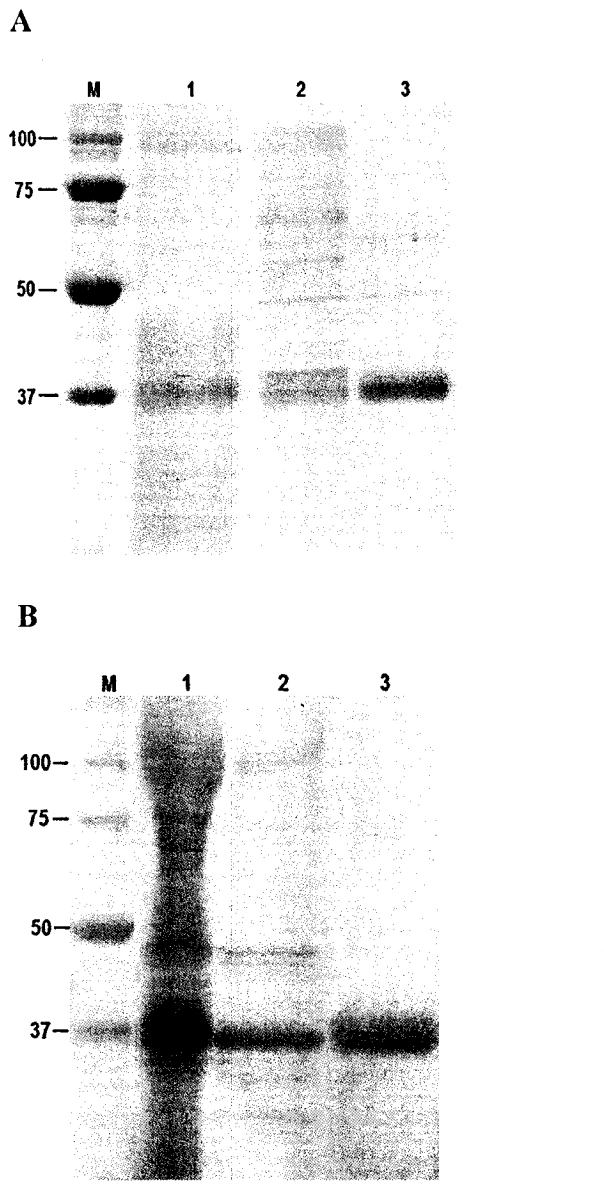


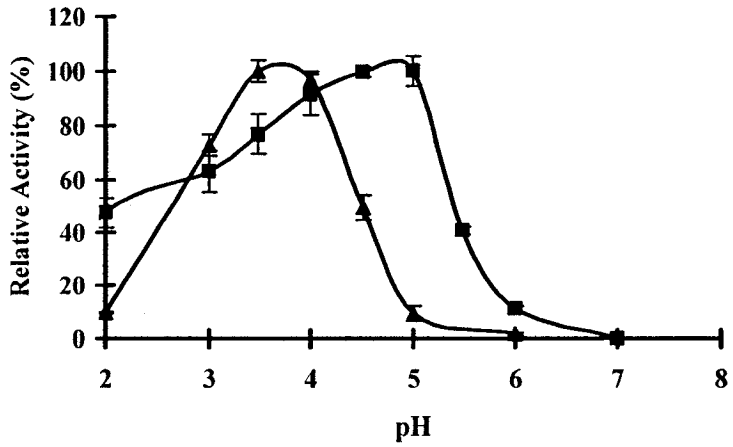
Figure 6. SDS-PAGE of the purified recombinant lipases.

Aliquots of different purification steps for LipA (panel A) and LipB (panel B) were resolved on 12% SDS polyacrylamide gels and stained with Commossie blue R250. Lane 1, crude samples; lane 2, fractions from the Superdex®75 column with lipase activity; lane 3, fractions with lipase activity from the Mono-Q column. The lanes on the left (M) show the molecular weight markers.

Table 2. Peptide fingerprints of recombinant lipases from mass spectrometry analysis

Amino acid position	Peptide sequence	Calculated mass	Observed mass
LipA			
77-102	MLLEFDLTNNFGGTAGFLAADNTNKR	2846.382	2845.374
103-114	LVVAFRGSSTIK	1277.715	1276.707
115-135	NWIADLDFILQDNDDLCTGCK	2526.274	2525.266
143-154	AWEAAADNLTSK	1277.143	1276.135
157-187	SAMSTYSGYTLYFTGHSLGGALATLGATVLR	3182.694	3181.686
188-202	NDGYSVELYTYGCPR	1793.786	1792.778
203-222	VGNYALAEHITSQGSGANFR	2092.024	2091.016
223-232	VTHLNDIVPR	1163.684	1162.676
LipB			
141-156	GFYEAWEVIADTITSK	1829.812	1828.804
157-189	VEAAVSSYPDYTLVFTGHSYGAALAAVAATVLR	3370.996	3369.988
190-204	NAGYTLDLYNFGQPR	1728.759	1727.751
205-224	IGNLALADYITGQNMGSNYR	2186.892	2185.884

A



B

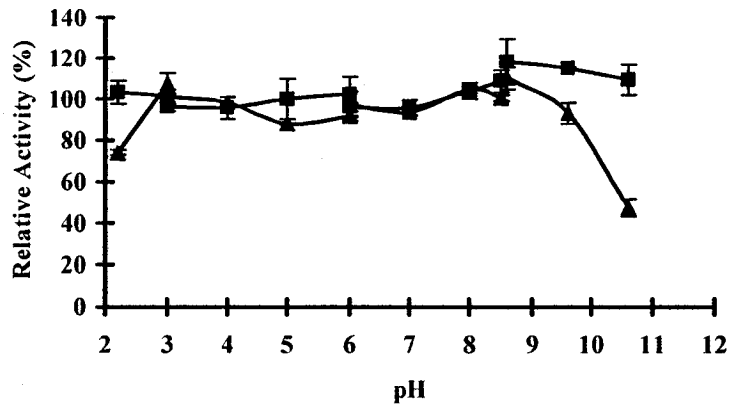
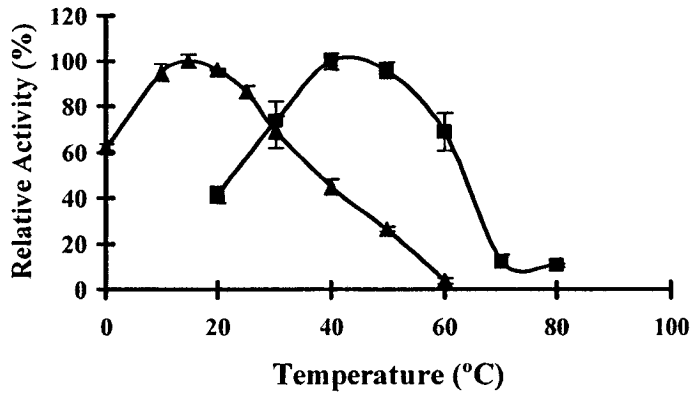


Figure 7. Effect of pH on the activities and stabilities of the lipases.

(A) The pH optima of the lipases were determined by incubating the enzymes at various pHs with 1 mM *p*-nitrophenyl caprate at 30°C for 30 min. (B) The pH stabilities of the lipases were assayed by incubating the enzymes at various pHs in different buffer systems at 22°C for 3 hours then measuring the activities at optimal pHs and temperatures. ■, LipA; ▲, LipB.

A



B

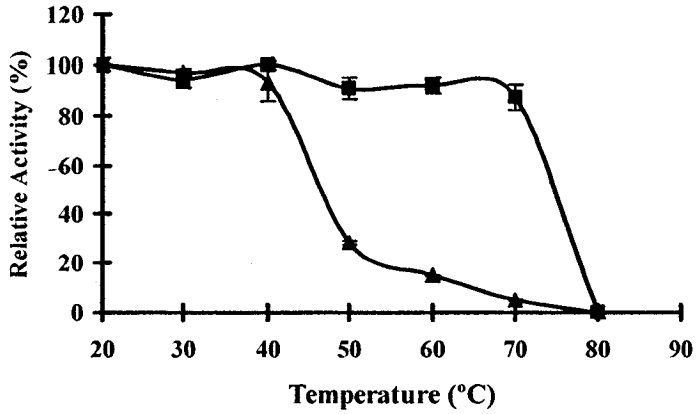


Figure 8. Effect of temperature on the activities and stabilities of the lipases. (A) The temperature optima of the lipases were determined by incubating the enzymes at various temperatures with 1 mM *p*-nitrophenyl caprate in 50 mM potassium acetate buffer at optimal pHs for 30 min. (B) The lipase thermostabilities were determined by incubating the enzymes at temperatures ranging from 20°C to 80°C for 60 min at optimal pH and then measuring the activities at optimal pH and temperature. ■, LipA; ▲, LipB.

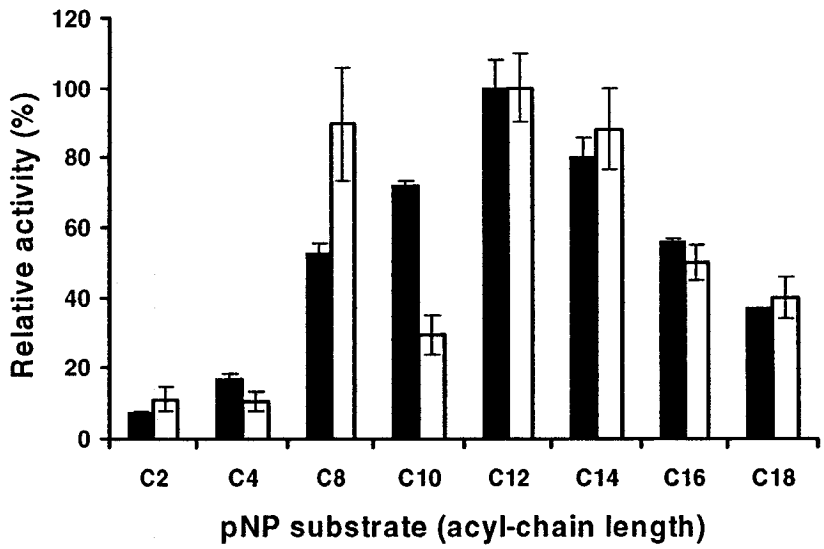


Figure 9. Substrate specificity of *A. niger* lipases towards *p*NP esters. Relative activities of the lipases toward *p*-nitrophenyl esters with different chain lengths were measured. Lipase Activities against *p*NPC12 represent 100%. Filled bars, LipA; open bars, LipB

Table 3. Properties of *A. niger* secreted lipases

Lipase (kDa)	Temp opt	pH opt	Stability[1]	Substrate specificity[2]	N-terminal sequence	Reference
19	n.d.	5.0-6.0	n.d.	C2-C8 (C2-C4)	n.d.	(Höfelmann et al., 1985)
31	n.d.	5.0-6.0	n.d.	C6-C12 (C10)	n.d.	(Höfelmann et al., 1985).
35	40°C	5.0-6.0	50°C	n.d.	AVLDFWLDNLQL FAQWAILA	(Namboodiri and Chattopadhyaya, 2000)
35	30°C	4.5-5.5	65°C	C4-C12 (C8)	SVT	(Sugihara et al., 1988)
37	n.d.	4.4-5.5	n.d.	n.d.	XVSTSTLDELQFA LQ	(Torossian and Bell, 1991)
37 LipA	40°C	4.5-5.0	70°C	C4-C18 (C12)	APTPLDVRSVSTS TLDELQLFSQ[3]	This study
37 LipB	15°C	3.5-4.0	40°C	C4-C18 (C12)	APAPAPMQRRDIS STVLD[3]	This study
43	40°C	7.0	n.d.	n.d.	n.d.	(Pokorny et al., 1997)
48	n.d.	n.d.	n.d.	n.d.	n.d.	(Tombs and Blake, 1982)
65	40°C	7.0	n.d.	C2-C18 (C2-C4)	n.d.	(Pokorny et al., 1997)
	25°C	5.6	45°C	n.d.	n.d.	(Fukumoto et al., 1964)
	25°C	7.0	40°C	n.d.	n.d.	(Hatzinikolaou et al., 1986)
	45°C	2.5-3.0	60°C	n.d.	n.d.	(Mahadik et al., 2002)

[1] After incubation for 30 min or longer at the indicated temperature, the enzyme can regain its activity when placed at the optimal temperature.
[2]Triglycerides or monoesters of different fatty acid chain lengths hydrolyzed by the enzyme. The numbers in parentheses refer to the substrate chain lengths at which the enzyme is most active.
[3]The N-terminal sequence deduced from the nucleotide sequence with the predicted 19-amino-acid signal peptide removed.
n.d., not determined

Discussion

We report here two genes of *A. niger* that display sequence similarity with known lipases. We confirmed that these two genes, *lipA* and *lipB*, encode secreted lipases by expressing them heterologously and by characterizing biochemically the recombinant proteins. They are the first two *A. niger* lipases characterized from cDNA clones so far. Further, we have recently expressed these two genes ectopically in *A. niger* with activity reaching 2 U/mL for LipA and as high as 10 U/mL for LipB (data not shown). As shown in the results section, sequences predicted to encode uncharacterized proteins with high degree of similarity to *lipA* and *lipB* are found in *A. oryzae*, *A. nidulans*, and *A. fumigatus*. Based on their similarity to *lipA* and *lipB*, we can extrapolate that the homologues in *A. oryzae*, *A. nidulans*, and *A. fumigatus* (Accession nos. *EAA59668*, *EAL86100*, *BAE56692*, *BAE58376*) also encode proteins with lipase activity.

Purified LipB had a relatively low specific activity of 6.8 U per mg protein (Table 1) when synthetic *p*-nitrophenyl monoesters were used as a substrate. Based on the sizes of the diameters formed on agar impregnated with various oils (see Fig. 5 for example), LipB was more active than LipA and the *C. rugosa* lipase. It is likely that LipB prefers triglycerides as substrates over synthetic monoesters of fatty acids.

The N-terminal sequence of the lipase identified by Torossian and Bell (Torossian and Bell 1991) matches perfectly to a region close to the N-terminal sequence predicted for LipA (Table 3). The molecular size and pH optimum of these two enzymes are also very similar. In addition, the catalytic properties and molecular size of the enzyme identified by Sugihara et al. (Sugihara et al. 1988) are very similar to those of LipA. The N-terminal sequences of the two proteins are also very similar though the threonine

residue in the third position of the reported peptide (Sugihara et al. 1988) is substituted with a serine residue in LipA. These results taken together indicate strongly that LipA is the same enzyme characterized previously by two independent groups (Sugihara et al.,1988; Torossian and Bell 1991). Despite the fact they were characterized by different researchers under non-identical conditions, the recombinant LipA produced in *P. pastoris* possesses properties that are nearly identical to that of the native *A. niger* enzyme.

The N-terminal sequences reported previously (Sugihara et al.1988; Torossian and Bell 1991) represent the beginning of the mature LipA protein. These N-terminal sequences start immediately after the first arginine residue, or 8 amino acids, downstream from the predicted cleavage site for the signal peptide. This observation implies that LipA is synthesized as a preproprotein of 297 amino acids. After processing of the pre- and propeptide, the mature polypeptide is 270 amino acids in length with a calculated molecular mass of 28966.14. The molecular size as determined by SDS-PAGE is about 37 kDa. The difference in size is most likely caused by posttranslational modification, probably glycosylation, a feature commonly observed for extracellular proteins.

The genome sequence for *A. niger* was recently made available. In this first version of the genome sequence, as many as 7 gene models can be predicted to encode secreted lipases. With several *A. niger* lipases characterized, it is tempting to match the characterized enzymes with the gene models. Based on molecular size and biochemical properties, however, it is difficult to make the correlation as secreted proteins are prone to posttranslational modifications. Peptide sequences and amino acid compositions may provide more reliable clues. Even these comparisons have yielded ambiguous or unexpected results. Though the N-terminal peptide sequence of Torossian and Bell

(Torossian and Bell 1991) matches perfectly to that of LipA, the determined amino acid composition of this enzyme (Torossian and Bell 1991) deviates appreciably with the predicted amino acid composition of LipA (unpublished observation). However, the amino acid composition of a lipase characterized by Tombs and Blake (Tombs and Blake 1982) shows a profile that is very similar to that predicted for protein model ASN53361 (http://genome.jgi-psf.org). Besides the sequence related to LipA (Sugihara et al. 1988; Torossian and Bell 1991), the N-terminal peptide has been reported for another *A. niger* lipase (Namboodiri and Chattopadhyaya, 2000). However, we were unable to map this sequence (listed in Table 3) to the genome sequence of *A. niger* or find a similar sequence in the NCBI databases. It is therefore likely that this lipase represents a new enzyme which is unique to the strain of *A. niger* used to produce the enzyme. Thus far, the matching of *lipA* and *lipB* to the genome sequence described in this study provides the only unambiguous correlation as entire sequences and extensive peptide fingerprints are available for comparison.

The most unexpected finding from this study is the demonstration that LipB is a cold-adapted enzyme. Cold-adapted enzymes are those that retain significant level of catalytic activity between 0°C to 30°C when compared to their mesophilic counterparts. Another characteristic for these enzymes is their lability at moderate temperatures (Siddiqui and Cavicchioli, 2006). The *A. niger* LipB exhibits these properties. The structures of cold-adapted enzymes are intrinsically interesting because of their ability to display high specificity and high K_{cat} at low temperatures (D'Amico et al. 2006; Siddiqui and Cavicchioli 2006). Moreover, psychrophilic enzymes can potentially be useful in applications that require low temperature of operation such as in detergents, and textiles,

and for environmental remediation (Gerday et al. 2000; Cavicchioli et al. 2002). A recent review summarizes in details the properties of cold-adapted enzymes (Siddiqui and Cavicchioli 2006). Most of these enzymes have temperature optima at 30°C or above. The exceptions are an isocitrate dehydrogenase from *Colwellia maris* (Watanabe et al. 2005), a lipase from *Photobacterium lipolyticum* (Ryu et al. 2006), and a lipase from *Pseudomonas fluorescens* (Luo et al. 2006), which have temperature optima between 20 and 25°C. With a temperature optimum at 15°C and retaining 70% of its peak activity at 0°C, LipB has the lowest temperature activity profile documented to our knowledge.

The cold-adapted enzymes identified to date originate mainly from psychrophilic organisms, especially bacteria, found in and around the Antarctic (Gerday et al. 2000). *Aspergillus niger* is generally considered as a mesophilic organism with the optimal temperature of growth at 35-37°C, though it has been known to grow at a broad range of temperature from 6°C to 47°C (Schuster et al., 2002). Two previous studies (Fukumoto et al. 1964; Hatzinikolaou et al. 1986) reported a lipase activity from *A. niger* which exhibits temperature optimum at 25°C and temperature inactivation above 40-45°C, suggesting that *A. niger* encodes at least one and possibly two more cold-adapted lipases. Together with LipB, these observations suggest that fungi, which thrive in a wide range of temperatures, may be a good source of cold-adapted enzymes for structural analysis and for the development of biotechnological applications.

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Chapter 3: Cloning, expression and characterization of an *Aspergillus niger* lysophospholipase

Keywords: lysophospholipase, lysophospholipid, *Aspergillus niger*, cloning, characterization

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Abstract

Two *Aspergillus niger* cDNA genes predicted to code for secreted phospholipases B/lysophospholipases, *plbA* and *plbB*, were cloned. The *plbA* and *plbB* cDNAs contained ORFs of 1917 bp and 1923 bp that coded for proteins of 638 and 640 amino acid residues, respectively. A catalytic triad consisting of Arg-127, Ser-166 and Asp-418 for PlbA and Arg-125, Ser-164 and Asp-416 for PlbB are proposed. Functional expression of both PlbA and PlbB was attempted, and a high level expression of PlbA was detected. However, PlbB could only be expressed at a very low level. We were unable to purify and characterize this enzyme. The molecular mass of the purified PlbA enzyme was estimated by SDS-PAGE to be about 90 kDa. PlbA exhibited optimal lysophospholipase activity at pH 3.0 and 50°C. It was stable in a pH range of 2.2 to 8.6 at 22°C for 2 hours, and up to 60°C for 60 minutes. Substrate specificity determination with various lysophospholipids showed this enzyme displayed a broad specificity toward lysophospholipids. For chain length specificity determined with lysophosphatidylcholine carrying acyl chains ranging in length from C12-C18, the enzyme preferred shorter chain substrates. The PlbA lysophospholipase activity hydrolyzed only lysophospholipids and

did not show phospholipase B, phospholipase A, lipase or general esterase activity. Kinetic analysis showed that the lysophospholipase activity of PlbA exhibited apparent cooperativity with $K_{0.5}$ of 19.27mM and Hill coefficient of 2.036.

Introduction

Phospholipases that catalyze fatty acid hydrolysis from both the sn-1 and sn-2 positions of glycerophospholipids are generally designated phospholipases B (PLBs, EC3.1.1.5). Lysophospholipases (LPLs, EC 3.1.1.5) catalyze the hydrolysis of the ester bonds of lysophospholipids (lyso-PLs) to free fatty acids and glycerolphosphate derivatives. Biochemical characterization of several fungal PLBs has revealed that they are remarkably versatile enzymes, which in addition to phospholipase B activity often possess lysophospholipase and transacylase activity enabling them to also cleave fatty acids from lysophospholipids (Gassama-Diagne et al. 1989; Lee et al. 1994) and catalyze phospholipid synthesis from lysophospholipids (Saito et al. 1991; Lee et al. 1994; Ghannoum 2000). Reflecting their phospholipase B and lysophospholipase activities PLBs are frequently called phospholipases B/lysophospholipases. PLBs are widely distributed in eukaryotes ranging from fungi to mammals; however, the biological function of PLBs remains poorly understood.

To date PLBs have been most extensively studied using the model yeast *Saccharomyces cerevisiae*, which has four genes that encode proteins displaying significant similarity to other PLBs (Lee et al. 1994; Merkel et al. 1999). Three of the yeast PLB genes, *PLB1*, *PLB2* and *PLB3,* are greater than 60% identical at the level of their protein sequences and localized to the plasma membrane, periplasmic space and

extracellular region (Lee et al. 1994; Merkel et al. 1999; Fyrst et al. 1999). *SPO1*, the fourth gene, codes for an intracellular protein that is about 30% identical with Plb1p, Plb2p and Plb3p.

Cell free extracts from vegetative yeast cultures of a triple deletion strain (*Δplb1 Δplb2 Δplb3*) showed no phospholipase B activity and lysophospholipase activity was reduced to less than 10% of the amount produced by a wild type control (Merkel et al. 1999). Plb1p, Plb2p and Plb3p catalyze the cleavage of both acylester bonds of glycerophospholipids. Nonetheless, lysophospholipids do not accumulate as intermediates, because the lysophospholipase activity of Plb1p and Plb2p greatly exceeds their phospholipase activities (Lee et al 1994; Merkel et al 1999). Plb1p and Plb2p also exhibit transacylase activity, catalyzing the synthesis of phosphatidylcholine (PtdCho) from two molecules of lysophosphatidylcholine (Merkel et al. 1999). Plb1p is mainly responsible for the deacylation of cellular PtdCho and phosphatidylethanolamine (PtdEtn), but not phosphatidylinositol (PtdIns). Plb2p has a broader substrate specificity, similar to Plb1p. Fyrst et al (1999) reported that Plb2p shows lysophospholipase activity toward PtdCho, PtdSer and PtdEtn. However, unlike PLB1, Plb2p does not contain significant transacylase activity. In contrast, Plb3p is similar to the two other extracellular Plbs regarding its transacylase activity and phospholipase activity with the PtdSer and PtdIns substrates; however, it does not detectably hydrolyze PtdCho and PtdEtn nor does it exhibit detectable lysophospholipase activity.

The enzymatic activity of Spo1p, which is localized to the nucleus, has not been reported; however *SPO1* is only expressed during meiosis (Tevzadze et al 2000). Moreover, both a *SPO1* deletion mutant and a *SPO1* mutant with conserved catalytic

triad residue Ser28 changed to Ala28 elicited a similar meiotic defect. This led the authors to suggest that Spo1p catalytic activity is essential for meiosis perhaps through nuclear membrane remodeling or the generation of a lipid signaling molecule.

The biological function of the three extracellular *S. cerevisiae* phospholipases remains obscure, since a *plb1 plb2 plb3* triple disruption strain did not have a detectable growth phenotype. Nonetheless, several lines of evidence demonstrate that they can play a significant role in cellular phospholipid metabolism. Plb1p, Plb2p and Plb3p participate in the breakdown of cell membrane and extracellular phospholipids, since membrane phospholipid turnover is dramatically reduced in a *plb1 plb2 plb3* deletion strain (Merkel et al. 1999). Additionally, the sensitivity of yeast to natural detergents such as lysophospholipds is dramatically enhanced by the deletion of *PLB1* and *PLB2* (Debets et al. 1986; Fyrst, H. et al. 1999), and a yeast strain with the three extracellular PLB genes deleted is unable to utilize palmitoyl-oleoyl-phosphatidylcholine as its sole source for unsaturated fatty acids under anaerobic conditions.

There is also evidence that extracellular PLBs are virulence factors in the human pathogenic fungi, *Cryptococcus neoformans* (Cox et al. 2001) and *Candida albicans* (Leidich et al. 1998). Secreted fungal PLBs also have applications in the food processing industries. Selected PLBs have been shown to improve the elasticity of bread and cake dough, to improve the filter-ability of aqueous solutions or slurries of carbohydrates containing phospholipids, and for the purification of edible oils containing phospholipids (www.freepatentsonline.com/6759225.html).

Only a few fungal PLBs have been studied (Kawasaki and Saito 1973; Merkel et al. 1999, Chen et al. 2000; Wright et al. 2004) and no A. niger PLBs have been reported. In

the present paper, we report the first cloning of two *A. niger* PLB cDNAs that we have designated *plbA* and *plbB*. Although both PlbA and PlbB were expressed as secreted proteins, PlbB could only be detected with a very low activity. PlbA had unique biochemical properties in that it exhibited high levels of lysophopholipase activity but unlike previously characterized fungal extracellular PLBs it did not exhibit significant phopholipase B activity.

Material and Methods

Materials

Aspergillus niger cDNA clones Asn_11385 and Asn_03295, which were predicted to harbour the complete ORFs for two lysophospholipases (Senova et al. 2006) were used as the sources of ORF DNA. The partial sequence of Asn_11385 and Asn_03295 are available at GenBank (DR709763 and DR703552). *Pichia pastoris* strain KM71 (Invitrogen) and *A. niger* strain NW171 (van de Vondervoort et al, 1997) were used as host strains for protein expression. *E. coli* strain DH5-α was used as host for the construction and preparation of recombinant plasmids. Plasmid vector ANEp7 was used for the expression of Asn_11385 and Asn_03295 in *A. niger* (Storms et al, 2005). For expression in *P. pastoris* we used pPID3.5, a derivative of pPIC3.5K (Invitrogen) where the multiple cloning site (BamHI, SnaBI, EcoRI, AvrII, NotI) was replaced with a multiple cloning site with restriction endonuclease sites for BamHI, PacI, AscI, SnaBI, FseI, AvrII and NotI. 1-stearoyl-2-hydroxy-*sn*-glycero-3- phosphoethanolamine, 1-stearoyl-2-hydroxy-*sn*-glycero-3-[phospho-*rac*-1-glycerol)] (sodium salt) and 1-oleoyl-2-hydroxy-*sn*-glycero-3-[phospho-L-serine](sodium salt), 1-lauroyl-2-hydroxy-*sn*-glycero-

3-phosphocholine, 1-myristoyl-2-hydroxy-*sn*- glycero-3-phosphocholine and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar Lipids, Inc (Alabama, USA). 1-stearoyl-*sn*-glycero-3-phosphocholine, L-α-phosphatidylcholine (from egg yolk), L-α-lysophosphatidylcholine (from egg yolk) and commercial phospholipase A_2 (PLA_2, from bovine pancreas) were purchased from Sigma-Aldrich (Toronto, Canada).

Sequence analysis

Asn_11385 and Asn_03295 were completely sequenced on both strands using an ABI 3730xl automated analyzer at the Génome Québec Innovation Centre. OrfPredictor (Min et al, 2005a) was used to identify the coding regions and the cDNA clones were assigned a tentative function using the tool TargetIdentifier (Min et al., 2005b). Signal peptides in the amino acid sequences were predicted using SignalP v3 (Bendtsen et al., 2004). Similarity searches were performed using BLAST algorithms (Altschul et al., 1990) to search the non-redundant NCBI nucleotide and protein databases. The search for protein families, domains, and active sites was performed using the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, v2.04). Multiple sequence alignments and analysis of relatedness were performed using CLUSTALW (Thompson et al., 1994) at the server provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw).

Cloning

The predicted Asn_11385 and Asn_03295 ORFs were amplified using primer pairs forward 5'- AGGCGCGCCATGAAGTTGCCTCTCTTTGCTGC -3', reverse 5'-AGCCTAGGCCGGCCCTACAGCATAAACGCAGCAAC-3' and forward 5'-

AGGCGCGCCATGAAGTTCAATGCACTCTTAACGACC-3' and reverse 5'-AGCCTAGGCCGGCCCCAACCTCCACTCACATCATCC-3' respectively. Both forward primers contained an AscI restriction site and both reverse primers contained an FseI restriction site. Following digestion with AscI and FseI, the ORFs were ligated into plasmids pPID3.5 and ANEp7 prepared by digestion with the same restriction endonucleases and the recombinant plasmids were transformed into *E. coli* DH5-α cells. Transformed DH5-α cells were plated on LB+ampicillin plates. Recombinant pPID3.5 and ANEp7 harbouring the Asn_11385 and Asn_03295 ORFs were identified by restriction analysis of plasmid DNA purified using QIAGEN miniprep columns and the method recommended by the manufacturer.

Expression of recombinant PLBs

Competent *P. pastoris* cells were prepared as described in the electroporation protocol provided by Invitrogen. Transformation of *P. pastoris* was achieved by electroporation using a Biorad Genepulser II. The transformed cells were plated on MD plates (1.34% yeast nitrogen base, 4X10-5 biotin, 2% dextrose, 1.5% agar) and incubated at 30°C for 3-4 days. His^+ transformants were selected and cultured in liquid GCM (1% Yeast extract, 2% peptone, 1.34% YNB, 4×10^{-5} % biotin and 1% glycerol) initially at 30°C with shaking at about 200 rpm, when the OD_{600} reached to 2-6, cells were collected and transferred to fresh MCM (same as GCM except that 1% glycerol was replaced by 0.5% methanol, which also serves as the main carbon source while inducing the expression) for induction of protein expression (Minning et al. 2001). Culture samples were taken at different time points for protein purification and LPL activity assays.

A. niger transformants with recombinant ANEp7 were generated essentially as described previously (Debets and Bos, 1986). Liquid cultures of *A. niger* were inoculated by transferring a piece of agar, which contained mycelia of the *A. niger* transformant of interest, from minimal medium (MM) agar plates into a flask containing 250 ml of liquid MM with 15% (w/v) glycerol as the carbon source. Both solid support and liquid cultures of *A. niger* were grown at 30°C. Liquid cultures were maintained at 150 rpm in a rotary shaker. Culture samples were removed at different time points, filtered through Miracloth to remove the mycelia and used for PLB purification and activity assays.

Purification

First, the proteins in 100 ml of filtered culture medium were concentrated using a Millipore Centricon YM-10 filter units. The concentrated proteins from the Centricon step (typically 1 ml) were loaded onto a Superdex®75 column (Pharmacia) equilibrated with 25 mM Tris-HCl buffer (pH 7.5). LPL activity was eluted at a flow rate of 0.4 ml min^{-1}. Each fraction (1.2 ml) was assayed for LPL activity and fractions with the highest activity were pooled for further purification. A 1 ml sample of the pooled fractions purified from the Superdex®75 column was loaded on a Mono-Q column equilibrated with 25 mM Tris-HCl buffer (pH 7.5). The column was run at a flow rate of 0.4 ml min^{-1}. After 15 minutes of washing with the same buffer, the column was eluted with a linear gradient (15 ml of 0 - 0.8 M NaCl). Fractions of 0.4 ml were collected. Electrophoresis was performed on 10% SDS-PAGE gels and stained with Coomassie Blue. Protein concentrations were determined using a protein assay kit (Bio-Rad) and a standard curve prepared using bovine serum albumin (Sigma).

Mass spectrometry

The major protein bands from the purified PlbA, identified by Coomassie Blue-staining following resolution by SDS-PAGE, were subjected to in-gel digestion with trypsin according to the method of Shevchenko et al. (Shevchenko et al., 1996) The trypsin-digested peptides were mixed with the matrices and prepared for MALDI-TOF analysis as described previously (Laugesen and Roepstorff, 2003). Peptide data was acquired in a positive-ion reflectron mode using a MALDI-LR mass spectrometer (Waters-Micromass).

Enzyme activity assay

LPL and phospholipase activity assays LPL and LPA activity were assayed using a modification of the turbidimetric assay for lipolytic enzymes described by Von Tigerstrom and Stelmaschuk (Von Tigerstrom and Stelmaschuk, 1989). Unless indicated otherwise assays were performed as follows. Reactions were performed in a 160 µl of 25 mM glycine-HCl buffer (pH 3.0) containing 9.38 mM substrate (PLs or lyso PLs), 0.625% Triton X-100 and 5 mM $CaCl_2$. Following incubation at 40°C for 15 min, the reaction was stopped by adding 50 µl of ice-cold 1M Tris base and stored on ice. Turbidity formed due to precipitation of the calcium salts was measured at 500 nm with a microplate reader (Power Wave HT, Bio-Tek Instruments, Winooski, Vermont). Activities against lyso-PLs with different length chains were calculated using standard curves prepared with the calcium salt of the corresponding fatty acid. One unit of LPL is defined as the amount of enzyme that releases 1 µmol of fatty acid from lyso-PC per minute under the assay conditions.

Lipase activity assays Lipase activity was quantified spectrophotometrically using *p*-nitrophenyl fatty acid esters as substrates. The lipase assays were performed in 150 µl

of 25 mM glycine-HCl buffer (pH 3.0) containing 1 mM *p*-nitrophenyl esters, 0.67% Triton X-100, 1% acetone, and appropriate amounts of enzyme. Unless otherwise indicated assay reactions were incubated at 40°C for 30 min and stopped by the addition of 60 µl of 300 mM Na_2CO_3. The absorbance was measured at 410 nm with a microplate reader (Power Wave HT, Bio-Tek Instruments, Winooski, Vermont). Lipase activity was calculated using a standard curve prepared with *p*-nitrophenol. One unit of lipase activity is defined as the amount of enzyme that releases 1 µmol of PNP per minute under the assay conditions.

Effect of pH and temperature on lipase activity

The pH optimum of the LPL activity was determined using the assay conditions described above except that different buffer systems at a final concentration of 25 mM were used (glycine-HCl for pH 2.2 to 3.6; sodium acetate for pH 3.6 to 5.6; Tris-malate for pH 5.6 to 8.6; Tris-HCl for pH 7.4 to 9.0; and glycine-NaOH for pH 8.6 to 10.6). For the determination of pH stability, enzyme samples were incubated at 22°C for 3 h at various pH intervals, and the activity was measured at optimal pH and temperature. The temperature optimum was determined by assaying at temperatures ranging from 30°C to 70°C. For determination of temperature stability, enzyme samples were preincubated at temperatures ranging from 20°C to 70°C for both 60 min and 180 min at the pH optimum, and following preincubation, the activities were determined as described above at optimal pH and temperature.

Substrate specificity and enzyme kinetics

Substrate specificity and kinetic determinations were performed with purified PLB at the optimal pH and temperature. The influence of the lipid polar head group on

lysophospholipase acitivty was assessed using lysophosphatidylcholine (lyso-PC), lysophosphatidyllethanolamine (lyso-PE), lysophosphatidylglycerol (lyso-PG) and lysophosphatidylserine (lyso-PS), each carrying a C18 fatty acid chain in position *sn*-1. The effect of fatty acid chain length on LPL specificity was assessed using lyso-PCs with C12:0, C14:0, C16:0 and C18:0 fatty acid chains located at position *sn*-1. LPL kinetic constants $K_{0.5}$ was obtained by non-linear regression performed with GraFit Version 4.0 software (Erithacus Software Limited, Surrey UK).

Results

Sequence analysis

A BLASTX search of the GenBank nonredundant protein database with fully sequenced Asn_11385 and Asn_03295 cDNA clones revealed significant sequence similarity with other fungal PLBs. Analysis of the Asn_11385 and Asn_03295 sequences revealed that they encoded ORFs of 1917 bp and 1923 bp, encoding proteins of 638 and 640 amino acid residues with predicted molecular masses of 68.7 kDa and 69.2 kDa. After cleavage of the putative 16 and 21 residue signal peptides, the two PLBs, designated PlbA and PlbB, have predicted molecular masses of 67.2 kDa and 67.1 kDa with theoretical pIs of 4.27 and 5.24.

A PLA2_B catylytic domain (pfam01735.12), which is found in lysophospholipases/phospholipases B (EC:3.1.1.5) and cytosolic phospholipases A2 (EC:3.1.4), was detected in the predicted sequence of both PlbA and PlbB. The PLA2_B domains cover 75% and 76% respectively of the predicted protein sequences of *plbA* and *plbB*. The amino acid sequences of PlbA and PlbB share 63% identity and 79%

similarity. Alignment of PlbA and PlbB with other fungal phospholipase B/lysophospholipase sequences showed that PlbA has highest similarity with *Aspergillus fumigatus* PlbA, with which it shares 65% identity and 80% similarity, PlbB has highest similarity with the *A. fumigatus* PlbAp (66% identity and 78% similarity) (Fig 10). The catalytic center, which consists of Arg-127, Ser-166 and Asp-418 for PlbA and Arg-125, Ser-164 and Asp-416 for PlbB, was proposed based on a sequence alignment with fungal PLBs from *Saccharomyces cerevisiae* (Merkel et al. 1999, results not shown).

Cloning, expression and purification

We wanted to express and characterize the lysophospholipase/phospholipase B enzymes encoded by *plbA* and *plbB*. For this, pPID3.5-plbA, and pPID3.5-plbB were transformed into *P. pastoris* and ANEp7-plbA and ANEP7-plbB were transformed into *A. niger*. Consistent with the predicted presence of signal peptides our preliminary studies showed that the great majority of recombinant PlbA activity expressed by both *P. pastoris* and *A. niger* was secreted into the culture medium. However, *P. pastoris* and *A. niger* were only able to express an extremely low levels of recombinant PlbB activity. The amount of lysophospholipase activity expressed by *P. pastoris* transformed with pPID3.5-plbA increased from day 2 to day 4 whereas the amount of activity expressed by *A. niger* transformed with ANEp7-plbA increased from day 1 to day 5 (Table 4). Lysophospholipase activity expressed by *A. niger* harboring ANEp7-plbA peaked at 68.9 units per ml, which was about 3.5 times higher that the maximum units per ml expressed by *P. pastoris* harboring pPID3.5-plbA (19.8 units/ml on day 4). For more detailed biochemical characterization, PlbA was purified from Centricon concentrated medium from 5 day cultures by sequential gel filtration chromatography and anion exchange

chromatography. The protein and activity profiles obtained following anion exchange chromatography are shown in Figure 11. The results of protein yield, specific activity, and fold enrichment with each purification step are summarized in Table 5. The specific activity of the final preparation was 4981U/mg protein. The molecular mass of purified PlbA was estimated to be 90 kDa by SDS-PAGE (Fig 12), a value was much greater than that calculated from the deduced amino acid sequence. PlbA was analyzed by MALDI-TOF (Fig 13), which identified 10 peptides covering 18% of the predicted of PlbA amino acid sequence (Table 6).

Effects of pH and temperature on lysophospholipase activity of PlbA

The effect of pH on lysophospholipase activity and stability was investigated (Figure 14). The results showed that the pH optimum for the lysophospholipase activity of PlbA was 3.0 (Fig 14A) and that the activity was stable during incubation from pH 2 to 8.6 (Fig 14B). These results indicated that the lysophospholipase activity of PlbA prefers acidic conditions but that it is stable over a wide pH range. The temperature optimum of the lysophospholipse activity of PlbA was 50°C (Fig 15A) and it retained at least 80% activity when incubated at temperatures up to 50°C for 3 h (Fig 15B).

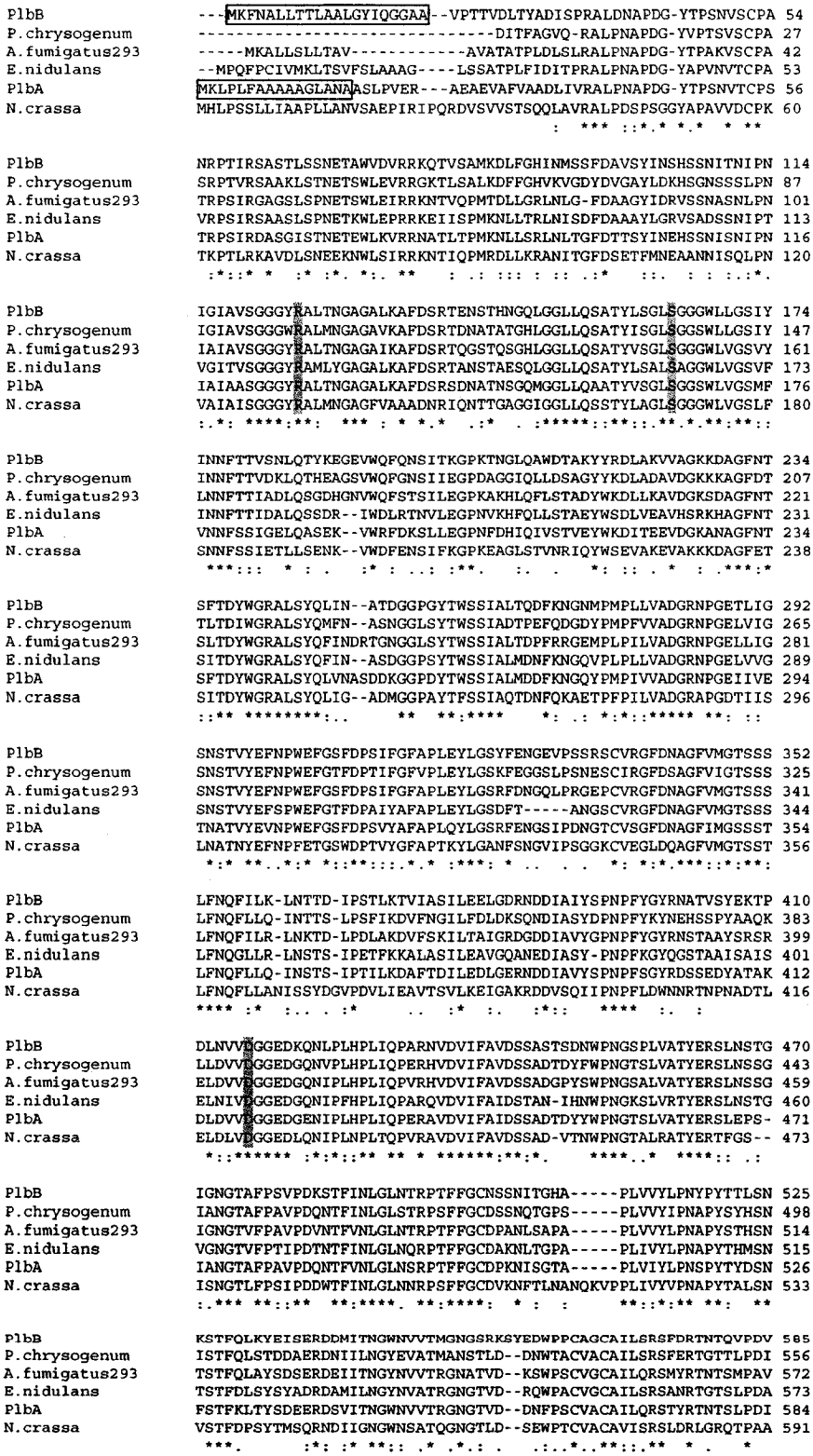
Substrate specificity and enzyme kinetics

The substrate specificity of PlbA was assessed using two sets of lyso-PLs. The first set, which varied the polar head group while keeping the fatty acid chain constant, showed that lyso-PS and lyso-PC were preferred substrates relative to lyso-PG and lyso-PE (Fig 16A). The second set of substrates kept the same choline containing polar head group but varied the length of the lyso-PC fatty acid group from C12:0 to C18:0 (Fig

16B). The results showed that PlbA preferred the shorter C12 chain substrate relative to substrates with C14 through C18 fatty acid chains.

Many lysophospholipases also exhibit phospholipase activity (Kawasaki and Saito 1973; Garsetti et al. 1992; Wright et al. 2004; De Jong 1973); however, we were unable to obtain measurable activity when PlbA was assayed for phospholipase activity using phosphatidylcholine as the substrate (data not shown). Based on these results, we conclude that PlbA does not have phospholipase B, A1 or A2 activity. PlbA also did not exhibit either esterase or lipase activity under the conditions tested, since it was unable to hydrolyze simple p-nitrophenyl esters with C2 to C16 fatty acid chains.

The substrate concentration dependence of PlbA was determined with lyso-PC. PlbA lysophospholipase activity displayed cooperative kinetics, which were best fitted to a Hill plot with a Hill coefficient of 2.036 (results not shown). The $K_{0.5}$ value was calculated to be 19.27 mM at 40°C and pH 3.0.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P.chrysogenum ----------DITFAGVQ-RALPNAPDG-YVPTSVSCPA 27
A.fumigatus293 -----MKALLSLLTAV----------AVATATPLDLSLRALPNAPDG-YTPAKVSCPA 42
E.nidulans --MPQFPCIVMKLTSVFSLAAAG----LSSATPLFIDITPRALPNAPDG-YAPVNVTCPA 53
PlbA MKLPLFAAAAAGLANAASLPVER---AEAEVAFVAADLIVRALPNAPDG-YTPSNVTCPS 56
N.crassa MHLPSSLLIAAPLLANVSAEPIRIPQRDVSVVSTSQQLAVRALPDSPSGGYAPAVVDCPK 60
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VAIAISGGGYRALMNGAGFVAAADNRIQNTTGAGGIGGLLQSSTYLAGLSGGGWLVGSLF 180
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SFTDYWGRALSYQLIN--ATDGGPGYTWSSIALTQDFKNGNMPMPLLVADGRNPGETLIG 292
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LFNQGLLR-LNSTS-IPETFKKALASILEAVGQANEDIASY-PNPFKGYQGSTAAISAIS 401
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ELDLVDGGEDLQNIPLNPLTQPVRAVDVIFAVDSSAD-VTNWPNGTALRATYERTFGS-- 473
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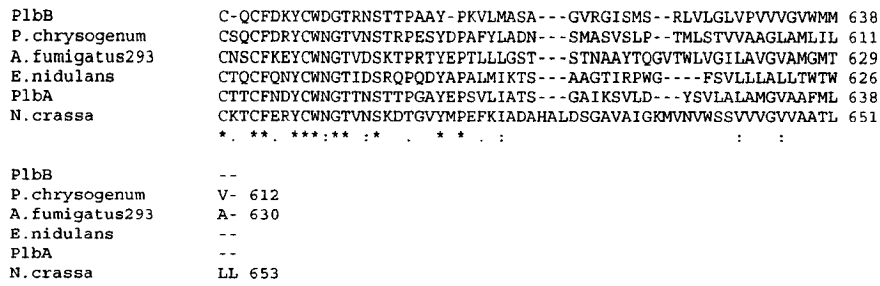


Fig. 10. Alignment of the amino acid sequences of *Aspergillus niger plbA* and *plbB* with the PLB sequences of *Aspergillus fumigatus* Af293 (EAL92119), *Emericella nidulans* (BAD95522), *Penicillium chrysogenum* (CAA42906) and *Neurospora crassa* (CAE76554). The residues belonging to the putative catalytic triad are shadowed gray. The predicted signal peptides are enclosed in the boxes.

Table 4. Activity of PlbA and PlbB on lyso-PC in *A. niger* and *P.pastoris* (units/ml)

	A. niger					P. pastoris		
	Day1	Day2	Day3	Day4	Day5	Day2	Day3	Day4
Control	0	0	0	6.331	0.597	0	0	00.036
PlbA	0	5.136	43.480	68.923	61.636	4.348	14.406	19.805
PlbB	0	0.119	1.433	14.215	3.942	-	-	3.548

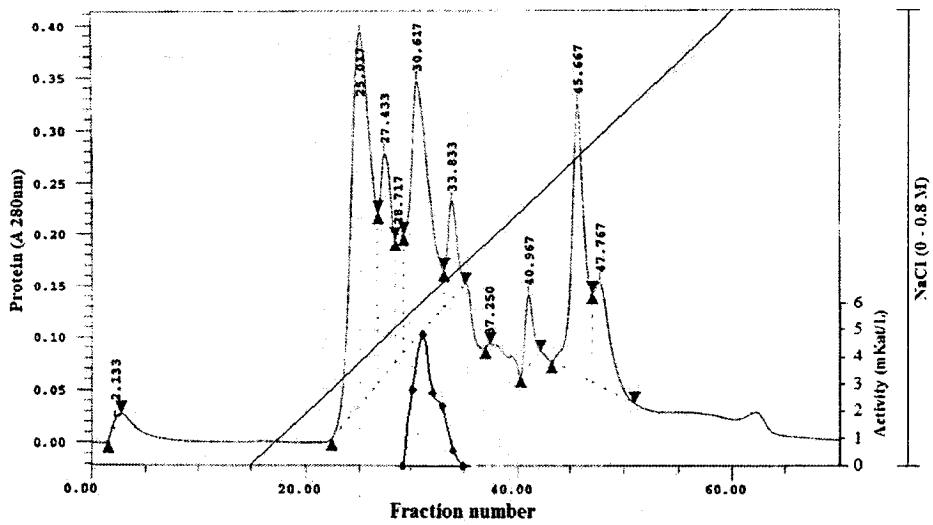


Fig. 11. Elution profile of PlbA on Mono-Q. Elution was carried out with a gradient of 0-0.8 M NaCl in 25 mM Tris-HCl buffer (pH 7.5). Flow rate is 0.4 ml/min. LPL activities of the fractions (—•—) were measured with lyso-PC.

Table 5. Summary of the purification of PlbA

Steps	Total protein (μg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Activity Yield (%)
Crude	1032.7	781	756.5	1	100
Superdex	402.1	674	1167	2.2	86.3
Mono-Q	23.2	116	4981	6.6	14.8

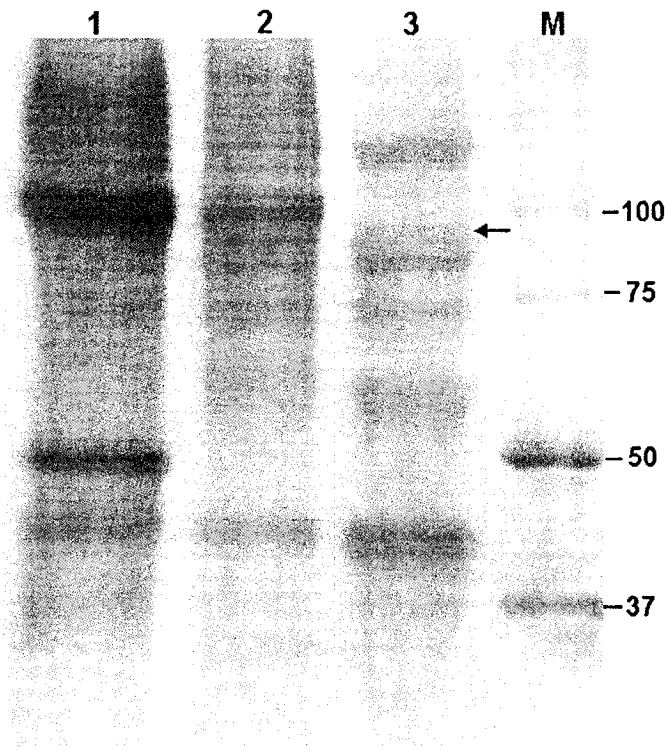


Fig. 12. SDS-PAGE of PlbA protein samples at various stages of purification. Lane 1. Crude sample following concentration on Centricom; Lane 2, Pooled lysophospholipase activity following Superdex®75 chromatography; Lane 3, Proetin sample after Mono-Q. 12% acrylamide gel is stained with Coomassie blue. PlbA is indicated with an arrow and its identity was confirmed by MALDI-TOF.

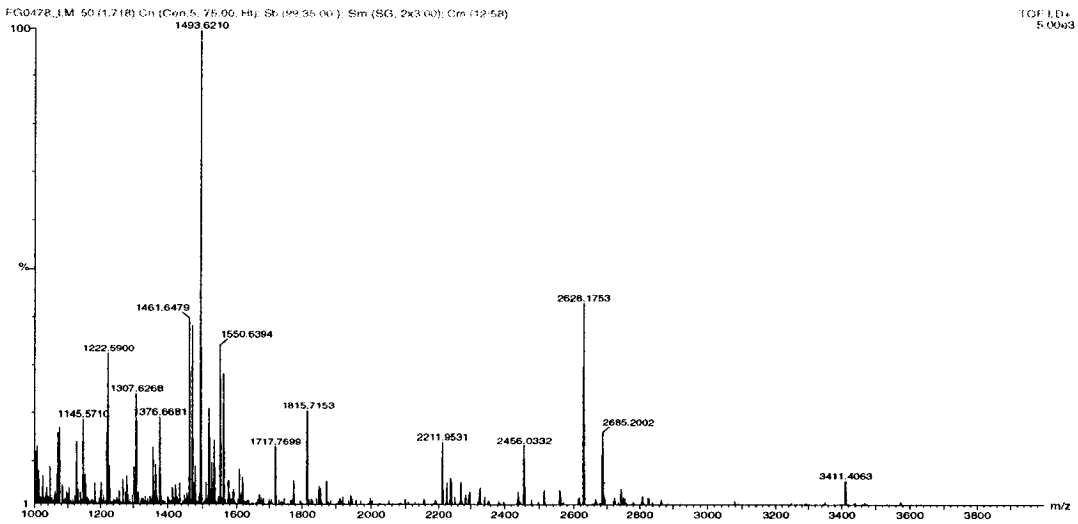


Fig 13. MALDI-TOF peptide mass fingerprinting maps of *A. niger* PlbA. The major protein bands from SDS-PAGE gel were subjected to in-gel digestion with trypsin. The digestion mixture was analyzed by MALDI-TOF mass spectrometry.

Table 6. Peptide fingerprints of recombinant LPL PlbA

Amino acid position	Peptide sequence	Calculated mass	Observed mass
63-76	DASGISTNETEWLK	1550.637	1549.629
80-92	NATLTPMKNLLSR	1474.606	1473.598
273-286	NGQYPMPIVVADGR	1516.642	1515.634
273-286	NGQYPMPIVVADGR	1532.609	1531.601
374-386	DAFTDILEDLGER	1493.618	1492.61
387-402	NDDIAVYSPNPFSGYR	1814.71	1813.702
413-436	DLDVVDGGEDGENIPLHPLIQPER	2627.155	2626.147
532-539	LTYSDEER	1012.407	1011.399
532-552	LTYSDEERDSVITNGWNVVTR	2454.003	2452.995
540-552	DSVITNGWNVVTR	1460.647	1459.639

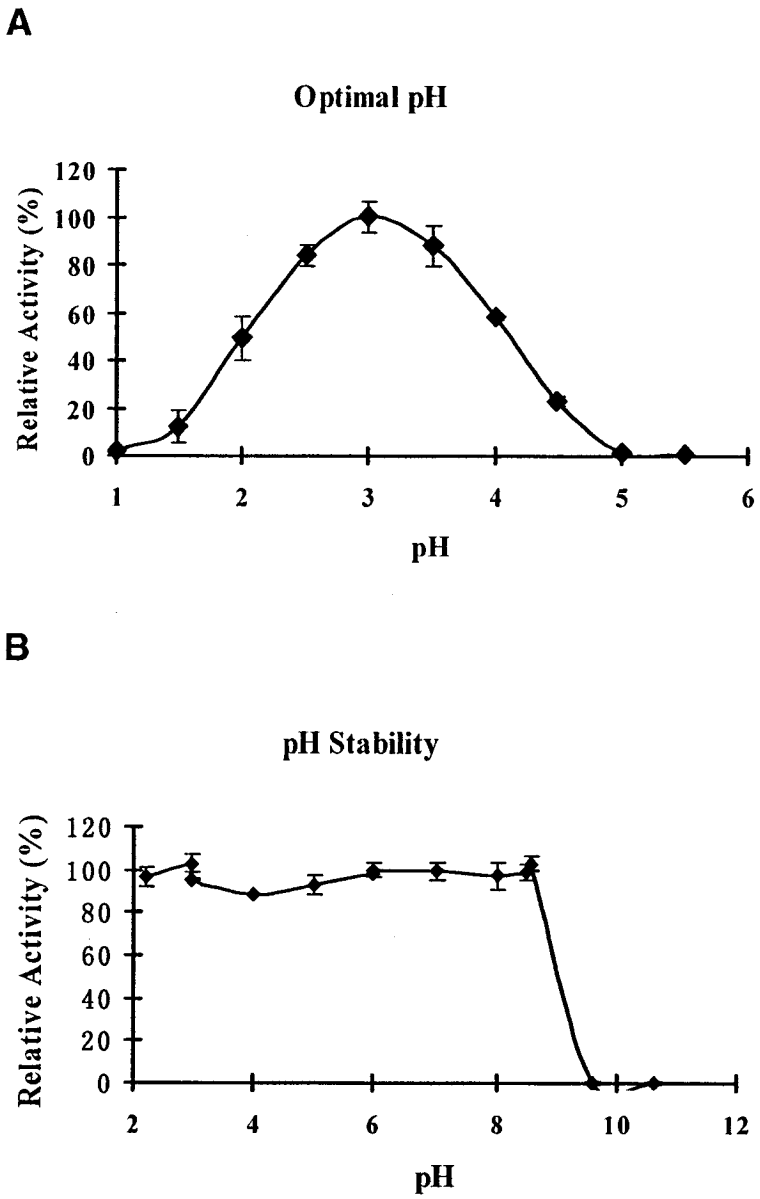
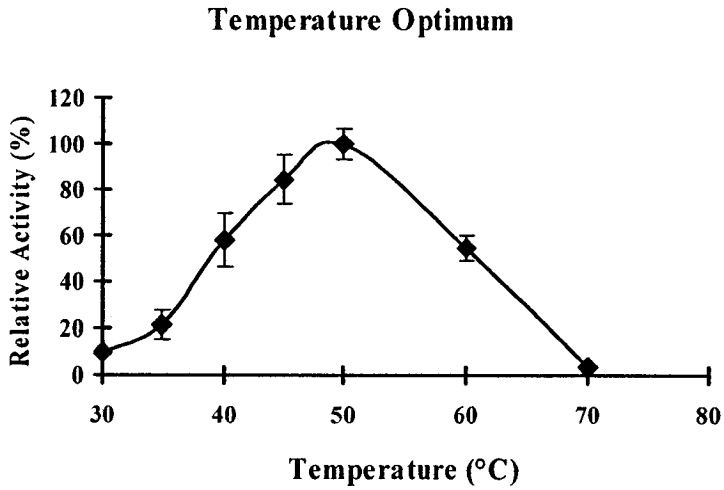


Fig 14. Effect of pH on the activity and stability of the *A. niger* PlbA. (A) The pH optimum of PlbA was determined by incubating the enzyme at various pHs with 9.38 mM lyso-PC and 5 mM $CaCl_2$ at 40°C for 15 min. (B) The pH stability of PlbA was determined by incubating the enzyme at various pHs in different buffer systems at 22°C for 3 hours and then measuring the activities at optimal pH and temperature.

A



B

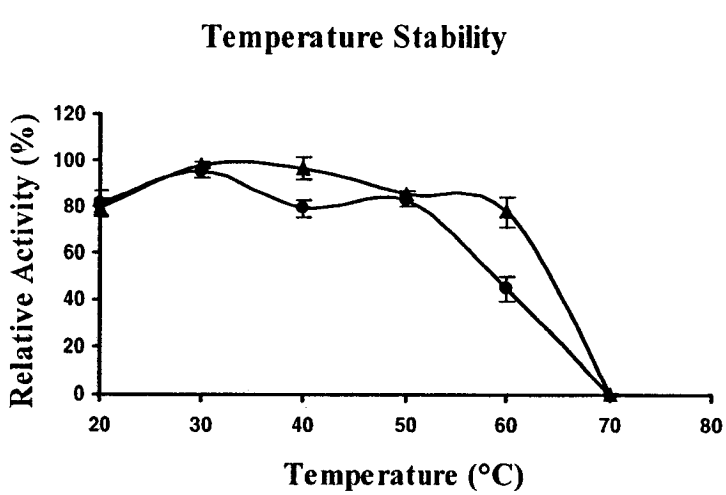


Fig 15. Temperature optimum (Panel A) and thermal stability (Panel B) of *A. niger* PlbA. Each assay contained 5 µl of enzyme and 1.5mmol of lyso-PC in 160 µl of 25 mM glycine-HCl buffer, pH 3. Samples were incubated for 10 min.
▲, 1 hour preincubation; ●, 3 hour preincubation.

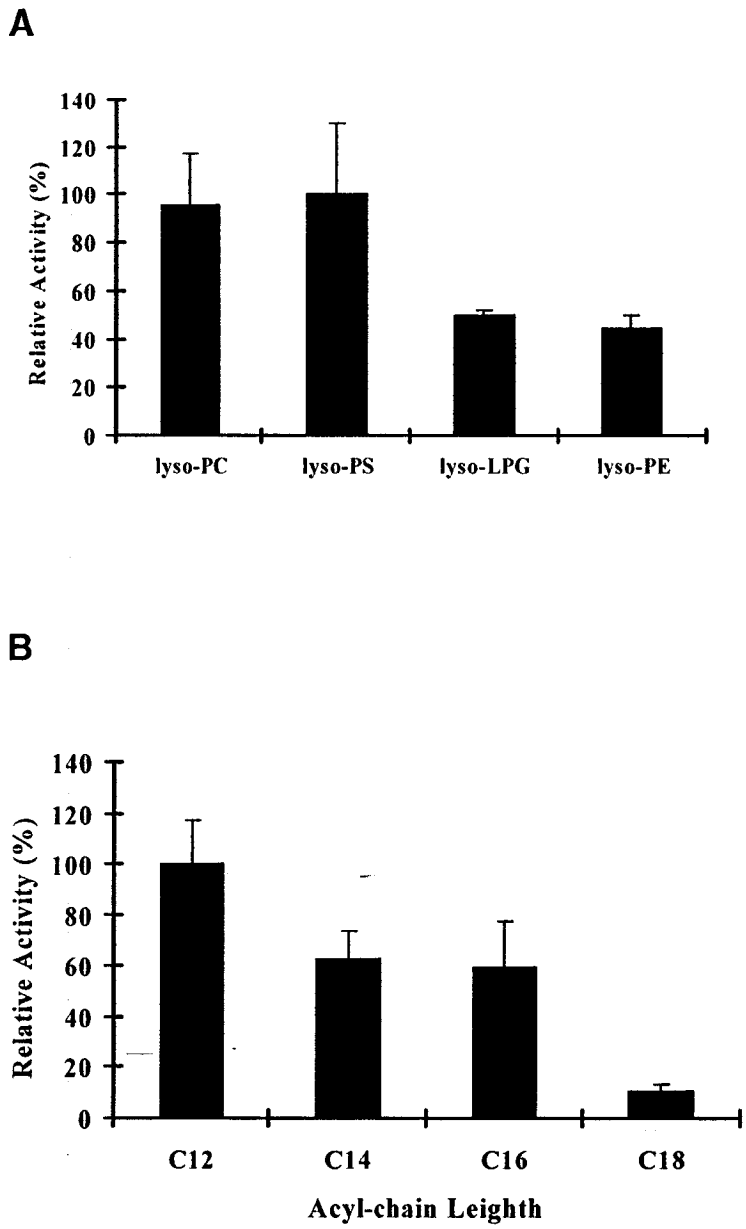


Fig. 16. Substrate specificity determination of *A. niger* PlbA. Relative activity of the enzyme towards various lyso-PLs with same chain lengths (panel A) and lyso-PC with different chain lengths (panel B).

Discussion

The predicted proteins PlbA and PlbB were compared by BLAST with sequences in the GenBank databases and found to possess sequence similarity to many other fungal LPLs with the highest homology to *Aspergillus fumigatus* phospholipase B /lysophospholipase. This *A. fumigatus* PLB is similar in size (630 amino acids) to the predicted *A. niger* proteins. PlbA and PlbB share 65% sequence identity. Analysis of the amino acid sequences by NCBI Conserved Domain Search revealed that both the proteins contains a LPL domain and a cytosolic PLA_2 domain. These analyses strongly suggest that Asn_11385 encodes a phospholipase B/lysophospholipase. PlbA contains a signal peptide, indicating that the protein is secreted. Sequence similarities between fungal phospholipases B/lysophospholipases, and mammalian cytosolic PLA_2 suggest that these enzymes may act by a common catalytic mechanism. Pickard *et al.* (Pickard et al. 1996) identified consensus motifs around putative catalytically competent amino acid residues of human cytosolic PLA_2. The authors postulated a catalyticc triad comprising Arg, Ser, and Asp that is conserved in cytosolic PLA_2 from a number of mammalian species and in several non-mammalian phospholipases B/lysophospholipases, including *S. cerevisiae* Plb1p. Recent data from x-ray analysis of cytosolic PLA_2 suggest that only the Ser and Asp residues are directly involved in catalysis, thus forming a catalytic dyad (Dessen et al. 1999). It will be of interest to fully identify the catalytic center used by fungal PLBs. According to Pickard *et al* (1996), the predicted catalytic center in the *S. cerevisiae* Plb1p consists of Arg-109, Ser-147, and Asp-403. These residues are also conserved in Plb2p and Plb3p at positions Arg-111 (115), Ser-149 (153), and Asp-405 (409), respectively (Merkel et al. 1999). Comparison of the protein sequences of *A. niger* PlbA with the

phospholipases B/lysophospholipases of *S. cerevisiae* revealed that PlbA shares 47% sequence identity and 64% similarity with the Plb1p and similar identity with Plb2p and Plb3p. Based on sequence alignments with the *S. cerevisiae* PLBs we predict that the catalytic center of PlbA consists of Arg-127, Ser-166 and Asp-418.

So far, there are no published reports describing the characterization of *A. niger* phospholipases B/lysophospholipases gene or protein sequences. The PlbA encoded by Asn_11385 is therefore the first *A. niger* phospholipase B/lysophospholipase to be functionally expressed, purified and biochemically characterized. Functional expression of secreted PlbA by *P. pastoris* and *A. niger* was confirmed by lysophospholipase assays and MALDI-TOF Mass Spectrometry for *A. niger*. The experimentally determined molecular mass, estimated as 90 kDa by SDS-PAGE, was significantly greater than the predicted mass of 67.2 kDa, resembling the results obtained with other secreted fungal phospholipases B/lysophospholipases (Kawasaki and Saito 1973; Merkel et al. 1999; Chen et al. 2000; Wright et al. 2004; Watanabe et al. 1994; Oishi et al. 1996; Leidich et al. 1998). Since the carbohydrate moiety has been shown to be important in the catalytic capability of another phospholipase B (Chen *et al*, 2000), it is possible that the molecular weight discrepancy occurring between purified mature enzyme and the deduced molecular mass is due to post-translational glycosylation.

Several differences between the enzymatic properties of the *A. niger* PLB, PlbA, and those of other fungi were observed. *A. niger* PlbA has a more acidic pH optimum than those of *Cryptococcus gattii* (pH 5.0) (Wright et al. 2004) and *Cryptococcus neoformans* (pH 4.0) (Chen et al. 2000) and is stable in a wide pH range. Both cryptococcal PLBs were optimally active at physiological temperatures (37°C) (Chen et

al. 2000), while our LPL exhibited optimal activity at around 50°C. Stability of *A. niger* PlbA activity to pre-incubation at high temperatures was also marked, with little change in activity after pre-incubation for 1 hour between 20–60°C and 3 hours between 20-50°C. The enzyme retained 100% of its activity for at least 24 hours during incubation at room temperature.

Substrate specificity determination of PlbA revealed that it is highly specific for lyso-PLs. First, we tested the influence of the lipid polar head group by a comparison of the susceptibility to hydrolysis of lyso-PC with lyso-PE, lyso-PG and lyso-PS, each carrying C16 fatty acid chain. The PlbA enzyme exhibited a rather broad specificity toward lyso-PLs with maximal activity on lyso-PC and lyso-PS. For chain length specificity, it exhibited marked substrate preference for substrates with shorter chains. We did not test its activity towards substrates with less than C12 chains due to the lower solubility of the calcium salts of the shorter fatty acids. Some characterized LPLs are non-specific and still catalyze other reactions in addition to the hydrolysis of lyso-PL (Kawasaki and Saito 1973; Garsetti et al. 1992, Wright et al. 2004; De Jong et al. 1973) and there are also multiple families of unrelated enzymes that display LPL activity (Sugatani et al. 1980; Saito and Kates 1974; Reynolds et al. 1993 Mirbod et al. 1995). In contrast to many non-specific lipolytic enzymes that exhibit LPL activity, the purified PlbA encoded by Asn_11385 did not hydrolyse diacylphosphatidylcholine and *p*-nitrophenyl esters at measurable rates under the conditions tested. These results suggest that PlbA did not exhibit phospholipase B, lipase or general esterase activity and appeared to be Lyso-PL specific. Similar results for LPLs from different sources were reported (Fujikura and Baisted 1985; Karasawa et al. 1985; Sunaga et al. 1995; Zhang

and Dennis 1988; Wang et al. 1999; Sugimoto et al. 1996). *A. niger* PlbA also differs from most PLB-type enzymes, which are able to hydrolyze not only lyso-PLs but also diacylphospholipids.

PlbA did not display typical Michaelis-Menten kinetics but rather strong positive cooperativity with respect to substrate binding, with a Hill coefficient (n(H)) of 2.036. Similar results were obtained with other LPLs (Chen et al. 2000; Sunaga et al. 1995; Zhang and Dennis 1988; Wang et al. 1999; Sugimoto et al. 1996; Jarvis et al. 1984; Stafford et al. 1993). PlbA seems to be a monomer according to the predicted and experimental molecular weight deduced from gel filtration experiments. The apparent cooperativity which is observed is likely due to the change of substrate presentation instead of a cooperative binding of two or more substrates to catalytic and/or allosteric sites (Wang et al. 1999)

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Chapter 4: Conclusion

The cDNAs from *Aspergillus niger* encoding two lipases (*lipA* and *lipB*) and two phospholipase B/lysophospholipases (*PlbA* and *PlbB*), were characterized. The *lipA* and *lipB* cDNAs encode polypeptides of 297 and 298 amino acid residues with predicted molecular masses of 31.7 and 32.0 kDa, respectively. The *PlbA* and *PlbB* cDNAs encode polypeptides of 638 and 640 amino acid residues with predicted molecular masses of 68.7 and 69.2 kDa, respectively. Sequence analysis showed that the deduced amino acid sequences of *lipA* and *lipB* share 53% identity and both exhibit 51-69% amino acid identity to four proteins predicted from the genome sequences of *Aspergillus fumigatus*, *Aspergillus nidulans*, and *Aspergillus oryzae.* The predicted catalytic triad for LipA is Ser173, Asp228, and His285; and for LipB is Ser175, Asp230, and His287. The serine at the active site of both enzymes resides in a conserved consensus Gly-X-Ser-X-Gly pentapeptide. The amino acid sequences of *Aspergillus niger* PlbA and PlbB share 63% identity and 79% similarity. *Aspergillus niger* PlbA shares 65% identity and 80% similarity with *Aspergillus fumigatus* PlbAp, whereas *Aspergillus niger* PlbB has the highest similarity (66% identity and 78% similarity) with the *Aspergillus fumigatus* PlbA. The catalytic center consists of Arg-127, Ser-166 and Asp-418 for PlbA and Arg-125, Ser-164 and Asp-416 for PlbB.

The recombinant *lipA*, *lipB* and *PlbA* proteins were purified by column chromatography. MALDI-TOF mass spectrometry analysis confirmed that the purified recombinant proteins extracted from SDS-PAGE gels corresponded to the predicted LipA, LipB and PlbA proteins. We could not obtain active preparations of recombinant PlbB from both *Pichia pastoris* and *Aspergillus niger* expression systems.

The optimal pH for LipA activity was found to be 4.5-5.0 while LipB optimal activity is obtained at a slightly more acidic pH of 3.5-4.0. LipA is stable from pH 2.2 to pH 10.6 whereas LipB is stable from pH 2.2 to pH 9.6. These results indicated that the two *Aspergillus niger* lipases prefer acidic conditions for activity and are stable in a broad pH range. LipA is most active at 40°C and is stable up to 70°C. In contrast, Lipase B is more active at low temperature, retaining 70% of its peak activity at 0°C. However, LipB is thermolabile, rapidly losing its activity above 40°C. Agar plate assays showed that culture filtrates of *Pichia pastoris* carrying the *lipA* or *lipB* genes reacted positively with tricaprin, triolein, olive oil and soy oil as substrates. Both LipA and LipB exhibited significant activities against *p*NPC4 – *p*NPC18, but preferred medium- to long-chain (C8 to C18) substrates with the highest activity towards *p*NPC12.

The pH optimum for PlbA lysophospholipase activity is 3.0 and the activity was stable from pH 2 to 8.6. These results indicate that PlbA prefers acidic conditions but is stable over a wide pH range. The temperature optimum of PlbA lysophospholipse activity was 50°C and it retained at least 80% activity when incubated at temperatures up to 50°C for 3 h. The substrate specificity determination of PlbA showed that lyso-PS and lyso-PC were preferred substrates relative to lyso-PG and lyso-PE when keeping the fatty acid chain constant. For fatty acid chain length specificity, PlbA preferred the shorter C12 chain substrate relative to substrates with C14 through C18 fatty acid chains. PlbA didn't exhibit phospholipase B, A1 or A2, esterase or lipase activity under the conditions tested. PlbA did not display typical Michaelis-Menten kinetics but rather strong positive cooperativity with respect to substrate binding, with a Hill coefficient of 2.036.