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The (phospho)lipase from *Staphylococcus hyicus*: Expression in *Escherichia coli*, large-scale purification and application in esterification reactions

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Abstract

The gene coding for the mature part of the (phospho)lipase from *Staphylococcus hyicus* has been cloned and overexpressed in *Escherichia coli*. The recombinant enzyme accumulated in the cytoplasm and was purified using a combination of cation- and anion-exchange chromatography and refolding from guanidine/HCl. The purification protocol yielded 0.46 g of pure *S. hyicus* (phospho)lipase from 100 gram wet *E. coli* cells. Although the recombinant enzyme has an N-terminal extension of 35 amino acids, the activity towards lipids and phospholipids of different chain-length is identical to that of the wild-type *S. hyicus* (phospho)lipase. Application of the (phospho)lipase in esterification reactions demonstrated that the enzyme is inactive in microemulsions of charged detergents, but active in the presence of the neutral detergent Triton X-100 and in the absence of a detergent. These results were compared to the performance of a series of other lipases from microbial origin. The results are discussed in view of the possible application of the *S. hyicus* (phospho)lipase in the modification of phospholipids.

Keywords: Staphylococcus hyicus (phospho)lipase; Overexpression; Purification; Esterification

1. Introduction

Under physiological conditions lipases (glycerol ester hydrolases, EC 3.1.1.3) hydrolyse the carboxyl ester bonds present in long-chain triglycerides to liberate fatty acids and glycerol. This hydrolysis is an equilibrium reaction and the direction of the reaction can be changed to ester synthesis by modification of the reaction conditions. Zaks and Klibanov [1,2] were the first to demonstrate that lipases are active in organic solvents and in these water-restricted environments they catalyse (trans) esterification reactions.

Since then, stimulated by the potential application in industrial processes, numerous researchers have explored the use of lipases, mainly those commercially available from microbial origin, in (trans)esterification reactions [3]. The most frequently used system in enzymatic (trans)esterification is a biphasic system

Abbreviations: CM-52, CM-Cellulose-52; DE-52, DEAE-Cellulose-52; L-diC8PC, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; D-diC8PC, 2,3-dioctanoyl-*sn*-glycero-1-phosphocholine; AOT, bis(2-ethylhexyl)sulphosuccinate; CTAB, cetyltrimethylammonium bromide; TX-100, Triton X-100; w_0, molar ratio of water to detergent; TLC, thin layer chromatography

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consisting of the solid enzyme (in powder form or adsorbed to a solid support) suspended in an organic solvent [4]. An alternative to these solid–liquid systems is the use of a water-immiscible organic solvent and an aqueous phase containing enzyme in solution (liquid–liquid system). A third possibility is the use of microemulsions or reverse micellar solutions, which are similar to biphasic liquid–liquid systems, except that the aqueous phase consists of water pools containing the enzyme in solution surrounded by a surfactant layer. These systems are thermodynamically stable and can be considered as monophasic systems [5]. In all three media with a low water content lipases were successfully applied to synthesise and modify triglycerides [6–10] and as a result a wealth of information on the behaviour of these enzymes in water-restricted environments is now available (see Ref. [11], and references therein).

In sharp contrast to the vast information about the application of lipases, the knowledge on the behaviour of a related class of enzymes, the phospholipases A_2 (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4), in organic solvents is scarce. There are only a few reports describing the application of phospholipases to synthesise phospholipids [12–14], albeit with very low yields. Only recently a more extended study on the behaviour of porcine pancreatic phospholipase A_2 in water-restricted environments [15] was published. As a consequence of this lack of information in combination with the thusfar disappointing performance of phospholipase A_2 in organic solvents, the use of lipases to modify phospholipids was explored [16–19]. The obtained (trans)esterification yields were low and this might directly be related to the observation that most microbial lipases are unable to hydrolyse phospholipids in aqueous environments. Interestingly, for the modification of phospholipids, the most successfully applied lipases are those isolated from *Rhizopus* species and *Mucor miehei*, which have a low hydrolytic activity towards phospholipids in aqueous solutions [20,21].

Recently, in our laboratory the lipase from *Staphylococcus hyicus* was purified. In *S. hyicus* this lipase is produced as a 76 kDa prepro-enzyme which is processed into the 46 kDa mature lipase after secretion into the growth medium [22]. Purification of the mature lipase from *S. hyicus* failed and therefore we expressed the lipase in *S. carnosus*. This heterologous host lacks the endopeptidase necessary for proteolytic processing and after purification of the pro-lipase we obtained the mature form by tryptic digestion; a tryptic cleavage site is present two amino acids after the natural processing site [23]. From a 10 l *S. carnosus* culture we obtained 10 mg of pure mature *S. hyicus* lipase. Characterisation revealed a broad substrate specificity and the *S. hyicus* lipase effectively hydrolysed a wide range of structurally different lipids, but was also extremely active towards both natural and synthetic phospholipids [23]. To the best of our knowledge such high phospholipase activity is unique among microbial lipases and the *S. hyicus* (phospho)lipase might therefore be the enzyme of choice for the (trans)esterification of phospholipids. Studying enzyme behaviour in organic solvents, however, requires large amounts of protein and therefore the purification as described before should be improved.

Here we report the large-scale purification of mature *S. hyicus* (phospho)lipase after overexpression in *E. coli*. The kinetic properties of the recombinant (phospho)lipase were compared to the wild-type enzyme purified from *S. carnosus* and in addition we present the results of an initial screening on the performance of this enzyme in organic solvents as compared to other microbial lipases.

2. Experimental procedures

2.1. Materials

All enzymes used in DNA manipulations were obtained from New England Biolabs.

Isopropyl-thio-β-D-galactopyranoside and lysozyme (from hen egg white) were bought from Boehringer. Ampicillin, taurodeoxycholate and olive oil were from Sigma. Tributyrin was bought from Aldrich. DEAE-Cellulose-52 (DE-52) and CM-Cellulose-52 (CM-52) were obtained from Whatman and QAE-Sephadex from Pharmacia. The detergents sodium bis(2-ethylhexyl)sulphosuccinate (AOT), cetyltrimethylammonium bromide (CTAB) and Triton X-100 (TX-100) were bought from Acros, Janssen, and Serva, respectively. All fatty acids and alcohols were obtained from Sigma. Crude lipase preparations were from Biocatalysts. 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (L-diC8PC) and 2,3-dioctanoyl-*sn*-glycero-1-phosphocholine (D-diC8PC) were synthesised as described by Bonsen et al. (1972). Organic solvents were of HPLC-grade and all other chemicals used of analytical grade.

2.2. Cloning and expression

Plasmid pLipP1 [24], containing the *S. hyicus* (phospho)lipase gene, was isolated from *S. carnosus* essentially as described before [25]. The cell pellet of 1.5 ml overnight culture was collected by centrifugation (3 min, 13,000 × *g*) and subsequently resuspended in 100 μl of 25 mM Tris/HCl, pH 8, 10 mM EDTA, 50 mM glucose and 4 mg/ml lysozyme followed by incubation at 37°C for 1 h. Cell lysis was induced by the addition of 200 μl 2 M NaOH containing 1% SDS (w/v). After 2 min of incubation at room temperature, proteins were precipitated by adding 160 μl 3 M NaAc, pH 4.5 and subsequent centrifugation for 10 min at 13,000 × *g*. To the DNA containing supernatant 1 ml ethanol was added followed by incubation at −20°C for 1 h. The precipitated DNA was collected by centrifugation for 15 min at 13,000 × *g*. After treatment with RNAse for 15 min at 37°C, the sample was extracted twice with a mixture of 50% phenol and 50% diethylether (v/v). Subsequently the DNA was again precipitated with 1 ml ethanol in the presence of 150 mM NaCl. The yield was approximately 0.3 μg of DNA from 1.5 ml cell culture. The isolated plasmid pLipP1 was digested with *Sal*I and *Pvu*II and the (phospho)lipase-encoding fragment isolated from agarose gel. This fragment was subcloned into vector pBR322 [26] using unique *Sal*I and *Eco*RV sites. The resulting plasmid pSHBR was transformed into *E. coli* DH5α [27] and positive clones were identified using restriction site analysis. Plasmid pSHBR was digested with *Sal*I and *Hin*dIII and the (phospho)lipase-encoding fragment subcloned into the expression vector pT7.7 [28] using unique *Sal*I and *Hin*dIII sites. The resulting expression plasmid pSHT7 (Fig. 1A) was transformed into the expression host *E. coli* BL21 (DE3) [29], which contains the structural gene for T7 RNA polymerase under control of the lac promotor.

Two 10 ml *E. coli* BL21 (DE3) cultures, containing plasmid pSHT7, were each diluted in 400 ml Luria–Broth medium supplemented with 100 mg/L ampicillin and incubated overnight at 37°C. These overnight cultures were added to 9.2 l medium containing per litre: 10.9 g tryptone, 5.4 g yeast extract, 9.2 g $Na_2HPO_4 \cdot 2H_2O$, 3.3 g KH_2PO_4, 0.54 g NaCl, 1.1 g NH_4Cl, 0.53 g $MgSO_4 \cdot 7H_2O$, 0.16 g $CaCl_2 \cdot 2H_2O$, 6.5 g glucose, 10.9 mg thiamine and 108.7 mg ampicillin. Cultivation took place in a fermentor (New Brunswick) at 37°C under vigorous stirring and aeration. When the optical density at 600 nm reached a value of 1.5 lipase expression was induced by adding isopropyl-thio-β-D-galactopyranoside to a final concentration of 0.4 mM. After another 5.5 h of growth the cells were collected by centrifugation (30 min, 5,000 × *g*, 4°C) and the cell pellet was subsequently stored at −20°C.

2.3. Purification

All purification steps took place at 4°C. In between steps the specific activity of the enzyme was routinely measured in the standard activity assay with mixed micelles of tributyrin

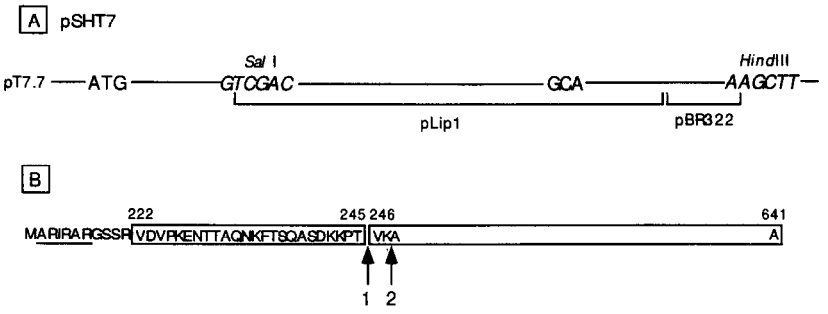

Fig. 1. Schematic representation of the constructed expression plasmid and the encoded enzyme. The plasmid for expression of recombinant *S. hyicus* (phospho)lipase in *E. coli*, pSHT7, is shown in panel A. Plasmid pSHT7 contains the T7 gene 10 promotor and a strong ribosome binding site. pLip1 indicates the (phospho)lipase-encoding fragment of pLip1 [24] and pBR322 a part of this vector present in the final construct because of the subcloning step. The start- and stopcodon for (phospho)lipase gene transcription are indicated as well as the restriction sites used in cloning. In panel B the encoded protein with amino acid numbering according to Götz et al. [24] is given. Valine 246 and alanine 641 are the first and last amino acids of the mature wild-type enzyme. The N-terminal extension consists of 11 amino acids due to the cloning procedure and 24 amino acids (residue 222 to 245) of the pro-peptide. The natural processing site (arrow 1) and the cleavage site of trypsin (arrow 2) are indicated. Amino acids identified by N-terminal sequencing are underlined.

(60 mM) and TX-100 (120 mM) as a substrate. *S. hyicus* (phospho)lipase concentrations were determined spectrophotometrically at 280 nm using an $E^{1\%}$ value of 14.5 calculated according to Mach et al. [30].

After suspending the cell pellet of a 10 l *E. coli* culture (~ 100 g wet cells) in 400 ml 50 mM Tris/HCl and 40 mM EDTA at pH 8, 100 g sucrose was added. After homogenisation of this suspension 300 mg lysozyme was added, followed by incubation with homogenisation at regular time intervals. After 30 min of incubation 400 ml of 50 mM Tris/HCl, 40 mM EDTA, pH 8 was added, with additional homogenisation, to induce an osmotic shock. The suspension was incubated for 30 min and subsequently sonicated three times for 2.5 min (with periods in between of 5 min) followed by centrifugation at 10,000 × *g* for 3 h. The resulting supernatant was directly loaded onto a 300 ml QAE-Sephadex column pre-equilibrated with 5 mM Tris, pH 8. The (phospho)lipase containing flow-trough fraction was dialysed against distilled water, the pH was set to 8 with ammonia, and subsequently lyophilised. The dry material was suspended in 200 ml 6 M guanidine/HCl and dialysed extensively against 5 mM Tris/HCl, pH 8. After dialysis the pH was adjusted to 6 with a 1 M succinic acid solution followed by centrifugation for 30 min at 5,000 × *g*. After removal of the clear supernatant (SupA), the pellet was resuspended in 200 ml 6 M guanidine/HCl and the procedure described before of subsequent dialysis, adjustment of pH and centrifugation was repeated. The obtained supernatant (SupB) was combined with SupA. The resulting solution (SupA + SupB) was loaded onto a 200 ml CM-52 column pre-equilibrated with 10 mM succinate, pH 6. After washing with 10 column volumes loading buffer, the (phospho)lipase was eluted with a linear NaCl gradient from 0 to 250 mM. The (phospho)lipase-containing fractions were pooled and after dialysis against distilled water again loaded onto a 200 ml CM-52 column pre-equilibrated with 10 mM succinate, pH 6. After elution with a linear NaCl gradient from 0 to 250 mM, the (phospho)lipase was dialysed against 10 mM Tris/HCl, pH 8.5 and subsequently loaded onto a 25 ml DE-52 column pre-equilibrated with 10 mM Tris/HCl, pH 8.5. The column was washed with 20 column volumes and elution of the (phospho)lipase was performed with a NaCl gradient from 0 to 150 mM.

2.4. Analytical protein techniques

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis [31] was performed to analyse the protein samples for purity. Acrylamide gels

of 15% were used and staining was performed with Comassie Brilliant Blue.

FPLC analysis was performed using a Pharmacia (Uppsala, Sweden) system consisting of a GP-200 gradient programmer, two P-5000 pumps, an UV-1 single path monitor and a REC-482 recorder. The (phospho)lipase (50 μg) was loaded onto a Mono-Q HR 5/5 column (Pharmacia) pre-equilibrated with 20 mM Tris/HCl, pH 8.5 and elution took place with a linear NaCl gradient from 0 to 250 mM at a flow rate of 1 ml/min.

Determination of the amino terminal sequence was carried out on a Perkin Elmer Applied Biosystems 476A protein sequencer according to the instructions of the manufacturer. The first six amino acids were identified by repetitive Edman degradation [32] followed by identification of the released amino acids on HPLC.

2.5. *(Phospho)lipase hydrolytic activity measurements*

Hydrolytic activity was measured by titrating released fatty acids with 5 mM NaOH under nitrogen. A Radiometer titration set consisting of a PHM-84 pH meter, a TTT-80 titrator, an ABU-80 autoburette, a TTT-60 titration assembly and a Rec-80 servograph was used. The reaction volume was 3 ml and the reaction temperature was 40°C. In the standard assay to measure lipase activity mixed micelles of tributyrin (60 mM) with TX-100 (120 mM) in 5 mM Tris/HCl, 10 mM $CaCl_2$ and 50 mM NaCl at pH 8 were used as a substrate. Activity with an emulsion of triolein as a substrate was assayed essentially as described before [22]: to 2 ml of 7.5 mM Tris/HCl, pH 8, 15 mM $CaCl_2$ and 7.5 mM taurodeoxycholate, 1 ml of an emulsion of triolein (4.4 ml olive oil in 40 ml of 10% gum arabic) was added. Activity towards phospholipids was determined with as substrate pure micelles of D-diC8PC or L-diC8PC at a concentration of 3 mM in 5 mM Tris/HCl, pH 8, 10 mM $CaCl_2$ and 50 mM NaCl. Activity on natural lecithins was measured using egg-yolk as substrate [33].

2.6. *Esterification reactions*

In all described systems the waterphase consisted of 0.05 M Tris/HCl, 0.01 M $CaCl_2$, pH 8 containing 0.5 mg crude lipase. AOT/isooctane microemulsions were prepared by dissolving 0.1 M AOT, 0.05 M heptadecanoic acid and 0.2 M 1-butanol in isooctane. In addition 46 μl lipase solution was added to yield a microemulsion with a molar ratio of water to detergent (w_0) of 10. When 1-butanol was replaced by glycerol (0.2 M) the water phase was reduced to 9 μl, containing 0.1 mg lipase, to obtain a stable microemulsion with a w_0 of 2 and a g_0 (molar ratio of glycerol to detergent) of 2. CTAB/1-hexanol/isooctane microemulsions ($w_0 = 10$) were obtained by dissolving 0.1 M CTAB, 1.62 M 1-hexanol and 0.05 M heptadecanoic acid in isooctane with subsequent addition of 46 μl lipase solution. CTAB/chloroform/isooctane microemulsions ($w_0 = 10$) were obtained by dissolving 0.1 M CTAB, 0.05 M heptadecanoic acid and 0.2 M 1-butanol in a mixture of 50% chloroform and 50% isooctane (v/v), followed by addition of 46 μl lipase solution. TX-100/1-hexanol/isooctane microemulsions were prepared by dissolving 0.35 M TX-100, 1.07 M 1-hexanol and 0.05 M heptadecanoic acid in isooctane, with subsequent addition of 167 μl lipase solution to give a w_0 value of 10. To prepare stable TX-100/hexane/toluene microemulsions with a w_0 of 5, 0.27 M TX-100, 0.05 M heptadecanoic acid and 0.2 M 1-butanol were dissolved in a mixture of 70% hexane and 30% toluene (v/v), followed by addition of 62 μl lipase solution. The (detergent-free) liquid–liquid system was prepared by adding 46 μl lipase solution to 2.5 ml isooctane containing 0.05 M heptadecanoic acid and 0.2 M 2-butanol. This two-phase system was stirred gently during incubation. The reaction volume was 2.5 ml in all cases and incubation was performed at room temperature

unless stated otherwise. To detect reaction products, samples (20 μl) from the incubations were applied as spots on thin layer chromatography (TLC) plates (Kieselgel 60, Merck). The solvent system used to separate free fatty acids, fatty acid–alcohol esters, monoglycerides and diglycerides consisted of: diethyl ether/hexane/acetic acid (50:50:3, by volume). Spots on the TLC plates were visualised by spraying the plates with a 50% sulfuric acid solution in water and charring.

3. Results

3.1. Expression

Cells transformed with plasmid pSHT7 were grown on a 10 l scale to reach a final OD_{660} value of 6.5, which corresponds to about 100 g of wet cells. Approximately 2.6 g of recombinant *S. hyicus* (phospho)lipase was produced and estimated from SDS-PAGE (Fig. 2A, lane 3) the expression level was about 20% of the total cellular protein.

3.2. Purification

Recombinant *S. hyicus* (phospho)lipase was isolated from 100 g of wet *E. coli* cells containing approximately 2.6 g of enzyme. The cells were suspended in 400 ml of lysis buffer and 100 g of sucrose was added. After incubation with lysozyme an osmotic shock was induced by increasing the volume to 800 ml by adding extra lysis buffer. This suspension was subsequently sonicated and cell debris were removed by centrifugation. The supernatant, containing about 80% of the lipase activity (1.6×10^6 U, measured in the standard assay with tributyrin as a substrate), was directly loaded onto a QAE-Sephadex column. Although the (phospho)lipase did not bind to this column, the dry mass of the solution was significantly reduced (Table 1) with a high recovery (1.5×10^6 U) of lipase activity. The specific activity increased from 73 to 136 U/mg as a result of this purification step. After dialysis at pH 8, the volume of the solution was reduced by lyophilisation and subsequent dissolving of the dry material in a small volume (200 ml) of 6 M guanidine/HCl. The (phospho)lipase was refolded by extensive dialysis at pH 8 yielding 1.2×10^6 U of lipase activity. Adjustment of the pH to 6 was followed by centrifugation: a large pellet was obtained and only 0.48×10^6 U (40%) were present in the supernatant (SupA). Therefore the pellet was resuspended in guanidine/HCl and the refolding procedure was repeated. In this

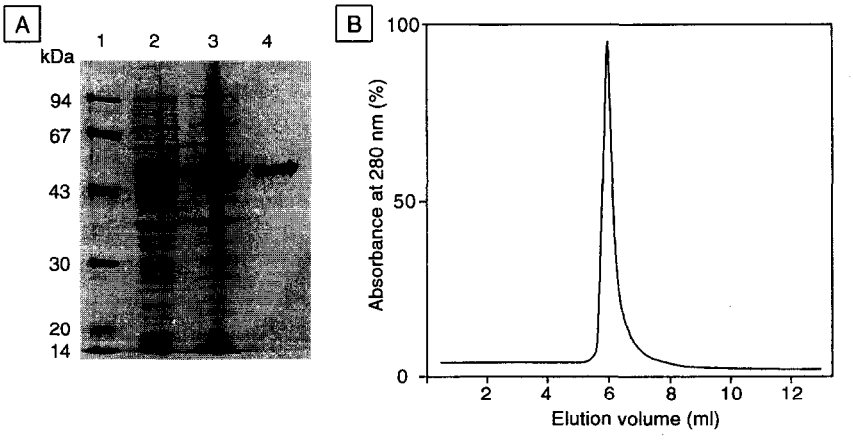

Fig. 2. SDS-PAGE (A) and FPLC (B) analysis of *S. hyicus* (phospho)lipase. (A) Lane 1: molecular weight marker proteins. Lane 2: non-induced *E. coli* cells. Lane 3: lysate of *E. coli* after induction. Lane 4: purified (phospho)lipase. (B) FPLC-chromatogram of the purified (phospho)lipase. The sample was loaded onto a mono-Q HR 5/5 column pre-equilibrated with 20 mM Tris/HCl at pH 8.5 and elution took place with a linear NaCl gradient from 0 to 250 mM at a flow rate of 1 ml/min.

Table 1
Purification of recombinant *S. hyicus* (phospho)lipase from a 10 l *E. coli* culture (~ 100 g wet cells)

Purification step	Protein (g)	Activity $\times 10^6$ (Units)	Specific activity (U/mg)	Yield (%)	Purification factor
Cell lysate	22 [a]	1.6	73	100	1
QAE-Sephadex	11 [a]	1.5	136	94	1.9
Refolding (from guanidine/HCl)	n.d.	1.2	n.d.	75	n.d.
Centrifugation (at pH 6)	n.d.	0.87	n.d.	54	n.d.
CM-52 (1)	1.6	0.58	363	36	5.0
CM-52 (2)	0.61	0.34	557	21	7.6
DE-52	0.46	0.28	609	18	8.3

n.d. not determined.
[a] Total dry mass.

step 0.39×10^6 U of lipase activity were recovered in the supernatant (SupB) and the pellet was discarded. Supernatant A and B were combined (0.87×10^6 U) and loaded onto a CM-52 column. The (phospho)lipase bound to this column and, after extensive washing, was eluted with a NaCl gradient resulting in a specific activity of 363 U/mg. Repeating this column gave a specific activity of 557 U/mg with a *S. hyicus* (phospho)lipase yield of 0.34×10^6 U. At this stage the enzyme was about 90% pure and the last impurities were removed by running a DE-52 column. The purified (phospho)lipase had a specific activity of 609 U/mg in the standard assay. It migrated as a single band as judged by SDS-PAGE analysis (Fig. 2A, lane 4) and eluted as one single peak from a mono-Q FPLC column (Fig. 2B). The yield of the purification was 0.46 g of pure *S. hyicus* (phospho)lipase which corresponds to almost 5 mg enzyme per g of wet cells. Results of a typical purification of *S. hyicus* (phospho)lipase from a 10 l *E. coli* culture are given in Table 1.

3.3. Characterisation of purified (phospho)lipase

The N-terminal amino acid sequence of the recombinant *S. hyicus* (phospho)lipase was identified as ARIRAR, which is in agreement with the DNA sequence (Fig. 1B). The N-terminal methionine was efficiently removed in vivo confirming the reported preference of *E. coli* methionine aminopeptidase [34] for an alanine or glycine residue at position two.

The substrate specificity of the enzyme expressed in *E. coli* was compared to that of the wild-type (phospho)lipase purified from *S. carnosus* (Table 2). Within experimental error the activities of both enzymes were identical with triglycerides of different chain-length and pure phospholipid enantiomers as a substrate. These data demonstrate that expression of *S. hyicus* (phospho)lipase in *E. coli* with an extra N-terminal extension of 35 amino acids does

Table 2
Comparison of the hydrolytic activities of recombinant *S. hyicus* (phospho)lipase expressed in *E. coli* and wild-type *S. hyicus* (phospho)lipase expressed in *S. carnosus*

Substrate	Activity (U/mg)	
	(phospho)lipase expressed in *E. coli*	(phospho)lipase expressed in *S. carnosus* [a]
Tributyrin	609	520
Triolein	60	60
Egg-yolk	175	n.d.
D-diC8PC	2022	2000
L-diC8PC	1079	1100

Activity with tributyrin (60 mM) as a substrate was measured in mixed micelles with TX-100 (120 mM). D-diC8PC and L-diC8PC were assayed as pure micelles at a substrate concentration of 3 mM. Activities with triolein or egg-yolk as a substrate were determined essentially as described before. The reaction volume was 3 ml and activities were measured in 5 mM Tris/HCl, 10 mM $CaCl_2$ and 50 mM NaCl, pH 8 at 40°C. Accuracy for each given value is about 10%.
n.d. not determined.
[a] Data taken from van Oort et al. [23].

not alter the kinetic properties as compared to the wild-type enzyme purified from *S. carnosus*. Because purification from *E. coli* is less time-consuming and results in an almost 50-times higher yield, this (phospho)lipase form was used in esterification experiments.

3.4. Esterification

An initial investigation of the esterification capacity of *S. hyicus* (phospho)lipase was performed in AOT/isooctane microemulsions ($w_0 = 10$) with heptadecanoic acid and 1-butanol as substrates. In this water-restricted environment no esterification by *S. hyicus* (phospho)lipase was observed. Therefore we decided (1) to replace the substrate heptadecanoic acid by octanoic acid or dodecanoic acid, (2) to replace the substrate 1-butanol by 1-hexanol, 1-octanol or glycerol, (3) to vary the substrate concentrations, (4) to vary the enzyme concentration, (5) to vary the water content of the reversed micelles ($w_0 = 5$ to $w_0 = 20$), (6) to vary the pH of the water phase (pH 5 to pH 11), (7) to vary the incubation temperature (20 to 50°C), (8) to replace the organic solvent isooctane by hexane and (9) to increase the incubation time (1 up to 14 days). Under all these conditions no esterification by *S. hyicus* (phospho)lipase was found. In contrast several other lipases, isolated from bacteria, yeasts or fungi, were able to esterify heptadecanoic acid with 1-butanol with moderate to high yields (Table 3). In addition these lipases (except *C. lipolytica* lipase) could also produce mono- and/or diglycerides from glycerol and fatty acid. Out of sixteen microbial lipases tested only four failed to catalyse esterification reactions in an AOT/isooctane microemulsion, the enzyme from *S. hyicus* being one of them.

Because of these negative results obtained in the presence of reversed micelles of the anionic detergent AOT, we also investigated esterification by *S. hyicus* (phospho)lipase in reversed micelles of the cationic detergent CTAB and the neutral detergent TX-100. To stabilise microemulsions of these (single-chain) detergents, 1-hexanol has to be added as a co-surfactant [35,36], to reduce the interfacial tension. In our experiments 1-hexanol was both co-surfactant and substrate at the same time. Another possibility to obtain stable CTAB or TX-100 reversed micelles is by changing the polarity of the organic phase [37]. As described before for the AOT reversed micelles, *S. hyicus* (phospho)lipase did not catalyse esterification reactions in CTAB reversed micelles either (Table 4). In sharp contrast a high esterification yield was observed in the TX-100/1-hexanol/isooctane microemulsion and a moderate yield in the TX-100/hexane/toluene mi-

Table 3
Esterification performance of microbial lipases in AOT/isooctane microemulsions

Lipase source [a]	1-Butanol esterification [b]	Glycerol esterification [c]
Aspergillus niger	−	+
Candida lipolytica	++	−
Candida rugosa	++	+
Geotrichum candidum	−	−
Humicola lanuginosa	−	−
Mucor javanicus	+	+
Mucor miehei	++	++
Penicillium cyclopium	+	+
Penicillium roquefort	−	−
Pseudomonas fluorescens	++	++
Pseudomonas glumae	++	++
Rhizopus delamar	+	++
Rhizopus japonicus	++	++
Rhizopus javanicus	++	++
Rhizopus niveus	+	++
Staphylococcus hyicus	−	−

Esterification of heptadecanoic acid (0.05 M) with 1-butanol (0.2 M) was carried out in 2.5 ml 0.1 M AOT in isooctane with as water phase 46 μl ($w_0 = 10$) 0.05 M Tris/HCl, 0.01 M $CaCl_2$, pH 8 containing 0.5 mg crude lipase. In the case of heptadecanoic acid (0.05 M)-glycerol (0.2 M) esterification the water phase was 9 μl ($w_0 = 2$) 0.05 M Tris/HCl, pH 8 containing 0.1 mg crude lipase. The reaction time was 24 h and incubation took place at room temperature.

[a] All crude lipase preparations were obtained from Biocatalysts. *S. hyicus* (phospho)lipase had an activity of 300 U/mg in the standard assay and was approximately 50% pure.

[b] Estimated % esterification of heptadecanoic acid by TLC-analysis. −, 20%; +20–80%; ++80–100%

[c] −, no esterification; +, formation monoglycerides; ++, formation diglycerides.

Table 4
Esterification performance of *S. hyicus* (phospho)lipase in low-water media of different composition

Organic phase [a]	1-Butanol esterification [d]	1-Hexanol esterification [d]
AOT/isooctane	−	n.d.
CTAB/1-hexanol/isooctane	n.d.	−
CTAB/isooctane/chloroform	−	n.d.
TX-100/1-hexanol/isooctane	n.d.	++
TX-100/hexane/toluene [b]	+	n.d.
Isooctane [c]	+	n.d.

Esterification of heptadecanoic acid (0.05 M) with 1-butanol or 1-hexanol was carried out in 2.5 ml organic phase with as water phase 0.05 M Tris/HCl, 0.01 M $CaCl_2$, pH 8, containing 0.5 mg of (phospho)lipase (activity in the standard assay was 300 U/mg). The microemulsions had a w_0-value of 10 and the detergent-free incubation a water content of 1.8% (v/v). The reaction time was 8 days and incubation took place at room temperature.
n.d. not determined.
[a] For exact composition: see Experimental procedures.
[b] $w_0 = 5$ (instead of 10).
[c] Gentle stirring during incubation.
[d] Estimated % esterification of heptadecanoic acid by TLC analysis. −, no esterification; +, about 50%; ++, > 75%.

croemulsion. In addition *S. hyicus* (phospho)lipase also catalysed the esterification of heptadecanoic acid with 1-butanol in the biphasic system, consisting of isooctane with 1.8% (by volume) water, with a moderate yield.

4. Discussion

Industrial applications of lipases are often hampered by the lack of sufficient amounts of enzyme. An increased knowledge in the field of recombinant DNA technology, however, resulted in the use of molecular cloning techniques to improve the production of lipases. Fungi [38], yeasts [39] and the gram-negative bacterium *E. coli* [40–42] have now successfully been applied to overexpress microbial lipases. In addition gram-positive bacteria are used for heterologous lipase expression; the *S. hyicus* (phospho)lipase was expressed in *S. carnosus* and purified from the culture medium after secretion. The overall yield was sufficient to characterise this enzyme, but too low for the screening of conditions for application in (trans)esterification reactions. To improve the expression level we decided to express the *S. hyicus* (phospho)lipase in *E. coli*. Because the expressed enzyme has a high phospholipase activity [23] it might be toxic for the host organism by degrading the cell membrane that consists mainly of phospholipids. To circumvent this problem we used the tightly regulated *E. coli* expression system as described by Studier and Moffatt [29]. Furthermore, in *S. carnosus*, the (phospho)lipase is produced as a prepro-enzyme and processed by tryptic digestion to yield the mature 46 kDa form. Because the mature species is the only one of interest we directly cloned the gene coding for the mature enzyme into an *E. coli* expression plasmid. Transformed *E. coli* cells grew to high cell density and the *S. hyicus* (phospho)lipase was expressed at a high level after induction. The enzyme was purified from the bacterial cytoplasm by a multistep protocol combining cation- and anion exchange chromatography and refolding from guanidine/HCl, resulting in 0.46 g of pure (phospho)lipase from 100 g of wet cells. The method described here has a 50-fold higher yield as compared to the original purification [23]. Although the recombinant enzyme has an N-terminal extension of 35 amino acids, the kinetic properties are identical to the wild-type *S. hyicus* (phospho)lipase.

An AOT/isooctane microemulsion was initially used to test whether *S. hyicus* (phospho)lipase can catalyse esterification reactions in a water-restricted environment. We chose this system because it is the most extensively studied reversed micellar system, and in addition AOT forms highly stable reversed micelles in isooctane upon addition of a water phase, without the need of a co-surfactant [35]. Moreover, there are numerous reports in literature on the successful application of this system in (trans)esterification reactions (Refs. [43,44], and references therein). In contrast to most other microbial lipases that we have tested, the *S. hyicus* enzyme was inactive in AOT reversed

micelles. In addition, upon a systematic modification of the composition of the system ('medium-engineering'), no esterification was found. We do not know the reason for this lack of esterification activity, but changes in enzyme structure upon solubilisation in AOT reversed micelles have been reported for the lipases from *Candida rugosa* and *Pseudomonas sp.* [45]. Although these two lipases catalyse esterification reactions in AOT reversed micelles (see Table 3), such a change in structure might inactivate the *S. hyicus* (phospho)lipase. Independent of the polarity of the organic phase, in reversed micelles of the cationic detergent CTAB, *S. hyicus* (phospho)lipase was again inactive. In sharp contrast a moderate to good esterification yield was found both in reversed micelles of the neutral detergent TX-100 and in a (detergent-free) system consisting of isooctane at low water content. These observations indicate that the esterification activity of the *S. hyicus* (phospho)lipase is negatively affected by the charged detergents AOT and CTAB.

The use of TX-100 reversed micelles for (trans)esterification reactions has severe disadvantages: (1) if 1-hexanol is used as co-surfactant one is restricted to this alcohol as substrate in esterification reactions, and in addition little is known about replacement of the alcohol by inert co-surfactants, (2) if hexane/toluene is used as organic phase, only a limited volume of water (and enzyme) is soluble because of instability of the reversed micelles, (3) TX-100 might be esterified itself by lipases with a broad substrate specificity. The disadvantage of the absence of detergent in the system is that stirring is necessary during the reaction because the enzyme is not solubilised in the organic phase, but an advantage is that no such additives have to be removed after completeness of the esterification reaction.

Although *S. hyicus* (phospho)lipase needs calcium for hydrolytic activity [23], the esterification yield was comparable in the absence and presence of calcium (data not shown). Interestingly, an identical behaviour has been reported before for porcine pancreatic phospholipase A_2 [13].

In conclusion we report here the large-scale purification of *S. hyicus* (phospho)lipase after overexpression in *E. coli*. Characterisation of the substrate specificity demonstrated that the recombinant enzyme behaves identically to the wild-type *S. hyicus* (phospho)lipase. An initial investigation revealed that the *S. hyicus* enzyme is inactive in reversed micelles of charged detergents, but is active in neutral TX-100 reversed micelles and in (detergent-free) organic solvent. We are now further optimising the conditions for (trans)esterification by *S. hyicus* (phospho)lipase and investigating the modifying activity towards phospholipids in organic solvents.

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