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Probing Enzyme Promiscuity of SGNH Hydrolases

Ivana Leščić Ašler,[a] Nives Ivić,[a] Filip Kovačić,[b] Sabrina Schell,[b] Janina Knorr,[b] Ulrich Krauss,[b] Susanne Wilhelm,[b] Biserka Kojić-Prodić,*[a] and Karl-Erich Jaeger[b]

Several hydrolases of the SGNH superfamily, including the lipase SrLip from *Streptomyces rimosus* (Q93MW7), the acyl-CoA thioesterase I TesA from *Pseudomonas aeruginosa* (Q9HZY8) and the two lipolytic enzymes EstA (from *P. aeruginosa*, O33407) and EstP (from *Pseudomonas putida*, Q88QS0), were examined for promiscuity. These enzymes were tested against four chemically different classes of a total of 34 substrates known to be hydrolysed by esterases, thioesterases, lipases, phospholipases, Tweenases and proteases. Furthermore, they were also analysed with respect to their amino acid sequences and structural homology, and their phylogenetic relationship was determined. The *Pseudomonas* esterases EstA and EstP each have an N-terminal domain with catalytic activity together with a C-terminal autotransporter domain, and so the hybrid enzymes $EstA_N$–$EstP_C$ and $EstP_N$–$EstA_C$ were constructed by swapping the corresponding N- and C-terminal domains, and their hydrolytic activities were compared. Interestingly, substrate specificity and kinetic measurements indicated a significant influence of the autotransporter domains on the catalytic activities of these enzymes in solution. TesA, EstA and EstP were shown to function as esterases with different affinities and catalytic efficacies towards *p*-nitrophenyl butyrate. Of all the enzymes tested, only SrLip revealed lipase, phospholipase, esterase, thioesterase and Tweenase activities.

Introduction

During the last decade, the phenomenon of enzyme promiscuity was discovered to challenge a central dogma of life sciences: the assumption that one protein usually exerts one function. Promiscuity associated with a multitude of functions and alternative activities of a particular protein is therefore of great importance for understanding life processes. Promiscuity, initially treated as an error in biological function, turned out to be one of Nature's approaches to the survival of living species and consequently their evolution.[1–3] Evolutionary, structural, mechanistic, physiological and biotechnological aspects of protein promiscuity have recently been reviewed.[1–5]

There are several definitions for the term *enzyme promiscuity*,[6–8] and at present three major types are distinguished:[7] 1) *enzyme condition promiscuity* for enzymes operating under various reaction conditions different from their natural ones, such as temperature, pH or non-aqueous media, 2) *enzyme substrate promiscuity* for enzymes with broad or relaxed substrate specificities, and 3) *enzyme catalytic promiscuity* for enzymes that catalyse distinct chemical transformations accompanied by different transition states.

The SGNH hydrolase family was recognised 15 years ago,[9] but still remains poorly characterised. From the conserved active site residues found localised in four blocks, the name SGNH hydrolases was proposed (block I–catalytic serine, block II–oxyanion hole glycine, block III–oxyanion hole asparagine, block V–catalytic histidine; the catalytic aspartate in block V is not entirely conserved throughout the family and so is not included in the family name).[10] By January 2010, 7582 protein sequences had been deposited in the InterPro database[11] and 29 structures of 13 different SGNH hydrolases could be found in the Protein Data Bank.[12] The SGNH family reveals poor overall sequence similarity, but the limited number of known 3D structures revealed significant structural homology.[10,13–15] A comprehensive review on SGNH hydrolases[16] referred to a diverse range of hydrolytic functions including lipase, protease, thioesterase, arylesterase, lysophospholipase, carbohydrate esterase and acyltransferase activities. These enzymes display broad substrate-specificities and regio- and enantiospecificities. They are involved in bacterial virulence, plant development and morphogenesis, and in plant defence mechanisms. An assumption that SGNH hydrolases are multifunctional enzymes with flexible binding sites was based almost exclusively on experimental data obtained with the protease I/thioesterase I/lysophospholipase L_1 from *Escherichia coli* (TAP).[14] Only limited experimental data on the substrate-specificities of other SGNH superfamily enzymes are available.[10,16]

This prompted us to test selected enzymes of the SGNH hydrolase family with regard to enzyme substrate promiscuity.[13,17–19] We also address a special type of multifunctionality involving two-domain proteins[1,2,20] through a study of two autotransporter esterases: namely the well-characterized enzyme EstA from *Pseudomonas aeruginosa* (EstA)[21–25] and the novel enzyme EstP from *P. putida* (EstP). These two-domain proteins

[a] *Dr. I. Leščić Ašler, N. Ivić, Dr. B. Kojić-Prodić*
Department of Physical Chemistry, Rudjer Bošković Institute
POB 180, 10002 Zagreb (Croatia)
Fax: (+385) 1-4680245
E-mail: kojic@irb.hr

[b] *Dr. F. Kovačić, Dr. S. Schell, Dr. J. Knorr, Dr. U. Krauss, Dr. S. Wilhelm, Prof. Dr. K.-E. Jaeger*
Institute of Molecular Enzyme Technology
Heinrich-Heine-University Düsseldorf, Research Centre Jülich

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each possess an N-terminal passenger domain harbouring the hydrolytic function together with a C-terminal β-barrel domain that functions as an autotransporter.[23–25]

Results and Discussion

Sequence homology, phylogenetic analysis and structural homology of tested enzymes

A multiple sequence alignment of selected bacterial GDSL esterase/lipase sequences (see Table S1 in the Supporting Information and the Experimental Section for details) revealed a low overall sequence similarity (data not shown). Figure 1 shows parts of the corresponding alignment, highlighting conserved sequence motifs characteristic of GDSL esterases/lipases of bacterial origin (clades I and II according to Akoh et al.[16]). Four blocks of homology (labelled I, II, III and V in Figure 1) can be identified, each containing a catalytically important amino acid (serine, glycine, asparagine, and histidine).[9] In contrast to Akoh et al.,[16] here we show the presence of all typical sequence motifs (blocks I, II, III and V) of the SGNH superfamily in all enzyme sequences examined.

Akoh et al.[16] have suggested that only clade I, and not clade II, contains all four motifs, thus limiting the SGNH superfamily of enzymes to clade I. We show, however, that both clade I and clade II sequences contain the appropriate sequence blocks and conserved residues. Upon closer inspection of the alignment, the presence of an inserted additional sequence block (block IIIa in Figure 1) characteristic for clade II sequences and absent in all clade I sequences can be detected. In order to evaluate whether the previously suggested separation of the clade I and clade II sequences[16] is still valid, phylogenetic tree computations were carried out as described in the Experimental Section.

The resulting phylogenetic tree (Figure 2) shows a clear separation into clade I and clade II sequences as suggested previously.[16] Bootstrap support is sufficiently high on most of the branches. The two autotransporter esterases (EstA and EstP) are grouped together with other two-domain autotransporter proteins in the lower part of the tree (clade II). In the upper part of the tree, enzymes such as the acyl-CoA thioesterase TesA of *P. aeruginosa*, as well as the corresponding enzyme of *E. coli* (TesA, (TAP) Uni*P*rot no. P0ADA1), are to be found in clade I. The fourth enzyme of this study—the lipase SrLip of *S. rimosus*—is found outside clade I as an early diverging branch

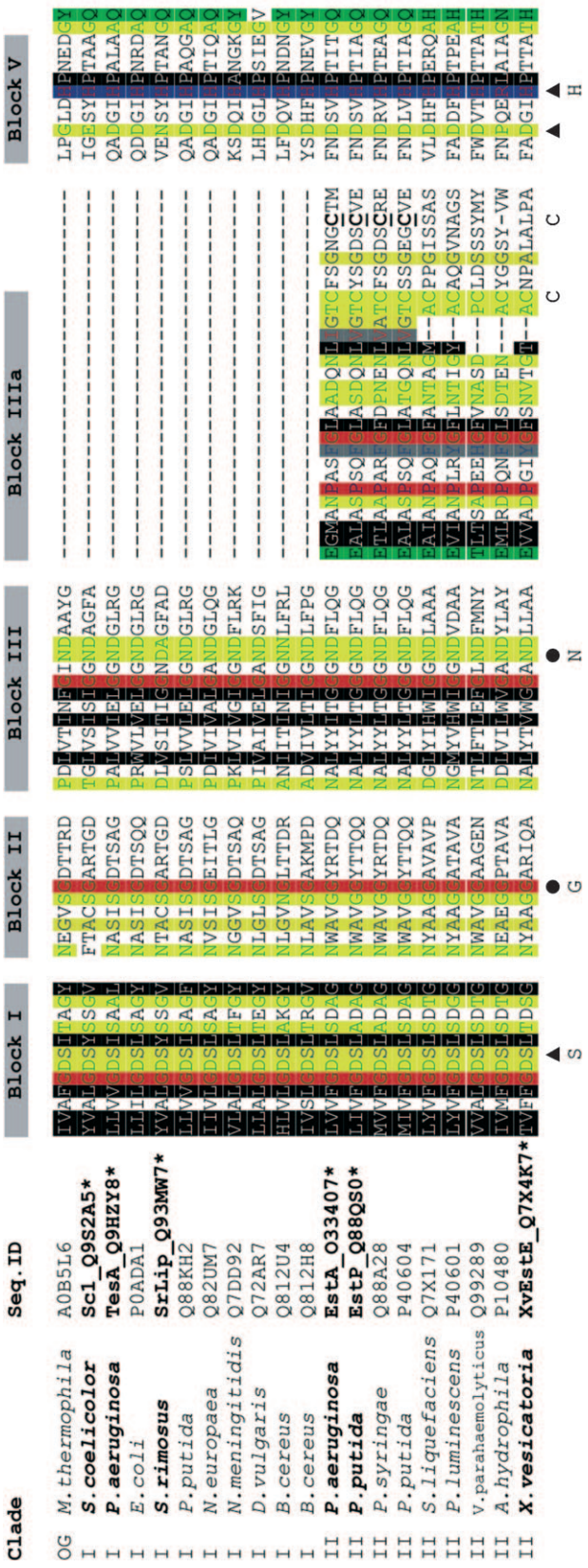


Figure 1. Multiple sequence alignment of selected bacterial GDSL esterase/lipase family sequences. Residues are coloured according to their physicochemical properties by using the GeneDoc[26] default colouring scheme. The four enzymes investigated in this study (EstA, EstP, SrLip1, TesA) as well as the three bacterial GDSL esterases/lipases (TesA-P0ADA1, Sc1 and XvEstE), for which substrate spectra are available in the literature, are highlighted in bold and marked by asterisks. The sequence blocks I, II, III and V previously suggested to be characteristic for enzymes of the SGNH family can readily be identified in all sequences. ▲ marks the residues of the catalytic triad and ● highlights residues of the oxyanion hole. In addition to the four motifs, block IIIa, present only in clade II sequences, was identified. Within this sequence block a totally conserved cysteine residue is found in all clade II sequences (marked by C). In EstA, Cys258 of block IIIa forms a disulfide bridge with Cys264. All autotransporter esterases are labelled with "AT" at the end of the sequence. The second cysteine residue (bold underlined) is only conserved in autotransporters originating from different Pseudomonadaceae, but is exchanged for serine in the *Serratia liquefaciens* autotransporter esterase EstA (Q7X171).

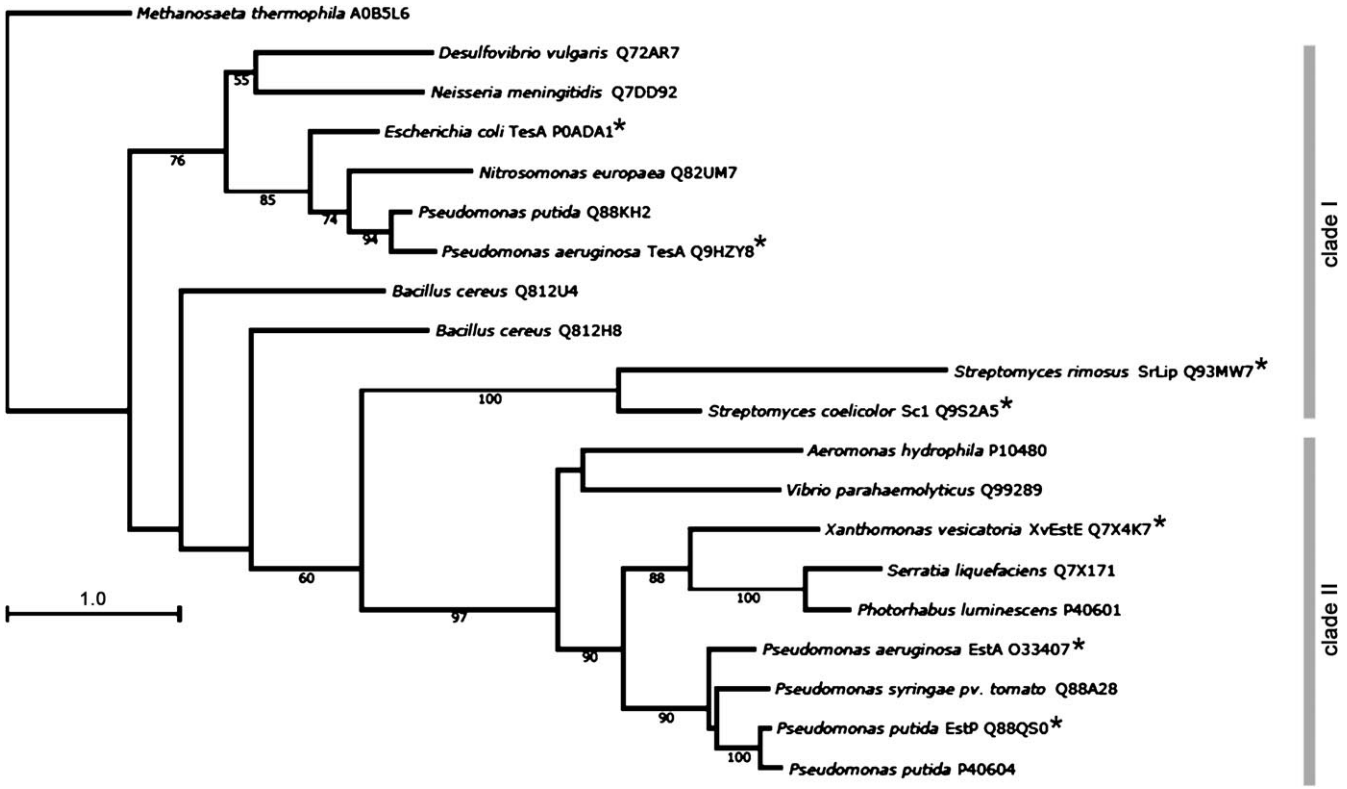


Figure 2. The phylogenetic tree of bacterial hydrolases with the GDSL motif shows clade I (monodomain proteins) and clade II hydrolytic (passenger) domains of two-domain proteins containing autotransporter domains. In cases in which proteins are marked with asterisks, substrate specificity data are provided in Table 1.

separated with moderate support from clade II sequences by a long branch. This separation of SrLip is not directly apparent from comparison of the corresponding sequence blocks (Figure 1) because SrLip clearly lacks the block IIIa motif. We thus cannot completely rule out long-branch attraction as a reason for the observed grouping.

For the newly identified block IIIa motif, the overall sequence conservation is limited and clear similarity is only detectable at the physicochemical level: that is, where residues with similar physicochemical properties are shaded (Figure 1). This low sequence conservation within block IIIa might be the reason why this motif has not been identified in previous alignment analyses. In the three-dimensional structure of the hydrolytic domain of the full-length autotransporter EstA[23] block IIIa constitutes parts of a surface helix (Gly238–Phe246) and a loop (Gly247–Cys258; Figures 1 and 3). Structurally it is located far from the active site and so should not be directly related to catalytic activity.

Substrate promiscuity can be related to variations in conformation around the active site, although the same active site configurations and essential structural features can also be preserved for natural and promiscuous activity.[1,2,27] For the bacterial enzymes that we have analysed in this study (Figure 2), the three-dimensional structures of two enzymes representing clades I and II—namely TesA (TAP) from *E. coli*[14] (PDB ID: 1ivn) and EstA from *P. aeruginosa*[23] (PDB ID: 3kvn)—are known. However, the only member of the SGNH superfamily thoroughly studied by biophysical and biochemical methods is the *E. coli* thioesterase I/protease I/lysophospholipase L_1 (TesA (TAP), Table 1). Considerable enzyme flexibility both in solution and in the solid state has been revealed by X-ray structure analysis,[28] NMR spectroscopy complemented by high-resolu-

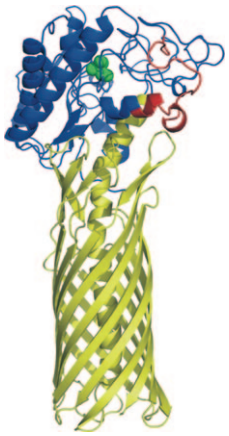


Figure 3. The structure of the full-length autotransporter of EstA from *P. aeruginosa* (PDB ID: 3kvn),[23] a member of clade II, showing amino acids Glu238–Cys258 of block IIIa (in red), which is located on the outer part of the hydrolytic passenger domain remote both from the catalytic site and from the substrate binding regions, with Cys258 located 6.4 Å away from the catalytic Asp286. The active site is located on the apical surface of the passenger domain (the catalytic Ser is depicted in green).

Table 1. Substrate specificities of studied enzymes and variants. Data for promiscuous enzymes TAP, Sc1 and XvEstE of the SGNH family were taken from the literature.[34,36,37]

Substrate fatty acid chain length	log *P*[b]	Enzyme activities[a]									
		SrLip	TesA	EstA	EstP	$EstP_N$	$EstP_N$–$EstA_c$	$EstA_N$–$EstP_c$	TAP	Sc1	XvEstE
p-nitrophenyl acetate (C2)	1.359	n.d.	18	3.0	56	498	5.4	4.9	395		*19*
p-nitrophenyl propionate (C3)	1.046	52	18	8.3	26	162	3.9	48			*29*
p-nitrophenyl butyrate (C4)	1.715	236	43	75	105	928	19	161	624		*23*
p-nitrophenyl valerate (C5)	2.275	135	9.3	32	86	503	16	86			*11*
p-nitrophenyl caproate (C6)	2.780	221	14	27	125	736	21	114	847		*28*
p-nitrophenyl caprylate (C8)	3.790	386	15	6.0	25	281	6.3	28	287		*7.9*
p-nitrophenyl caprate (C10)	4.801	368	9.2	9.5	126	1263	18	18	49		
p-nitrophenyl laurate (C12)	5.811	365	6.2	7.0	28	281	6.4	73	72	70	*1.5*
p-nitrophenyl myristate (C14)	6.821	299	1.2	6.0	5.6	23	2.1	89	47	101	*0.1*
p-nitrophenyl palmitate (C16)	7.832	299	0.4	4.2	0.8	4.1	0.5	72	7.6	76	*0.0*
p-nitrophenyl stearate (C18)	8.611	132	n.d.	4.0	0.1	1.4	0.1	51			
tributyrin (C4)	3.275	27	1.7	0.8	3.6	48	0.7	2.4	4.0		++
tricaprylin (C8)	8.849	453	0.6	0.0	0.8	4.7	0.6	0.9			+
tristearin (C18)	10.862	28	0.0	0.0	0.0	0.0	0.0	0.0			–
triolein (C18:1)	10.775	171	0.0	0.4	0.8	1.0	0.3	1.0		92	
Tween 20		271	85	22	156	687	25	88		138	
Tween 40		251	33	18	106	618	20	96		110	
Tween 60		233	15	12	108	510	19	86		80	
Tween 80		203	15	18	104	438	18	94		147	
α-naphthyl acetate (C2)	2.649	117	2.9	0.6	2.7	32	0.5	0.5	13		++
β-naphthyl acetate (C2)	2.673	66	4.6	0.9	2.6	22	0.7	3.0			++
α-naphthyl butyrate (C4)	3.877	113	2.7	3.0	31	157	5.5	15	5.9	++++	+
β-naphthyl butyrate (C4)	3.901	104	17	31	51	336	13	214		+	+
α-naphthyl laurate (C12)	7.918	459	1.6	1.0	4.8	40	1.6	3.4	0.0	++++	
β-naphthyl laurate (C12)	7.942	226	0.0	1.0	5.2	34	2.4	2.9		++	
cholesteryl butyrate	8.808	2.7	0.0	0.0	0.2	0.0	0.2	0.6	0.0		
diheptanoyl glycerophosphocholine	0.344	1196	0.9	0.5	3.5	46	0.9	1.3			
dioleoyl glycerophosphocholine	9.249	18	0.8	0.0	0.0	0.0	n.d.	n.d.			
acetyl coenzyme A	−4.096	0.1	0.0	0.0	0.0	0.0	0.0	0.0			
palmitoyl coenzyme A	3.024	262	7.8	3.8	98	696	24	161	21		
egg yolk		230	2.4	1.2	1.9	4.4	1.6	3.1			
resorufin acetate	2.143	2.5	0.8	0.0	0.1	0.7	0.0	0.1			
resorufin butyrate	3.371	11	3.6	0.8	12	33	1.2	7.0			
Azocoll		+	–	–	–	–	–	–			

[a] Regular numbers refer to activity in $U\,mg^{-1}$, italic numbers to activity in $U\,mL^{-1}$. Symbols indicate enzyme activities with ++++: strong, ++: moderate, +: weak , −: no activity. N.d.: not determined. All values for enzymes investigated in this study represent means of at least three measurements with standard deviations typically less than 10–15%. Abbreviations are the same as in Table 2, TAP: *Escherichia coli* thioesterase I/protease I/lysophospholipase L_1;[34] Sc1: *Streptomyces coelicolor* SGNH hydrolase;[36] XvEstE: *Xanthomonas vesicatoria* esterase EstE.[37] [b] log *P*: partition coefficient of substrate.[38]

tion molecular dynamics simulations[29,30] and site-directed mutagenesis,[31] providing insight into the active site and catalytic mechanism of the enzyme.[31,32] The mobility and the shape of the binding pocket obviously enables the enzyme to tolerate a certain substrate diversity.[14] Conformational diversity can thus increase functional diversity, facilitating the evolution of new proteins and functions from previous ones.[1,2,27,33]

Substrate specificity

Our previous findings on the broad substrate specificity of the lipase from *S. rimosus* (SrLip),[17,18] together with reports on the multifunctionality of the protease I/thioesterase I/lysophospholipase L_1 from *coli* (TAP),[11,12,14,28–31,34,35] prompted us to investigate enzyme promiscuity among SGNH hydrolases further. A large number of substrates (Table 1) were therefore tested against SrLip, acyl-CoA thioesterase I (TesA), the esterase (EstA) from *P. aeruginosa* and the lipase/esterase from *P. putida* (EstP). EstA and EstP are two-domain proteins, each with the N-terminal domain harbouring the hydrolytic function and the C-terminal β-barrel domain functioning as an autotransporter.[21,22] We also attempted to examine whether these domain pairs interact with each other and how such interaction might influence their catalytic activities. To this end, we constructed

Table 2. Enzymes and variants used in this study.

Enzyme	Abbreviation	UniProt no.	No. of residues[a]	M_W[a]
lipase from *Streptomyces rimosus*	SrLip	Q93MW7	268	27607
acyl-CoA thioesterase I from *Pseudomonas aeruginosa*	TesA	Q9HZY8	201	21037
esterase EstA from *Pseudomonas aeruginosa*	EstA	O33407	646	69609
lipase/esterase from *Pseudomonas putida*	EstP	Q88QS0	629	67175
N-terminal domain of EstP	$EstP_N$		296	30539
N-terminal domain of EstP + C-terminal domain of EstA	$EstP_N$–$EstA_C$		633	68082
N-terminal domain of EstA + C-terminal domain of EstP	$EstA_N$–$EstP_C$		642	68686

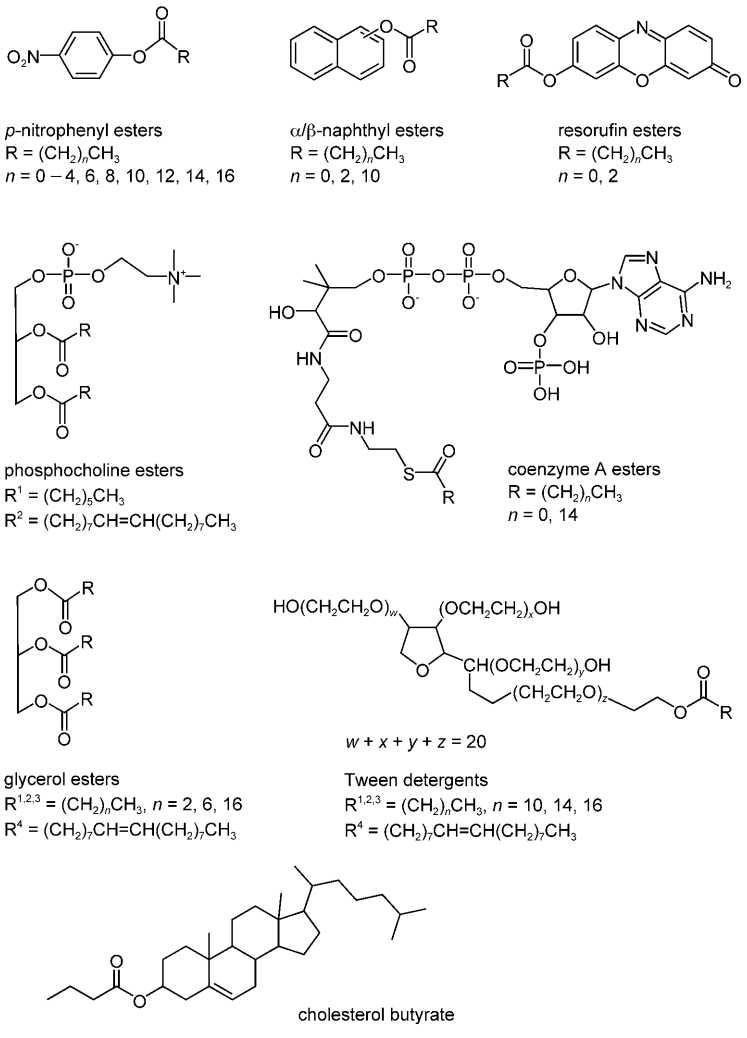
[a] No. of residues and molecular weight refer to unprocessed enzymes.

hybrid enzymes by exchanging the corresponding N- and C-terminal domains between EstA and EstP, resulting in the hybrid constructs $EstP_N$–$EstA_C$ (consisting of the N-terminal domain of EstP and the C-terminal domain of EstA) and $EstA_N$–$EstP_C$ (consisting of the N-terminal domain of EstA and the C-terminal domain of EstP). Furthermore, we also constructed a truncated esterase consisting exclusively of the N-terminal catalytically active domain of EstP ($EstP_N$). All enzymes and variants (Table 2) were purified prior to determination of activities.

In total, 34 substrates, including esterase, lipase, thioesterase, phospholipase and protease substrates, and also Tween detergents, were tested. Comparison of the obtained results with those available for other SGNH hydrolases revealed remarkable differences in the activity profiles in the SGNH hydrolase family (Table 1). Figure 4 shows a comparison of the activities of the investigated enzymes and enzyme constructs towards selected substrates for the identification of esterase, lipase, phospholipase and thioesterase activities.

In addition to the previously observed high activities of SrLip towards *p*-nitrophenol and glycerol esters of medium-chain acids (C_8–C_{12}), as well as towards Tween detergents and several natural oils,[17,18] we can now report significant activities of this enzyme towards thioesterase and phospholipase substrates (Figure 4). Although for triglycerides and natural oils this lipase prefers substrates with unsaturated fatty acids,[18] diheptanoyl glycerophosphocholine was more readily hydrolysed than dioleoyl glycerophosphocholine. As is also the case for the other studied enzymes, there seems to be no correlation between preferred substrates and their lipophilicities (expressed as log *P*[38]). Still, our attempts to measure the activity of SrLip towards the chymotrypsin-like substrates *N*-carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester (L-NBPNPE) and its D enantiomer (D-NBPNPE) failed. These substrates had been used to show enantioselective proteolytic activity of *E. coli* thioesterase I/protease I/lysophospholipase L_1 (TAP), a typical example of a promiscuous enzyme.[14,34] A paper on the properties of the SGNH hydrolase from *Streptomyces coelicolor* (Sc1),[36] an enzyme with significant sequence similarity to SrLip, has very recently been published. It is interesting that, notwithstanding their sequence similarity (66% sequence identity), the activity profiles of these two enzymes are different (Table 1). Whereas SrLip prefers substrates of medium acyl chain length, Sc1 shows its highest activity towards a long-chain *p*-nitrophenyl ester (C14). SrLip cleaved Tween 20 and Sc1 Tween 80 at highest rate. SrLip shows no significant difference in the hydrolysis of α- or β-naphthyl esters, whereas Sc1 clearly prefers α-naphthyl esters. The *S. coelicolor* enzyme (Sc1) thus exhibits (aryl)esterase and lipase activities. In summary, we have shown that *S. rimosus* lipase clearly shows substrate promiscuity according to literature definitions.[6–8]

TesA exhibited the highest activity towards Tween detergents and *p*-nitrophenyl esters of short acyl chain length (Table 1). Surprisingly, although it was named acyl-coenzyme A thioesterase I by sequence similarity to TAP (42% sequence identity), its activity towards palmitoyl-coenzyme A was rather low under the test conditions (Figure 4). Still, the activity profiles



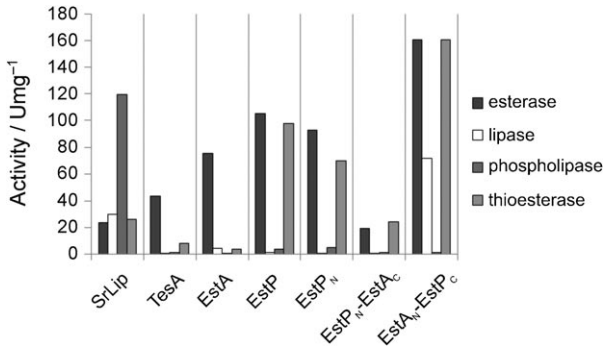


Figure 4. The specific activities (U mg^{-1}) of studied SGNH hydrolases toward selected substrates: esterase (*p*-nitrophenyl butyrate), lipase (*p*-nitrophenyl palmitate), phospholipase B (diheptanoyl glycerophosphocholine) and thioesterase (palmitoyl coenzyme A) activities. The SrLip and EstP$_N$ are much more active than the other enzymes, and so their activities are each expressed as one tenth of the measured activities.

of these two enzymes are similar (Table 1). Although TAP was discovered and identified three times—as a thioesterase I, as a protease I and finally as a lysophospholipase L$_1$—Karasawa and co-workers suggested that its primary physiological role would be phospholipolytic.[35] The activity of the homologous enzyme TesA towards the phospholipase substrates used in this study was very low.

According to our results, EstA and EstP are typical esterases with pronounced activities towards *p*-nitrophenyl esters of short acyl chain length and Tween detergents (Table 1). EstA also showed relatively high activity towards β-naphthyl butyrate, whereas its activities toward other substrates are negligible. EstP exhibited a somewhat broader substrate specificity: it cleaved *p*-nitrophenyl esters of acyl chain lengths C4–C10 and also tributyrin and resorufin butyrate at significant rates. Its activity towards palmitoyl-coenzyme A was pronounced. The differences in the substrate activity profiles of EstA and EstP are somewhat surprising in view of the high sequence similarity between these two enzymes (61.5%). The 3D structure of EstA shows the active site on the apical surface of the passenger domain at the entrance of a large hydrophobic pocket.[23] The common catalytic triad Ser14, Asp286 and His289 shares its topology with the triads of other SGNH hydrolases with the same catalytic amino acids. Primary structure comparison of EstA (from *P. aeruginosa*) and EstP (from *E. coli* strain O157:H7, PDB ID: 2QOM) for both domains revealed close similarity of the β-barrel domains, whereas the catalytic domains showed substantial differences, particularly in the loops and turns.[23] Most probably, these differences, together with differences in the active sites' architectures (shape, size and polarity), explain the substrate profiles of these two enzymes. The characterised enzymes in the Uni*P*rot database—the esterases EstE from *Xanthomonas vesicatoria* (XvEstE)[37] and ApeE from *Salmonella typhimurium*[39]—display sequence similarities to EstA of 27% and 26%, respectively. Their preferred substrates are similar to those of EstA (*p*-nitrophenyl and α- or β-naphthyl esters of short acyl chains), although ApeE cleaved *p*-nitrophenyl esters up to C16 and XvEstE hydrolysed both α- and β-naphthyl butyrate with the same efficacy (Table 1).

A striking observation was that the N-terminal esterase domain of EstP (EstP$_N$) showed activities five to ten times higher than those of the complete enzyme EstP towards all tested substrates (Table 1), whereas the activity profiles were still the same (Figure 4). The EstP$_N$–EstA$_C$ hybrid enzyme consisting of the N-terminal domain of EstP and the C-terminal domain of EstA, however, showed all activities lower than EstP by factors of 3–7 (Table 1). We thus conclude that in solution the C-terminal domain hinders access to the active site of the esterase domain, and that this effect is even more pronounced for the heterologous C-terminal domain. Surprisingly, the EstA$_N$–EstP$_C$ hybrid enzyme showed activities three to seven times higher than those of EstA towards all substrates (Table 1).

An autotransporter domain can influence the folding of the hydrolytic domain and its catalytic ability without preventing translocation itself, as shown for PalA, an autotransporter lipolytic enzyme from *Pseudomonas* sp.[40] Although the mechanism underlying this phenomenon remains elusive, it is possible that the autotransporter domain might serve as a foldase or it might affect proper folding of the catalytic domain. Similar behaviour was also observed for *P. aeruginosa* EstA.[41]

There are as yet no data that would demonstrate in vivo interaction between the two domains of an autotransporter esterase. However, the 3D structures of N-terminal or C-terminal domains of autotransporter proteins[42–47] (available in the Protein Data Bank[12]) suggest that the catalytic domains might interact with the β-barrel structures of transmembrane domains when the membrane is not present, thus altering the 3D arrangement of residues in and around the hydrolytic active site.

Kinetic analysis of *p*-nitrophenyl butyrate hydrolysis

In our previous study[17] we showed that the SrLip enzyme is a true lipase, exhibiting the characteristic property of interfacial activation (that is, pronounced activity at the substrate solubility limit). The other enzymes investigated here were designated as esterases by sequence analysis. To verify this assignment the activities of all studied enzymes and enzyme constructs were measured at different concentrations of *p*-nitrophenyl butyrate (*p*NPB) with the solubility limit defined at a substrate concentration of 1.5 mM. The results revealed Michaelis–Menten kinetics and enabled calculations of kinetic parameters (Table 3).

TesA showed the lowest affinity (highest K_m value) for the substrate used and also the lowest catalytic efficacy (lowest

Table 3. Kinetic parameters of enzymes and variants.

Enzyme	V_m [U mg^{-1}]	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} M^{-1}]
TesA[a]	37	1.0	12	1.2×10^4
EstA	220	0.7	247	3.4×10^5
EstP	85	0.3	91	3.3×10^5
EstP$_N$	735	0.3	345	1.2×10^6
EstP$_N$–EstA$_C$	11	0.5	12	2.5×10^4
EstA$_N$–EstP$_C$	43	0.5	47	9.4×10^4

[a] For abbreviations see legend to Table 2.

k_{cat}). EstA also had rather low affinity for *p*NPB, but its efficacy was more than 20 times higher. Hybrid $EstA_N$–$EstP_C$, however, showed a somewhat higher affinity than EstA but a fivefold lower efficacy. These ratio values indicate interactions between heterologous N-terminal (hydrolytic) and C-terminal (autotransporter) domains presumably resulting in a modification of the active site with respect to its substrate binding capacity and possibly affecting the substrate approach and/or product-releasing pathways.

EstP showed the highest affinity for *p*NPB of all the investigated enzymes. The fact that the isolated N-terminal domain of this enzyme had the same affinity but a much higher efficacy suggests that, in vivo, the C-terminal domain of this enzyme somehow hinders the active site from converting the substrate, thereby diminishing enzyme efficacy, but does not change the interactions of active site residues with the substrate. However, the $EstP_N$–$EstA_C$ hybrid enzyme showed both lower affinity and lower efficacy, thus indicating different interactions of the heterologous C-terminal domain with the N-terminal catalytic domain, changing both the binding of substrate in the active site and the accessibility of the active site. The altered kinetic properties we have observed are presumably the result of modified interactions between swapped hydrolytic and autotransporter domains.

Conclusions

Alignment analyses and phylogenetic-tree computations of several bacterial SGNH hydrolases revealed the presence of all four conserved blocks and a clear separation into two clades, as described previously.[16] Additionally, we have newly identified another motif named block IIIa, which is characteristic for the enzymes of clade II only. The recently solved crystal structure of the full-length autotransporter EstA from *P. aeruginosa* suggests that the loop formed by block IIIa is not directly involved in catalysis. Two esterases—namely, EstA from *P. aeruginosa* and EstP from *P. putida*—revealed different activities and catalytic efficacies towards *p*-nitrophenyl butyrate, with EstP showing the most pronounced activity towards this substrate out of all the tested enzymes. $EstP_N$, representing the isolated hydrolytic domain of EstP, had the same substrate affinity but a much higher efficacy of hydrolysis (turnover number). The high efficacy of the stand-alone hydrolytic domain $EstP_N$ suggests that, in vivo, the C-terminal domain of EstP might shield the active site from converting a substrate, thereby diminishing enzyme efficacy, but does not change the interactions of active site residues with the substrate. The $EstP_N$–$EstA_C$ hybrid of these two enzymes shows lower affinity and efficacy, thus indicating different interactions between its domains that affect both accessibility to the active site and substrate binding. Surprisingly, the $EstA_N$–$EstP_C$ hybrid obtained by domain swapping of these enzymes showed activities three to seven times higher than those of EstA towards all substrates used. The 3D structure of EstP is not available, so it is currently difficult to predict how the autotransporter domain $EstP_C$ affects the hydrolytic activity of $EstA_N$.

Of the enzymes studied, SrLip showed catalytic activity towards a wide range of different substrates (Table 1), clearly revealing enzyme substrate promiscuity. Moreover, SrLip catalysed distinctive chemical reactions, exhibiting lipase, phospholipase, esterase, thioesterase and Tweenase activities and thus also revealing enzyme catalytic promiscuity. *E. coli* TAP and the acyl-CoA thioesterase I TesA from *P. aeruginosa*, which share about 42% identity, exhibit largely similar activity profiles, although *P. aeruginosa* TesA showed only low activity towards palmitoyl-coenzyme A. Evidently, two homologous enzymes from two entirely different bacteria possess similar catalytic properties with respect to their substrate spectra. We thus predict that the high degree of conformational flexibility revealed for *E. coli* TAP and thought to be responsible for the this enzyme's promiscuity will be discovered in *P. aeruginosa* TesA as well.

Experimental Section

Phylogenetic analysis: Initial tree computations on the full dataset (clades I–V) reproduced the subgrouping into clades I to V as reported by Akoh et al.[16] Clade I and clade II sequences represent the bacterial esterases/lipases (see Table S1 in the Supporting Information for details), whereas clades III–V are represented by plant esterases mainly made up of sequences of *Arabidopsis thaliana* and *Oryza sativa*. It has previously been suggested that these last three clades have evolved by convergent evolution independently from the bacterial GDSL esterases/lipases (clades I and II).[16] To obtain more detailed information on the evolution of bacterial GDSL esterase/lipase families (clades I and II) we have excluded all plant GDSL esterase/lipase sequences from further tree computations and analyses.

Representative amino acid sequences of the GDSL family of esterases/lipases were obtained from the SwissProt/TreEMBL database. Initially the sequences of clades I–V selected according to Akoh et al.[16] were aligned with the aid of the structure-guided alignment tool Expresso (3D-Coffee).[48] As an outgroup the sequence of an uncharacterized archaeal GDSL-lipase of *Methanosaeta thermophila* (A0B5L6) was included in all analyses. The subgroup alignment for the microbial GDSL lipases (clades I and II) was generated with the same tool. Other alignment programs such as T-Coffee[49] and ClustalW[50] were tested and alignment quality was judged from alignment scores and visual inspection with respect to the correct alignment of key amino acid motifs previously suggested to be important for enzymatic function.[16] Expresso invariably generated the best alignment, resulting in the highest scores and the smallest amount of gap positions. Auxiliary regions outside the conserved esterase/lipase sequences (the autotransporter domain of EstA, for example) were removed by use of the alignment editor GeneDoc.[26] In order to retain as much phylogenetic information as possible, no gap columns were removed from the resulting trimmed alignments. ProtTest[51] suggested the Whelan and Goldman (WAG)[52] amino acid substitution matrix with estimated proportions of invariable sites (+I), four gamma rate categories (+G) and empirical amino acid frequencies (+F) as best suited for inferring the phylogeny of the dataset. Phylogenetic tree inference was performed with the aid of the RAxML webserver[53] with 100 bootstrap replicates. Tree editing and visualization were carried out either with ATV[54] or with the ITOL web-resources (http://itol.embl.de).[55]

Protein expression and purification

DNA manipulation: Recombinant DNA techniques were performed as described by Sambrook et al.[56] Restriction endonuclease and bacteriophage T4 DNA ligase reactions were carried out as recommended by the manufacturers. DNA fragments were analysed on agarose gels (0.8%, *w/v*). Plasmid DNA was purified with the aid either of the HiSpeed plasmid purification midi kit (Qiagen) or, for genomic DNA from *P. aeruginosa* and *P. putida*, of the DNeasy tissue kit (Qiagen).

DNA fragments were amplified by standard PCR methods. Genes *estA* and *tesA* from *P. aeruginosa*, as well as *estP* and *estP*$_N$ from *P. putida*, were amplified by PCR with use of the corresponding chromosomal DNA as template. Specific primers encoding restriction sites for NdeI (up), XhoI (down) and SacI (down) for *tesA* and *estP*$_N$, respectively, were used. All primers were synthesised by MWG Biotech (Ebersberg, Germany).

PCR mixtures were composed of template DNA (1 ng), each primer (5 pmol), buffer (1×), dNTPs (0.2 mM) and Triple Master polymerase (2.5 U, Eppendorf) in a reaction volume of 50 μL. PCR conditions included initial denaturation (5 min 98 °C), 40 repeating cycles (50 s 98 °C, 50 s at T_m 64 °C and 2 min 72 °C) and final elongation (10 min at 72 °C) with a Mastercycler ep gradientS (Eppendorf).

The resulting products were cloned into pET22b+ for a bacteriophage T7-RNA polymerase-dependent expression from the T7 promoter, yielding the plasmids pET22estA, pET22tesA$_{His6}$, pET22estP and pET22estP$_N$.

The hybrid proteins EstP$_N$–EstA$_C$ and EstA$_N$–EstP$_C$ were constructed by insertion of an NheI restriction site downstream of the region encoding the passenger domains of *estA* and *estP*, respectively, by overlap extension PCR with plasmids pET22estA and pET22estP as templates.

The PCR products were cloned into plasmid pET22b+ with use of the NdeI/XhoI restriction sites resulting in plasmids pETEstA*Nhe*I and pETEstP*Nhe*I. Finally, the gene regions encoding the corresponding passenger domains were interchanged by use of NdeI/NheI restriction sites, yielding plasmids pET22A$_N$P$_C$ and pET22P$_N$A$_C$. PCR-amplified DNA fragments were verified by DNA sequencing (Sequiserve, GmbH, Vaterstetten, Germany).

Expression: *E. coli* BL21(DE3) was transformed with expression plasmids pET22tesA$_{His6}$, pET22estP$_N$, pET22estA, pET22estP, pET22A$_N$P$_C$ and pET22P$_N$A$_C$. Each culture was grown at 37 °C in Luria–Bertani (LB) medium containing glucose (0.4%, *w/v*) to a cell density corresponding to an $OD_{580\,nm} = 0.5$. Gene expression was induced by addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.4 mM. After 2 h of growth (5 h for *E. coli* harbouring pET22*tesA*), cells were harvested by centrifugation at 4000 *g* for 20 min (Sorvall RC 5B Plus, SLC 4000, Thermo Scientific).

Purification: Cells expressing esterases were resuspended in Tris·HCl buffer (pH 8.0, 20 mM), and disrupted with a French press (Thermo Electron Corporation, three passages at 2000 psi). Inclusion bodies were collected by centrifugation for 30 min at 16000 g, washed twice with Tris·HCl buffer (pH 8.0, 20 mM) and denatured in urea (8 M)/sodium chloride (0.1 M)/Tris·HCl (pH 8.0, 20 mM) over 15 min at room temperature. Residual debris was removed by ultracentrifugation at 175000 *g* for 60 min. The supernatant contained denatured EstA, EstP, EstA$_N$–EstP$_C$ and EstP$_N$–EstA$_C$. Refolding was initiated by rapid tenfold dilution with buffer containing *N*-dodecyl-*N*,*N*-dimenthyl-1-ammonio-3-propanesulfonate (SB-12, 0.5%, *w/v*)/Tris·HCl (pH 8.0, 20 mM)/sodium chloride (1 M) and incubation at 37 °C for 72 h. EstP$_N$ was diluted with Tris·HCl buffer (pH 8.0, 20 mM)/EDTA (5 mM) and refolding was carried out for a minimum of 24 h at room temperature. Enzyme samples were concentrated by centrifugation (Viva Science concentrators, exclusion limit 10 kDa) and purified by anion-exchange chromatography on a Q-Sepharose FF column (Pharmacia) with use of a linear NaCl gradient (0–1 M) for elution. Fractions were collected, EstP$_N$-containing fractions were combined, and proteins were concentrated as before and analysed by SDS-PAGE.

Cells expressing TesA were resuspended in Tris·HCl buffer (pH 8.0, 100 mM) containing imidazole (20 mM)/NaCl (300 mM) and disrupted by sonication (ultrasonifier Bandelin Sono Plus HD 60, 2 cycles of 5 min at 48 W with samples cooled on ice). TesA was purified from the soluble cell fraction obtained after the centrifugation for 30 min at 20000 *g* by affinity chromatography on Ni-NTA agarose (Qiagen). After removal of non-specifically bound proteins by washing with Tris·HCl buffer (pH 8.0, 100 mM) containing imidazole (50 mM) and NaCl (300 mM), TesA was eluted with the same buffer containing imidazole (250 mM).

Lipase from *Streptomyces rimosus* (SrLip) was expressed in the homologous host and purified as described previously.[17]

The protein concentration was determined by the method of Bradford[57] with bovine serum albumin as a standard.

Purities of protein samples were verified by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) on PhastSystem apparatus (GE Healthcare) by the manufacturer's procedures. PhastGel homogeneous 12.5% plates were used. Protein bands were visualised by silver staining.[58]

Enzyme assays: Hydrolytic activities of samples towards different substrates were measured by various methods. All chemicals used (substrates, buffers, additives) were obtained from Sigma–Aldrich unless otherwise stated. Assays were performed at room temperature. The unit of enzyme activity (1 U) is defined as the amount of enzyme catalysing the release of 1 μmol of (fatty) acid per minute.

Spectrophotometric methods: Activities towards several types of esters were determined spectrophotometrically with a CamSpec M-501 single-beam spectrophotometer (Cambridge, UK).

Activities towards esters of *p*-nitrophenol were determined as described previously.[17] Final concentrations in substrate emulsions were: substrate (1 mM), sodium deoxycholate (5 mM), dioxane (Merck, 2.5%), sodium phosphate buffer (pH 8.0, 50 mM, Kemika, Zagreb, Croatia). The activities were followed by measuring the absorbance at 410 nm. The enzyme activities were calculated with use of the molar extinction coefficient of *p*-nitrophenolate ($\varepsilon = 17000\ M^{-1}\,cm^{-1}$).

The activities towards resorufin esters were determined as described by Kitson and Kitson.[59] Final concentrations in substrate solutions were: substrate (25 μM), ethanol (0.7%, Kemika), sodium phosphate buffer (pH 7.4, 50 mM). The activities were followed by measuring the absorbance at 571 nm. The enzyme activities were calculated by use of the molar extinction coefficient of resorufin ($\varepsilon = 70000\ M^{-1}\,cm^{-1}$).

Activities towards acetyl-coenzyme A and palmitoyl-coenzyme A were determined as described by Bonner and Bloch.[60] Substrate solutions contained substrate (25 μM) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB ,0.5 mM) in Tris·HCl buffer (pH 8.4, 50 mM). The activities were followed by measuring the absorbance at 412 nm. The enzyme activities were calculated by use of the molar extinction coefficients ($\varepsilon = 13600\ M^{-1}\,cm^{-1}$).

Titrimetric methods: Activities towards several types of esters were determined by pH-stat titration with a 718 STAT-Titrino instrument (Metrohm, Herisau, Switzerland). The assays were performed in closed vessels (stirring conditions) and enzyme activities were followed by titration of the released acid with NaOH (10 mM).

Reaction conditions for esters of α- or β-naphthol were:[18] substrate (10 mM), gum arabic (1%, *w/v*), NaCl (Kemika, 0.1 M), dioxane (6%) and Tris·HCl buffer (pH 8.0, 2 mM).

Activities towards glycerophosphocholine esters were determined as described for activities towards α- or β-naphthyl esters, except that the final concentration of substrate was 2.5 mM.

Reaction conditions for triacylglycerols, cholesteryl butyrate and Tween detergents were: substrate (10 mM), gum arabic (1%, *w/v*), NaCl (Kemika, 0.1 M), dioxane (4.8%), Tris·HCl buffer (pH 8.0, 2 mM).

Thioesterase activities with egg yolk as a substrate were determined as described by Nieuwenhuizen et al.[61] In short, one egg yolk was vigorously mixed with calcium chloride (Kemika, 17 mM, 106 mL). The suspension was filtrated and diluted threefold with Tris·HCl buffer (pH 8, 3 mM) containing sodium deoxycholate (3.75 mM).

Proteolytic activity: To detect proteinase activities, enzymes were tested with a qualitative Azo-coll (the Azo dye-impregnated collagen) assay.[62]

Determination of kinetic parameters: Investigation of the influence of a substrate concentration on the hydrolytic activities of enzymes was performed with *p*-nitrophenyl butyrate as the substrate, as described previously.[17] Accordingly, the substrate stock solution of appropriate concentration in dioxane was added to a sodium phosphate buffer (50 mM) with gum arabic (pH 7.2, 0.33%) so that dioxane concentration was 1%, and the obtained emulsion was sonicated for two minutes. Kinetic parameters were calculated with the aid of a Microsoft Excel program from linearizations of Michaelis–Menten equations.

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