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Biochemical properties and three-dimensional structures of two extracellular lipolytic enzymes from *Bacillus subtilis*

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Abstract

This article reviews our present knowledge on the extracellular lipolytic enzymes LipA and LipB from *Bacillus subtilis*. Growth of *B. subtilis* to the late logarithmic growth phase results in a total lipolytic activity of 12–18 units per liter of culture supernatant. Immunodetection with LipA- and LipB-specific antibodies indicated a differential expression of both lipolytic enzymes depending on the composition of the growth medium. LipA was produced in rich and in minimal medium, whereas LipB was present only in rich medium. The *lipA* and *lipB* genes were cloned and overexpressed in *B. subtilis* and *Escherichia coli*, the corresponding proteins purified to electrophoretic homogeneity and their substrate specificities, pH- and temperature stabilities were determined. The active site residue Ser₇₈ of LipB is located in the consensus sequence Ala–X–Ser–X–Gly where the alanine replaces a glycine found in most of the bacterial lipases. The role of this Ala-residue was investigated by constructing LipB variant A76G thereby restoring the canonical lipase consensus motif. When compared with wild-type LipB this variant showed a markedly reduced thermostability at pH 11 but an increased stability at pH 5–7. These findings were rationalized by building a three-dimensional structural model of LipB using the atomic coordinates of the LipA crystal structure, which was solved recently. The LipB model structure revealed that 43 out of 45 residues, which are different from LipA, were located on the surface of LipB. The surface-exposed amino acids including those located at the rim of the active site cleft may cause the differences in specific activities between LipA and LipB. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Bacillus subtilis*; Esterase/lipase; 3D-structural model; Catalytic triad; Lipase consensus motif

1. Introduction

Bacillus subtilis is one of the best studied microorganisms with respect to its genome, proteome and secretome [1–3]. Its genome sequence published in 1997 by Kunst et al. [1] represented

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the first complete genome sequenced from a Gram-positive bacterium. The capability of *B. subtilis* to secrete homologous and heterologous proteins at gram per litre amounts into the growth medium and its classification as a generally regarded as safe (GRAS) organism by the US Food and Drug Administration (FDA) have made it an attractive expression host to produce proteins of commercial interest [4,5]. This bacterium can also efficiently utilize complex nutrient sources because it produces several extracellular hydrolytic enzymes [6]. Among them, three lipolytic enzymes have been identified: a phospholipase [7,8], a lipase LipA [9,10] and an esterase LipB [11,12].

Lipolytic enzymes are widely distributed throughout animals, plants and microorganisms. Bacteria produce different classes including carboxylesterases [EC 3.1.1.1] which hydrolyze esters of short-chain carboxylic acids ($C \leq 12$) and lipases [EC 3.1.1.3] which display maximum activity towards water-insoluble long-chain ($C \geq 12$) acylglycerides. The third group of lipolytic enzymes includes phospholipases [EC 3.1.4.3] with activities towards polar phospholipids. Lipolytic enzymes and especially lipases presently attract enormous attention because of their biotechnological potential, i.e. their ability to catalyze both hydrolysis and synthesis reactions with high regio- and enantioselectivity. Lipolytic enzymes have, therefore, developed into the most widely used class of enzymes in synthetic organic chemistry [13–16]. Consequently, the genes of many bacterial lipolytic enzymes have been identified, cloned, expressed and the corresponding enzymes have been characterized [17] which allowed the classification of more than 50 bacterial esterases and lipases into eight homology families [18]. Several of these enzymes have been crystallized and their three-dimensional structures were determined revealing a conserved folding pattern called the α/β -hydrolase fold [19–21]. Their active sites form a catalytic triad consisting of a nucleophilic serine, a histidine and an acidic residue (glutamate or aspartate) with the catalytic serine located at a sharp turn between an α -helix and a β -strand called the nucleophilic elbow. Another characteristic structural element found in many lipases is an α -helix named the ‘lid’ which covers the active site in the absence of a

substrate. The lid can move away upon contact with a lipid–water interface giving rise to the so-called open conformation of the enzyme. This observation served to explain the phenomenon of interfacial activation, i.e. a sharp increase in lipase enzymatic activity as soon as the substrate molecules start to self aggregate (for review see [17,19,20]).

2. Lipolytic enzymes from *Bacillus* species

Six extracellular lipases and two esterases have been described so far to occur in *Bacillus* species ([9,12,22–24,26–36], GenBank accession no. AJ297356) which share a conserved Ala–X–Ser–X–Gly pentapeptide containing the catalytic serine residue. The alanine residue replaces a glycine residue which is present in the canonical lipase consensus motif Gly–X–Ser–X–Gly (Fig. 1 (A)). Table 1 lists biochemical properties of extracellular lipolytic enzymes from *Bacillus* species.

Four closely related lipolytic enzymes originating from *B. subtilis* (LipA, LipB) [9,12], *Bacillus pumilus* [26] and *Bacillus licheniformis* [GenBank accession no. AJ297356] were grouped to form subfamily 4 of true lipases [18] because they share an average of 75% identical amino acid residues. The active residues Ser, His, and Asp are located at conserved positions in these protein sequences (Fig. 1B). These proteins are now believed to form a group of minimal α/β -hydrolase fold enzymes because they are the smallest lipases known having molecular masses of 19 kDa [41]. Furthermore, they have similar biochemical characteristics, namely a maximum activity and stability at alkaline pH values [9,10,12,26].

3. Lipolytic enzymes from *B. subtilis*

B. subtilis secretes at least two different lipolytic enzymes, LipA and LipB, into the extracellular medium. The *lipA* gene encoding an extracellular lipase was cloned, overexpressed and the corresponding enzyme was purified and characterized by the group of Colson [9,10]. LipA is a small protein with a molecular mass of 19.3 kDa which

(A)

Source of <i>Bacillus</i> lipase / esterase	overall sequence similarity [%]	conserved pentapeptide sequence
<i>B. subtilis</i> LipB	100	A - H - S - M - G
<i>B. pumilus</i>	77,3	A - H - S - M - G
<i>B. licheniformis</i> lipase	74,6	A - H - S - M - G
<i>B. subtilis</i> LipA	74,0	A - H - S - M - G
<i>B. licheniformis</i> esterase	19,8	A - H - S - M - G
<i>B. stearothermophilus</i>	14,3	A - H - S - Q - G
<i>B. thermoleovorans</i>	12,6	A - H - S - Q - G
<i>B. thermocatenulatus</i> BTL2	12,6	A - H - S - Q - G
bacterial lipase consensus motif		G - X - S - X - G

(B)

1	E S V HNPVV LVHGI S GAS YNFFA I KNYLI SQGW QSNKLYA I	40	<i>B. subtilis</i> LipB
1	AE - HNPVV MVHGI GGAS FNFAG I KSYLV SQGW SRDKLY AV	39	<i>B. subtilis</i> LipA
1	AE - HNPVV MVHGI GGAS YNFFS I KSYLA T QGWDRN QLYA I	39	<i>B. pumilus</i>
1	AE - HNPVV MVHGI GGAS YNFFAS I KSYLV G QGWDRN QLF A I	39	<i>B. licheniformis</i>
*			
41	DF Y DKTGN L NNGP Q LA S YV DR VLKETGAKKVDIVAH S MG	80	<i>B. subtilis</i> LipB
40	DFW DKTGT NYNNGP VLSRFVQK VLDETGAKKVDIVAH S MG	79	<i>B. subtilis</i> LipA
40	DF I DKTGN RRNNGP RLS R FVKDVL DK T GAKKVDIVAH S MG	79	<i>B. pumilus</i>
40	DF I DKTGN RRNNGP RLS R FVKDVL DK T GAKKVDIVAH S MG	79	<i>B. licheniformis</i>
*			
81	GANTLYYI KYLGGG NK I QNVVTLGGANGLVSS T ALPGT DP	120	<i>B. subtilis</i> LipB
80	GANTLYYI KNLDGG NK V ANVVTLGGAN R L T T G K ALPGT DP	119	<i>B. subtilis</i> LipA
80	GANTLYYI KNLDGG D KI ENVV T I GGANGLVSS RALPGT DP	119	<i>B. pumilus</i>
80	GANTLYYI KNLDGG D KI ENV I P I GGANGLVSS RALPGT DP	119	<i>B. licheniformis</i>
*			
121	NQK ILYTS IYSS LND Q I V I NSLS RL QGARN I Q L Y G I G H I G L	160	<i>B. subtilis</i> LipB
121	NQK ILYTS IYSSAD M I V M N Y L S R L D GARN V Q I H G V G H I G L	159	<i>B. subtilis</i> LipA
120	NQK ILYTS VYSSAD L I V V N S L S R L I GARN I L I H G V G H I G L	159	<i>B. pumilus</i>
120	NQK ILYTS VYSSAD L I V V N S L S R L I GARN V L I H G V G H I G L	159	<i>B. licheniformis</i>
*			
161	L S N S Q V N G Y I K E G L N G G G L N T N	182	<i>B. subtilis</i> LipB
160	L Y S S Q V N S L I K E G L N G G G Q N T N	181	<i>B. subtilis</i> LipA
160	L T S S Q V K G Y I K E G L N G G G Q N T N	181	<i>B. pumilus</i>
160	L T S S Q V K G Y I K E G L N G G G Q N T N	181	<i>B. licheniformis</i>

Fig. 1. Sequence alignment of lipases and esterases from different *Bacillus* species. (A) The strictly conserved amino acids of the consensus pentapeptides are boxed. The overall protein sequence similarities of the mature enzymes were determined with the program MegAlign of the software package DNA-Star (Lasergene). Amino acid sequences were retrieved from the GenBank database <http://www.ncbi.nlm.nih.gov/> and have the following accession numbers: C69652 (*B. subtilis* LipB), M74010 (*B. subtilis* LipA), A34992 (*B. pumilus* lipase), AJ297356 (*B. licheniformis* lipase), U35855 (*B. licheniformis* esterase), AF134840 (*B. thermoleovorans* lipase), X95309 (*B. thermocatenulatus* lipase) and U78785 (*B. stearothermophilus* lipase). (B) Amino acid sequence alignments of four closely related extracellular lipases and esterases from *B. subtilis*, *B. pumilus* and *B. licheniformis*. The residues forming the catalytic triad are marked with an asterisk.

Table 1
Biochemical properties of extracellular lipases and esterases from *Bacillus* species

Source of lipolytic enzyme	Molecular mass (kDa)	pH _{opt.} ^a	Temp _{opt.} ^b (°C)	Temp _{stab.} ^c (°C)	Substrate specificity ^d	Conserved pentapeptide sequence	Comments	References
<i>B. subtilis</i> 168								
LipA	19.3	10	35–40	45	pNP-C8, C14 TG-C8	A-H-S-M-G	Tendency to form aggregates	[9,10,22–24]
LipB	19.5	10	35–40	45	pNP-C8, C14 TG-C8	A-H-S-M-G	Tendency to form aggregates	[12]
<i>B. subtilis</i> NRRL 365								
Esterase I	36.0	8.0	n.d.	n.d.	pNP-C2-C3	n.d.	Heterodimer subunits with M _r 48 kDa and M _r 57 kDa	[25]
Esterase II	105.0	8.0	n.d.	n.d.	pNP-C2	n.d.	80.1 and 77.3% similarity to <i>B. subtilis</i> LipA and LipB, respectively	[25]
<i>B. pumilus</i>								
	19.3		30	40	n.d.	A-H-S-M-G		[26]
<i>B. thermocatenulatus</i>								
BLT1	16.0	7–8	60–70	40	pNP-C10 12	n.d.	Tendency to form aggregates (>750 kDa)	[27,28]
BLT2	43.0	8–9	60–70	50	pNP-C10 TG-C4	A-H-S-Q-G	Tendency to form aggregates (>500 kDa)	[28–31]
<i>B. stearothermophilus</i> L1								
	43.0	9–10	60–65	55	pNP-C8 TG-C3, C12	A-H-S-Q-G	94.2% similarity to <i>B. thermocatenulatus</i> BLT2	[32,33]
<i>B. thermoleovorans</i> ID-1								
	43.0	7–8	70–75	50	pNP-C6 TG-C8	A-H-S-Q-G	95.4% similarity to <i>B. thermocatenulatus</i> BLT2	[34,35]
<i>B. licheniformis</i>								
Lipase	19.2	n.d.	n.d.	n.d.	n.d.	A-H-S-M-G	96.1% similarity to <i>B. pumilus</i> lipase	Accession no. AJ297356
Esterase	81.3	8–8.5	55	50	pNP-C6-C8	A-H-S-M-G		[36,37]
<i>B. circulans</i>								
	30.0	8.5–9.5	60	70	pNP-C3-C4	n.d.	Forms trimers of M _r 95 kDa	[38,39]
<i>B. spec.</i> THL027								
	69.0	7	70	n.d.	TG-C8	n.d.	Very low activity (4.9 Umg ⁻¹ towards TG-C8); tendency to form aggregates	[40]

n.d., Not determined.

^a pH optimum.

^b Temperature optimum.

^c Maximum tolerable temperature.

^d Specificity towards *p*-nitrophenyl-ester (pNP-) and triacylglyceride substrates (TG-) of various chain length; the number of alkyl chain carbon atoms is given.

did not show interfacial activation when tested with triacetin as the substrate [10] suggesting the absence of a lid domain. The second lipolytic enzyme LipB has been identified from the *B. subtilis* 168 genome sequence [1,11] and showed high homology to the known lipases from *B. subtilis* [9] and *B. pumilus* [26]. The *lipB* gene was amplified by PCR using chromosomal DNA from *B. subtilis* 168 as the template, overexpressed in the homologous host and the corresponding enzyme was purified to electrophoretic homogeneity [12]. An extracellular location of LipA and LipB was predicted because these enzymes contained putative N-terminal signal sequences of 31 or 28 amino acid residues, respectively. Purification of the mature enzymes from bacterial culture supernatants and determination of their N-terminal sequences confirmed this prediction [10,12].

4. Differential regulation of LipA and LipB production

The maximum lipolytic activity of the wild-type strain *B. subtilis* 168 was detected at the transition from the late logarithmic to the stationary growth phase [42]. An extracellular lipase activity of 12–18 U l⁻¹ of culture supernatant was determined with *p*-nitrophenyl-palmitate as the substrate. Either LipA- or LipB-specific antibodies were used for Western-blotting experiments to detect the corresponding enzymes in culture supernatants from *B. subtilis* grown to the late logarithmic growth phase. As shown in Fig. 2 both LipA and LipB were expressed and secreted in rich medium. Interestingly, the extracellular lipolytic activity of *B. subtilis* grown in minimal medium reached the same level as in rich medium, however, no LipB protein was detectable [42].

5. Substrate specificities of LipA and LipB

The specific enzyme activities of LipA and LipB were determined with various lipase and esterase substrates. In Table 2 the catalytic profiles of both *B. subtilis* enzymes are summarized as determined with triacylglyceride and *p*-nitrophenyl-ester sub-

strates with varying sizes of the fatty acid site chains. Triacylglycerides with short chain fatty acids (\leq C12) were preferentially hydrolyzed with maximum activity towards the C8 substrate tri-caprylin (C8:0) [12]. It became obvious that LipA exhibited higher specific activities than LipB towards substrates with longer chain lengths, namely tricaprylin (C10:0), trilaurin (C12:0), and triolein (C18:1). The specific activity profiles determined with *p*-nitrophenyl-ester substrates were comparable with LipA and LipB. Both enzymes were active against all esters tested with fatty acid chain lengths from C6 to C18 with maximum activities against the esters of fatty acid chain lengths C8 and C14. The absolute specific activities of LipB were always higher than those of LipA [12]. LipB does not hydrolyze the typical lipase substrates such as triolein (C18:1) or a triacylglyceride isolated from tung oil (C18:3) or a naturally fluorescent triacylglyceride extracted from seeds kernels of *Parinari glaberrimum* (C18:4) suggesting to classify LipB as an esterase rather than a lipase (Table 2) [12]. The highest specific activities of LipA and LipB were detected using short chain vinyl-esters vinyl-propionate and -butyrate. No activities were found against various phospholipase substrates (Table 2).

6. Three-dimensional structures of LipA and LipB

The X-ray structure of *B. subtilis* lipase LipA shows that the enzyme has a single, globular compact domain with dimensions of 35 × 36 × 42 Å³. Its fold consists of six β -strands in a parallel β -sheet, surrounded by α -helices, two on one side of the β -sheet and three on the other. Like other lipases, its fold conforms to the α/β hydrolase fold [19–21], although it is much smaller. It lacks the first two β -strands (β 1 and β 2) of the canonical fold and helix α D is replaced by a small 3_{10} helix. Furthermore, helix α E is exceptionally small with only one helical turn. Fig. 3A shows a schematic drawing of the three-dimensional structure of *B. subtilis* lipase. The position of the catalytic triad residues in LipB were predicted from a sequence alignment of three closely related lipases from *B. pumilus* [26] and *B. subtilis* [9,11] as being Ser78,

Asp134 and His157 (Fig. 1B). This prediction was confirmed by constructing the variants S78C, D134N and H157N by site directed mutagenesis. All variant enzymes were expressed at protein levels comparable with the wild-type LipB protein in the

heterologous host *Escherichia coli*, but they were all enzymatically inactive [12]. The prediction was furthermore confirmed by the 3D-structure of LipA, which showed that the Ser, Asp and His residues are close together, and form a catalytic triad.

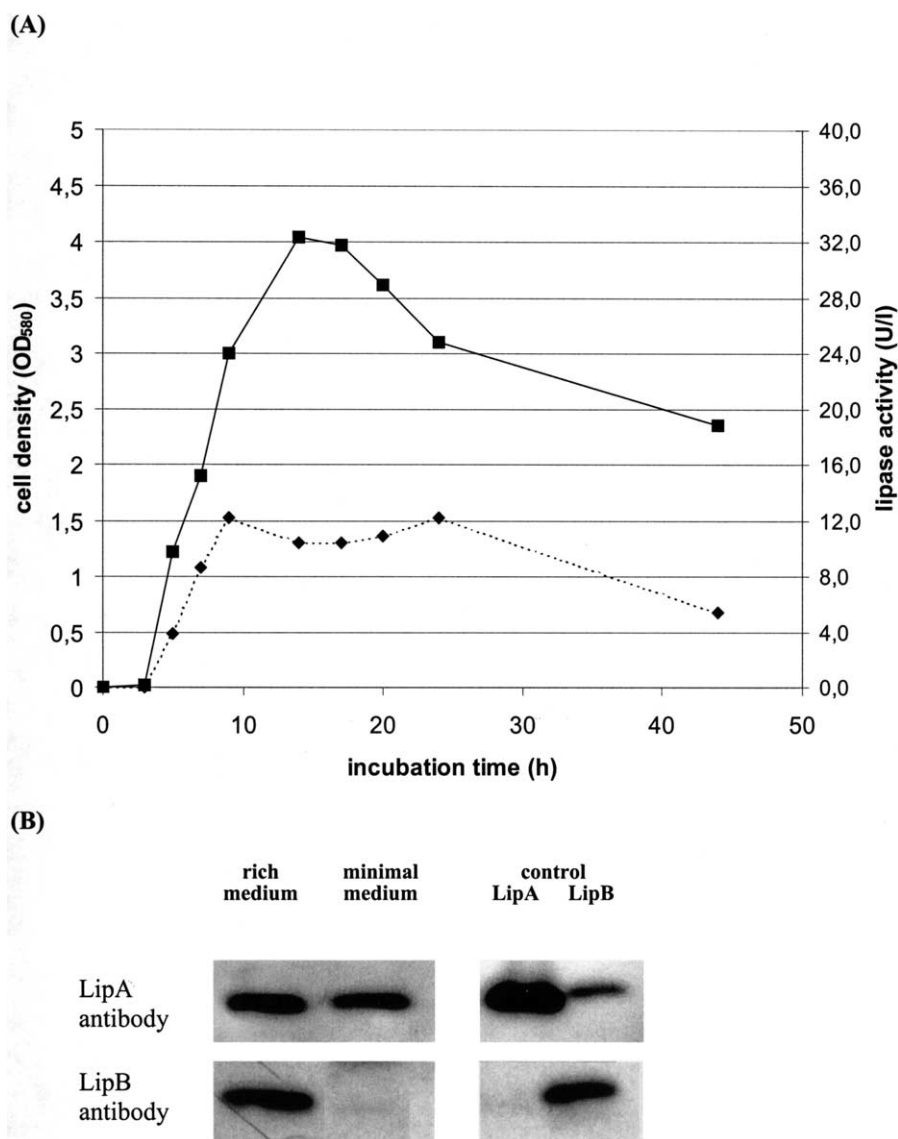


Fig. 2. Extracellular lipolytic activity of *B. subtilis* 168. (A) Lipase activity was determined with a spectrophotometric assay using *p*-nitrophenyl-palmitate as the substrate in supernatants obtained from cultures grown in rich medium (Luria broth). The cell density of the cultures grown at 37 °C (■) and the lipolytic activity in the culture supernatant (◆) were determined over a time period of 45 h. (B) Western-blot with LipA- and LipB-specific antibodies of *B. subtilis* culture supernatants from cultures grown to the end of the logarithmic growth phase in rich and minimal medium. Ten nanogram each of purified LipA or LipB protein were used as a control.

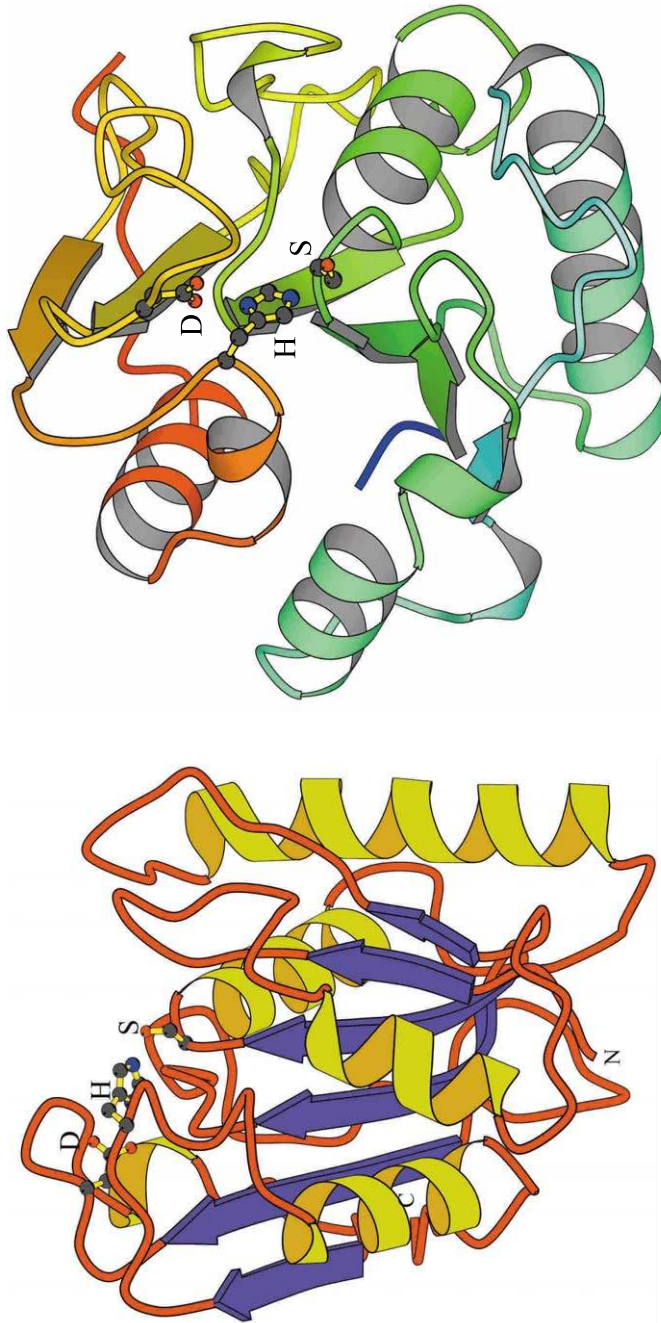


Fig. 3. 3D structure of LipA and 3D structural model of LipB. (A) Schematic drawing of the structure of *B. subtilis* lipase LipA. The catalytic triad residues Ser77, His156 and Asp133 are labeled with S, H and D, respectively. The letters N and C indicate the N- and C-termini, respectively. (B) Schematic drawing of the model of LipB. The view is from the solvent into the active site. The catalytic residues Ser78 (S), Asp134 (D) and His157 (H) are indicated in a ball-and-stick representation.

Table 2
Specific activities of *B. subtilis* LipA and LipB towards various substrates

Substrate	Specific activity (U mg ⁻¹)	
	LipA	LipB
Triacetin ⁽¹⁾ (C2:0)	12.2	6.8
Tripropionin ⁽¹⁾ (C3:0)	125.7	51.8
Tributyryn ⁽¹⁾ (C4:0)	262.2	322.2
Tricaproin ⁽¹⁾ (C6:0)	301.9	422.6
Tricaprylin ⁽¹⁾ (C8:0)	337.0	512.8
Tricaprin ⁽¹⁾ C10:0)	265.8	183.0
Trilaurin ⁽¹⁾ C12:0)	46.0	3.0
Triolein ⁽¹⁾ (C18:1)	16.23	0
TG from tung oil ⁽²⁾ (C18:3)	22	0.05
TG-PnA ⁽³⁾ (C18:4)	2.6	0.01
Monoolein ⁽⁴⁾ (C18:1)	0.025	<10 ⁻⁴
<i>p</i> -NP-butyrate ⁽⁵⁾ (C4:0)	92.5	99.3
<i>p</i> -NP-caproate ⁽⁵⁾ (C6:0)	123.5	252.1
<i>p</i> -NP-caprylate ⁽⁵⁾ (C8:0)	149.5	325.5
<i>p</i> -NP-caprate ⁽⁵⁾ (C10:0)	108.5	253.6
<i>p</i> -NP-laurate ⁽⁵⁾ (C12:0)	144.7	305.8
<i>p</i> -NP-myristate ⁽⁵⁾ (C14:0)	163.5	319.9
<i>p</i> -NP-palmitate ⁽⁵⁾ (C16:0)	129.5	202.5
<i>p</i> -NP-stearate ⁽⁵⁾ (C18:0)	86.5	156.9
DGGR ⁽⁵⁾	0.9	0.1
Vinyl propionate ⁽¹⁾	1690	2700
Vinyl butyrate ⁽¹⁾	1400	650
Di C12-phosphatidylglycerol ⁽⁴⁾	0	0
Di C12-phosphatidylethanolamine ⁽⁴⁾	0	0
Di C12-phosphatidylcholine ⁽⁴⁾	0	0
<i>p</i> -NP-phosphorylcholine ⁽⁵⁾	0	0

The S.D. was about 10% for each given value. Activities were determined using the following assays, ⁽¹⁾, pH-stat; ⁽²⁾, UV-spectrophotometric; ⁽³⁾, fluorescent; ⁽⁴⁾, monolayer; ⁽⁵⁾, spectrophotometric.

LipB is 74% identical to LipA (see Fig. 1B for a sequence alignment). Therefore, the X-ray structure of LipA (see Fig. 3A) allowed us to build a model of LipB with confidence [42]. This model revealed that the residues in the core of the LipB molecule are mostly identical to those of LipA. Two substitutions occur, which only slightly affect the volume of the side chain (Val96 of LipA into Ile97 in LipB, and Met8 of LipA into Leu9 in LipB). These substitutions are, therefore, not likely to affect the 3D-structure of LipB to a large extent. The other substitutions are at the surface

of the molecule, and are remote from the active site. Ser14 of LipB, (Gly13 in LipA) and Gln135 of LipB, (Met134 in LipA) are closest to the catalytic serine at a distance of about 9 Å. Fig. 3B shows the structural model of LipB.

The assignment of Ser78, Asp134 and His157 as the active site residues of LipB is in agreement with the location of these residues in the structural model. Ser78, Asp134 and His157 are arranged in a catalytic triad-like configuration, with Ser78 positioned at the very sharp turn between strand β5 and helix αC, the so-called nucleophilic elbow. The active site is located at the bottom of a small cleft between two loops consisting of residues 11–16 and 132–138. It is freely accessible from the solvent; and, in contrast to other (larger) lipases, no lid is present. As mentioned above the active sites of LipA and LipB are identical. The nearest amino acid substitutions are at 9 Å distance or more from the active site; they occur at the rim of the putative substrate binding surface [41]. This explains the minor differences in activity on short chain substrates, and rationalizes the different activities towards substrates with longer fatty acid chains [41].

7. *Bacillus* lipases have an unusual consensus pentapeptide

Most *Bacillus* lipases have in common that an Ala replaces the first Gly in the conserved pentapeptide Ala–X–Ser–X–Gly (Fig. 1A). To investigate a potential function of this structural motif, LipB variant A76G was constructed by site-directed mutagenesis thereby restoring the canonical lipase consensus motif. Enzyme activity and stability of the purified A76G variant protein were compared with the wild-type LipB enzyme by recording heat denaturation profiles at different temperatures. The variant lipase displayed a marked temperature sensitivity with a half-life of 8 min at 45 °C, whereas the wild-type enzyme remained stable [12].

Substrate specificities of the A76G variant were comparable with those of the wild-type enzyme, with maximum activities against *p*-nitrophenyl-esters with chain lengths of C8 and C14 and against

Table 3

Ranking of ratios of specific activities towards various substrates of wild-type *B. subtilis* esterase LipB and its variant A76G

Substrate	Wild-type LipB	Variant A76G	Ratio A76G/wt
<i>p</i> -nitrophenyl caprate (C10:0) ⁽¹⁾	253.6	126.9	0.50
<i>p</i> -nitrophenyl myristate (C14:0) ⁽¹⁾	319.9	183.1	0.57
<i>p</i> -nitrophenyl stearate (C18:0) ⁽¹⁾	160.0	137.1	0.87
Tripropionin (C3:0) ⁽²⁾	53.3	46.7	0.88
Tributylin (C4:0) ⁽²⁾	316.7	453.3	1.43
Tricaprylin (C8:0) ⁽²⁾	507.0	583.0	1.15
1,3-dioctanoylglycerol (C8:0) ⁽²⁾	130.4	305.1	2.34
1-monocapryloylglycerol (C8:0) ⁽²⁾	0.1	2.6	26.0
Tricaprin (C10:0) ⁽²⁾	180.0	233.3	1.30
Monoolein (C18:1) ⁽³⁾	< 10 ⁻⁴	0.138	> 10 ³

Catalytic activities were determined spectrophotometrically and titrimetrically and in a monolayer assay as previously reported. Activities were determined using ⁽¹⁾, spectrophotometric; ⁽²⁾, pH-stat; ⁽³⁾, monolayer assays.

the triacylglyceride tricaprylin (C8:0). However, the specific activities differed from those of the wild-type enzyme. All *p*-nitrophenyl-ester substrates were hydrolyzed with about 50% of the wild-type activity. Interestingly, the specific activities against triacylglyceride substrates were at least 40% higher than the wild-type activities. The most obvious differences in catalytic activities of wild-type LipB and its variant A76G were found when monoolein was used in a monolayer assay. The specific activity of variant A76G was at least 10³-fold higher than that of the wild-type enzyme [12]. The same tendency was also detectable when tricaprylin, 1,3-dioctanoylglycerol and 1-monocapryloylglycerol were used as substrates for both enzymes in a pH-stat assay (Table 3). These results indicate that the mutation A76G has led to a shift in substrate preference converting an esterolytic enzyme into a monoacylglycerol hydrolyase (Table 3) [12].

8. Conclusions

A number of publications describe the cloning, expression, purification and biochemical characterization of lipases from *Bacillus* species ([9–12,22–39]). More recently, the first 3D structure of a *Bacillus* lipase was solved [41]. These enzymes will remain attractive research objects mainly for the following reasons (1) their production seems to be a regulated process which obviously requires

further investigation; (2) they appear to have biotechnologically interesting properties including alkaline pH optima, a broad substrate specificity and also a small size; (3) they can be produced with high yields by overexpression both in *E. coli* and in *B. subtilis* making them interesting candidates for optimization, e.g. by directed evolution.

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