Bile Salt Stimulated Lipase: Comparative Studies in Ferret Milk and Lactating Mammary Gland[1]

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Bile salt stimulated lipase (BSSL) activity is 10–20 times higher in ferret milk than in human milk. We have used the ferret to study BSSL activity in lactating mammary gland and in mammary cells isolated by hyaluronidase-collagenase treatment followed by Ficoll gradient centrifugation. Furthermore, we have compared the characteristics of BSSL in the tissue preparations (homogenate or cells) to BSSL of ferret milk and to BSSL purified from ferret and human milk. The characteristics of BSSL in ferret mammary gland preparations and milk were similar to those of human milk BSSL—absolute requirement of primary bile salts, pH optimum of 7.5–9.0, stability at pH 3–9 and inhibition by eserine (physostigmine) and by serum. Purified ferret milk BSSL had a lower molecular weight (90kD) than did human milk BSSL (125 kD). There was an 86% homology of the *N*-terminal amino acid sequence between BSSL of ferret and of human milk. The marked similarity in characteristics between BSSL in ferret and human milk and the high activity of BSSL in ferret milk (520 U/mL colostrum and 250 U/mL mature milk) indicate that this species is an ideal animal model for the study of the synthesis and secretion of this digestive lipase which constitutes a significant portion (1–2%) of total milk protein.
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The presence and functional significance of bioactive components in human milk have been elucidated (1–3) only recently. Although enzyme activities of human milk have been demonstrated by *in vitro* studies (1), *in vivo* studies of the mechanisms of synthesis and secretion of these milk components have not been possible.

Bile salt stimulated lipase (BSSL), an enzyme previously found in the milk of humans (4,5), high primates (6), and carnivores such as the dog and cat (7), represents a significant proportion of total milk protein (1–2%) (8,9) and is probably the most abundant of the enzyme components in milk. BSSL is functionally similar and immunologically homologous to pancreatic carboxyl esterase (10). Because pancreatic lipase activity is low in the infant during the first months of life (11), it has been suggested that BSSL serves a compensatory role in fat digestion in the infant (5,12–15). Although several studies have assessed the possible compensatory role of this enzyme in the digestion of fat in the newborn (12–15), only one study has addressed localization of the enzyme in the lactating mammary gland (16).

In the present study, we have used a new animal model (the ferret) to quantitate and to compare the characteristics of the activity of BSSL in milk, in mammary gland and in cells isolated from the lactating mammary gland to those of the enzyme purified from ferret milk, as well as to known characteristics of BSSL activity of human milk.

MATERIALS AND METHODS

Two primiparous ferret jills, species *Mustela putoris furo* (Marshall Farms, North Rose, NY) were obtained in late pregnancy. The animals were housed separately in 18 × 10-inch stainless steel cages containing a four-inch bed of cedar shavings (17). Both jills received Purina Ferret Chow (Ralston Purina, St. Louis, MO) and water *ad libitum*. Kits were born at term on day 43 of gestation.

Milk collection. Milk was collected from each jill on days 3 (colostrum), 7, 10 and 15 of lactation. The jills were lightly sedated with acepromazine (TechAmerica Group Inc., Elwood, KS) and 2–5 units of oxytocin (pitocin, Parke Davis Division of Warner Lambert Co., Morris Plains, NJ) were injected intramuscularly to initiate milk let-down. Milk was manually expressed from each teat, collected by capillary action into a pasteur pipette and immediately transferred to a collection tube held on ice. Milk specimens were frozen at −70°C within 45 min from initiation of collection.

Mammary biopsy/tissue collection. Samples of mammary tissue were taken from each jill on day 10 (by sterile surgery) and on day 14 (sacrifice). Three hours prior to surgery, kits were separated from jills and placed in an incubator (70% humidity; 32°C). Milk was fully expressed from each teat prior to surgery. Ferrets were sedated with telezol (Fort Dodge Labs, Fort Dodge, IA) (14 mg/kg) delivered intramuscularly. The entire lobe of the left anterior mammary gland was removed through a half-moon incision and immediately placed on ice. Subsamples of the gland were rinsed in saline and frozen at −70°C, or used for cell dissociation. On day 14 *postpartum*, jills were sacrificed by an overdose of pentobarbital and exsanguination by cardiac puncture. Mammary tissue in the right inguinal area was perfused through the right femoral artery with 30 mL cold saline. The gland was removed and subsampled for frozen storage at −70°C. The left inguinal and right anterior glands were then removed, rinsed in saline and either frozen at −70°C or used for cell dissociation.

Mammary cell isolation. A mammary cell suspension enriched in secretory epithelial cells was prepared as previously described (18). Briefly, 0.25 to 10 g of mammary tissue were minced and subjected to enzymatic digestion with hyaluronidase and collagenase (100 U/mL and 150 U/mL, respectively; Worthington, Freehold, NJ) during 25–30 min incubation at 37°C with constant infusion of O_2/CO_2 (95:5%). The isolated cells were counted under light microscopy using a Neubauer homocytometer and

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Abbreviations: BSSL, bile salt stimulated lipase; HPLC, high-performance liquid chromatography; SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis.

crystal violet staining. When approximately 1×10^6 cells per mL of suspension were present, the digestion process was stopped by addition of trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) and slow cooling on ice. The suspension was filtered and subjected to a series of three washings and sedimentations on a 30% discontinuous Ficoll gradient (18). The cell band was collected and diluted to approximately 1×10^6 cells/mL before freezing. DNA from both tissues and cells was extracted by the method of Schneider (19) and quantitated by the method of Burton (20). Protein content of milk and purified enzyme was determined by the method of Lowry *et al.* (21).

Purification of BSSL. BSSL was purifed from ferret milk using a small-scale version of the method developed by Blackberg and Hernell (18). Molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on both 11–23% gradient (22) and 7.5% total acrylamide concentrations according to the methods of Laemmli (23). *N*-Terminal amino acid sequence was determined using an Applied Biosystems (Foster City, CA) Gas Phase Protein Sequencer (Model 470A) and a Waters Associates (Milford, MA) HPLC (high-performance liquid chromatography) System.

Assay of BSSL. Activity of BSSL was measured using glycerol tri[9,10-(n)^{3}H]oleate as substrate (24). The triglyceride was emulsified at 4°C with a Branson Model 200 Sonifier (Branson Sonic Power, Danbury, CT) for two 2-min intervals at setting 3, 50% of time pulsed. The standard assay system contained 1.6 mM triolein, 60 mM Tris-HCl buffer pH 8.5, 150 mM NaCl, 12 mM sodium taurocholate, 2.8% bovine serum albumin and dilute enzyme in a total volume of 200 μL. After incubation at 37°C for 15 min, the reaction was stopped by the addition of a mixture of chloroform/methanol/heptane (1.25:1.41:1, vol/vol/vol). The [^{3}H]oleic acid released was separated by liquid-liquid partition (25) and quantitated by liquid scintillation spectrometry with automatic external standardization as previously described (7). One unit of lipase activity is the release of 1 μmol of fatty acid per min. A milk sample of known BSSL activity was included in each assay to verify accuracy and reproducibility of the assay.

RESULTS

Concentration of BSSL in ferret milk during lactation. The activity of BSSL in ferret colostrum and milk collected on days 3, 7, 10 and 15 of lactation is shown in Figure 1. Additional samples of ferret milk (Marshall Farms) at various points throughout lactation were tested for comparison and are included in Figure 1. Marked differences were evident between the activity of BSSL in colostrum produced by each jill (310–634 units mL). However, beyond day 7 of lactation, activity of BSSL remained relatively constant in a range of 250 units/mL among different jills.

Purification of BSSL. Data for the purification of BSSL from ferret milk are presented in Table 1. There were differences in the partition of BSSL into whey and casein fractions between ferret milk and human milk. Initial purification of BSSL from human milk in this laboratory has resulted in 80–90% of total BSSL activity remaining in the whey fraction. Furthermore, loss of enzyme protein was appreciable only after acidification and incubation at 40°C for removal of casein. However, only 51% of the

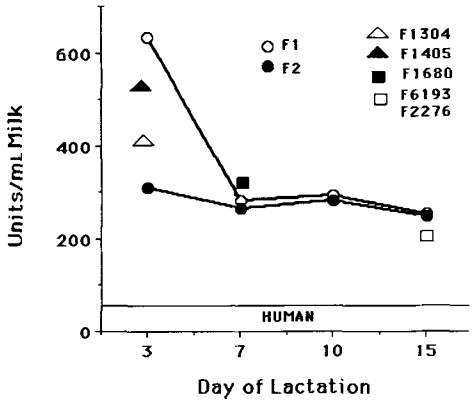

FIG. 1. Bile salt stimulated lipase (BSSL) activity in ferret colostrum and milk. Longitudinal data are presented for two ferrets. Additional data are provided for individual specimens of ferret colostrum and milk. Average activity of BSSL in human milk during the first two weeks of lactation is given for comparison.

BSSL in ferret milk was associated with the whey fraction. Centrifugation (10,000 × *g* for 1 h) alone resulted in significant precipitation of BSSL protein (17%) from ferret milk, whereas acidification and incubation at 40°C resulted in additional loss of enzyme in the precipitating fraction. The remaining (16%) BSSL activity not accounted for is most likely associated with the lipid fraction as this amount was lost after the defatting stage.

BSSL purified in this laboratory (26), as well as by others (8,9), has been shown to represent 1–2% of total milk protein. Data from the purification of BSSL in ferret milk indicate that the enzyme represents 8% and 3% of total protein in colostrum and in milk collected at day 18 of lactation, respectively. The molecular weight of BSSL in ferret milk as determined by SDS-PAGE is 90,000 Daltons. The molecular weight of the BSSL in ferret milk is identical to the molecular weight of BSSL in the milk of the cat (27), a close relative of the ferret, but is somewhat smaller than the human form [determined in this and other laboratories to be 125,000 Daltons (8,28,29) (Fig. 2)].

The *N*-terminal amino acid sequence determined for BSSL purified from ferret milk is shown in Table 2. The amino acid sequence of BSSL in the milk of the ferret shows 86% homology to the known amino sequence of human BSSL (9). There were differences between ferret and human BSSL at amino acids 2, 11 and 18 (Ser, Gly and His, respectively). In all cases these amino acids substituted for lysine residues in the BSSL of human milk.

Enzyme kinetics. Kinetics of BSSL purified from ferret milk and those of BSSL in whole ferret milk are presented in Figure 3. The substrate concentration curves for BSSL in ferret and in human milk are shown in Figure 4. While BSSL purified from ferret milk showed a hyperbolic response to increasing substrate concentration, the response of human milk BSSL was sigmoidal with a lag phase at substrate concentrations below 0.8 mM and inhibition at substrate concentrations greater than 3.2 mM. The apparent Km of BSSL in ferret milk was 1.67 mM. The apparent Km of human milk BSSL was close to that of the ferret enzyme, ranging between 1.6–1.7 mM.

Characterization of BSSL in ferret milk, in mammary tissue and in mammary cells and comparison to BSSL

TABLE 1

Purification of Ferret Milk BSSL[a], Comparison with the Purification of BSSL in Human Milk

Purification step	Milk fraction	Ferret Volume (mL)	Ferret Total activity (units; U)	Ferret U/mg protein	Human Volume (mL)	Human Total activity (units; U)	Human U/mg protein
Whole milk		5.6	1256	3.53	58	1393	1.1
Defatted	Skim	3.5	841	4.26			
	precipitate		212				
Acidified	Whey	2.7	650	6.64	83	139	2.2
(40°C)	casein	2.7	180	2.80			
Heparin Sepharose I		15.3	293	15.75	26.5	609	15.6
Heparin Sepharose II		11.1	194	108.80	9.7	54	66.6

[a]BSSL, bile salt stimulated lipase.

TABLE 2

***N*-Terminal Amino Acid Sequence of Bile Salt Stimulated Lipase (BSSL) Purified from Human or Ferret Milk**

Human[a]	Ala	Lys	Leu	Gly	Ala	Val	Tyr	Thr	Glu	Gly	Lys	Phe	Val	Asn	Lys	Lys	Leu	Gly	Leu			
		2									11				15							22
Ferret	Ala	Ser	Leu	Gly	Ala	Val	Tyr	Thr	Glu	Gly	Gly	Phe	Val	Asn	His	Lys	Leu	Gly	Leu	Phe	Gly	Asp

[a]Data on human milk from ref. 9.

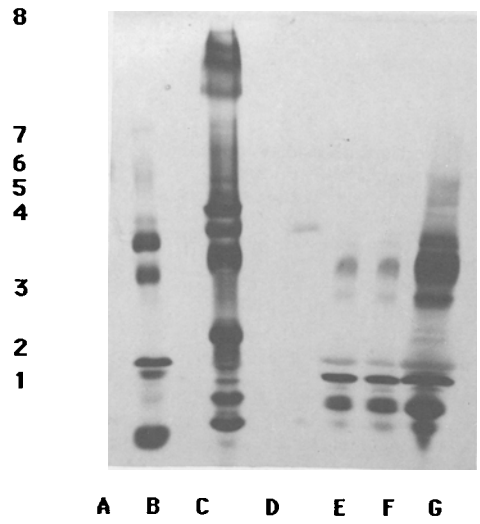

FIG. 2. Sodium dodecyl sulfate polyacylamide gel electrophoresis of purified ferret and human milk bile salt stimulated lipase (BSSL). Lanes: A, BSSL purified from ferret milk; B, BSSL purified from human milk; C, human milk, skim milk fraction; D, standards for molecular weight; E, BSSL purifed from ferret milk; F, ferret milk casein pellet, second centrifugation; G, ferret milk casein pellet, first centrifugation; H, ferret milk, skim milk fraction. Standards for molecular weight (kD), lane D: 1, lysozyme (14.4 kD); 2, trypsin inhibitor (22 kD); 3, carbonic anhydrase (31 kD); 4, ovalbumin (45 kD); 5, serum albumin (66 kD); 6, phosphorylase B (97 kD); 7, β-galactosidase (116 kD); 8, myosin (200 kD).

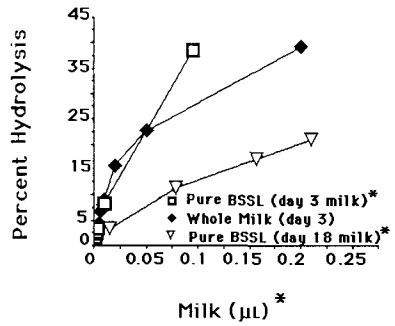

FIG. 3. Bile salt stimulated lipase (BSSL) enzyme concentration curve. Comparison of activity of pure ferret milk BSSL (expressed in equivalent milk volume based on purification data, Table 1) with BSSL activity in whole milk.

purified from ferret milk. The effect of bile salts on the activity of BSSL purified from ferret milk, in whole ferret milk, mammary tissue and mammary cells (0–30 mM taurocholate or glycocholate) is shown in Figure 5. Maximal enzyme activity for whole milk and for purified BSSL was achieved at taurocholate concentrations of 12–15 mM, whereas BSSL in mammary tissue and mammary cells was maximally activated at 10 mM taurocholate. In terms of absolute values, at 10 mM taurocholate, BSSL activity in the mammary tissue was 6.2-fold greater than cellular BSSL. Inhibition of BSSL activity in mammary tissue and mammary cells occurred at concentrations greater than 12 mM taurocholate, with 51–57.5% inhibition of maximum activity at 30 mM taurocholate. Likewise, purified BSSL and BSSL in whole milk showed inhibition in activity at taurocholate concentrations greater than 20 mM; however, at 30 mM taurocholate inhibition was somewhat less (34.2 and 46%, respectively) than that of the enzyme in mammary tissue and mammary cells.

BSSL activity in whole milk, in purified enzyme, in mammary tissue and in mammary cells was maximal in the presence of 10 mM glycocholate but declined in activity at glycocholate concentrations greater than 10 mM.

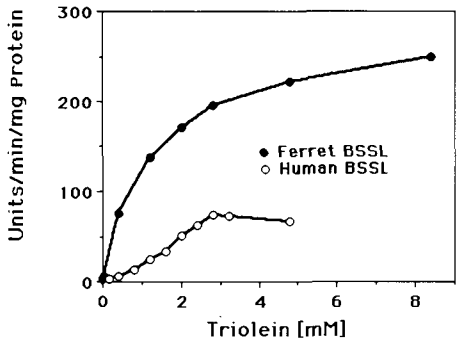

FIG. 4. Substrate concentration curve for bile salt stimulated lipase in ferret milk and in human milk.

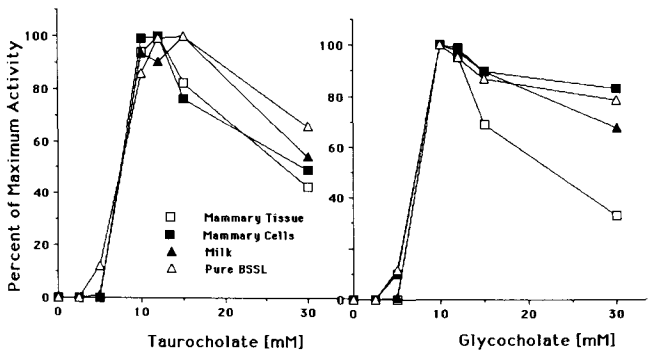

FIG. 5. Effect of bile salts, glycocholate and taurocholate, on bile salt stimulated lipase activity of ferret mammary tissue, mammary cells, milk and the purified enzyme. No enzyme activity was detected in the presence of secondary bile salts.

BSSL activity in mammary tissue was most affected with 67% inhibition at 30 mM glycocholate as compared to 17-32% inhibition of purified, whole milk and cellular BSSL. The response of BSSL was greater to glycocholate than to taurocholate at all bile salt concentrations: enzyme activity in cells, tissue and whole milk was 120-170% greater in the presence of glycocholate as compared to taurocholate; purified BSSL was 300-415% more active with glycocholate than with taurocholate. Secondary bile salts did not elicit BSSL activity in milk, mammary tissue or cells. In human milk, BSSL activity was slightly higher with glycocholate than taurocholate in the range of 10-15 mM, and no activity was detected in the presence of secondary bile salts.

Effect of pH. The effect of pH on the activity of BSSL in milk, purified enzyme, mammary tissue and mammary cells is shown in Figure 6. Maximal activity of purified BSSL and BSSL in whole milk was achieved at pH 8.0, and mammary tissue and mammary cells reached optimum activity at pH 8.5. At pH 7.5-9.0, greater than 75% of BSSL was present in whole milk, purified enzyme and mammary cells. No activity was detected in mammary tissue homogenate below pH 8.0 or above pH 9.0.

Stability of BSSL as function of pH. Dilute samples of purified BSSL, ferret milk, mammary tissue and mammary cells were incubated at 37°C for up to 60 min at pH 1.5-9.5. Samples were assayed after 0, 30 and 60 min of pre-incubation at optimal conditions (pH 8.5, 12 mM taurocholate) to determine the stability of enzyme activity. Stability of BSSL to 60 min pre-incubation is shown in Figure 7. Greater than 75% of BSSL was present in whole milk after 60 min of preincubation at pH 4.0-9.0. BSSL purified from ferret milk retained 75% of control activity when pre-incubated for 60 min at pH 8.5, but only 25-30% of initial activity was measurable after pre-incubation at pH above or below optimum. In comparison to purified BSSL and whole milk BSSL, the BSSL activity in mammary tissues and mammary cells was more stable at extremes of pH. As compared to initial activity, mammary cells showed enhanced activity after 60-min pre-incubation at pH 3.0.

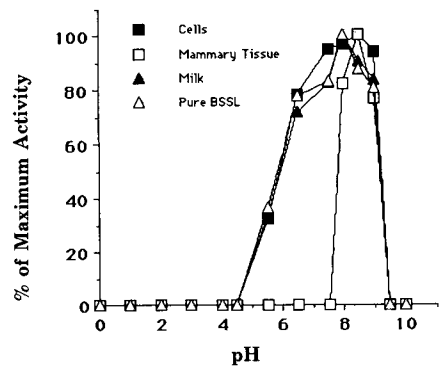

FIG. 6. The effect of pH on bile salt stimulated lipase (BSSL) activity. Comparison of the activity of purified ferret milk enzyme with that of BSSL in ferret mammary tissue, mammary cells and ferret milk. The standard assay system was adjusted to the various pH values using buffers at a final assay concentration of 60 mM. Buffers used were: pH 2.4-3.6 glycine-HCl; pH 3.6-6.0 Na acetate; pH 6.0-7.5 Na phosphate; pH 7.5-8.5 Tris-HCl; and pH 8.5-9.5 glycine-NaOH.

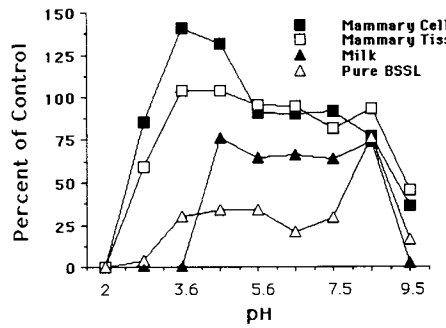

FIG. 7. Stability of bile salt stimulated lipase (BSSL) as a function of pH. Mammary tissue, mammary cells, milk and purified enzyme were incubated in 0.05 M buffer (see legend to Fig. 6) for 60 min at a pH range of 2.0-9.5, followed by incubation in the standard assay system (pH 8.5) for quantitation of BSSL activity. Note: the X axis is not drawn to scale.

Inhibition of BSSL activity by eserine hemisulfate and serum. Both eserine (physostigmine) and serum are potent inhibitors of milk BSSL (24). Activity of BSSL in milk, mammary tissue and mammary cells was measured in the presence of 0.1 to 10 mM eserine hemisulfate (Fig. 8). In all cases, less than 10% of maximum activity remained when 2 mM eserine was included in the assay.

Activity of non-perfused mammary tissue homogenate was compared to activities of purified BSSL, ferret milk and serum-free mammary cells in the presence of 0.5-5% heat-inactivated serum (vol/vol) (Fig. 9). At concentrations

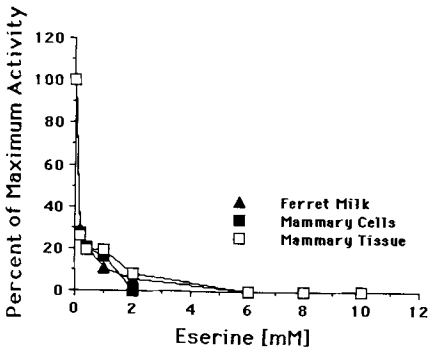

FIG. 8. Effect of eserine (physostigmine) on bile salt stimulated lipase activity in ferret milk, mammary cells and mammary tissue.

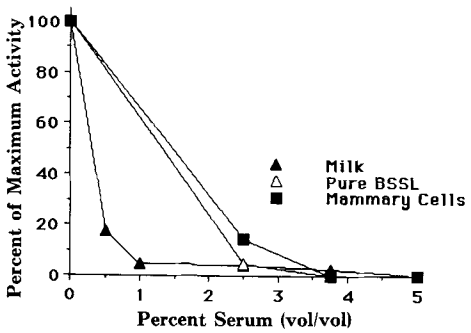

FIG. 9. Effect of serum on bile salt stimulated lipase activity in ferret milk, mammary cells and mammary tissue.

of 0.5% serum, less than 20% of maximum activity was measured in whole milk, and complete inhibition was reached with 4% serum. The nature of the BSSL inhibitor in serum is not known. Recent studies have established that the serum inhibition of acid and neutral cholesteryl ester hydrolases is due to serum apoprotein A-I (30). Whether milk BSSL, an enzyme identical to pancreatic carboxylester hydrolase (10,31), is likewise inhibited by apoprotein A-I remains to be established. It is of interest that while BSSL activity is inhibited by serum, a lipolytic activity stimulated by taurocholate was measured in the serum of the ferret (275 nmol/min/mL serum). This serum lipase did not contribute to mammary homogenate lipolytic activity at the concentration of tissue assayed (0.01 mg).

DISCUSSION

This study describes the strong similarities in both structural and functional characteristics of BSSL produced by the ferret and by the human. Ferret milk contains 10–20 times higher BSSL activity than human milk. This difference does not appear to be a result of higher quantities of BSSL protein in mature ferret milk (after day 7 of lactation) but is more likely due to the greater Vmax (160% of human) and higher turnover number (5800 sec^{-1}) of BSSL in ferret milk as compared to human BSSL. The data demonstrate the presence of BSSL in the mammary gland and in the mammary cells of the lactating ferret. Although this study does not delineate the site of synthesis for BSSL, the data suggest that the enzyme is synthesized in the mammary gland. Although a bile-salt activated lipase is present in the serum of the lactating ferret (probably pancreatic lipase or carboxylester hydrolase), it is unlikely that this is the source of BSSL in milk as there is a greater than 1000-fold difference in milk BSSL activity as compared to serum bile salt stimulated lipolytic activity. Comparison of the activity of BSSL in mammary tissue homogenate to activity of cellular BSSL shows a 3–5-fold greater activity of BSSL in tissue homogenate (expressed per μg DNA). This observation may indicate that synthesis of BSSL is specific to a particular cell type or that enzyme activity might be lost during the cell isolation procedure. A cell preparation enriched in epithelial cells was used in this study. However, the BSSL in mammary cells also may be in a bound form either at the cellular or subcellular membrane or may somehow be modified once transported from the cell. The time dependent increase of BSSL activity in mammary cells at low pH may represent the lysis of Golgi vesicles transporting protein from the cell or release of BSSL from a membrane-bound receptor.

The physiological mechanisms of lactation are poorly understood, particularly with respect to specialized bioactive components in milk. BSSL is a particularly unique component of milk since it possesses no known function in milk nor in the lactating mammary gland. Comparison of species with and without BSSL shows a strong evolutionary link in species secreting BSSL. Milk of ruminants, rodents and omnivorous mammals has no BSSL activity (32,33), whereas BSSL is secreted in milk produced by carnivores (7,33). Further study of this protein is necessary to elucidate the reasons for species differences. Human milk also contains large quantities of amylase (34,35) which, likewise, has no known function in milk or in the lactating mammary gland. Study of genetic similarities in mammary and pancreatic cells may provide evidence of an evolutionary expression of DNA coding for these enzymes as the mammalian species evolved.

While the evolution of lactation and the origin of bioactive components in human milk are still unknown, the functional significance of BSSL to the neonate cannot be ignored. Once ingested by the infant, BSSL is active in the digestion of milk lipid in the intestine. Because pancreatic function of the human infant is not fully developed in early life, this bioactive component is important for the neonate, especially the premature (36). Wang *et al.* (27) reported a greater rate of growth in kitten fed milk replacer supplemented with purified human BSSL. Similarly, addition of human milk to low-birth-weight infant formulas resulted in a lower excretion of fat by very premature infants as compared to infants fed formula alone (37). Furthermore, fat absorption is lower in preterm infants fed pasteurized as compared to fresh human milk (38).

Use of the ferret as an animal model will allow detailed investigation of the mechanisms of BSSL synthesis in the mammary gland and secretion into milk throughout lactation. Furthermore, animal studies will enable the investigation of the regulation of *prepartum* synthesis and secretion of BSSL. Preliminary studies indicate that BSSL is present in human *prepartum* mammary secretions as early as three months before full term delivery (39). In addition, the use of the ferret will allow study of the *in vivo* role of BSSL in fat digestion in the newborn's

intestine and its impact on normal growth and development.

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