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Human bile salt-dependent lipase efficiency on medium-chain acyl-containing substrates: control by sodium taurocholate

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Bile salt-dependent lipase was purified to homogeneity from lyophilized human milk and used to screen the influence of the acyl chain length (2–16 carbon atoms) on the kinetic constants k_{cat} and K_m of the hydrolysis of *para*-nitrophenyl (pnp) ester substrates in the presence or absence of sodium taurocholate (NaTC: 0.02–20 mM). The highest k_{cat} value (~3,500 s^{-1}) was obtained with $pnpC_8$ as substrate, whereas the lowest K_m (<10 μM) was that recorded with $pnpC_{10}$. In the absence of NaTC, the maximal catalytic efficiency (k_{cat}/K_m) was obtained with $pnpC_8$, while in the presence of NaTC k_{cat}/K_m was maximal with $pnpC_8$, $pnpC_{10}$ or $pnpC_{12}$. The bile salt activated the enzyme in two successive saturation phases occurring at a micromolar and a millimolar concentration range, respectively. The present data emphasize the suitability of this enzyme for the hydrolysis of medium-chain acyl-containing substrates and throw additional light on how BSDL is activated by NaTC.

Keywords: bile salt activation/human milk BSDL/kinetic constants/purification and characterization/substrate chain length.

Bile salt-dependent lipase (BSDL: EC 3.1.1.13)—also referred to as bile salt-activated lipase, bile salt-stimulated lipase, carboxylester lipase or cholesterol esterase—is secreted at a significant level by the pancreas and the mammary glands, and contributes to the intestinal digestion of lipids. Comparison of the enzyme in mammary glands and pancreas showed that they were identical (*1*), and their concomitant presence in the infant small gut was thought to be required for efficient fat digestion. BSDL is provided to newborns and infants by maternal milk and makes it possible for them to wait for the maturation of their own lipolytic system (*2*, *3*). BSDL is distinguished from other esterases by its dependence on bile salts for optimal activity and by its ability to catalyse the hydrolysis of long- and short-chain glycerol acyl esters, cholesterol esters, lipid soluble vitamin esters and aryl esters (*4–9*).

Kinetic and crystallographic studies afforded valuable data on BSDL substrate specificity (*3–9*) and bile salt activation (*1*, *6*, *10–13*). In addition to their solubilizing role, bile salts, especially primary bile salts, have the property of specifically binding to the enzyme. Two basic clusters located in the vicinity of the active site (proximal site) and on the back side of the molecule (distal site) have been identified and assigned to this function (*12*, *13*). Concerning the enzyme-substrate specificity, with focus on the substrate acyl length, the preferential activity of BSDL on monoacylglycerols was in the rank order $C_8 > C_{10} > C_{12} > C_{14} > C_{16}$, whereas with acyl esters of nitrophenol (C_2, C_3, C_4, C_5 and C_6), the highest turnover values were obtained with *para*-nitrophenyl (pnp) butyrate as substrate (*8*).

Generally, only one bile salt concentration and limited substrate chain lengths were used and, so far, none of the previous studies used k_{cat}, K_m and k_{cat}/K_m for analysis of the enzyme kinetic behaviour. Also, due to the concerns about enzyme purity and the variations in the experimental conditions, it was quite difficult to get reference values for the kinetic constants of this enzyme.

In this study, BSDL was purified to homogeneity from lyophilized human milk and was used to screen the effect of the substrate acyl chain length (C_2–C_{16}) and that of NaTC (0.02–20 mM) on the kinetic constants k_{cat}, K_m and k_{cat}/K_m of the BSDL-catalysed hydrolysis of nitrophenyl esters. The aim was to probe the substrate specificity and the specific role of BSDL in the intestinal lipolysis processes.

Materials and Methods

Materials

Sodium taurocholate (NaTC), *para*-nitrophenyl-acetate ($pnpC_2$), *para*-nitrophenyl-butyrate ($pnpC_4$), *para*-nitrophenyl-caprylate ($pnpC_8$), *para*-nitrophenyl-caprate ($pnpC_{10}$), *para*-nitrophenyl-laurate ($pnpC_{12}$) and *para*-nitrophenyl-palmitate ($pnpC_{16}$) were purchased from Sigma and Fluka-Biochemika. Pnp was purchased from Biomedicals. *Para*-nitrophenyl-caproate ($pnpC_6$) was not commercially available. The other reagents, including ampholines (Pharmacia) and molecular mass markers (BioRad), were of the highest available purity. Lyophilisate from human milk was a gift from Hôpital la Timone (Marseille, France) and BSDL polyclonal antibody pAbL64 directed against human pancreatic BSDL was home-made.

Purification procedure

Fresh nursery milk was stored at 4°C until a fat cake formed at the surface. The cake was carefully removed and the remaining milk phase was lyophilized and stored at −20°C. For the purification procedure, 0.5 g of the lyophilisate was solubilized in 10 ml of the homogenization buffer (Tris–HCl 10 mM, pH 8, containing 10 mM NaCl and 1 mM NaN_3). After delicate mixing in ice using a glass rod, the homogenate was subjected to anion exchange chromatography on a quaternary methylamine gel (QMA) in a cold room. Before applying the homogenate, the anion exchange gel was equilibrated with the homogenization buffer and washed until the absorbance at 280 nm became null. Elution was performed at 0.5 ml mn^{-1} using the same buffer as above but containing 150 mM instead of 10 mM NaCl. The protein-containing fractions, detected at 280 nm, as well as those active on $pnpC_4$, were pooled and dialysed overnight against a 50-fold volume of the homogenization buffer and then applied to a 1-ml Hi-Trap Heparin affinity gel using a FPLC system (Pharmacia Biotech). The elution was carried out at 1 ml mn^{-1} in the same buffer and a NaCl gradient (from 10 mM to 1 M). The fractions active on $pnpC_4$ were pooled and dialysed overnight against the buffer used for the activity assay and then stored at −20°C.

BSDL identification procedure

Protein concentration was determined by means of the Bradford method (*14*), using bovine serum albumin as a reference protein. Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 10% of polyacrylamide following the Laemmli method (*15*). When necessary, the enzyme solution was concentrated using a speed vacuum system. Isoelectrofocusing analysis was performed as previously described (*16*) using a mixture of pH 5–7 ampholine and pH 3–10 pH ampholine. Western blot analysis was performed using polyclonal antibodies pAbL64 as positive control for the purified BSDL, as described elsewhere (*17*).

Enzyme activity assay

Aliquots of the enzyme stock solution were stored at 4°C until activity recording. All the nitrophenyl-acyl ester substrates were solubilized in ethanol. Adequate dilutions were made so that the ethanol concentration in the reaction medium was 10%, which was previously found to have no significant effect on the activity of BSDL (*18*). The enzyme activity assay was performed in 100 mM Tris–HCl (pH 7.5) containing 150 mM NaCl at 37°C. The reaction was allowed to proceed in a 96-well microplate containing 160 µl of buffer and 20 µl of enzyme by adding 20 µl of substrate by means of a multichannel pipette. The release of pnp was then continuously monitored using a microplate reader (Kirial International) for 3 min at 405 nm. The molar extinction coefficient of pnp determined for those experimental conditions was 8,333 $M^{-1}cm^{-1}$. This value is slightly lower than the value of 11,500 $M^{-1}cm^{-1}$ previously reported (*8*). The same experiments were achieved but in the absence of BSDL to provide control experiments, and the amounts of pnp released were subtracted from those released in the presence of the enzyme.

All the experiments were repeated at least three times, and the repeatability was further checked after 1 year or so, using freshly purified enzyme. The mean values of the initial velocity (v_i expressed as µM mn^{-1}) are given. From these values, the kinetic constants k_{cat} (s^{-1}) and K_m (µM) were determined with the Graph Pad Program, using the substrate concentration ranges best fitting the Michaelis–Menten model. The BSDL molecular mass of 100 kDa was used. Additional details are given in the legends of the figures and tables.

Results and Discussion

Purification and characterization of BSDL

The results of the purification and characterization of hmBSDL are summarized in Fig. 1 and Table I. Three major protein peaks were eluted from QMA gel, accounting for ~65% of the protein loaded onto the gel, whereas ~100% of the initial activity on $pnpC_4$ was found in the first elution peak. The second purification step gave a single protein peak containing 77% of the initial activity but only ~0.5% of the total protein of the milk homogenate. The recovery was slightly higher than that (0.25%) previously obtained using fresh milk (*19*). The purified protein seemed to be identical to the pancreatic enzyme. Indeed, using SDS–PAGE followed by Coomassie blue staining (Fig. 1C) and western blot analysis (Fig. 1D) with pAbL64, two protein bands were detected at ~110 and 90 kDa probably corresponding to glycovariants of the protein or to allelic forms (*20*). In addition, the 90 kDa form seems to exist in two molecular species (Fig. 1C), suggesting minor differences in glycosylation. The isoelectric point (about 4.8) was similar to that previously reported for the porcine pancreatic form (*21*). It is worth noticing that the isoelectric migration was the same for the two forms of the enzyme, meaning that the two variants bore the same charges, whatever the origin of this variability.

Kinetic behaviour of hmBSDL with nitrophenyl-acyl esters as substrates in the presence or absence of NaTC

Figure 2 shows the plots of initial velocity versus substrate concentration with or without NaTC. All the curves well fit the Michaelis–Menten model, showing an hyperbole, except for $pnpC_8$. In the case of $pnpC_8$ and $pnpC_{10}$ a slowing down of velocity is observed at high concentration of substrate (>15 µM) and low taurocholate concentration (<0.6 mM) but not at higher NaTC concentration (>0.6 mM). This is a typical inhibition by excess of substrate that was suppressed by increasing concentration of NaTC, in agreement with the role of the bile salt as acyl acceptor (*22*), the efficiency of which depends on the initial molar ratio between NaTC and the substrate. The specific activating role of NaTC is also visible and its efficiency is intimately linked to the chain length of the acyl moiety, suggesting the formation of optimal micelles or other interfacial structures that present the substrate to BSDL. The sigmoid profile of the BSDL kinetic plots with $pnpC_8$ as substrate quite resembles that for interfacial enzymes or for allosteric enzymes. In this case, whatever its concentration, NaTC inhibits BSDL when the $pnpC_8$ concentration is <5 µM and activates it for a $pnpC_8$ concentration >5 µM, with a sharp increase in the activation efficiency when the NaTC concentration ranges between 0.02 and 0.20 mM, as observed with $pnpC_{10}$ and $pnpC_{12}$. The sigmoidal profile observed with $pnpC_8$ likely reflects progressive incorporation of lipid substrate with increasing concentration in micelles of NaTC that are best recognized by BSDL (*5*). Concerning the inhibition by NaTC observed <5 µM of $pnpC_8$, it is possible that the binding of monomers of $pnpC_8$ is hindered by monomeric NaTC (low NaTC concentration) due to competition at the active site area, and at this low substrate concentration, it is incorporated in NaTC micelles (high NaTC concentration) in such a way that it is less accessible to BSDL.

Kinetic constants of hmBSDL

Table II gives the values of k_{cat} and K_m obtained for all the experimental sets. The catalytic constant of

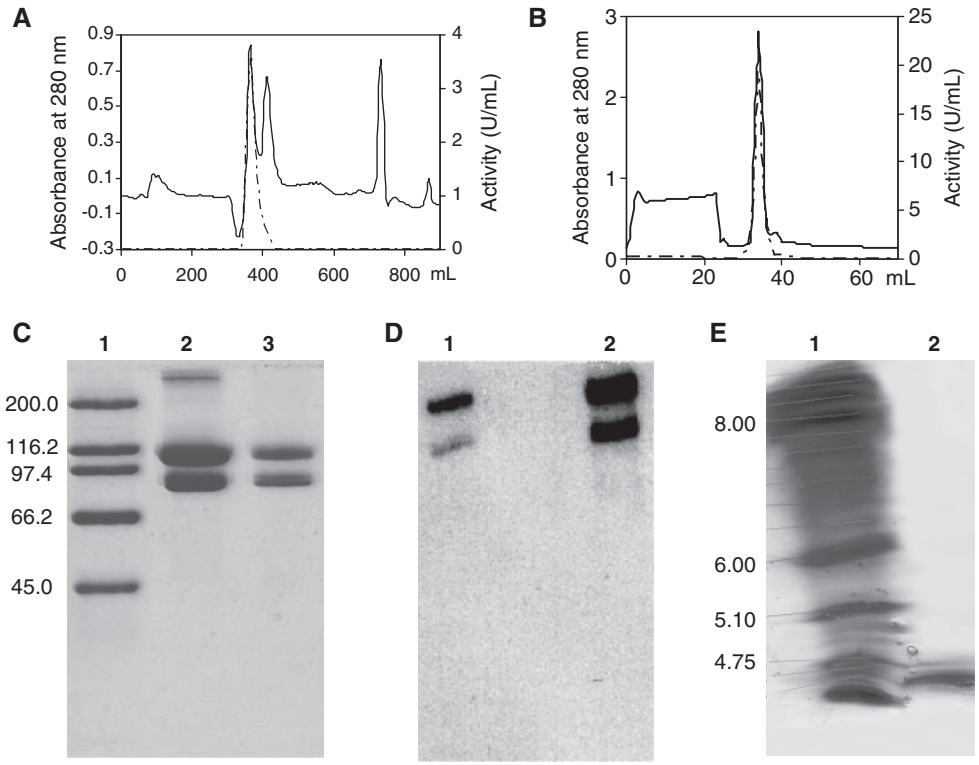

Fig. 1 Purification and characterization of human milk BSDL. (A and B) Chromatography profiles on QMA and heparin gels, respectively (broken line, activity expressed as μmol pnp.min^{-1}.ml^{-1}; full line, absorbance at 280 nm); (C) PAGE–SDS (1, molecular mass markers; 2, control human pancreatic BSDL; 3, purified hmBSDL); (D) western blot using polyclonal antibody pAbL64 (1, purified hmBSDL; 2, control human pancreatic BSDL); (E) isoelectrofocalisation (1, pH standards; 2, purified hmBSDL).

Table I. Purification assessment of BSDL from lyophilized human milk.

	Total proteins (mg)	Total activity (U)	Purification factor
Crude homogenate	56 ± 4.6	164 ± 15	1
QMA	38 ± 2.2	164 ± 31	1.5
High trap heparin	0.3 ± 0.09	126 ± 48	137

BSDL was found to be the highest with $pnpC_8$ (between 900 and 3,500 s^{-1}) and $pnpC_4$ (between 800 and 3,400 s^{-1}) as substrates. It decreases down to the range of hundreds and dozens of turnovers per second with the shorter substrate $pnpC_2$ and the longer substrates $pnpC_{10}$, $pnpC_{12}$ and $pnpC_{16}$. The k_{cat} values here obtained with $pnpC_2$ and $pnpC_4$ are in the range of those previously obtained for the same enzyme in the absence or the presence of 20 mM of NaTC (*7*), whereas the k_{cat} of bovine, rat, porcine and human pancreatic BSDL, with $pnpC_2$ as substrate, were in the range 20–164 s^{-1}, whatever the NaTC concentration (*3*, *23–26*). Such a discrepancy in the k_{cat} values of BSDL may be linked to the enzyme origin, in which case the mammary form is more active than the pancreatic form, or linked to the experimental conditions, especially during the purification procedure.

On the other hand, as the length of the acyl moiety of the substrate was increased, K_m of BSDL decreased from >7 mM with $pnpC_2$ down to <10 μM with $pnpC_{10}$, and then increased with $pnpC_{12}$ and $pnpC_{16}$ up to ~250 μM. The K_m values with $pnpC_2$ and $pnpC_4$ are once again in the same order of magnitude as those previously obtained for the human milk BSDL, with or without NaTC (*8*).

The catalytic efficiency of BSDL increases from ~0.1 10^6 M^{-1} s^{-1} (with $pnpC_2$ as substrate in the absence NaTC) up to 135 10^6 M^{-1} s^{-1} (with $pnpC_{10}$ in the presence of 6 mM NaTC). The latter value is much higher than the catalytic efficiency of the porcine pancreatic alpha-amylase (~5.10^6 M^{-1} s^{-1}) (*27*). This highlights the important digestive role that BSDL plays among the hydrolases working in the intestine lumen.

Effects of NaTC on the kinetic constants of hmBSDL

The k_{cat} increase induced by NaTC (mean value for all the concentrations) was 2- to 3-fold with substrates with less than eight carbons and 6- to 10-fold with those containing more than eight carbons, whereas its effect on K_m did not exceed one order of magnitude. Consequently, the bile salt especially affected k_{cat}/K_m for the short substrates $pnpC_2$ and $pnpC_4$ (2- to 4-fold increase) and the medium-chain substrates $pnpC_{10}$ and $pnpC_{12}$ (8- and 15-fold increase, respectively). NaTC was found to activate BSDL through two saturation phases (Fig. 3). The apparent dissociation constant of NaTC for these phases ranged between 8 μM (with $pnpC_8$ as substrate) and 800 μM

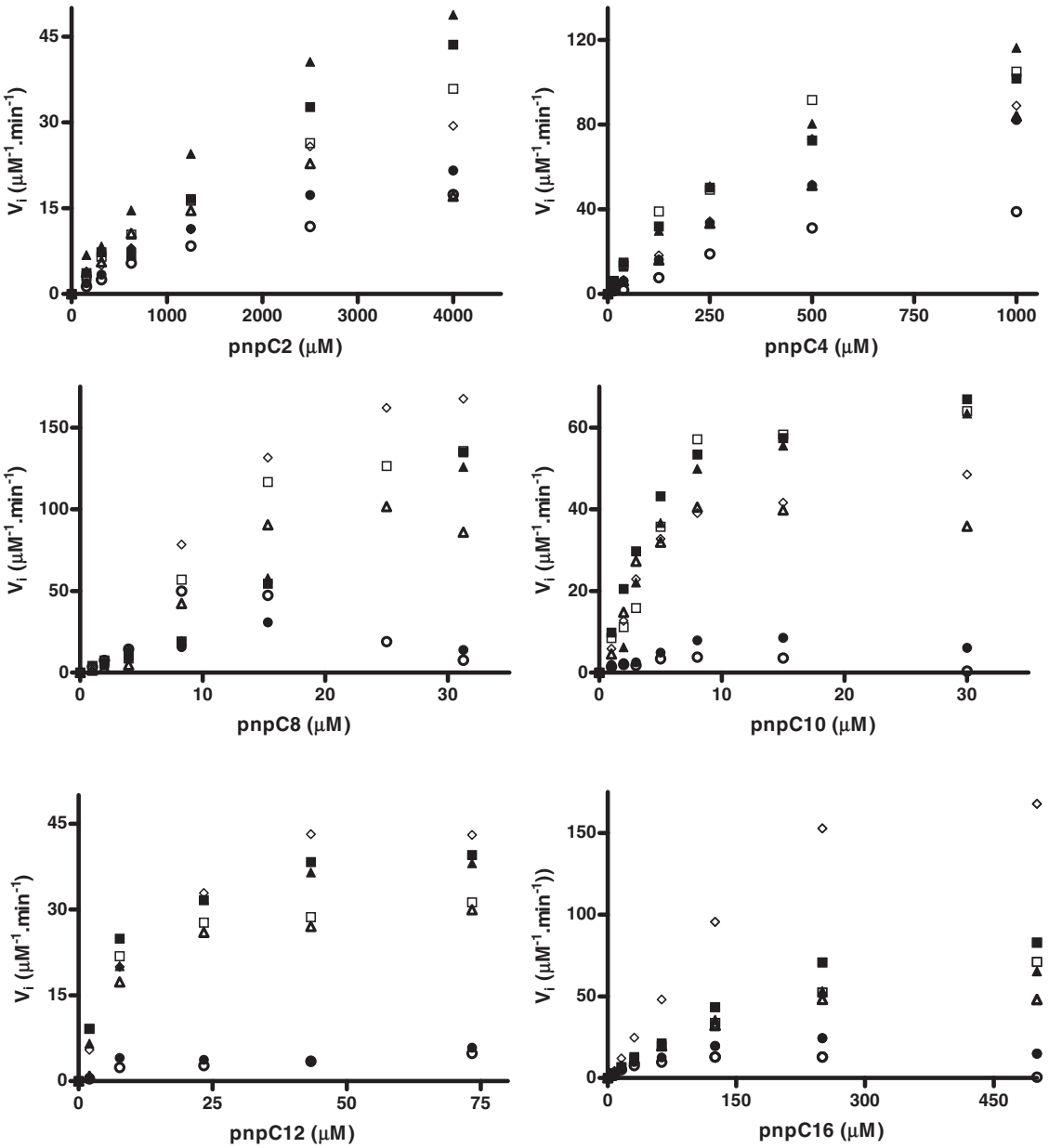

Fig. 2 Michaelis–Menten graphs for hmBSDL with nitrophenyl esters as substrates, with or without NaTC. Initial velocities (v_i), determined as described in the 'Materials and Methods' section, are represented versus substrate concentration with or without NaTC; the number of carbons in the substrate chains ranged between 2 and 16 (NaTC concentration: ○ 0, ● 0.02, Δ 0.2, ▲ 0.6, □ 2, ■ 6 and ◇ 20 mM).

(with $pnpC_{16}$ as substrate) for the first phase, and for the second phase between 0.6 mM (with $pnpC_4$) and 6 mM (with $pnpC_{16}$). Two NaTC binding sites have been previously identified (*12*, *13*): the proximal site ($K^{31,56,58,61,62}$, R^{63}) also called specific site, and the distal one (K^{336}, R^{423}, K^{429}, $R^{454,458}$, $K^{462,503}$) also called non-specific or pre-micellar site. The movements of loop 120 (amino acids 115–126) induced by the bile salt binding is considered as playing a key role in the accessibility of the substrate ester bonds to the catalytic triad Ser^{194}, Asp^{320} and His^{435} (*10*, *28*). It is therefore likely that the first saturation phase observed here should correspond to the binding of monomeric NaTC to each of the two bile salt-binding sites, whereas the second saturation, which occurs above the critical micellar concentration of NaTC (*13*), corresponds to the binding of micellar NaTC to one or both of the two bile salt-binding sites. Note that NaTC micelle may be sufficiently large to allow interactions of NaTC with both the proximal and distal bile salt sites at the surface of BSDL.

Effects of the substrate acyl chain length

Whether NaTC was present or not, k_{cat} first increased when the acyl chain length was increased from two to eight carbons and then decreased markedly for more than eight carbons (Table II). Such a dramatic decrease in k_{cat} of BSDL between eight- and nine-carbon containing substrates was also observed with + tri-acyl glycerols in the absence of bile salt (*5*). Thus, BSDL hydrolyses more rapidly the esters containing one or three acyl chain with less than

Table II. Kinetic constants of hmBSDL for the hydrolysis of nitrophenyl esters with 2–16 carbons in the acyl chain moiety, in the presence or absence of NaTC.

	NaTC (mM)						
	0	0.02	0.2	0.6	2	6	20
K_{cat} (s^{-1})							
$pnpC_2$	261 ± 7	297 ± 15	261 ± 11	691 ± 30	569 ± 53	1,049 ± 95	575 ± 93
$pnpC_4$	821 ± 158	1,258 ± 104	3,335 ± 198	1,663 ± 60	1,223 ± 31	1,251 ± 36	1,566 ± 118
$pnpC_8$	877 ± 289	2,566 ± 14	3,490 ± 1,096	2,248 ± 691	2,629 ± 371	2,762 ± 610	3,672 ± 1,193
$pnpC_{10}$	51 ± 3	122 ± 6	577 ± 89	655 ± 8	650 ± 40	641 ± 9	496 ± 13
$pnpC_{12}$	49 ± 3	65 ± 11	289 ± 5	365 ± 11	273 ± 10	381 ± 17	414 ± 6
$pnpC_{16}$	13 ± 0.8	27 ± 1.1	75 ± 4	78 ± 3.1	94 ± 1.5	108 ± 5.1	212 ± 14.5
K_m (μM)							
$pnpC_2$	3,407 ± 159	2,588 ± 259	1,280 ± 79	2,732 ± 225	3,744 ± 558	7,474 ± 962	5,225 ± 1,090
$pnpC_4$	1,093 ± 313	1,160 ± 152	750 ± 74	724 ± 49	387 ± 22	492 ± 37	941 ± 125
$pnpC_8$	14 ± 7	60 ± 0.4	69 ± 28	29 ± 14	36 ± 8	39 ± 13	42 ± 18
$pnpC_{10}$	4 ± 0.6	10 ± 1.6	6 ± 1.8	6 ± 0.4	6 ± 0.9	5 ± 0.4	5 ± 0.5
$pnpC_{12}$	20 ± 4	33 ± 13	10 ± 0.8	9 ± 0.7	4 ± 0.5	8 ± 1.1	11 ± 0.6
$pnpC_{16}$	35 ± 6	87 ± 8	222 ± 20	209 ± 18	288 ± 9	252 ± 25	219 ± 32

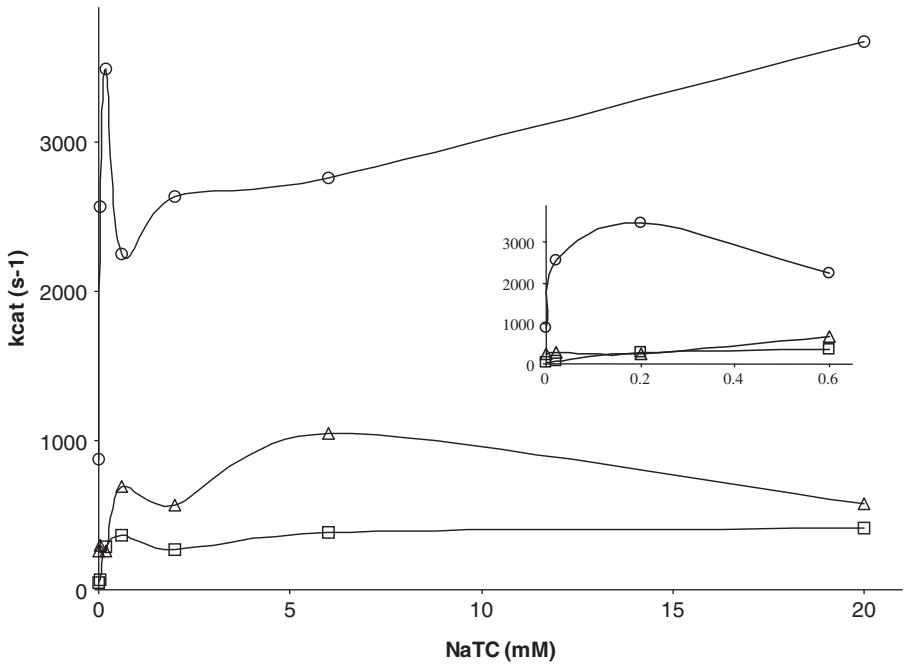

Fig. 3 NaTC activates hmBSDL following two saturating phases. The graph is based on the data from Table II; triangles, circles and squares correspond to pnpC2, pnpC8 and pnpC12, respectively. The inset shows the plots at low concentrations of NaTC.

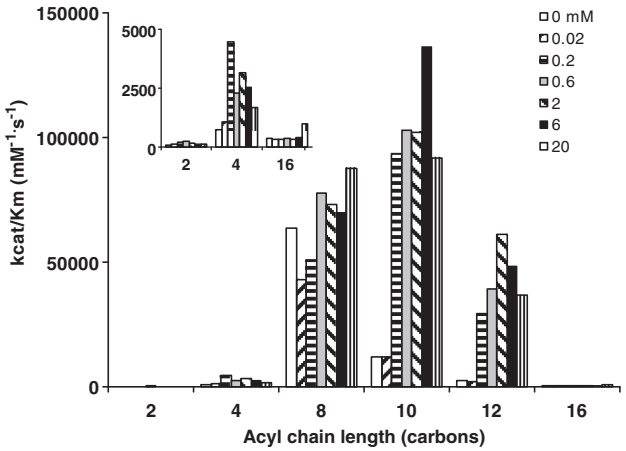

Fig. 4 The catalytic efficiency of hmBSDL is maximal on the medium-chain length substrates with subtle control by the bile salt.

eight carbons. Note that the decrease of k_{cat} between $pnpC_8$ and $pnpC_{10}$ is more pronounced in the absence of NaTC than in its presence. Furthermore, the preferential activity of BSDL on monoacylglycerols decreased continuously from C_8 up to C_{16} (*8*). Unfortunately, the shorter monoacylglycerols were not examined in the latter study. Instead, with short aryl esters ($pnpC_{2–6}$), the highest turnover value was that with $pnpC_4$ (*8*). On the other hand, phospholipids with eight carbons in the acyl chain had been found to be preferred by BSDL (*4*), as observed here with nitrophenyl esters. Taken together, these data show that the highest BSDL turnovers are those with short- or medium-chain acyl-containing substrates. In line with this, according to crystallographic data, the binding pocket made from loop 270 and alpha helix

H has been supposed to nicely match six-carbon substrates (*28*).

As regards the effect of the acyl chain length on K_m, the lowest values were definitely those obtained with $pnpC_{10}$ as substrate in the micromolar range, with a different-fold increase at each side of this concentration range (Table II). This suggests that the medium-chain acyl-containing substrates have the highest affinity for BSDL. In the absence of the bile salt, the enzyme is mostly efficient on the eight-carbon substrate and k_{cat}/K_m dramatically decreased for an acyl chain containing either more or less than eight carbons (Fig. 4). Interestingly, in the presence of the bile salt, the highest catalytic efficiencies were recorded for all the medium-chain length substrates, $pnpC_8$, $pnpC_{10}$ and $pnpC_{12}$. This indicate that NaTC permits to BSDL to be still highly efficient $pnpC_{10}$ and $pnpC_{12}$, likely by mean of control of the substrate accessibility and binding to the catalytic triad. One may think that this enzyme is adapted to milk lipids, because, among other food products, milk has the unique characteristic of containing short and medium (4–12 carbons) fatty acyl-containing lipid esters.

Conclusion

This article puts emphasis on how useful it is to simultaneously use k_{cat}, K_m and k_{cat}/K_m to screen the kinetic behaviour of BSDL as a function of the bile salt concentration and the substrate acyl chain length. The use of k_{cat} as a response marker to the effectors on the kinetics of BSDL, or of any enzyme, allows only partial kinetic analysis and should be limited by the enzyme purity criteria.

The Michaelis and catalytic constants obtained here for $pnpC_2$ and $pnpC_4$ are close to those previously published, thus affording reference values of these kinetic constants for hmBSDL with these two substrates and with those having longer acyl chain. The acyl chain lengths of the aryl esters used are well representative of those of the usual substrates of this enzyme, and the concentrations of NaTC used cover the physiological concentrations of bile salts. The enzyme exhibits similar kinetic behaviour regarding the acyl chain length whether the substrates are glycerol esters or aryl esters. The present data throw additional light on the mechanism of BSDL activation by NaTC and show that this enzyme is the most efficient on substrates whose acyl chain moiety is of medium length. This suggests high suitability of hmBSDL for hydrolysis of milk lipids, because milk has the unique property, among foodstuffs, to contain significant amounts of short- and medium-chain acyl-containing triacylglycerols. The topic needs further investigations, including re-examination of the pancreatic form of BSDL based on an integrative approach, such as that used here, thus completing previous data (*29*) for getting more information on the specific roles of pancreatic and milk BSDL regarding intestinal lipolysis.

Conflict of interest
None declared.

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