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Purification and Substrate Specificity of *Staphylococcus hyicus* Lipase†

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Received February 6, 1989; Revised Manuscript Received June 21, 1989

ABSTRACT: The *Staphylococcus hyicus* lipase gene has been cloned and expressed in *Staphylococcus carnosus*. From the latter organism the enzyme was secreted into the medium as a protein with an apparent molecular mass of 86 kDa. This protein was purified, and the amino-terminal sequence showed that the primary gene product was indeed cleaved at the proposed signal peptide cleavage site. The protein was purified from large-scale preparations after tryptic digestion. This limited proteolysis reduced the molecular mass to 46 kDa and increased the specific activity about 3-fold. Although the enzyme had a low specific activity in the absence of divalent cations, the activity increased about 40-fold in the prepsence of Sr^{2+} or Ca^{2+} ions. The purified lipase has a broad substrate specificity. The acyl chains were removed from the primary and secondary positions of natural neutral glycerides and from a variety of synthetic glyceride analogues. Thus triglycerides were fully hydrolyzed to free fatty acid and glycerol. The enzyme hydrolyzed naturally occurring phosphatidylcholines, their synthetic short-chain analogues, and lysophospholipids to free fatty acids and water-soluble products. The enzyme had a 2-fold higher activity on micelles of short-chain D-lecithins than on micelles composed of the L-isomers. Thus the enzyme from *S. hyicus* has lipase activity and also high phospholipase A and lysophospholipase activity.

Lipases (glycerol ester hydrolase, EC 3.1.1.3) and bacterial lipases in particular are widespread in nature. These enzymes hydrolyze triglycerides to diglycerides, monoglycerides, glycerol, and fatty acids. Interest in bacterial lipases has increased markedly in the last two decades. A large number of lipases have been screened for applications in medicine (digestive enzymes) or as food additives (flavor-modifying enzymes), industrial reagents (glyceride-hydrolyzing enzymes), and cleaners (detergent additives). Several lipases have been isolated and biochemically analyzed (Muruoka et al., 1982; Tyski et al., 1983; Rollof et al., 1987). These enzymes are characterized by a wide substrate specificity since they hydrolyze tri-, di-, and monoglycerides as well as poly(oxyethylene) sorbitan fatty acyl esters (Tweens).

The lipase gene of *Staphylococcus hyicus* has been cloned and expressed to high levels in *Staphylococcus carnosus* (Götz et al., 1985; Lechner et al., 1988). A preprotein with 641 amino acids was predicted from the DNA sequence with a molecular mass of 71.4 kDa, although the apparent molecular mass from SDS-PAGE gels was about 86 kDa. In *S. hyicus*, but not in *S. carnosus*, the 86-kDa protein was rapidly transformed into a 46-kDa form. In this paper we describe

† This work has been supported by the Dutch Organization for Advancement of Pure Science (NWO) and by the European Economical Community (BAP Contracts 0071-NL and 0196-D).
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0006-2960/89/0428-9278$01.50/0

the isolation and purification of the 86- and 46-kDa forms of the *S. hyicus* lipase. By use of synthetic substrates, the enzyme was characterized with respect to substrate specificity and kinetic properties.

Materials and Methods

Bacterial Strains and Media. *S. carnosus* TM 300 was used as the cloning host for lipase gene expression. Cells were cultivated in PYS (10 g of peptone, 5 g of yeast extract, and 5 g of NaCl per liter, at pH 7.2) as previously described (Götz et al., 1985). Cultures were grown overnight in the presence of 10 μg/mL chloramphenicol. After cultivation, cells were removed by centrifugation for 30 min at 4000*g*. Solid ammonium sulfate was added to the supernatant to 70% saturation, and the precipitate was collected by centrifugation for 30 min at 5000*g*.

Small-Scale Purification of the 86-kDa Lipase. After dialysis the ammonium sulfate precipitate was chromatographed on DEAE-Sepharose CL-6B (Pharmacia) in 20 mM Tris-HCl, pH 8. Proteins were eluted with a linear NaCl gradient (0–0.5 M) in 5 column volumes. The lipase-containing fractions were desalted and concentrated in an Amicon concentration cell equipped with a YM 10 membrane. HPLC chromatoraphy on a Mono Q HR 5/5 column (Pharmacia) was carried out in 20 mM Tris-HCl buffer, pH 7. Proteins were eluted with a NaCl gradient (0–1.5 M) in 44 min at a flow rate of 1 mL/min. Final purification was achieved with a HPLC Superose HR 10/30 column (Pharmacia) at a flow rate of 0.4 mL/min in 20 mM Tris-HCl and 0.1 M NaCl, pH 7. The fractions containing pure 86-kDa lipase were concentrated in a Centricon microconcentrator (Amicon).

Large-Scale Purification of the 46-kDa Lipase. All purification steps were carried out at 4 °C. The ammonium sulfate precipitate from 10 L of culture medium was dissolved in 25 mL of 5 mM Tris-HCl at pH 8 containing 10 mM EDTA. This solution was applied to a Sephadex G-100 fine column (3 × 70 cm) with a layer of Sephadex G-25 coarse ($^1/_4$ column volume) placed on top of the G-100 layer. The lipase-containing fractions were combined and incubated with 0.1 μg of trypsin/mg of lipase for 4–5 h at room temperature. After this incubation, the solution was applied directly to a DEAE-cellulose column (2.5 × 30 cm) equilibratd with 5 mM Tris and 10 mM EDTA buffer, pH 8. The active lipase was eluted with a linear NaCl gradient from 0 to 0.2 M with 10 column volumes. After dialysis, the lipase was eluted from a second DEAE-cellulose column equilibrated at 10 mM histidine and 10 mM EDTA, at pH 6.5, by using a linear NaCl gradient (0–0.1 M) with 10 bed volumes. The lipase-containing fractions were pooled, dialyzed, and concentrated by partial lyophilization. Protein concentration was determined by absorbance using $E_{280nm}^{1\%}$ of 11.0.

Other Enzymes. Pancreatic prophospholipase A_2 (pro-PLA_2) was isolated from porcine pancreas and converted into the active enzyme (PLA_2) as described before (Nieuwenhuizen et al., 1974). Porcine pancreatic lipase (PPL) was purified according to Verger et al. (1969).

Enzyme Assays. Lipase activity was measured by determining the free fatty acids released by hydrolysis of the substrate emulsion using 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. Tributyrin was used as the substrate in a 2.5-mL solution containing 5 mM Tris-HCl buffer, pH 8.5, 10 mM $CaCl_2$, and 67 mM tributyrin. The assay was carried out under nitrogen with 10 mM sodium hydroxide solution. In some cases, triolein was used instead of tributyrin. To 20 mL of 5 mM Tris-HCl buffer, pH 8.5, containing 10 mM $CaCl_2$ and 5 mM deoxycholate, 10 mL of an emulsion of triolein (44 mL of olive oil in 400 mL of 10% arabic gum) was added.

Quantitative Determination of Enzymatic Activity on Water-Insoluble Phospholipids. The same titration equipment as described above was also used for the determination of the specific activity using a variety of substrates. In order to obtain reproducible results, the water-insoluble phospholipids were transformed to mixed micelles with the aid of deoxycholate. When enzymatic activity was measured as a function of substrate concentration, the ratio of substrate and deoxycholate was kept constant.

Qualitative Determination of Enzymatic Activity. For the determination of enzymatic activity with triglycerides, 10–20 mg of the substrate was mixed with 500 μL of 5% sodium deoxycholate, 100 mg of arabic gum, and 3 mL of 25 mM Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$. These mixtures were incubated with 5–200 units of lipase. The arabic gum was omitted when phospholipids were used as substrates. Control experiments were carried out under the same conditions without enzyme. The hydrolysis was followed by thin-layer chromatography using silicic acid plates (DC-60 Merck, Darmstadt, West Germany) developed in ether–hexane mixtures (90/10 v/v and 50/50 v/v) for neutral lipids and chloroform–methanol–water (65/35/8 v/v) for phospholipids.

To determine the positional specificity of the enzyme(s), the released fatty acids were analyzed by gas–liquid chromatography. Incubations were carried out as described above in the presence of pentadecanoic acid as an internal standard. At regular time intervals 0.5-mL fractions were withdrawn, acidified, and lyophylized. The samples were dissolved in chloroform–methanol (1/1 v/v), and the hydrolysis products were separated by TLC using chloroform–methanol–water (65/35/8 v/v) and petroleum ether–ether–formic acid (120/80/3 v/v) for phospholipids and triglycerides, respectively. The fatty acids were visualized by spraying with a 0.1% Rhodamine G solution, and fatty acids were converted to their methyl esters by heating at 70 °C in methanol containing 5% (v/v) sulfuric acid. After cooling, an equal volume of water was added and the fatty acid methyl esters were extracted with *n*-hexane. The organic phase was washed with 5% sodium bicarbonate and water, after which it was dried under vacuum. The samples were dissolved in trimethylpentane prior to injection into the gas chromatograph. Analyses were performed on a Packard Becker 519 gas chromatograph using a Chrompack 15% Cp-tm-Sil 84 Chrom WHP mesh 100–120 column at 200 °C, an injector temperature of 230 °C, and a flame ionization detector at 210 °C.

SDS–PAGE. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970) using 1-mm slab gels at pH 8.8 containing 11% acrylamide. Proteins were stained with Coomassie Brilliant Blue G-250.

Synthesis of Substrates. In general, substrates and substrate analogues were synthesized by established procedures; see Bonsen et al. (1972) and Chandrakumar et al. (1983; and references cited therein). Briefly the neutral glycerides were prepared as follows. *rac*-1-Lauroyl-2-myristoyl-3-palmitoylglycerol was synthesized by consecutive acylation of the primary and secondary hydroxyl group of *rac*-1-*O*-benzylglycerol with palmitic and myristic acids. After removal of the benzyl group by catalytic hydrogenolysis, a lauric acid chain was introduced. *rac*-1-Palmitoyl-2-elaidoyl-4-myristoylbutane-1,2,4-triol was prepared by acylation of 4-*O*-benzyl-1,2,4-butanetriol at the 1-position with palmitoyl chloride. After re-

moval of the benzyl group by hydrogenolysis, the 4-position was acylated with myristoyl chloride and the purified product was finally acylated with elaidoyl chloride.

Compounds **1–3**, **5**, and **8** (Table II) were prepared essentially as described by Bonsen et al. (1972). Compound **4** (Table II) was prepared from compound **1** by degradation with *Crotalus atrox* phospholipase A_2 in ether as described by Hanahan et al. (1952). Compound **6** was prepared by monotritylation of *rac*-1,2-propanediol followed by acylation of the secondary hydroxyl group. After detritylation with methanolic BF_3, the phosphocholine moiety was introduced as described by Bonsen et al. (1972). Compound **7** was also obtianed from *rac*-1,2-propanediol. In this case monoacylation of the primary hydroxyl group was followed by introduction of the phosphocholine group.

The compounds listed in Table III were synthesized according to Bonsen et al. (1972). The optically pure β-lecithins **11** and **12** were prepared from the parent 1,2-dipalmitoyl- and 1,3-dimyristoylglycero-2-phosphocholine by degradation with *C. atrox* phospholipase A_2, followed by reacylation with myristoyl and palmitoyl chloride, respectively.

The phosphorylation procedure described by Chandrakumar et al. (1983) was used for the synthesis of the amide-containing phospholipids **15–17**.

Results

Purification of S. hyicus Lipase. After growth, the cells were removed by centrifugation, and ammonium sulfate was added to the supernatant to a final concentration of 70% saturation. In this procedure most of the protein and all the lipase precipitates. Using this ammonium sulfate precipitate, the 86-kDa form of the lipase was quickly isolated on a small scale by HPLC. For larger scale purifications, the precipitate was applied to a combined Sephadex G-100/G-25 column. The lipase eluted just after the void volume of the column with a yield of 95–100% based on enzymatic activity, well separated from salt and most of the colored material. During this gel filtration step, a partial degradation of the 86-kDa form occurred with a concomitant appearance of several active lipase bands in the range of 46–86 kDa. The extent of this proteolytic degradation decreased with shorter separation times on the Sephadex column, but for larger scale isolations no conditions of pH or absence or presence of metal ions, etc., could be found that completely stopped this degradation with retention of lipase activity (data not shown). In order to facilitate the isolation of larger amounts of a homogeneous lipase, the preparation obtained after the Sephadex step was incubated with various proteolytic enzymes. Trypsin, papain, staphylococcus V8 protease, thermolysin, and chymotrypsin all yielded bands with molecular masses, between 46 and 50 kDa (data not shown). In further studies we have used tryptic digestion since it yielded a single active band and the enzymatic activity of the product was stable over a long period of time. In fact, tryptic digestion resulted in a 2-fold increase in lipase activity. The 46-kDa lipase was purified to homogeneity by using two DEAE-cellulose columns developed at pH 8.0 and 6.5, respectively (Table I). As the protein was purified, its stability toward lyophilization decreased. For this reason, pure preparations were concentrated by lyophilization to 0.2–0.1 times the initial volume.

The amino-terminal sequences of the 86-kDa form and the 46-kDa forms were determined by automated Edman degradation on an Applied Biosystem gas-phase sequenator equipped with an on-line PTH-amino acid analyzer, using the 03 RPTH program. For the 86-kDa form the following sequence was found: NH_2-Asn-Asp-Ser-Thr-Thr-Gln-Thr-Thr-Thr-Pro-.

Table I: Flow Sheet for Purification of *S. hyicus* Lipase

purification step	protein[a] (mg)	activity (μmol/min)	sp act. [μmol/(min·mg)]	purification (x-fold)
ammonium sulfate precipitate		3967		
Sephadex G-100/G-25	97.7	3875	40	1
proteolytic breakdown	97.7	7972	80	1
DEAE, pH 8.0	12.5	6043	483	12.1
DEAE, pH 6.5	11.2	5856	520	13.1

[a] Based on $E_{280nm}^{1\%} = 11$.

1	M	K	E	T	K	H	Q	H	T	F	S	I	R	K	S	A	Y	G	A	A	S	V	M
24	V	A	S	C	I	F	V	I	G	G	G	V	A	E	A	N	D	S	T	T	Q	T	T
47	T	P	L	E	V	A	Q	T	S	Q	Q	E	T	H	T	H	Q	T	P	V	T	S	L
70	H	T	A	T	P	E	H	V	D	D	S	K	E	A	T	P	L	P	E	K	A	E	S
93	P	K	T	E	V	T	V	Q	P	S	S	H	T	Q	E	V	P	A	L	H	K	K	T
116	Q	Q	Q	P	A	Y	K	D	K	T	V	P	E	S	T	I	A	S	K	S	V	E	S
139	N	K	A	T	E	N	E	M	S	P	V	E	H	H	A	S	N	V	E	K	R	E	D
162	R	L	E	T	N	E	T	T	P	P	S	V	D	R	E	F	S	H	K	I	I	N	N
185	T	H	V	N	P	K	T	D	G	Q	T	N	V	N	V	D	T	K	T	I	D	T	V
208	S	P	K	D	D	R	I	D	T	A	Q	P	K	Q	V	D	V	P	K	E	N	T	T
231	A	Q	N	K	F	T	S	Q	A	S	D	K	K	P	T	V	K	A	A	P	E	A	V
254	Q	N	P	E	N	P	K	N	K	D	P	F	V	F	V	H	G	F	T	G	F	V	G
277	E	V	A	A	K	G	E	N	H	W	G	G	T	K	A	N	L	R	N	H	L	R	K
300	A	G	Y	E	T	Y	E	A	S	V	S	A	L	A	S	N	H	E	R	A	V	E	L
323	Y	Y	Y	L	K	G	G	R	V	D	Y	G	A	A	H	S	E	K	Y	G	H	E	R
346	Y	G	K	T	Y	E	G	V	L	K	D	W	K	P	G	H	P	V	H	F	I	G	H
369	S	M	G	G	Q	T	I	R	L	L	E	H	Y	L	R	F	G	D	K	A	E	I	A
392	Y	Q	Q	Q	H	G	G	I	I	S	E	L	F	K	G	G	Q	D	N	M	V	T	S
415	I	T	T	I	A	T	P	H	N	G	T	H	A	S	D	D	I	G	N	T	P	T	I
438	R	N	I	L	Y	S	F	A	Q	M	S	S	H	L	G	T	I	D	F	G	M	D	H
461	W	G	F	K	R	K	D	G	E	S	L	T	D	Y	N	K	R	I	A	E	S	K	I
484	W	D	S	E	D	T	G	L	Y	D	L	T	R	E	G	A	E	K	I	N	Q	K	T
507	E	L	N	P	N	I	Y	Y	K	T	Y	T	G	V	A	T	H	E	T	Q	L	G	K
530	H	I	A	D	L	G	M	E	F	T	K	I	L	T	G	N	Y	I	G	S	V	D	D
553	I	L	W	R	P	N	D	G	L	V	S	E	I	S	S	Q	H	P	S	D	E	K	N
576	I	S	V	D	E	N	S	E	L	H	K	G	T	W	Q	V	M	P	T	M	K	G	W
599	D	H	S	D	F	I	G	N	D	A	L	D	T	K	H	S	A	I	E	L	T	N	F
622	Y	H	S	I	S	Y	L	M	R	I	E	K	A	E	S	T	K	N	A				

FIGURE 1: Deduced amino acid sequence of the *S. hyicus* lipase and the cleavage sites of the signal peptidase, of an unidentified staphylococcal endoprotease, and of trypsin. For details see text.

This sequence corresponds with the sequence around the proposed (Götz et al., 1985) cleavage site of the signal peptidase between residues Ala-38 and Asn-39 of the preproenzyme (Figure 1). The amino-terminal sequence of the 46-kDa form that was isolated (data not shown) from *S. hyicus* supernatant was NH_2-Val-Lys-Ala-Ala-Pro-Glu- (Figure 1). This shows that in the culture supernatant of this organism an endopeptidase occurs that cleaves between Thr-245 and Val-246. In *S. carnosus*, the expression host, no such cleavage occurs, but as mentioned above, several proteases yield 46-kDa cleavage products. We determined the amino-terminal sequence of the trypsin cleavage product as NH_2-Ala-Ala-Pro-Glu-Ala-Val-, indicating that cleavage had occurred between residues Lys-247 and Ala-248 (Figure 1). The calculated molecular weight of the 86-kDa form (603 amino acids) is thus 67 406 and that of the 46-kDa form (394 amino acids) is 44 263.

Calcium Dependence and pH Profile. Since *S. hyicus* lipase was dependent on Ca^{2+} ions for its enzymatic activity, the role of calcium was further investigated. As shown in Figure 2, a saturation curve was obtained from which a dissociation constant of about 25 μM was calculated. It should be noted that in the presence of EDTA or EGTA a basal activity of 2.5% was found. Only Sr^{2+} ions were found to be able to replace Ca^{2+} ions with full retention of activity. Addition of other divalent ions such as Mg^{2+}, Ba^{2+}, Cu^{2+}, Cd^{2+}, etc. or

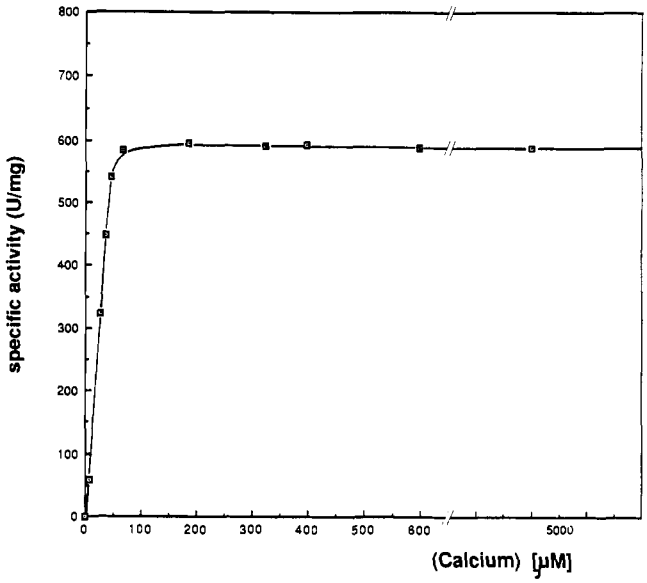


FIGURE 2: Activity of *S. hyicus* lipase as a function of the calcium concentration measured with 67 mM tributyrin. Conditions: 5 mM Tris-HCl, pH 8.5 at 25 °C.

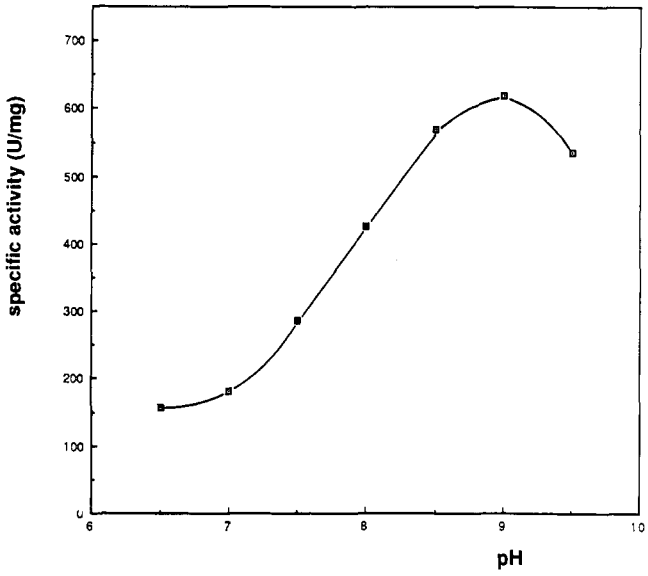


FIGURE 3: pH dependence of tributyrin hydrolysis by *S. hyicus* lipase. Conditions: 5 mM Pipes (pH 6–7.5), 5 mM Tris-HCl (pH 7.5–9), 10 mM $CaCl_2$, tributyrin concentration 67 mM, 25 °C.

trivalent ions such as Eu^{3+} or Gd^{3+} to the enzyme in the presence of traces of EDTA further lowered the activity to levels below 0.5%. Whether these ions compete for the same binding site as Ca^{2+} or Sr^{2+} ions was not tested.

The *S. hyicus* lipase activity was strongly dependent on pH (Figure 3). No activities below pH 6 could be tested since the enzyme was unstable at low pH and irreversibly lost activity. The enzyme has optimal activity around pH 9.

Activity on Neutral Lipids. Triglycerides containing short-chain fatty acids are sparingly soluble in water. Above a certain concentration, substrate droplets are formed. These droplets are the preferred substrate for porcine pancreatic lipase, and this property clearly distinguishes this lipolytic enzyme from other esterases (Sarda & Desnuelle, 1958). The dependence of the *S. hyicus* lipase activity on tributyrin concentrations is shown in Figure 4. At tributyrin concentrations up to 400 μM the enzyme acts on substrate monomers with a relatively low maximal activity of 100 units/mg. At the solubility limit a break in the activity profile was observed,

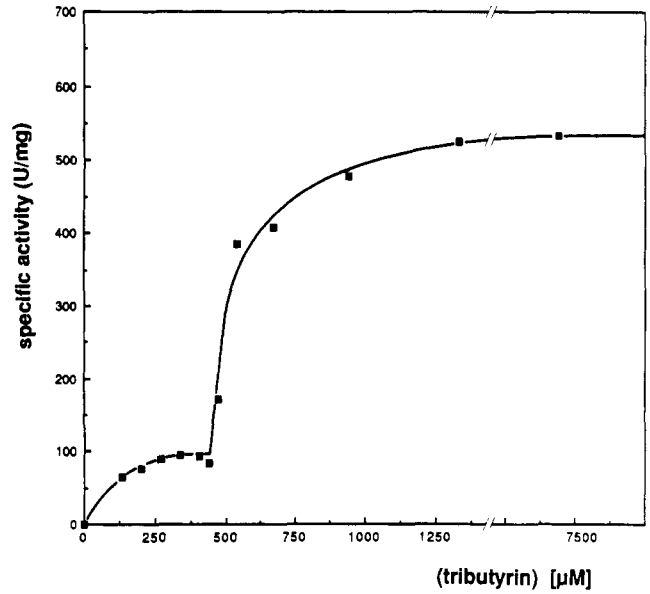


FIGURE 4: Activity of *S. hyicus* lipase as a function of the tributyrin concentration. Conditions: 5 mM Tris-HCl, pH 8.5, 10 mM $CaCl_2$, 25 °C.

and at high substrate concentration, the activity rose to about 520 units/mg. The activity of *S. hyicus* lipase was also tested on other neutral lipids. By use of triolein as a substrate in the presence of deoxycholate or arabic gum to obtain dispersions, a much lower activity of about 60 units/mg was found.

Specificity of S. hyicus and Pancreatic Lipase. Initial experiments suggested that *S. hyicus* lipase hydrolyzed natural triglycerides to glycerol and fatty acids. Therefore, the activity of this enzyme was further investigated by using synthetic lipids with a well-defined structure and fatty acid distribution. The release of fatty acids was followed with the aid of GLC. *S. hyicus* lipase and porcine pancreatic lipase (PPL) were used for comparison. The activity of both lipases was tested on the following substrates: *rac*-1-lauroyl-2-myristoyl-3-palmitoyl-glycerol (I), *rac*-1-palmitoyl-2-elaidoyl-4-myristoylbutane-1,2,4-triol (II), and triheptanoyltris(hydroxymethyl)methane (III). As expected, PPL released only the fatty acids linked via primary ester bonds from compounds I and II. The enzyme from *S. hyicus* rapidly released all fatty acids from both compounds without a significant preference for a given position (data not shown). Compound III, containing three short-chain fatty acids linked to primary hydroxyl groups, was a good substrate for both lipases, which completely hydrolyzed this compound to free fatty acid and tris(hydroxymethyl)methane. Thus *S. hyicus* lipase is a very aspecific lipase.

Activity on Phospholipids. Since it is known that some lipases are able to slowly hydrolyze phospholipids, several lecithins and lecithin analogues were tested as substrates for *S. hyicus* lipase. In these experiments porcine pancreatic phospholipase A_2 (PLA_2) was included for comparison. In Figure 5 the dependence of the enzymatic activity on the substrate concentration is plotted for *S. hyicus* lipase and PLA_2 with 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine as substrate. The expected activity profile for PLA_2 action (Figure 5, lower curve) was found: a low activity at submicellar concentrations and a sharp rise in activity upon passing the critical micelle concentration (cmc) of this lipid. The upper curve in Figure 5 shows that *S. hyicus* lipase had high enzymatic activity on this substrate but without a jump in activity around the cmc.

In Figure 6 the V_{max} values of *S. hyicus* lipase and PLA_2 on a series of short-chain lecithins are represented as a function of the acyl chain length. Both the natural L-isomers and the

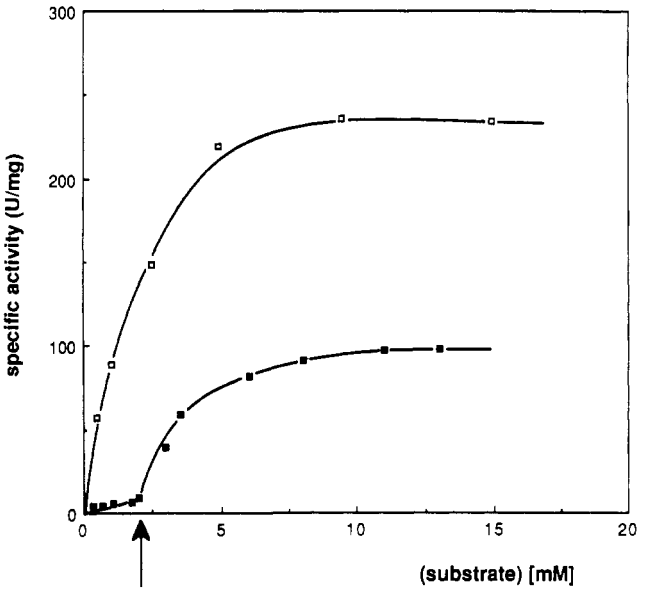


FIGURE 5: Activity of *S. hyicus* lipase (□) and porcine pancreatic PLA_2 (■) as a function of the 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine concentration. The critical micellar concentration is indicated by an arrow.

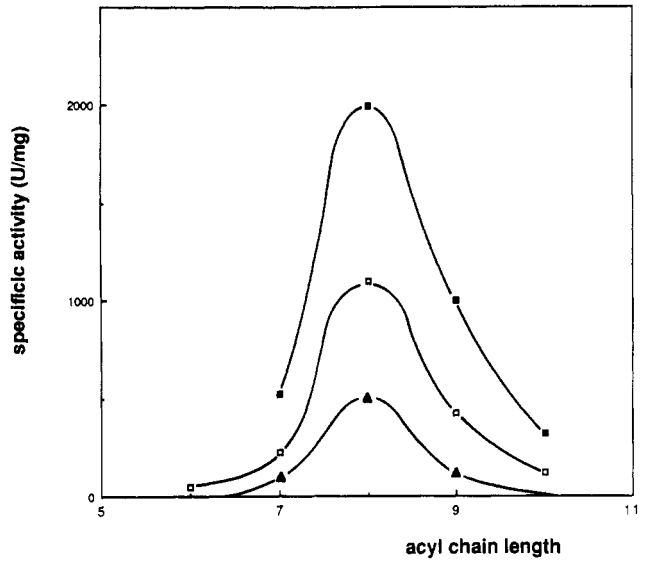


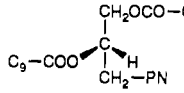
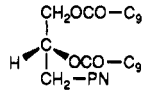
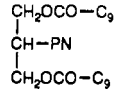
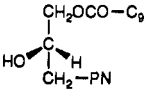
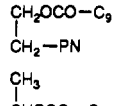
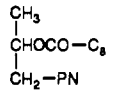
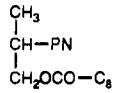
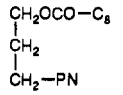
FIGURE 6: Specific activity of *S. hyicus* lipase on L-α-lecithins (□) and D-α-lecithins (■) and specific activity of porcine pancreatic phospholipase on L-α-lecithins (▲) as a function of acyl chain length. Conditions: 5 mM Tris-HCl, pH 8.5, 10 mM $CaCl_2$, 25 °C. Maximal activities were determined separately as shown in Figure 4.

D-isomers were tested. In agreement with the known specificity of PLA_2, this enzyme only hydrolyzed the L-isomers with a clear preference for dioctanoyl lecithin. For the *S. hyicus* enzyme the dioctanoyl lecithin also appeared to be the optimal substrate. Surprisingly, it was found that not only L-lecithins (Figure 6, middle curve) were hydrolyzed, but also D-lecithins (Figure 6, upper curve) were hydrolyzed and even more efficiently than the L-lecithins. Remarkably, the specific activity of *S. hyicus* lipase on the D-lecithins was about twice as high as on L-lecithins independent of the acyl chain length. Lecithins with acyl chains shorter than C_6 or longer than C_{10} were poor substrates. This is probably due to the fact that short-chain ($<C_6$) lecithins are too soluble to form micelles and that lecithins containing longer acyl chains ($>C_9$) form bilayer structures in the absence of detergents.

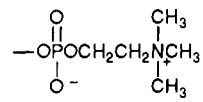
The positional specificity of *S. hyicus* lipase was investigated by GLC analysis of the fatty acids released from the positional isomeric lecithins: 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-myristoyl-*sn*-glycero-3-phosphocholine. The curve for the former substrate is shown in Figure 7, but an indistinguishable picture was obtained with the latter compound. Both fatty acids were released at the same rate. One explanation might be that the lysolecithin was hydrolyzed at a much higher rate than the lecithin itself. A second possibility is that the enzyme has no preference for primary or secondary ester bonds.

In order to test the first possibility, the activity of the enzyme on mono- and diacylglycerophosphocholine analogues was tested. The results are shown in Table II. Since the diacyl lecithins **1–3** form bilayer-like structures in water, whereas the lysolecithin analogues **4–8** yield micellar solutions, the activity of *S. hyicus* lipase on these substrates was measured both in the presence and in the absence of sodium deoxycholate. For comparison, the activity of PLA_2 on the same phospholipids was tested. It was evident that for *S. hyicus* lipase mixed micelles of lecithins **1–3** and deoxycholate were better substrates than the bilayer form of these pure lecithins. On lysolecithin analogues **4–8** which form micelles by themselves, *S. hyicus* lipase displayed high activity. Addition of deoxycholate, which induces the formation of mixed micelles, reduced the activity. The β-lecithin **3**, containing two primary

Table II: Activity of *S. hyicus* Lipase and Pancreatic Phospholipase A_2 on Various (Lyso)lecithins and (Lyso)lecithin Analogues Containing Decanoic Acid (Compounds **1–5**) or Nonanoic Ester Bonds (Compound **6–8**)[a]

compd	structure	V_{max} (units/mg) *S. hyicus* lipase −DOC	*S. hyicus* lipase +DOC	PLA_2 +DOC
1		110	220	68
2		220	460	0
3		300	740	1
4		547	244	0
5		1712	520	<1
6		200	98	8
7		1050	611	<1
8		660	274	0

[a] Conditions: T = 25 °C, 5 mM Tris-HCl, pH 8.5, 10 mM $CaCl_2$. −DOC = activity measured in the absence of deoxycholate. +DOC = activity measured in equimolar amounts of deoxycholate. −PN stands for



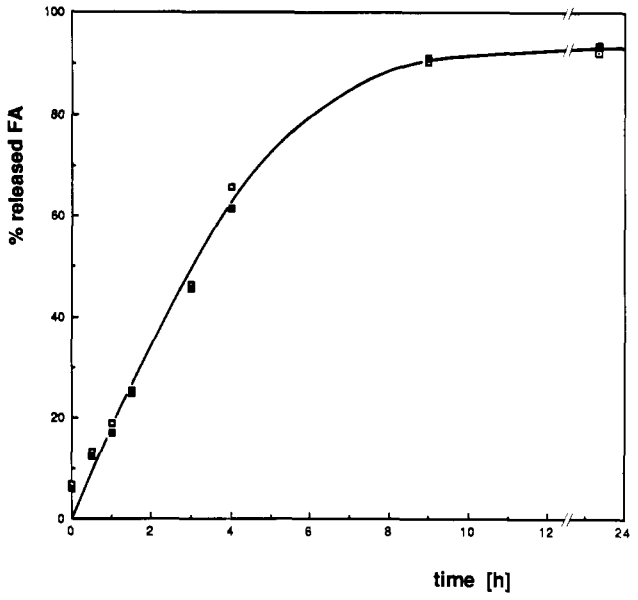


FIGURE 7: Percentage fatty acid release as a function of time by *S. hyicus* "lipase" action on 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine. Fatty acids were determined by GLC. (□) Myristic acid; (■) palmitic acid. Conditions: 100 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 25 °C.

ester bonds, was a much better substrate for *S. hyicus* lipase than the naturally occurring isomeric α-lecithins **1** and **2**. This was in sharp contrast with the behavior of PLA_2 on these substrates. Comparison of the substrate properties of compounds **4**–**8**, all lysolecithin analogues, showed that *S. hyicus* lipase prefered a primary ester bond adjacent to the C–O–PN linkage (compounds **5** and **7**). Good activity was still found for compounds **4** and **8** containing a primary ester bond but not adjacent to the C–O–PN linkage. However, if a secondary ester bond was present, even adjacent to the C–O–PN linkage (compound **6**), the enzymatic activity further dropped. These effects were seen both in the presence and in the absence of deoxycholate.

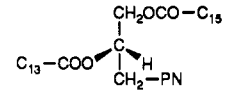
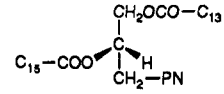
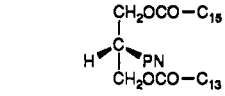
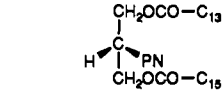
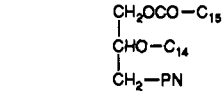
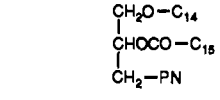
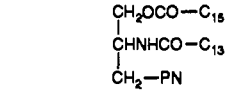
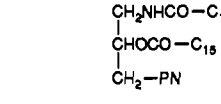
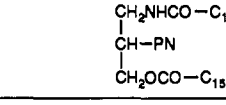
The second possibility, i.e., a lack of preference for primary or secondary ester bonds, was investigated by comparing the activity on several isomeric diacyl phospholipid analogues containing one amide or ether bond and with lecithins containing two ester bonds. The degradation of these long-chain lipids was carried out in the presence of equimolar amounts of deoxycholate. The results are summarized in Table III. *S. hyicus* lipase degraded the isomeric compounds **9** and **10** at comparable rates, showed that the enzyme had no clear preference for the length of the acyl chain. As was seen for the short-chain lecithins (Table II), the long-chain β-lecithins were hydrolyzed faster than the α-lecithins. All compounds containing primary ester bonds were good substrates. In contrast, compounds **14** and **16** with a secondary ester bond and a nonhydrolyzable group at the 1-position were poor substrates. This showed that the *S. hyicus* lipase has a high phospholipase A_1 activity and only low (<1%) phospholipase A_2 activity.

S. hyicus lipase rapidly hydrolyzed lecithins to free fatty acids and glycerophosphocholine. The data of Tables II and III indicate that the degradation of lecithins proceeds via removal of the first fatty acid by a phospholipase A_1 action followed by a rapid hydrolysis of the intermediate 2-acyl lysolecithin.

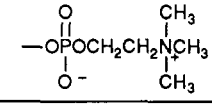
Discussion

The lipase which is secreted into the culture medium by *S. hyicus* has an apparent molecular mass of about 46 kDa. The gene of *S. hyicus* lipase has been cloned in *S. carnosus*, and from the latter organism the enzyme is secreted with an apparent molecular mass of about 86 kDa. The sequence of the *S. hyicus* lipase was predicted from the sequence of its cloned gene (Götz et al., 1985). A putative signal sequence of 38 amino acids and a lipase sequence comprising 603 amino acids corresponding to a theoretical molecular mass of 71 kDa were determined. This study illustrates that the protein was processed in *S. carnosus* at the proposed signal peptide cleavage site. It furthermore shows that the correct molecular mass of this protein was not obtained from SDS gels. In *S. hyicus* but not in *S. carnosus*, the protein is then rapidly further processed to the 46-kDa form during or after export to the culture medium. We have shown that trypsin cleaved at a site that is near the *S. hyicus* cleavage site. The shorter protein was twice as active as the 86-kDa form, resulting in a 3-fold higher specific activity; it contained 420 amino acids and its calculated molecular mass was close to the experimental 46-kDa value. The molecular mass of *S. hyicus* lipase (46 kDa) falls well within the range that is generally reported for lipases

Table III: Maximal Activities for *S. hyicus* Lipase and Porcine Pancreatic Phospholipase A_2 on Several Lecithins and Lecithin Analogs, All Containing One Palmitic and One Myristic Acid Acyl Chain[a]

compd	substrate	*S. hyicus* lipase [μmol/(min·mg)]	PLA [μmol/(min·mg)]
9		101	310
10		112	250
11		362	4.8
12		337	2.7
13		140	0
14		<1	27
15		127	0
16		<1	3.2
17		191	<1

[a] Conditions: T = 25 °C, 5 mM Tris-HCl, pH 8.5, 10 mM $CaCl_2$. All activities were measured in the presence of equimolar amounts of deoxycholate. -PN stands for



S.H.	F	V	F	V	H	G	F	F	I	G	H	S	M	G	G
P.F.	I	L	L	V	H	G	L	L	I	G	H	S	Q	G	A
M.L.P.	F	V	V	I	H	G	W	L	L	G	Y	S	L	G	A
A.E.	V	L	L	L	H	G	Y	V	I	G	H	S	M	G	S
P.P.L.	R	F	L	I	H	G	F	V	I	G	H	S	L	G	S
H.L.	V	M	I	I	H	G	W	F	A	G	S	S	I	G	G
H.G.	V	F	L	Q	H	G	L	Y	V	G	H	S	Q	G	T
H.S.L.	V	V	H	I	H	G	G	L	A	G	D	S	A	G	G

FIGURE 8: Amino acid sequence containing the active site histidine and serine of several lipases from different sources. S.H. (*S. hyicus* lipase): residues 90–196 and 190–197, Götz et al. (1985). P.F. (*Pseudomonas fragi* lipase): residues 12–18 and 80–87, Kugimuya et al. (1986). M.L.P. (mouse lipoprotein lipase): residues 38–45 and 107–113, Wion et al. (1987). A.E. (*Pseudomonas putida* atropinesterase): residues 50–56 and 129–136, Hessing (1983). P.P.L. (porcine pancreatic lipase): residues 71–77 and 148–155, de Caro et al. (1981). H.L. (human postheparin plasma hepatic lipase): residues 62–68 and 154–161, Martin et al. (1988). H.G. (human gastric lipase): residues 62–68 and 150–157, Bodmer et al. (1987). H.S.L. (hormone-sensitive lipase): residues 345–351 and 419–426, Holm et al. (1988).

from prokarytoic and eukaryotic sources.

Like most of the lipases, the *S. hyicus* lipase had a basic pH optimum. Only lipases isolated from lingual or gastric tissue have an acidic pH optimum (Verger et al., 1986). Calcium ions greatly enhanced the activity of *S. hyicus* lipase whereas most lipases do not need calcium as a cofactor. Whether this Ca^{2+} is involved in the catalytic site of *S. hyicus* lipase remains to be determined, but we propose that Ca^{2+} ions have a structural role rather than being involved in catalysis. This is supported by the observation that activity in the presence of EDTA or EGTA never dropped to zero but remained at a value of 2–3%. In addition, Sr^{2+} ions could replace Ca^{2+} ions to yield a fully active enzyme. A sequence comparison of the *S. hyicus* lipase with lipases from several sources indicated two conserved domains which may be part of the active site for these lipases. Most striking was the presence of conserved sequences surrounding a histidine and a serine residue (Figure 8). Despite the fact that little consistant data on the modification of active site residues in lipases are available, this sequence homology suggests an important role for these two residues.

Lipases are secreted by several staphylococci, and their activity on a wide range of neutral substrates including Tweens and tri-, di-, and monoglycerides has been reported (Götz et al., 1985; Muruoka et al., 1982; Rollof et al., 1987). *S. hyicus* lipase also hydrolyzed these substrates, but it also attacked phospholipids, and in many cases the phospholipase activity was considerably higher than the lipase activity. This distinguishes the *S. hyicus* enzyme from lipases from various sources where the phospholipase activity in general is 5–100 times lower than the lipase activity (de Haas et al., 1968; Durand et al., 1978; Fauvel et al., 1981). Despite its aspecific character permitting the hydrolysis of both primary and secondary ester bonds in compounds of a large structural variety, the enzyme should be considered as a true lipolytic enzyme. This is illustrated by Figure 4, from which it can be concluded that the enzyme was more active on triglyceride emulsions than on monomolecularly dispersed molecules of this substrate. Such a definition of lipolytic enzymes has been introduced by Sarda and Desnuelle (1958). A break in the activity profile did not occur with diheptanoyl lecithin as a substrate (Figure 5), but this does not necessarily exclude an activation of *S. hyicus* lipase by micelles. A similar smooth progressive curve has been reported for PLA_2 from *Naja melanoleuca* venom, and the high activity of this enzyme was explained by the authors as a high capacity of this enzyme to form lipid–protein aggregates via a comicellization pathway at concentrations well below the cmc (van Eijk et al., 1983). We suppose that *S. hyicus* lipase also has a high affinity for monomers of diheptanoyl lecithin and that binding of a monomer initiates a comicellization pathway similar to that described for the venom enzyme. The affinity of the enzyme for dissolved single tributyrin molecules apparently is insufficient to start such a complex formation below the solubility limit of this substrate. A high molecular weight lipid–protein complex is only formed if triglyceride droplets are already present.

When the *S. hyicus* enzyme degraded mixed-acid lecithins, both fatty acids were released with the same speed (Figure 7). At least three explanations are possible for this remarkable phenomenon. First, it could be that the enzyme has no preference for either of the two acyl ester bonds and thus is equally active as a phospholipase A_1 and as a phospholipase A_2. We do not believe that this explanation is very likely since it conflicts with the results obtained with phospholipid analogues carrying a nonhydrolyzable bond at the 1-position (i.e., compounds **14** and **16**, Table III) compared to those containing a blocked 2-position (compounds **13** and **15**). These data suggested that the phospholipase A_1 activity is about 100 times higher than the phospholipase A_2 activity. A second explanation could be a preference of the enzyme for lysophospholipids over phospholipids. Inspection of the data in Table II shows that in the absence of deoxycholate, where diacyl phospholipids form bilayer structures, the enzyme had a preference for micellar aggregates of lysophospholipids. In the presence of deoxycholate, where both lysophospholipids and diacyl phospholipids are present in mixed micelles, this preference disappeared, suggesting that in the absence of deoxycholate the difference in rates is mainly caused by physicochemical factors. Because the enzyme has a 100 times higher phospholipase A_1 than A_2 activity the first product to be formed will be the 2-acyl lysophospholipid. Because the acyl chain in the 2-acyl lysophospholipid can migrate to the primary ester position, kinetic results obtained with this substrate could be due to the presence of either isomer in the resulting mixture. For this reason compound **6**, a structural analogue of the 2-acyl lysolecithin where no acyl migration can take place, was used instead. This compound was a relatively poor substrate. We therefore conclude that the results presented in Figure 7 cannot be explained on the basis of sustrate specificity. A third explanation for the simultaneous release of two fatty acids is that, after release of the first fatty acid, the resulting lyso compound remains bound to the active site rather than being released. A subsequent small change in the orientation might then be sufficient to bring about hydrolysis of the second acyl ester bond. Although this is an attractive assumption, it might conflict with a possible involvement of serine (Figure 8) in catalysis since it is generally accepted that, in serine esterases, the first product to be released is the alcohol. A more definitive conclusion must therefore await further data on the amino acids involved in catalysis.

A remarkable property of this enzyme is its preference for α-lecithins of the D-configuration over the natural L-configuration (Figure 6 and Table II). The enzyme also shows high activity on β-lecithins. PLA_2 from pancreas or snake venom shows high stereospecificity and positional specificity. This specificity has been explained (de Haas et al., 1968) by assuming that these enzymes very efficiently fix and orient the

scissle ester bond, one acyl chain, and the phosphate group. Our data obtained with *S. hyicus* lipase indicate that binding of substrate in the active site of this enzyme was not as strictly defined as in the case of PLA_2. The fact that tri-, di-, and monoglycerides as well as many analogues of phospholipids were hydrolyzed efficiently suggests that, for substrate binding, interaction of one acyl chain and the scissle ester bond with the active center is sufficient. Apparently, binding of D-phospholipids and/or D-lysophospholipids is sterically less hindered than that of L-(lyso)phospholipids. The question then arises whether this enzyme is an aspecific esterase or a (phospho)lipase. On the basis of the high activity on aggregated substrates we believe that this enzyme must be considered as a lipolytic enzyme. This conclusion is particularly based on the increase that was observed in the rate of hydrolysis of tributyrin upon passing the solubility limits.

ACKNOWLEDGMENTS

We thank Drs. Karen Brune and Peter Thomas for critically reading and correcting the manuscript.

Registry No. **1**, 3436-44-0; **2**, 63700-87-8; **3**, 61444-33-5; **4**, 22248-63-1; **5**, 99165-85-2; **6**, 123330-39-2; **7**, 123330-40-5; **8**, 123330-41-6; **9**, 69441-09-4; **10**, 69525-80-0; **11**, 121382-71-6; **12**, 121382-72-7; **13**, 123330-42-7; **14**, 123330-43-8; **15**, 123357-70-0; **16**, 123357-71-1; **17**, 123357-72-2; Sr, 7440-24-6; Ca, 7440-70-2; lipase, 9001-62-1; phospholipase A, 9001-84-7; lysophospholipase, 9001-85-8; prolipase (*Staphylococcus hyicus* reduced), 98726-84-2; preprolipase (*Staphylococcus hyicus* reduced), 98726-85-3; lipase (*Staphylococcus hyicus* reduced), 123411-16-5; 3-395 lipase (*Staphylococcus hyicus* reduced), 123411-17-6.

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