

Lipolytic enzymes LipA and LipB from *Bacillus subtilis* differ in regulation of gene expression, biochemical properties, and three-dimensional structure

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Abstract *Bacillus subtilis* secretes the lipolytic enzymes LipA and LipB. We show here that they are differentially expressed depending on the composition of the growth medium: LipA is produced in rich and in minimal medium, whereas LipB is present only in rich medium. A comparison of biochemical characteristics revealed that LipB is thermostable at pH 11 but becomes thermolabile at pH 5. However, construction of a variant carrying the substitution A76G in the conserved lipase pentapeptide reversed these effects. The atomic coordinates from the LipA crystal structure were used to build a three-dimensional structural model of LipB, which revealed that 43 out of 45 residues different from LipA are surface-located allowing to rationalize the differences observed in the substrate preferences of the two enzymes. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Regulation of gene expression; Esterase/lipase; Three-dimensional structural model; *Bacillus subtilis*

1. Introduction

Bacillus subtilis is a Gram-positive, aerobic and endospore-forming bacterium commonly found in soil, aquatic habitats and associated with plants. Its genome, proteome and secretome have been studied in great detail [1–3]. The capability of *B. subtilis* to secrete homologous and heterologous proteins at g/l amounts into the growth medium and its classification as a GRAS (generally regarded as safe) organism have made it an attractive expression host to produce proteins of commercial interest [4,5]. Lipolytic enzymes produced and secreted by *B. subtilis* include a phospholipase [6], a lipase LipA (EC 3.1.1.3) [7,8], and an esterase LipB (EC 3.1.1.1) [9].

These enzymes are of substantial biotechnological interest and many have therefore been identified, cloned, and characterized [10–13]. Recently, more than 50 bacterial esterases and lipases were grouped into eight homology families [14] with true lipases in family I, and six presently known extracellular *Bacillus* lipases in subfamilies 4 and 5. Subfamily 4 contains the 19-kDa lipases and esterases from the mesophilic or mod-

erately thermophilic strains *B. subtilis* [7,9], *Bacillus pumilus* [15] and *Bacillus licheniformis* (GenBank accession no. AJ297356) with protein sequence identities of around 75%. Three lipases from the thermophilic *Bacillus* strains *Bacillus stearothermophilus* [16], *Bacillus thermocatenulatus* [17] and *Bacillus thermoleovorans* [18] with sequence identities up to 95% were grouped together with *Staphylococcus* lipases into subfamily 5. All these *Bacillus* lipases share a conserved Ala-X-Ser-X-Gly pentapeptide that contains the catalytic serine residue. The alanine in this pentapeptide replaces the first glycine residue of the canonical Gly-X-Ser-X-Gly lipase consensus motif.

The *B. subtilis* lipase gene *lipA* was cloned, expressed [7] and the corresponding 19.3-kDa enzyme purified [8]. Its three-dimensional (3D) structure revealed a compact minimal α/β hydrolase fold with a six-stranded parallel β -sheet flanked by five α -helices, two on one side of the sheet and three on the other side [19].

Here, we report the biochemical characterization of LipB, the second extracellular lipolytic enzyme from *B. subtilis* classified as an esterase, which is 74% identical to LipA. Interestingly, we found a differential regulation of *lipA* and *lipB* gene expression depending on the medium composition. By modeling its 3D structure we have investigated structural features of LipB in comparison to LipA to explain the different substrate preferences of these related enzymes.

2. Materials and methods

2.1. Bacterial strains and plasmids

B. subtilis 168 (*Bacillus* genetic stock center, OH, USA) was used as the wild-type strain. The strains *B. subtilis* BCL1051 [20] and *B. subtilis* TEB1003 [9] were used for overexpression of *lipA* and *lipB* in the homologous host. *Escherichia coli* BL21(DE3) [21] was used as a heterologous expression host for plasmid-encoded enzymes. The plasmids for heterologous expression of wild-type LipB and its variant A76G were pET22lipB and pET22A76G [9], respectively. The *lipA-lacZ* and *lipB-lacZ* fusions were constructed by cloning the 185-bp (*lipA*) and 500-bp (*lipB*) upstream region to a promoterless *lacZ* gene using the pDG268 plasmid [22]. Resulting plasmids pDGlipA1, pDGlipB1 and as a negative control pDG268 were integrated into the *B. subtilis* chromosome at the *amyE* locus by transformation and homologous recombination. The resulting strains were named *B. subtilis* TEB1040 (pDG268), *B. subtilis* TEB1041 (pDGlipA1) and *B. subtilis* TEB1042 (pDGlipB1).

2.2. Media and growth conditions

For immunodetection of LipA and LipB in the culture supernatant the *B. subtilis* 168 cultures were grown in 30 ml rich or minimal

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Abbreviations: TCA, trichloroacetic acid; X-Gal, 5-bromo-4-chloro-3-indolyl- β -D-galactoside

medium in 100-ml Erlenmeyer flasks at 37°C. The cultures were inoculated with 30 µl of an overnight culture grown either in rich (Luria broth (LB) supplemented with 1% (w/v) glucose) or minimal medium (Spizizen's minimal salts (SMS) (125 mM K-PO₄ buffer pH 7, 15 mM (NH₄)₂SO₄, 3.4 mM Na-citrate, 0.8 mM MgSO₄) supplemented with 50 µg/ml tryptophan and 1% (w/v) glucose). LB-β-galactosidase indicator plates contained 0.03% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and 1.5% (v/v) each of *n*-hexadecane or tributyrin which were emulsified with an ultraturrax.

2.3. Enzyme expression and purification

The wild-type enzymes, LipA and LipB, as well as the LipB variant A76G were expressed and purified as described previously [9].

2.4. Immunodetection of LipA and LipB

The supernatants from *B. subtilis* 168 cultures grown in rich or minimal medium to the late logarithmic growth phase were isolated by centrifugation. The proteins were concentrated by trichloroacetic acid (TCA) precipitation [23] of 1 ml culture supernatant and separated using SDS-PAGE with a 5% stacking gel and a 15% separating gel [24] and Western blotted onto a polyvinylidene difluoride (PVDF) membrane using carbonate buffer (pH 9.3) containing 20% methanol [25]. LipA protein was detected using a polyclonal antiserum (1:10 000 diluted) kindly provided by W.J. Quax and M. Dröge (Department of Pharmaceutical Biology, University Centre for Pharmacy, Groningen, The Netherlands). For the detection of LipB, a polyclonal antiserum was raised against purified LipB protein as the antigen by using a standard immunization protocol for rabbits (Eurogentec, Herstal, Belgium). The LipB antiserum was diluted 1:20 000 in TBST (50 mM Tris-HCl pH 6.8, 150 mM NaCl, 1 mM MgCl₂, 0.2% (v/v) Tween 20). Horseradish peroxidase-labeled goat-anti-rabbit antibody (Bio-Rad, Munich, Germany) was used as the second antibody. Detection was performed with the ECL system (Amersham Pharmacia Biotech, UK) under conditions recommended by the manufacturer.

2.5. Effect of temperature on enzyme activity and stability

The thermal stability of LipB and its A76G variant was determined spectrophotometrically with *p*-nitrophenyl-palmitate as the substrate after incubation for at least 60 min at 45°C. The enzyme samples were diluted in 50 mM glycine/HCl (pH 5) and glycine/NaOH buffer (pH 11).

2.6. Computer software

Multiple sequence alignments were performed with the program MegAlign of the software package DNASTar (Lasergene). A homology model of LipB was built manually starting from the LipA crystal structure [19] using the program O [26]. 43 of the 45 residues that differ between LipA and LipB are located on the surface of the molecule. The other two substitutions (Ile97 for Val96 and Leu9 for Met8) are in the core. The small differences in size of the side chains could easily be accommodated by slight adjustments of nearby residues.

3. Results

3.1. LipA and LipB are regulated differentially

The wild-type strain *B. subtilis* 168 secretes at least two different lipolytic enzymes, LipA and LipB. Their extracellular location was demonstrated by purification from the culture supernatant and N-terminal sequence analysis of the isolated enzymes. [8,9]. The extracellular lipolytic activity reached 12–15 U/l in rich medium and 15–18 U/l in minimal medium (Fig. 1). The lipolytic activity was maximal in both media at the end of the logarithmic growth phase, which was reached after 7 h in rich and 14 h in minimal medium. Western blotting experiments performed with samples taken at the transition from the logarithmic to the stationary growth phase, revealed that both enzymes were present in rich medium. Interestingly, the extracellular lipolytic activity in minimal medium was the same as in rich medium, but no LipB protein was detectable (Fig. 1). To investigate whether the differential regulation of

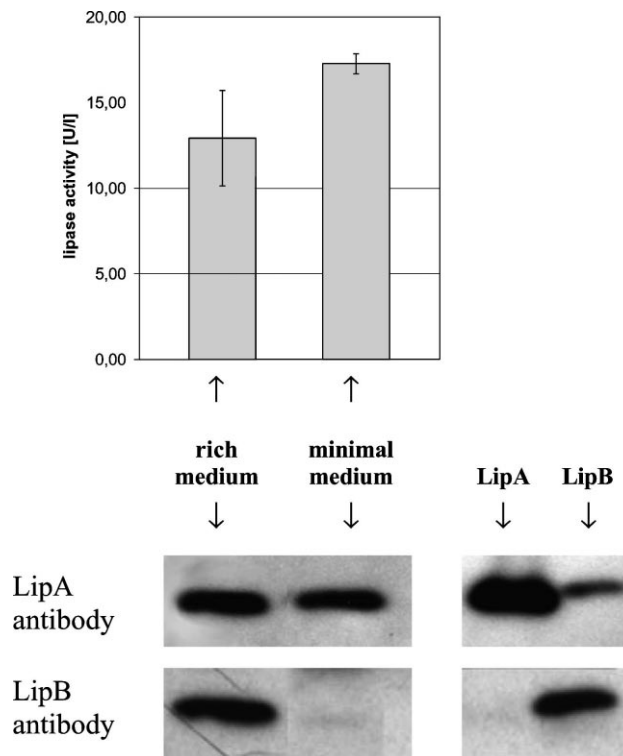


Fig. 1. Lipolytic activity of wild-type *B. subtilis* 168 cultured in rich and minimal medium. Extracellular lipase activities of late logarithmic cultures were determined with *p*-nitrophenyl-palmitate as the substrate. Corresponding Western blots of *B. subtilis* culture supernatants using LipA- and LipB-specific antibodies show that both lipolytic enzymes are secreted into the culture supernatant when cultured in rich medium whereas in minimal medium only LipA is present. Purified LipA and LipB proteins served as a control (lower right panel).

lipase production occurred at the transcriptional level we have constructed the reporter gene fusions *lipA-lacZ* and *lipB-lacZ*. When the fusion strains were grown on LB agar plates containing the synthetic β-galactosidase substrate X-Gal we observed a significant increase of *lipB* gene expression when *n*-hexadecane or tributyrin were emulsified in the growth medium (Fig. 2) whereas *lipA* gene expression remained the same.

3.2. The Ala to Gly mutation in the pentapeptide affects the pH stability of the enzyme

Eight extracellular lipases and esterases from *Bacillus* species have in common that an alanine residue replaces the first glycine in the conserved lipase pentapeptide Gly-X-Ser-X-Gly. The function of this structural motif was investigated by biochemical characterization of an A76G variant of *B. subtilis* LipB in which the canonical lipase consensus motif had been restored. The wild-type enzyme LipB was stable at alkaline pH (around pH 11), whereas the residual activity after 1 h of incubation at pH 5 and 45°C dropped to 50%. The heat denaturation profile of variant A76G revealed a marked temperature sensitivity at pH 11 and 45°C with no residual activity left after 1 h of incubation. However, this LipB variant was absolutely stable at low pH values. At pH 5 the variant enzyme activity remained unchanged after incubation for 1 h at pH 5 and 45°C (Fig. 3).

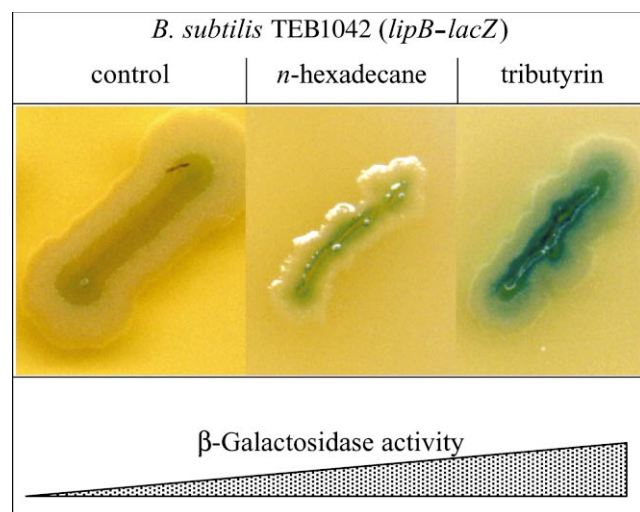


Fig. 2. Effect of *n*-hexadecane and tributyrin on the expression of the *lipB* gene as determined in *B. subtilis* TEB1042 (*lipB-lacZ*) grown on LB agar plates containing 0.03% (w/v) X-Gal. The intensity of the blue color indicates the expression level of the *lipB-lacZ* fusion.

3.3. A 3D structural model for LipB

LipB is 74% identical to LipA. The X-ray structure of LipA [19] showed that the enzyme has a globular shape with dimensions of $35 \times 36 \times 42 \text{ \AA}^3$. It consists of a single compact domain, with six β -strands in a parallel β -sheet surrounded by five α -helices, two on one side of the β -sheet and three on the other side. In view of the high homology between LipA and LipB, a model of LipB could be built with confidence. The residues in the core of the LipB molecule are almost 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 \AA . Fig. 4 shows the structural model of LipB.

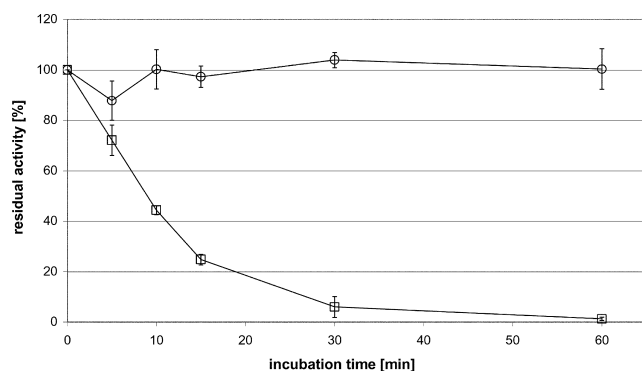


Fig. 3. Thermal stability profiles of LipB variant A76G. Enzyme activities were determined with *p*-nitrophenyl-palmitate as the substrate. Error bars give the standard deviations from three independent assays. 4.0 $\mu\text{g/ml}$ of purified LipB variant A76G were incubated at 45°C for 60 min in 50 mM glycine buffer at pH 11 (□) or pH 5 (○).

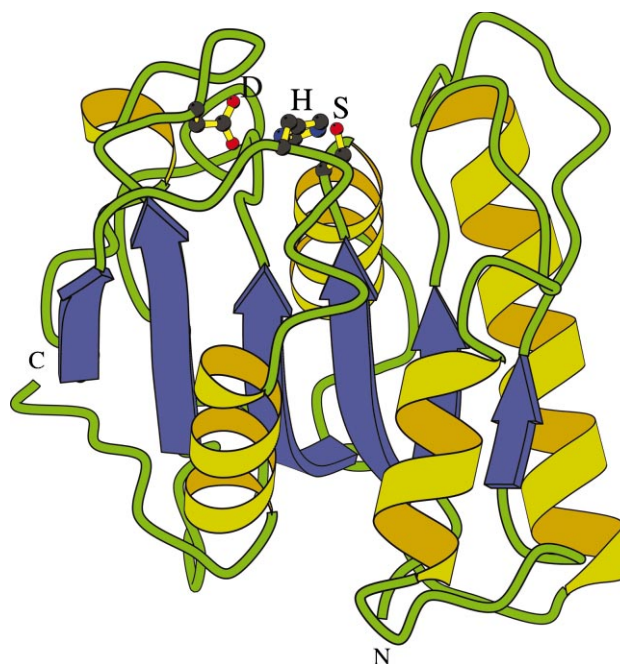


Fig. 4. 3D model of LipB from *B. subtilis*. The catalytic triad residues Ser78, His157 and Asp134 are labeled with S, H and D, respectively. The N- and C-termini of the enzyme are indicated.

3.4. The active site cleft of LipB is exposed to the surface

The active site of *B. subtilis* LipB consists of residues Ser78, Asp134 and His157 as previously suggested [9]. In the structural model of LipB these residues are arranged in a catalytic triad-like configuration as expected, with Ser78 positioned at the very sharp turn between strand β_5 and helix α_C , the so-called nucleophile elbow. The active site is located at the bottom of a small cleft between two loops consisting of residues 11 to 16 and 132 to 138. It is freely accessible from the solvent, and, in contrast to other (larger) lipases, no lid is present. The active sites of LipA and LipB are identical. The nearest amino acid substitutions are at 9 \AA distance or more from the active site; they occur at the rim of the putative substrate-binding surface. This explains the minor differences in activity on short-chain substrates, and rationalizes the different activities towards substrates with longer fatty acid chains [9,19].

4. Discussion

The *Bacillus* genome project revealed an open reading frame with homology to the previously identified *lipA* gene, which was therefore named *lipB* [1]. The corresponding enzyme was purified and classified as an esterase rather than a lipase [9]. The coexistence of two closely related lipolytic enzymes in one organism raised the question whether both genes would be coexpressed and the corresponding enzymes secreted under the same growth conditions. The immunoblotting experiments (Fig. 1) showed that both LipA and LipB were expressed and secreted when *B. subtilis* was grown in rich medium whereas no extracellular LipB protein was detectable upon growth in minimal medium with glucose as the sole carbon source. Thus, the lipolytic activity of *B. subtilis* grown in minimal medium is caused by LipA alone. LipB pro-

tein was not detectable even when the culture was grown up to 40 h (data not shown), indicating that the extracellular esterase LipB was not expressed in minimal medium at any time.

The experiments with *lacZ* reporter gene fusions revealed an up-regulation of *lipB* but not *lipA* transcription upon addition to the growth medium of hydrophobic compounds as *n*-hexadecane or tributyrin (Fig. 2). This result suggests that LipB may be involved in an adaptation of *B. subtilis* cells to hydrophobic environments, e.g. by altering the lipid structure or the lipid composition of the membrane.

The lipases from *B. stearothermophilus* [16], *B. thermocatenulatus* [17], *B. thermoleovorans* [18], *B. licheniformis* (GenBank accession no. AJ297356) *B. subtilis* [7], *B. pumilus* [15] and the esterases from *B. subtilis* [9] and *B. licheniformis* [27] share several biochemical properties. They all have pH optima in the alkaline range (pH 8–10) where they also show maximum stability. In addition, all these *Bacillus* lipases preferentially hydrolyze substrates with medium or short-chain fatty acids [8,9,15–18,27]. A unique feature of these enzymes is the replacement of the first glycine residue in the canonical lipase consensus motif Gly-X-Ser-X-Gly by an alanine.

The substrate specificity of the LipB variant A76G was comparable to that of the wild-type enzyme with maximum activity against *p*-nitrophenyl-esters with chain lengths of 8 and 14 carbon atoms, and against the triacylglycerol substrate tricaprylin (C8:0). However, the specific activities were only about one half of the wild-type enzyme when *p*-nitrophenyl-esters were used as the substrate. The specific activities against triacylglycerol substrates were at least 40% higher [9]. In contrast, a T103G variant of *Candida antarctica* lipase B (CALB), in which the canonical lipase consensus motif had also been restored, showed a 5-fold decrease in specific activity towards vinyloctanoate, whereas the specific activity towards *p*-nitrophenyl-caprylate was higher than that of the wild-type enzyme [28]. These results indicated that the first residue of the conserved pentapeptide (Ala for *B. subtilis* LipB and Thr for CALB) might play an important role for substrate recognition. Furthermore, the pentapeptide variants of CALB [28] and LipB (Fig. 2) showed an altered thermostability. At alkaline pH, the LipB variant A76G was thermostable whereas the same variant was stable at pH 5. The structural model we have built for LipB suggests an indirect role for the first residue of the conserved pentapeptide: a redirection of the helix α C and the strand β 5 by the introduction of a Gly at position 76 caused the observed differences in stability and activity.

In summary, our results suggest that two extracellular lipolytic enzymes have evolved in *B. subtilis* based on a conserved core structure. Lipolytic activity was conserved for both LipA and LipB by maintaining the α/β hydrolase fold for the protein core whereas the protein surfaces are different thereby allowing for different substrate specificities which, in combination with the differentially regulated gene expression indicate different physiological functions for both enzymes in *B. subtilis*.

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