Further characterization of the bile salt-stimulated lipase in human milk

Lars Bläckberg* and Olle Hernell

*Departments of *Physiological Chemistry* and *Pediatrics, University of Umeå, S-901 87 Umeå, Sweden*

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Bile salt-stimulated lipase is a milk enzyme unique to the higher primates. Its molecular and kinetic characteristics differ greatly from other lipolytic enzymes; e.g., pancreatic lipase and lipoprotein lipase. It has a much higher app. M_r, 310000 on gel filtration and 100000 after denaturation. It requires primary bile salts for optimal activity and bile salts also protect the enzyme from proteolytic and heat inactivation. It may, due to its low substrate specificity, contribute to the utilization of a variety of milk lipids. Since it lacks positional specificity, digestion of milk triglycerides should be complete, which may explain why fat absorption is more efficient in breast-fed than in formula-fed infants.

Bile salt-stimulated lipase *Human milk* *Lipid digestion* *Newborn infant*

1. INTRODUCTION

Every animal species has a unique composition of its own milk. This probably reflects differences in various needs of their respective offspring. One example of this is the bile salt-stimulated lipase (BSSL). This enzyme is present in milk only from the highest primates. In his original studies, Freudenberg [1] investigated milk from several species, but found a high, bile salt-stimulated lipase activity only in human milk. In a later report, he described this enzyme activity also in gorilla milk [2]. The situation was further clarified when Hernell and Olivecrona demonstrated that human milk, unlike milk from most species, contain not one but two lipases [3]. One lipase activity has the same characteristics as that found in milk from most mammals and was identified as due to a lipoprotein lipase [3]. In addition, human milk was found to contain a bile salt-stimulated lipase (BSSL) [4]. More recently, we made a thorough search for a bile salt-stimulated lipase in milk from Rhesus monkeys, but found no evidence for this lipase or a protein related to it [5]. Thus, it was concluded that BSSL as a milk enzyme, has appeared late in evolution. It has, however, been demonstrated that the milk enzyme is closely related to the pancreatic non-specific lipase. The fact that antibodies raised against pure BSSL from milk react with immunochemical identity with a pancreatic protein was the first indication of such a pancreatic counterpart [5]. Further studies together with Lombardo and Guy led to the identification of this protein as the non-specific lipase (also called the carboxyl ester hydrolase) [6]. Direct comparison of the enzymes from milk and human pancreas revealed that they are indistinguishable in kinetic and immunochemical properties as well as in amino acid composition [6]. However, the lipase from milk has a slightly slower mobility on electrophoreses in dodecyl sulphate, indicating a larger molecular size. Whether this difference is in the primary gene product or in its processing is not yet known. Although BSSL is relatively new as a milk protein its counterpart is phylogenetically old as a pancreatic secretory protein. A lipase activity stimulated by bile salts is present in shark pancreas whereas the colipase-dependent lipase is absent [7]. In the rat, which is devoid of bile salt-stimulated lipase activity in milk, the pancreatic counterpart, carboxyl ester hydrolase, dominates over the colipase-dependent

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pancreatic lipase as long as the offspring suckles [8]. Thus, as long as milk is the main food carboxyl ester hydrolase is the essential enzyme for lipid digestion. In the newborn infant pancreatic lipase activities are low compared with adults [9]. Estimation of enzyme activities in duodenal contents after meals with raw human milk has demonstrated that 2/3rds of the non-specific lipase activity in duodenal contents originates in milk [10].

2. PURIFICATION AND MOLECULAR CHARACTERISTICS

BSSL can be purified from human milk by sequential chromatographies on heparin immobilized on Sepharose and on Affi-Gel blue [11]. An alternative method based on chromatography on taurocholate immobilized on Sepharose has also been published [12]. The enzyme is a single-chain glycoprotein with a pI of about 4. The apparent M_r is ~100000 as determined by SDS–polyacrylamide gel electrophoresis (fig.1). On gel filtration the pure enzyme protein elutes at a volume corresponding to an M_r of 310000 (fig.2). The reason for this behaviour is unknown. There are at least two possibilities. Studies done on carboxyl ester hydrolase from human pancreas indicate that this enzyme has an ellipsoid shape [15], which could result in an abnormal gel filtration behaviour. This might be the explanation also for the milk enzyme. However, there is also a possibility that BSSL forms aggregates. The fact that the enzyme peak (fig.2) is not symmetrical may speak in favour of that. At any rate, compared with other lipolytic enzymes (i.e., colipase-dependent pancreatic lipase and lipoprotein lipase), BSSL is a much larger molecule.

The amino acid composition of BSSL shows a high percentage of acidic residues reflecting the comparatively low pI. An unusual feature is that proline constitutes more than every tenth residue [11]. The N-terminal residue is alanine.

In contrast to pancreatic lipase but similar to acetylcholine esterase BSSL is completely inhibited by eserine [10]. It is also inhibited by low concentrations of organophosphates such as diisopropylfluorophosphate [16]. In the experiments shown in fig.3, the molar ratio between inhibitor and enzyme was 0.75–0.85 in the peak fractions. The

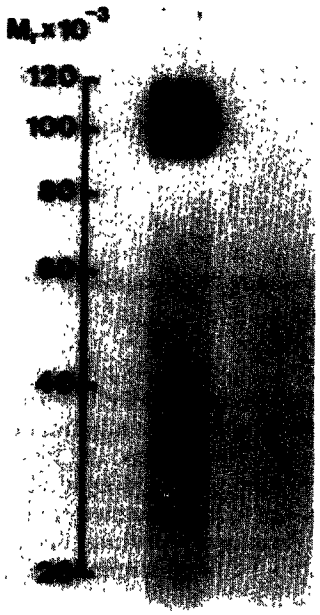

Fig.1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of bile salt-stimulated lipase; 25 μg purified [11] enzyme was subjected to electrophoresis according to Weber and Osborn [13]. Protein was stained with Coomassie brilliant blue as in [14]. Pharmacia low-molecular weight calibration kit (Pharmacia Fine Chemicals, Uppsala) was used for determination of M_r. The cathode is at the top of the photo.

deviation from an equimolar ratio could be explained by a minor fraction of inactive enzyme in the purified enzyme preparation. This is supported by the fact that heat-inactivated BSSL coelutes with active enzyme on a heparin–Sepharose column but does not bind diisopropylfluorophosphate (not shown). These data suggest that the active site of BSSL contains a serine residue essential for catalysis. This is similar to acetylcholine esterase and many proteases [18] but different from lipoprotein lipase and colipase-dependent pancreatic lipase which do not show the same inhibition by organophosphates.

3. INTERACTION WITH BILE SALTS

One of the more interesting characteristics of BSSL concerns its interaction with bile salts. BSSL is active against certain substrates (e.g., tributyrin and *p*-nitrophenyl acetate), in the absence of bile

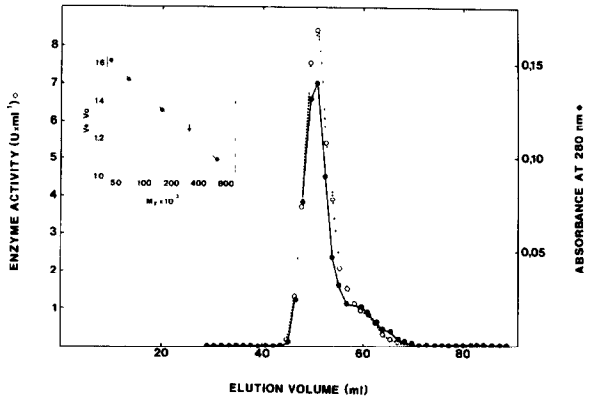

Fig.2. Gel filtration of bile salt-stimulated lipase. Purified bile salt-stimulated lipase (0.5 mg) was dissolved in 0.05 M Tris–HCl buffer (pH 7.5), 0.25 M NaCl and applied to a column (1.0 × 110 cm) of Sephacryl S-300 equilibrated in the same buffer. The flow rate was 12 ml/h. Aliquots of the fractions were assayed for lipase activity with an emulsion of long-chain triglyceride as substrate as described in [11]. One unit (U) is defined as the amount of enzyme that releases 1 μmol fatty acid/min. The app. M_r of the lipase was determined (inset), the void volume (V_o) was determined by chromatography of blue dextran. The standard proteins used were ovalbumin, bovine serum albumin, aldolase and thyroglobulin (Sigma, St Louis MO). About 5 mg of each protein was run individually and the elution volume (V_e) was determined. The arrow indicates the elution volume of BSSL.

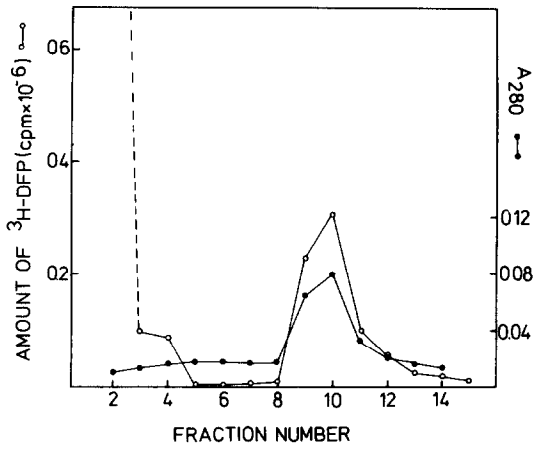

Fig.3. Binding of diisopropylfluorophosphate to bile salt-stimulated lipase. Purified BSSL (0.2 mg) was incubated with an excess radiolabelled diisopropylfluorophosphate (DFP) (Radiochemical Centre, Amersham) for 30 min at 30°C in 5 mM veronal buffer (pH 7.4). The mixture was applied to a column of heparin–Sepharose [17], about 2 ml settled gel. The column was washed with 6 ml of 5 mM veronal (pH 7.4), 0.05 M NaCl and then eluted with 1.0 M NaCl in the same buffer. Each fraction contained 1 ml. Radioactivity was determined on aliquots of each fraction. No lipase activity could be determined in any fraction.

salts, but the activity is enhanced by bile salts. The bile salt requirement is absolute for activity when long-chain triglyceride is the substrate and only the primary bile salts, i.e., those containing a 7α-hydroxyl group are effective [4]. However, bile salts may have effects not only on the enzyme but also on the substrate. This complexity is shown in fig.4. Taurocholate at 2 or 4 mM gave in this system very slow hydrolysis of a long-chain triglyceride emulsion. If deoxycholate, which does not have the 7α-hydroxyl group and therefore in itself cannot activate the enzyme, was included, a many-fold increase in the rate of triglyceride hydrolysis was observed. In other words, the simultaneous presence of deoxycholate lowered the molar requirement of taurocholate. It is evident that taurocholate can interact directly with the enzyme protein since BSSL binds to cholate immobilized on Sepharose [12,16]. The enzyme can only be eluted with primary (taurocholate) but not

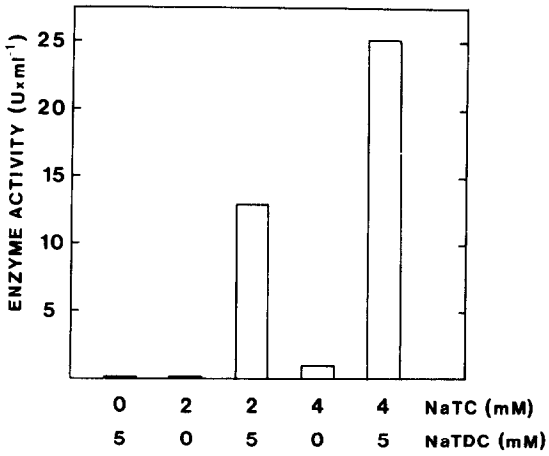

Fig.4. Effect of different bile salts on the activity of bile salt-stimulated lipase. The activity of BSSL against long-chain triglyceride was determined [11] in the presence of sodium taurocholate (NaTC) and sodium taurodeoxycholate (NaTDC) as indicated.

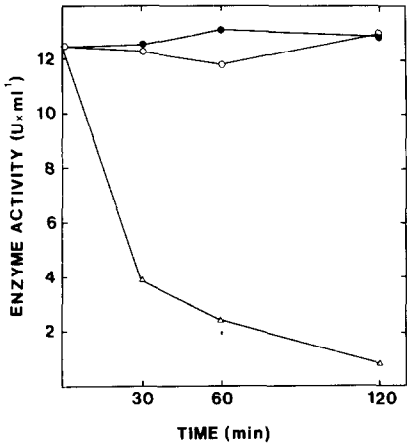

Fig.5. Protective effect of bile salts on tryptic inactivation of bile salt-stimulated lipase. Purified BSSL was mixed with trypsin (Sigma, St Louis MO) in a molar ratio of 10 mol trypsin/mol BSSL. Incubations were carried out in 0.05 M Tris–HCl (pH 7.5) in the presence (●) or absence (△) of 5 mM sodium taurocholate. BSSL was also incubated in the absence of trypsin as a control (○). Incubations were at 37°C and at times indicated aliquots were withdrawn and assayed for BSSL activity [11]. The activity is expressed as μmol fatty acid released/ml incubation mixture.

with secondary (deoxycholate) bile salt. Thus, binding of bile salt to the enzyme shows the same specificity as does activation of enzyme activity.

Bile salt not only has effects on the catalytical ability of the enzyme but also on its stability. In the absence of bile salt BSSL is inactivated at >50°C [19]. In the presence of bile salt the enzyme is protected against such heat inactivation. To have a function in duodenal fat digestion it is of course essential that BSSL is not degraded and inactivated by intestinal proteolytic enzymes. Fig.5 shows an experiment where BSSL was mixed with an excess of trypsin (10 mol/mol) in the presence or absence of 5 mM taurocholate. In the absence of bile salt the activity decreased and after 2 h of incubation only about 5% remained, whereas if bile salt was included no activity was lost during the incubation period. Thus, bile salts protect BSSL also from proteolytic degradation which allows the enzyme to be fully active in the duodenum of the newborn.

4. SUBSTRATE SPECIFICITY AND PHYSIOLOGICAL ROLE

BSSL is a non-specific lipase regarding the chemical structure of the substrate as well as the physical state (table 1). It hydrolyzes short- and long-chain triglycerides, diglycerides, monoglycerides, cholesteryl esters, retinol esters and *p*-nitrophenyl esters. It also catalyzes synthesis of cholesteryl esters. There is no great difference in enzyme activity against emulsified, micellar or soluble substrates. Thus, the enzyme is able to carry out complete fat digestion and, after activation by bile salts in duodenal contents, it can supplement low endogenous colipase-dependent pancreatic lipase and carboxyl ester hydrolase concentrations and contribute to an efficient hydrolysis of a variety of milk lipids. It can, however, also serve as a complement to the colipase-dependent pancreatic lipase. Due to its positional specificity the latter enzyme hydrolyzes each triglyceride molecule to two free fatty acids and one 2-monoglyceride [20]. It can also induce acylation forming di- and triglyceride from fatty acid and monoglyceride [21]. Thus, unless monoglycerides are removed the

Table 1

Substrate specificity of the bile salt-stimulated lipase

Substrate		
Chemical structure	Physical state	Spec. act.[a]
Trioleate	Emulsion	50[b]
sn-1(3)monooleate	Micellar solution	21[b]
sn-2-monooleate	Micellar solution	16[b]
Cholesteryl ester	Micellar solution	0.5[b]
Cholesterol + fatty acid	Micellar solution	2[c]
Retional palmitate	Emulsion	6–8[b]
p-Nitrophenyl acetate	Aqueous solution	30[d]

[a] Enzyme activities were determined as previously described: triglyceride hydrlysis [11]; monoglyceride hydrolysis [24]; cholesteryl ester hydrolysis and synthesis [6]; retinol ester hydrolysis [10]; and *p*-nitrophenyl acetate hydrolysis [10]

[b] Expressed as μmol fatty acid released.min^{-1}.mg protein^{-1}

[c] Expressed as μmol cholesteryl ester formed.min^{-1}.mg protein^{-1}

[d] Expressed as μmol *p*-nitrophenol released.min^{-1}.mg protein^{-1}

lipase reaction will stop when equilibrium concentration are formed. Recently, we have shown that BSSL can enhance the forward reaction in triglyceride hydrolysis while hydrolysing the 2-monoglyceride to glycerol and fatty acid [20]. Therefore, BSSL can drive intraluminal lipolysis towards completion. It is reasonable to assume that this will improve the overall process of fat absorption [22] although it is not yet clear exactly how this is accomplished. In support of this point is a report that fat absorption in preterm infants was about 1/3 lower from pasteurized compared with raw human milk (pasteurization completely inactivates BSSL) [23].

5. CONCLUSIONS

Human milk, in contrast to milk from most other species, contains a lipase stimulated by bile salts. This lipase is in many respects different from other well studied lipolytic enzymes; i.e., lipoprotein lipase and pancreatic colipase-dependent lipase. The apparent M_r is much higher. In an active state (e.g., during gel filtration) the M_r was about 310000. BSSL is inhibited by low concentrations of typical serine-hydrolase inhibitors suggesting a serine residue in its active site. It requires primary bile salts for optimal activity. Bile salts also protect the enzyme from inactivation caused by heat or proteolysis. The enzyme protein interacts directly with primary bile salts although the stoichiometry is not established. BSSL is fully active in the intestine of the breast-fed newborn. Due to its low substrate specificity it can be of great significance for the utilization of a variety of milk lipids. Furthermore, it has no positional specificity and therefore it may confer a last step to triglyceride hydrolysis in breast-fed infants. This may be the explanation of why absorption of fat from human milk is superior to that from most artificial formulae.

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