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Production of a lipolytic enzyme originating from *Bacillus halodurans* LBB2 in the methylotrophic yeast *Pichia pastoris*

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Abstract A gene encoding a lipolytic enzyme amplified from the alkaliphilic bacterium *Bacillus halodurans* LBB2 was cloned into the pPICZαB vector and integrated into the genome of the protease deficient yeast strain *Pichia pastoris* SMD1168H. This previously undescribed enzyme was produced in active form, and cloning in frame with the *Saccharomyces cerevisiae* secretion signal (α-factor) enabled extracellular accumulation of correctly processed enzyme, with an apparent molecular mass of 30 kDa. In shake-flask cultivations, very low production levels were obtained, but these were significantly improved by use of a "batch-induced" cultivation technique which allowed a maximum enzyme activity of 14,000 U/l using *p*-nitrophenyl butyrate (C-4) as a substrate and a final extracellular lipolytic enzyme concentration of approximately 0.2 g/l. Partial characterization of the produced enzyme (at pH 9) revealed a preference for the short-chain ester (C-4) and significant but lower activity towards medium (C5-C6) and long (C16 and C18) fatty acid chain-length esters. In addition, the enzyme exhibited true lipase activity (7,300 U/l) using olive oil as substrate and significant levels of phospholipase activity (6,400 U/l) by use of a phosphatidylcholine substrate, but no lysophospholipase activity was detected using a lysophosphatidylcholine substrate.

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Introduction

A diverse variety of lipolytic enzymes are produced by bacteria. These include, e.g. carboxylesterases (hydrolysing small partly water soluble molecules), true lipases (displaying maximal activity towards water-insoluble long-chain triglycerides) and various types of phospholipases. This group contains enzymes with a wide range of industrial applications and are also considered as interesting catalysts in organic media (Jaeger and Reetz 1998). With this background, they have attracted great attention, resulting in the isolation of a number of novel variants, mainly from microbial species. Enzymes of extremophilic origin have been of special interest in this search due to their extreme properties, allowing function in a range of conditions other than that is normally the case for enzymes from mesophiles. Thus far, the main interest has been focused on thermophiles, as their enzymes show extreme stability at elevated temperatures, and also in organic solvents (Jaeger et al. 1994; Breithaupt 2001). Less attention has been given to alkaliphiles, which are organisms with optimal pH for growth at or above pH 9 (Grant and Horikoshi 1992). However, lipolytic enzymes isolated from alkaliphiles can have a great potential in, e.g. biotransformations, where their extreme pH for activity can well exert selective pressure on one chiral form, or improve solubility of certain classes of compounds (Grant and Horikoshi 1992).

The Gram-positive bacterium *Bacillus halodurans* is an alkaliphilic but also halo-tolerant and moderately thermophilic bacterium that produces a number of alkaline active enzymes such as amylase, xylanase, protease, galactosidase, pectinase and pullalanase (Gessesse and Gashe 1997; Kurono and Horikoshi 1973; Hashim et al. 2005). Recently, lipolytic enzymes produced under alkaline conditions have also been reported in *B. halodurans* strains originating from a Kenyan alkaline soda lake (Vargas et al. 2004).

As the complete genome sequence has been determined for the closely related strain *B. halodurans* C-125 (Takami et al. 2000), direct design of primers for amplification of genes of interest was possible. We utilized this possibility

in the current work to evaluate the methylotrophic yeast *Pichia pastoris* as an expression host for production of the lipolytic enzyme. This expression system was chosen after a first attempt to produce the enzyme in an *Escherichia coli* system which solely resulted in inactive inclusion bodies (Vargas et al., unpublished data). Moreover, the *P. pastoris* system has gained an increased interest for the production of a variety of heterologous proteins, and efficient expression is currently attainable, employing one of the alcohol oxidase promoters (the *AOX1* promoter) (Cregg et al. 1993). Many lipases have been overexpressed as extracellular proteins in *P. pastoris*, but none of these are, to our knowledge, originating from an alkaliphilic source. Thus, to evaluate the expression system also for an alkaliphilic representative of the lipolytic enzymes, we have, in the present investigation, cloned and produced a previously uncharacterized lysophospholipase homologue from *B. halodurans* LBB2 and partially characterized the produced enzyme.

Materials and methods

Strains, plasmid and reagents

Pichia pastoris strain SMD1168H and the expression vector pPICZαB were from Invitrogen (San Diego, CA). *Escherichia coli* NovaBlue (Novagen, Madison, WI) was used as the host during plasmid construction. Expand High Fidelity DNA polymerase was purchased from Roche Diagnostics (Mannheim, Germany). Restriction enzymes were from New England Biolabs Ltd. (Beverly, MA) and used according to the manufacturer's recommendations. Oligonucleotides were synthesised by MWG Biotech Scandinavia A/S (Denmark).

Construction of the yeast expression vector

The DNA sequence encoding the *B. halodurans* lipase sequence was cloned in frame with a secretion signal (*Saccharomyces cerevisiae* α-factor) into the expression vector pPICZαB under the control of the *AOX1* promoter. Total genomic DNA from *B. halodurans* LBB2, isolated from Lake Bogoria in Kenya (Vargas et al. 2004), was used as a template in a polymerase chain reaction (PCR) amplification of the sequence encoding the lipolytic enzyme using standard conditions. Primers were designed using the reported *B. halodurans* C-125 gene sequence (BH3288) encoding a putative lysophospholipase (http://www.ncbi.nlm.nih.gov). The primers are listed below, with the gene-specific sequence in capital letters, and the introduced cleavage sites for *Pst*I and *Xba*I underlined. In the primer design the start codon (ATG) of the mature native enzyme is replaced by a triplet (GGA) encoding a glycine (Gly) residue. The reverse primer (Rev) used for the construction includes a sequence coding for a hexa-histidine tag and a stop codon directly after the triplet encoding the final Ser residue (S260) of the wild-type enzyme.

- (Fd) 5′ tatgaccga*ctgcag*gaTGGAAATGGGAAGTTG C 3′
- (Rev) 5′ cgcctag*tctaga*tcaatgatgatgatgatgTGATAA TTGCTGTTCG 3′

After DNA amplification the resulting PCR product was purified using the Qiaquick PCR purification kit (Qiagen Inc., Chatsworth, CA). The PCR product and plasmid pPICZαB were digested overnight with restriction enzymes *Pst*I and *Xba*I and ligated using T4 DNA ligase (Invitrogen Life Technologies, Frederick, MD) at room temperature for 12 h followed by incubation at 4°C for 3 h. The resulting plasmid was transformed into *E. coli* NovaBlue (Novagen) by electroporation (Gene Pulser II; Bio-Rad, Hercules, CA) using standard conditions and selected on Luria–Bertani (LB) plates (1% tryptone, 1% NaCl, 5% yeast extract, and 1.2% Agar) containing 25 μg/ml zeocin. Transformants were selected and screened by direct PCR using the above primers. Positive transformants were grown in 100-ml liquid LB containing zeocin (25 μg/ml) for 12 h, and the recombinant plasmids were isolated using a Mini-Prep kit (Qiagen).

Transformation, selection and analysis of *P. pastoris* clones

Plasmids were linearized with *Sac*I, and transformation to electrocompetent cells of the protease deficient strain (SMD1168H) was made by electroporation according to the Invitrogen manual with some modifications. A 15-μg amount of linearized DNA was transformed into 60 μl electrocompetent cells using Gene Pulser II (Bio-Rad) with the following settings: T (2.5 kV), C (50 μF) and R (200 Ω). After a 1.5-h recovery period, 200 μl [of the 1-ml transformed cell culture in yeast extract peptone dextrose plus sorbitol (YPDS)] was spread for growth and selection on YPDS plates (1% yeast extract, 2% peptone, 2% glucose, 1 M Sorbitol and 1.2% Agar) containing 100 μg/ml zeocin and incubated for 50 h at 30°C. Screening for positive lipase inserts were carried out by the direct PCR technique. Preparation of the template DNA involved resuspending a colony in 20 μl Millipore quality H_2O, addition of 10 μl lyticase (3,000 U/ml) followed by incubation at 30°C for 30 min. Thereafter, the samples were frozen at −80°C for 10 min, and thawed by the addition of 20 μl H_2O, after which about 5–10 μl was used as template in the PCR reactions.

Sequence analysis

Similarity searches by basic local alignment search tool (BLAST) were performed on the National Center for Biotechnology Information (NCBI) server (http://www.

ncbi.nlm.nih.gov). The ClustalW tool on the European BioInformatics Institute (EBI) server (http://www.ebi.ac.uk/clustalw) was used to create multiple sequence alignments, which were displayed using Gene doc 2.6.02 (Nicholas et al. 1997). Secondary structure was analysed by the prediction programme PROF (Rost et al. 1996). N-glycosylation sites were predicted using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc).

Expression optimisation

Shake-flask cultivations

Cultivations in shake flasks at 30°C were performed according to Ramchuran et al. (2005), employing four different media formulations (Table 1). Absence of endogenous and exogenous lipase was confirmed using plasmid-free *P. pastoris* strain SMD1168H in the same media.

Batch-induced fermentations

Batch fermentations were performed according to Ramchuran et al. (2005) to evaluate protein expression in a more controlled environment with regard to pH and dissolved oxygen (DO). The inoculum was prepared using 100 ml YPD (1% yeast extract, 2% peptone and 2% glucose) medium in a 1-l baffled Erlenmeyer flask. Mid-log cultures (1 ml), in 20% glycerol (stored at −80°C) of the *P. pastoris* clones expressing the lipase, were used to inoculate the flasks which were subsequently incubated at 30°C in a water bath (Heto, Allerod, Denmark) with rotary shaking at 200 rpm. After 20 h (late exponential phase of growth), methanol (0.1%) was added to the flasks, and the cells were grown for a further 12 h. Cells were then harvested by centrifugation (3,000 rpm, 15 min), and the resulting pellet was re-suspended in 150 ml of YPTM medium (Table 1) to facilitate transfer into the reactor. The cultivations were performed in 2 l medium (YPTM) in a 3-l fermenter (Belach Bioteknik AB, Stockholm, Sweden). The medium components, yeast extract, peptone and tryptone soy broth, were sterilised in the vessel at 121°C for 45 min, and thereafter, methanol (100%) was aseptically added. Cultivation temperature was controlled at 28°C, and the pH was maintained at 6.0 by titration with 6.7 M aqueous ammonia and 0.5 M H_2SO_4. Aeration rate was set at 3 l/min. Dissolved oxygen concentration was measured using a polarographic electrode calibrated to 100% at 800 rpm and at 28°C. The DO was automatically controlled at 35% saturation by adjusting the stirrer speed.

Analytical methods

Sampling and sample treatment

Samples (30 ml) were withdrawn from the fermenter at various time intervals and kept on ice during further processing. For determination of extracellular enzyme activity, samples were centrifuged (11,000×*g*, 15 min, at room temperature), and the resulting supernatants were stored on ice or kept frozen (−20°C) until analysis.

Cell density measurements

Cell density during the cultivations was measured as the optical density (OD) at 600 nm. Samples with OD values exceeding 0.5 were appropriately diluted with 0.9% (w/v) NaCl prior to the measurement.

Table 1 Compositions of the media used in shake-flask cultivations

Component	Growth media (ml/l)				Induction media (ml/l)			
	MG	BMG	BMGY	YPTG	MM	BMM	BMMY	YPTM[a]
Buffer[b]		100	100			100	100	
YNB[c]	100	100	100		100	100	100	
Biotin (0.02%)	2	2	2	2	2	2	2	2
20% yeast extract			50	50			50	50
20% peptone			50	100			50	100
10% glycerol	100	100	100	100				
5% methanol					100	100	100	100
2% TSB[d]				20				20
H_2O	798	698	598	728	798	698	598	728

The medium used in the batch-induced cultivations corresponds to induction medium YPTM (Yeast extract, Peptone, Tryptone soy broth, Methanol) but with the methanol concentration increased to 1%

MG Minimal glycerol, *BMG* buffered minimal glycerol, *BMGY* buffered glycerol complex, *YPTG* complex (TSB) glycerol, *MM* minimal methanol, *BMM* buffered minimal methanol, *BMMY* buffered methanol complex, *YPTM* complex (TSB) methanol

[a]For batch-induced cultivations, a final methanol concentration of 1% (from a 100% stock solution) and 5 ml/l PTM_1 trace metal solution (Clare et al. 1991) was used, and the added H_2O volume was correspondingly corrected

[b]1 M potassium phosphate buffer, pH 6.5

[c]Yeast nitrogen base with ammonium sulphate and without amino acids (13.4%)

[d]Tryptone soy broth

Cell dry weight determination

Cell dry weight (CDW) was determined after centrifuging (1,400×*g*, 10 min, at room temperature) triplicate samples (3 ml) of cell suspension in pre-weighed glass tubes, drying overnight (105°C) and subsequently weighing the tubes with the dried biomass.

Electrophoresis and N-terminal sequencing

Protein production by *P. pastoris* was analysed by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) and stained with Coomassie Brilliant Blue G250 (Merck, Darmstadt, Germany). Densitometry measurements were performed using a Gel Doc 2000 system (Bio-Rad).

Protein samples for N-terminal sequencing were separated electrophoretically as above and subsequently transferred to a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, MA) by semi-dry electroblotting using Sartoblot IIS electroblotting unit (Sartorius, Göttingen, Germany). The membrane was stained with Coomassie Brilliant Blue, and selected protein bands were sequenced by Edman degradation at a commercial laboratory (Karolinska Institutet, Stockholm, Sweden).

Estimation of total protein

Protein concentration was determined using the bicinchoninic acid (BCA) method (Sigma, St. Louis, MO) with bovine serum albumin (0.2–1.0 mg/ml) as a standard. Measured contribution of the peptide-rich medium components to total protein concentration was 2.3 g/l and was subtracted from the values obtained.

Quantification of lipolytic activity in batch-induced cultivation

Lipolytic activity was estimated by a spectrophotometric assay method with *p*-nitrophenyl butyrate (*p*-NPB) as substrate (Winkler and Stuckmann 1979). One enzyme unit is defined as the amount required to release 1 μmol of *p*-nitrophenol from the substrate per millilitre per minute (ml^{-1} min^{-1}) at 37°C and pH 8.0. Since the *p*-NPB was not stable at a pH of more than 8.3, all enzyme assays at higher pH values were carried out using *p*-nitrophenyl palmitate (*p*-NPP) as substrate.

Substrate specificity

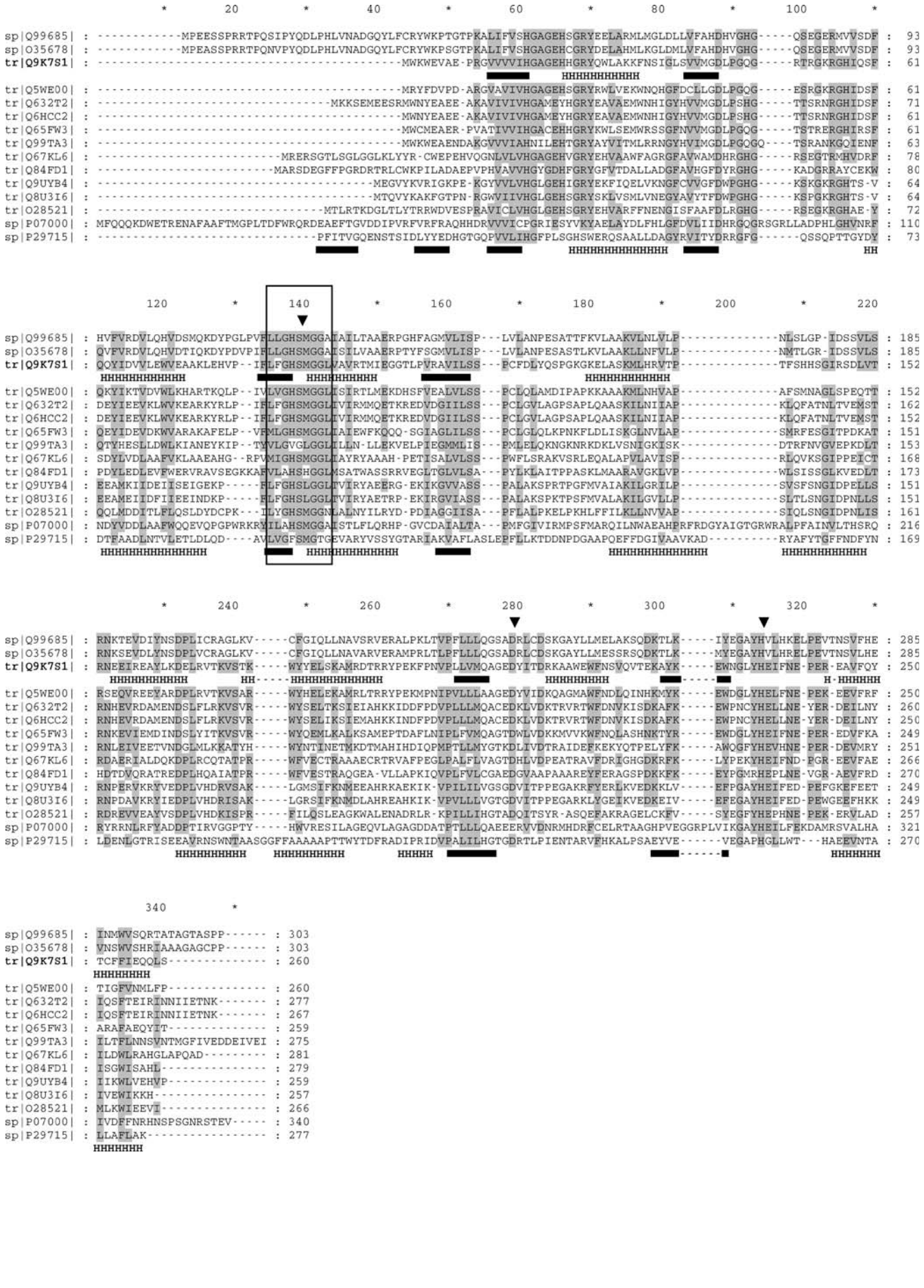
Substrate specificity of the recombinant lipase towards different *p*-nitrophenyl esters (*p*-NPEs) was analysed spectrophotometrically as describe above. The substrates used were equimolar amounts of *p*-NPB, *p*-NP-valerate, *p*-NP-caproate, *p*-NPP and *p*-NP-stearate. The highest activity of the enzyme assay using the different substrates was defined as the 100% level.

Activity towards insoluble substrate was tested using a stabilized olive oil emulsion prepared by mixing 0.3% (w/v) arabic gum, 1 ml of olive oil and 9 ml of 50 mM Gly/NaOH buffer, pH 10 by sonication. The reaction mixture was composed of 500 μl of substrate and 500 μl enzyme solution. Hydrolysis of olive oil was determined by measuring released free fatty acids (see below). One international unit (U) of activity was defined as the amount of enzyme releasing 1 μmol of product per minute under the assay conditions.

Phospholipase activity of the enzyme was evaluated using phosphatidylcholine (PC, Sigma, egg yolk) or lysophosphatidylcholine (LPC, Sigma, egg yolk) as substrates. The relative hydrolytic activity of the enzyme towards both substrates was determined by a spectrophotometric assay using copper complexation for the detection of free fatty acids released (see below). Palmitic and stearic acids were used as calibration standards. In each case, 1 mg of substrate in 0.5 ml of 50 mM Gly/NaOH buffer pH 9.0 was mixed with 0.5 ml of culture supernatant containing the lipolytic enzyme.

The end products of PC and LPC hydrolysis were analysed by thin-layer chromatography (TLC). The reaction mixture was extracted using a protocol by Bligh and Dyer (1959). The products in the chloroform phase were dried under nitrogen and dissolved in 50 μl chloroform/methanol (2:1, v/v), and 10 μl were analysed by TLC on Silica Gel 60 plates using $CH_3Cl/CH_3OH/CH_3COOH/H_2O$ (65:25:6:4, v/v/v/v).

Fig. 1 Amino acid sequence alignment of the lipolytic enzyme from *B. halodurans* with several related proteins. The alignment was made ▶ using ClustalW, and displayed by the programme Genedoc. The predicted secondary structure (by PROF) of the *B. halodurans* enzyme (Q9K7S1-identical sequence from *B. halodurans* C-125) is shown *below* the primary sequence in the alignment. The secondary structure elements of the evolutionary related bromoperoxidase (P29715) are also shown *below* the primary sequence of that enzyme. Beta-strands are shown as *black boxes*, while α-helices are displayed with the letter *H*. The residues of the putative catalytic triad are marked by *arrowheads*, the conserved motif surrounding the catalytic serine residue is *boxed*, and similar residues in more than 50% of the aligned sequences are *shaded*. Sequences are named using the Swiss-Prot/Trembl accession numbers Q99685, human monoglyceride lipase homologue; O35678, monoglyceride lipase, *Mus musculus*; Q9K7S1, lipolytic enzyme *B. halodurans*; Q5WEO0, putative lysophospholipase (genome seq) *Bacillus clausii*; Q632T2, putative lysophospholipase (genome seq) *Bacillus cereus*; Q6HCC2, putative lysophospholipase (genome seq) *Bacillus thuringiensis*; Q65FW3, ytpA (genome seq) *Bacillus licheniformis*; Q99TA3, lysophospholipase homologue (genome seq) *Staphylococcus aureus*; Q67KL6, putative lysophospholipase (genome seq) *Symbiobacterium thermophilum*; Q84FD1, AgmH *Myxococus xantis*; Q9UYB4, putative lysophospholipase (genome seq) *Pyrococcus abyssi*; Q8U3I6, putative lysophospholipase (genome seq) *Pyrococcus furiosus*; O28521, putative lysophospholipase (genome seq) *Archaeoglobus fulgidis*; P07000, characterized lysophospholipase *E. coli*; and P29715, bromoperoxidase *Streptomyces aureofaciens*



Free fatty acids determination

The free fatty acids were quantitated spectrophotometrically in the form of their copper salts as follows: 200 µl reaction mixture was immediately transferred to a test tube containing 2.3 ml toluene. Then, 500 µl cupric acetate–pyridine solution [consisting of copper(II)-acetate-1-hydrate aqueous solution (90 mM), adjusted to pH 6.1 with pyridine], was added to the mixture and vortexed for 30 s. After centrifugation (9,000×*g*, 1 min, at room temperature), 10 µl of a chromogenic reagent containing 4-(2-pyridylazo)-resorcinol (1.5 µM) dissolved in absolute ethanol was added, and the absorbance was measured at 500 nm. One unit of activity was defined as the amount of enzyme releasing 1 µmol of free fatty acids per minute under the assay conditions.

Results

Sequence analysis

Sequence analysis of the *B. halodurans* LBB2 gene revealed that its open reading frame (ORF) encoded a protein of 260 amino acids (corresponding to a theoretically calculated molecular weight of 30 kDa), with a 100% identity to the putative lysophospholipase of *B. halodurans* C-125 (entry BH3288 in the KEGG Database at http://www.genome.jp). A BLAST search using the deduced amino acid sequence (260 residues) of the native enzyme yielded significant hits to a number of sequences (the output limited to 100), electronically annotated as lysophospholipases or hypothetical proteins (ranging from 52 to 23% identity), and included 11 ORFs from *Bacillus* species. Most of the sequences were the results of genome projects (including all the bacilli sequences), and by manually going through the 40 highest similarity hits in the search (E values $\leq 1\times10^{-20}$, scores $\geq$100), only one characterized enzyme was identified, which was a monoglyceride lipase from *Mus musculus* (Karlsson et al. 1997), exhibiting 26% identity with the *B. halodurans* enzyme. Karlsson and coworkers had, by mutations, identified the catalytic triad, and by primary and secondary structure similarities, established an evolutionary relationship between esterases, lysophospholipases and haloperoxidases (Karlsson et al. 1997), which points out the importance of analysing substrate specificity prior to classifying novel lipolytic enzymes.

Sequence alignment and secondary structure prediction of the *B. halodurans* enzyme allowed identification of its catalytic triad (S88, D206 and H236). The alignment included the *B. halodurans* enzyme, the *M. musculus* enzyme, an evolutionary related *Streptomyces aurefaciens* bromoperoxidase with a known 3D structure, and some uncharacterized putative homologues (Fig. 1). Secondary structure prediction using PROF showed that the positions of the β-strands and α-helices coincided well with the evolutionary related bromoperoxidase (Fig. 1), despite an identity on the sequence level being as low as 8%. The serine residue (S88) appears in the well-conserved pentapeptide G-X-S-X-G (in this case G-H-S-M-G), while the aspartate (D206) and His residues (H236) were located in areas of lower sequence conservation, difficult to locate without a combination of the alignment and prediction data. Enzymes displaying the α-/β-hydrolase fold can have varying numbers of β-strands and α-helices, but the catalytic domains of all lipolytic enzymes are shown to share a minimal fragment of the fold (Cygler and Schrag 1997) containing five β-strands (β3–β7) and two helices (B and C) (Fig. 2). In addition to β3-7, strand β8, preceding H236 of the catalytic triad, was also predicted in

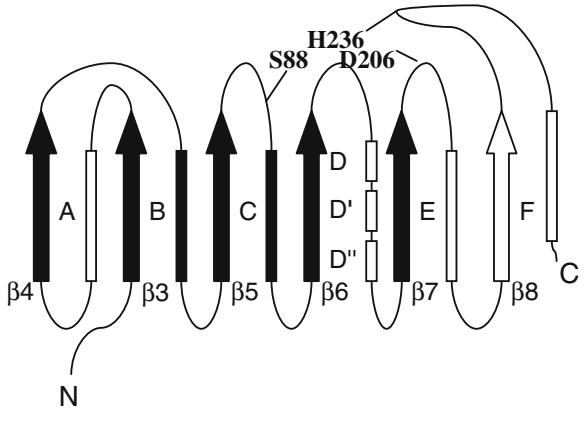


Fig. 2 A folding diagram illustrating the secondary structure elements of the α-/β-hydrolase fold predicted by PROF for the *B. halodurans* lipolytic enzyme. Strands and helices are *numbered* according to the nomenclature of the α-/β-hydrolase fold (Ollis et al. 1992). The secondary structure elements shown in *black* represent the strands and helices present in all structurally known lipases. The position and numbering of residues in the putative catalytic triad of the enzyme is indicated

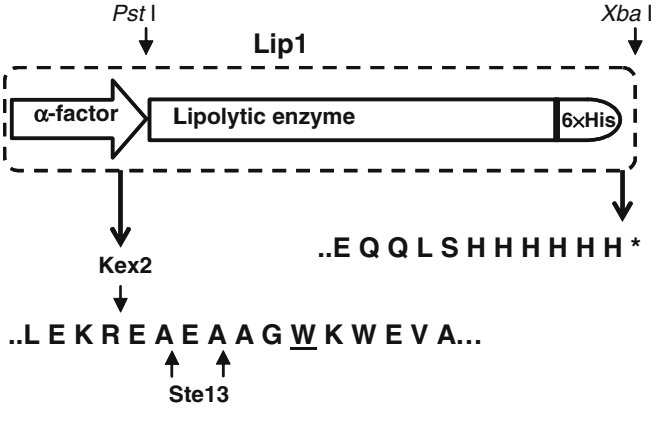


Fig. 3 Schematic representation of the primary structure of the unprocessed lipolytic enzyme (*Lip1*), showing the positions of the *S. cerevisiae* α-factor secretion signal, the sequence encoding the lipolytic enzyme and the C-terminal primer-introduced histidine tag and stop codon. The positions of the recognition sites for *Pst*I and *Xba*I, used to clone the PCR-amplified gene from *B. halodurans*, are indicated. The part of the primary sequence containing the *Kex*2 and *Ste*13 signal cleavage sites is shown together with the C-terminal part of the primary sequence

the *B. halodurans* lipase (Fig. 2). Helices A, B, C, E and F were predicted with high probability, while the helix content (three predicted helices) in the area of helix D is predicted with lower accuracy (and is located in an area with low sequence conservation).

Vector construction and selection of clones

An ORF (termed *Lip1*, following yeast nomenclature) was constructed for expression in vector pPICZαB, encoding the 85-residue *S. cerevisiae* α-factor followed by the 260-residue sequence of the *B. halodurans* gene and a 6-residue His tag (Fig. 3). By correct processing, the α-factor will be cleaved off during secretion at the *Kex*2 cleavage site, leaving an N-terminal six-residue insertion in the primary sequence (E-A-E-A-A-G), which can be further processed at the *Ste*13 sites (leaving 2–4 of the inserted N-terminal residues) prior to W2 (wild-type enzyme numbering) that marks the start of the lipolytic enzyme primary sequence (Fig. 3). The theoretically calculated molecular mass of the resulting enzyme is 31.4 kDa (including the N-terminal six-residue insertion and the C-terminal 6× His tag but excluding the 10-kDa α-secretion factor). No N-glycosylation sites (with the signature N-X-S/T) were predicted in the sequence (again excluding the α-secretion factor).

Electroporation of the plasmid construct to *P. pastoris* strain SMD1168H and selection on YPDS/zeocin plates resulted in approximately 300 transformants per plate, and by direct PCR screening for the insert (encoding the lipolytic enzyme), three positive clones were picked for sequence and expression studies.

Expression during the batch-induced mode of cultivation

Lipolytic activity of the three positive clones picked by PCR was first assessed in shake-flask cultivations employing four different media formulations according to Ramchuran et al. (2005). The absence of endogenous and exogenous lipases was confirmed using plasmid-free *P. pastoris* strain SMD1168H in the same media. Extracellular lipolytic activity was detected only in one of the three selected clones, and at very low levels (corresponding to approximately 2 U/l). Expression optimisation using this clone was carried out in a stirred tank bioreactor, which provided a more stable environment in terms of controlled pH and

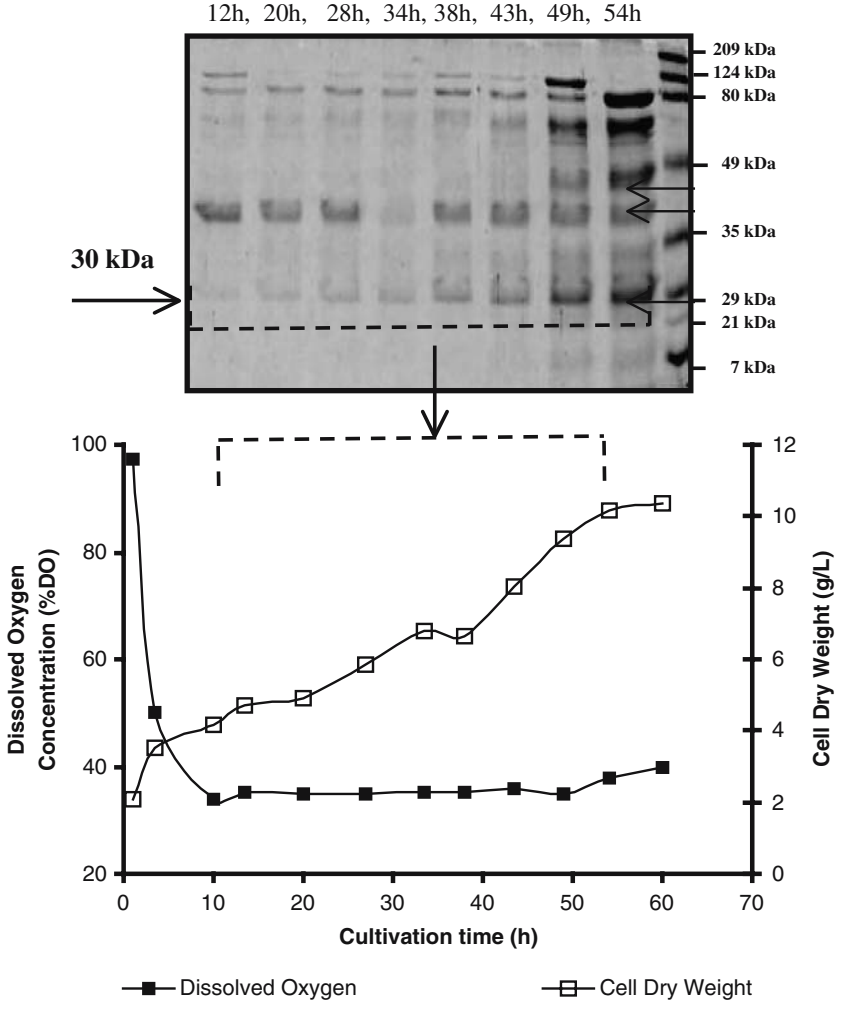


Fig. 4 Diagram displaying off-line analysis of cell dry weight and online data capture of dissolved oxygen (*DO*) concentration during the batch-induced cultivation together with an SDS-PAGE gel of extracellular samples at the corresponding sampling times. The samples taken at increasing cultivation times are shown from *left* to *right* and *numbered* with the sampling times starting at *12 h*. The three bands (30, 37 and 48 kDa) selected for N-terminal sequencing are indicated by *thin arrows* on the *right side*. The resulting N-terminal sequences were (residue alternatives in parenthesis; X, any residue) A(E)-A(E)-E-A-G-W-K-W-E-V (30 kDa), M-R-I-N-T-N-I-X-X-T (37 kDa) and A(E)-L(E)-S(Q)-I (A)-F-E-D-V(L)-E-K (48 kDa). The 30-kDa protein corresponding to the recombinant lipolytic enzyme is indicated by an *arrow* on the *left side*

increased aeration as compared to shake flasks. The "batch-induced" strategy used (Ramchuran et al. 2005) involves a pre-induction phase at the inoculum stage (12 h prior to inoculum transfer to the reactor) to start transcription of the *AOX1* promoter-controlled genes. Methanol was hence added during the late exponential phase of growth in the inoculum shake flask, and cultivation was allowed to proceed for a further 12 h. Using this strategy, no apparent lag phase was observed upon transfer into the reactor (in which methanol was the sole carbon source), as indicated by the immediate decrease in the DO concentration (Fig. 4). A DO concentration of 35% was attained at 10 h after inoculum transfer and was maintained by increasing the stirrer speed throughout the entire cultivation time (60 h), resulting in a cell mass concentration of 10 g/l (Fig. 4).

The extracellular lipolytic activity of the clone was followed during cultivation using two substrates: *p*-NPB (C-4) and *p*-NPP (C-16). Increase in activity was observed only after about 38 h of growth and continued to increase for about 12 h (Fig. 5). The activity measured after 60 h of cultivation with *p*-NPB was approximately 14,000 U/l, while the activity measured with *p*-NPP at the corresponding pH was 2,383 U/l (Fig. 5), indicating a preference for short-chain esters.

Judging from the SDS-PAGE (Fig. 4), three proteins accumulated extracellularly, with apparent molecular masses of 30, 37 and 48 kDa, respectively. The latter (48 kDa) was only seen during the last 10 h of the cultivation, concomitant with an increase in the number of higher and lower molecular weight proteins, implying an increase in extracellular host-specific enzymes (maybe as a result of cell lysis due to the lower nutrient availability during the later stages of the batch cultivation). To identify the recombinant lipolytic enzyme, and to investigate if an intracellularly produced (unprocessed) enzyme was released as a consequence of lysis, all three extracellular protein bands (Fig. 4) were subjected to N-terminal sequencing. The 30-kDa band was identified as the lipolytic enzyme, and N-terminal sequencing of the ten first amino acids yielded the sequence E(A), A(E), E, A, G, W, K, W, E, V. This showed that the secretion tag was removed at the *Kex*2 site, but that the protein was not processed further by the *Ste*13 gene product, thus leaving five residues introduced in the cloning design before the start of the native enzyme sequence (W, K, W, E, V). The theoretically calculated molecular mass of 31.4 kDa corresponded well to the apparent mass of 30 kDa estimated by SDS-PAGE. The sequences of two remaining bands (37 and 48 kDa) were not corresponding to any part of the sequence or to unprocessed products of the produced lipolytic enzyme and were judged to be host-specific.

Substrate specificity

The enzyme specificity was preliminarily characterized from the culture supernatant, since attempts to utilize the C-terminal His tag for purification of the enzyme by immobilized metal–ion affinity chromatography (IMAC) were not successful. A pH profile using *p*-NPP (data not shown) showed pH 9 to be optimal for activity, yielding 3,400 U/l, and this pH was thus used to screen activity on *p*-nitrophenyl alkanoate esters of varying chain lengths (Fig. 6). *p*-NPB proved to be the best substrate despite the slightly lower pH required for substrate stability. A rather low affinity towards medium-chain-length (C5–C6) fatty acids was observed, but there was no further activity decrease when the fatty acid chain length was extended (C16 and C18) (Fig. 6). True lipase activity was confirmed

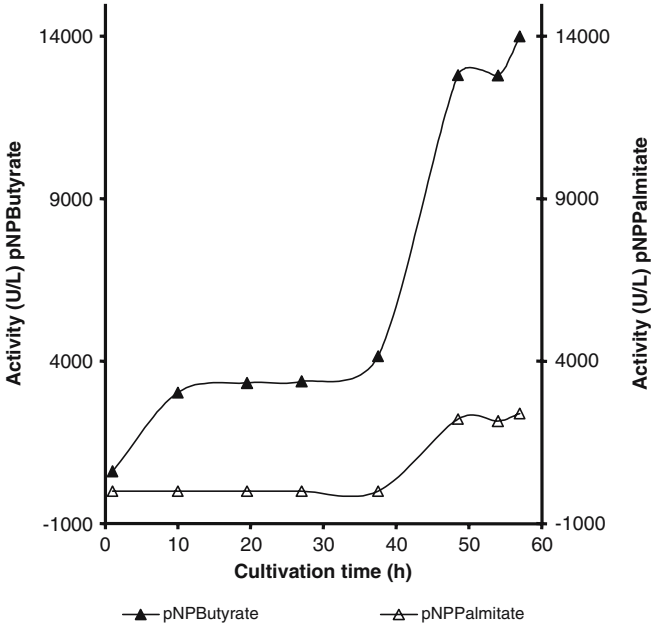


Fig. 5 Quantification of extracellular lipolytic activity during the batch-induced cultivation using *p*-nitrophenyl esters of two different chain lengths [*pNPPalmitate* (C-16) and *pNPButyrate* (C-4)]. Experimental details are stated in the text

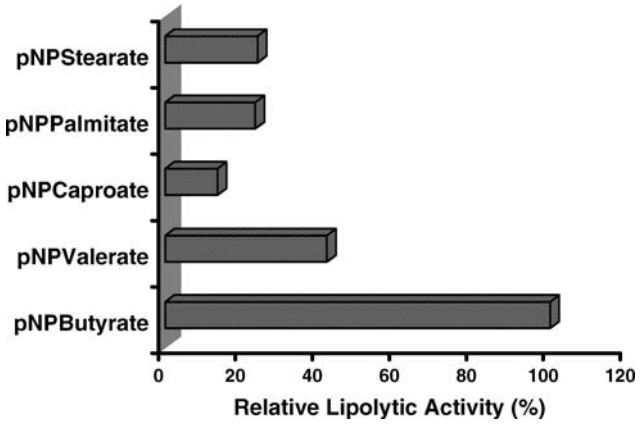


Fig. 6 Relative extracellular lipolytic activity using synthetic *p*-nitrophenyl alkanoate esters of varying chain lengths (between C2 and C18), with 100% defined as the highest measured activity. The 100% activity corresponded to 14,000 U/ml. All activities were measured at pH 9 (apparent optimum using *pNPPalmitate*) except for *pNPButyrate*, which was measured at pH 8.3 due to substrate instability at higher pH values

using olive oil as substrate and yielded an activity of 7,300 U/l.

Remarkably, with the electronic annotation of the enzyme in mind, the heterologous enzyme showed no activity towards LPC, but an evaluation of phospholipase activity resulted in an activity of 6,400 U/l when PC was used as substrate. This was confirmed by TLC showing hydrolysis of PC to free fatty acids and phospholipids (data not shown).

Discussion

Over the years, the methylotrophic yeast *P. pastoris* has been developed into a highly successful host–vector system for the production of a variety of heterologous enzymes (Cereghino and Cregg 2000) capable of both intra- or extracellular accumulation of the target enzyme (Cereghino et al. 2002; Berrin et al. 2000). This work shows this system to be successful also for the production of a lipolytic enzyme of alkaliphilic Gram-positive origin. *P. pastoris* has lately become an expression system of choice for enzymes (e.g. lipolytic enzymes) that are generally problematic to produce in active form in the well-known *E. coli* systems. This was also the case when production of the *B. halodurans* LBB2 encoded lipolytic enzyme was attempted using an *E. coli* system, in which it was solely produced as inactive inclusion bodies (Vargas et al., unpublished data).

Our first attempts to express the lipolytic enzyme in the *P. pastoris* system using shake-flask cultivations yielded active enzyme, but very poor activity levels were reached despite the use of a range of growth media. This pinpoints towards the importance of having controlled cultivation conditions when evaluating production possibilities. The batch-induced mode of cultivation represents a rather simple technique, yet gaining tighter control of pH and aeration during the production. The use of this technique and peptide-rich media components, with methanol as the sole carbon source, has, in a previous investigation in our laboratory, resulted in good yields of the target protein without spending much time on process optimisation (Ramchuran et al. 2005). In the current work, the batch-induced mode resulted in a dramatically improved production, yielding a maximum extracellular enzyme activity of approximately 14,000 U/l (*p*-NPB) and a total extracellular lipolytic enzyme production of approximately 0.2 g/l (judged by densitometry). These concentrations are good compared to many other reported values (Kademi et al. 2003). For example, Holmquist et al. (1997) reported the production of a lipase originating from *Geotrichum candidum* to approximately 60 mg/l, and Rotticci-Mulder et al. (2001) have produced lipase B from *Candida antarctica* to 25 mg/l, both using the *AOX1* promoter. Further development of the production using fed-batch technology will most likely boost the production levels. Jahic et al. (2003) have reported protein concentrations of 1 and 2 g/l using methanol- and temperature-limited fed-batch techniques, respectively.

The *P. pastoris*-produced lipolytic enzyme could not be purified by IMAC. We can speculate that the absence of amino acid residues between the enzyme and the His tag resulted in too low flexibility and was thus a hindrance for exposure of the His tag for purification, as helix F is predicted to be very close to the C-terminal-end of the peptide, leaving only two residues between the predicted helix and the start of the tag. On the other hand, the protein content on SDS-PAGE showed that if the cultivation is terminated 10 h earlier, the production time would be shortened, and an enzyme of higher electrophoretic purity would be produced, hence rendering purification tags unnecessary. The main drawback in this case would, however, be a risk of lower total activity, as good activity levels were only obtained during the last 15 h. This activity pattern was also shown to be reproducible when the batch-induced cultivation of the same clone was repeated.

From the sequence analysis, the *B. halodurans* LBB2 enzyme was identified as a lysophospholipase. The enzymes classified as phospholipases and lysophospholipases (Fischer and Pleiss 2003; Arpigny and Jaeger 1999) use phospholipids as their natural substrates and release fatty acids from either or both the *sn*-1 and *sn*-2 positions of phospholipids. However, as the various lipase classes contain enzymes with overlapping specificities, this makes the classification of a novel enzyme complex. For example, carboxylesterases (EC 3.1.1.1) hydrolyse small ester-containing molecules that are at least partly soluble in water, but these enzymes are reported to have a wide specificity also capable of catalysing arylesterase, lysophospholipase and acetylesterase reactions (http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/1/1/1.html). Monoglyceride lipases or acylglycerol lipase (EC 3.1.1.23), which catalyse hydrolysis of glycerol monoesters of long-chain fatty acids, have also been shown to possess carboxylesterase activity (acting on smaller esters like *p*-NPB) (Karlsson et al. 1997). In addition, the wide diversity of methods used for lipase assays (such as the hydrolysis of *p*-NPEs, the pH stat method and the monolayer technique) prevents a direct comparison of results on substrate specificity (Rogalska et al. 1993; Gupta et al. 2003).

With this background, a definite classification of the *B. halodurans* enzyme will have to await further more detailed specificity studies. However, it is quite clear, based on the lack of activity on LPC, that the electronic annotation is not a good description of the enzyme, which, based on the activity tests done so far (showing good activity on short-chain esters and PC), would better fit among either carboxylesterases or phospholipases.

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