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Bile salt-stimulated lipase in human milk: evidence for its synthesis in the lactating mammary gland

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Human milk contains many enzymes and other biologically active proteins. One of the enzymes, the bile salt-stimulated lipase, constitutes as much as 1% of the milk proteins. Its importance for efficient utilization of milk lipids by the breast-fed infant is now well established. However, whether the lipase protein is a product of protein synthesis within the mammary gland has up till now been an unanswered question. Using biopsy material from lactating human mammary gland we have now demonstrated that the enzyme is synthesized within the gland. This was done by immunoprecipitation of [^{35}S]methionine-labelled protein from tissue pieces. By activity determination we could also determine the amount of enzyme stored in the gland. It was concluded that bile salt-stimulated lipase accounted for 1.3 μg/mg tissue protein. Finally, from this figure it could be calculated that about 10–15% of the total protein present in the tissue was milk protein.

Bile salt-stimulated lipase; Protein synthesis; Immunoprecipitation; Lactation; (Human mammary gland)

1. INTRODUCTION

Human milk contains a lipase that, after activation by primary bile salts in duodenal contents, contributes to the digestion of milk lipids in the breast-fed infant [1–4]. Hence, with regard to its function, and the fact that it is not present in milk from most species [1,4,5], it is of particular interest among the milk enzymes [6]. Therefore, in comparison with most of them, it is also well characterized [2,7].

Because, as judged by immunochemical and functional studies, this bile salt-stimulated lipase is identical with or at least very similar to carboxylic ester hydrolase secreted from the pancreas [8], it has been argued that the enzyme found in milk may in fact originate in the pancreas [9]. Indirect evidence has contradicted this hypothesis [10,11] but until now there has been no direct evidence of synthesis of bile salt-stimulated lipase within the mammary gland. We now report, from studies on a biopsy from lactating human mammary gland, on active protein synthesis in general, and of bile salt-stimulated lipase in particular.

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2. MATERIALS AND METHODS

2.1. *Preparation of enzyme and antiserum*

Bile salt-stimulated lipase was purified from human milk as described [7]. To minimize formation of contaminating antibodies the preparation thus obtained was run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroeluted [12] prior to injection into rabbits. 25 μg purified enzyme, together with an equal volume of Freund's complete adjuvant, was used for the first injection and the same amount of enzyme with incomplete adjuvant in the subsequent monthly booster injections. The animals were bled monthly about 2 weeks after each booster. Serum collected from a non-immunized rabbit was used as control serum.

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Iodination of total skim milk protein and purified enzyme protein, omitting the SDS-PAGE step, was done using lactoperoxidase and glucose oxidase as described by Peterson et al. [13]. Free and protein-bound ^{125}I was separated by chromatography on Sephadex G-25. Only fractions where more than 98% of the radioactivity was trichloroacetic acic precipitable was used.

2.2. *Tissue preparation and [^{35}S]methionine incorporation*

A mammary gland biopsy was taken under local anesthesia from a healthy volunteer 2 h after her baby had been fed. She had been breast-feeding for 2 months. The tissue piece (wet wt 150 mg) was immediately placed in 4 ml cold Eagle's minimal essential medium with Earle's salts and 2 g/l sodium bicarbonate without glutamine and methionine. The medium was supplemented with 5% bovine serum albumin, 5 mM glucose, 1 mM glutamine, 10^{-7} M insulin (bovine, Sigma, St. Louis, MO), 0.149 ng/ml methionine and 1.25 mM Hepes, pH 7.4, and kept on ice. Within 30 min the tissue piece was transferred to the same medium including [^{35}S]methionine (1075 Ci/mmol, 45 μCi/ml; NEN, Du Pont, Scandinavia AB) and incubated for 3 h at 37°C. The tissue was removed and homogenized (Polytron) in 3 ml buffer [0.025 M ammonia, adjusted to pH 8.2 with HCl, 5 mM EDTA and, per ml, 8 mg Triton X-100, 0.4 mg SDS, 1 μg pepstatin (Boehringer Mannheim), 10 μg leupeptin (Boehringer) and 25 KIE Trasylol^{R} (Sigma)]. Aliquots of tissue homogenate and medium were withdrawn for determination of enzyme activity. The remaining tissue homogenate was made 1% in SDS and boiled for 5 min. The boiled homogenate was used for the immunoprecipitation experiments.

2.3. *Immunoprecipitation*

For test experiments 10 μl ^{125}I-bile salt-stimulated lipase, or ^{125}I-labelled skim milk protein, were diluted 10-fold with phosphate-buffered saline, pH 7.4 (PBS), with 1% SDS and heated to 95°C for 6 min. Samples were further diluted 5-fold with a buffer containing per ml: 3.564 mg $Na^2HPO_4 \cdot 2H_2O$, 55 μg NaN_3, 8.766 mg NaCl, 10 mg Triton X-100, 1 mg SDS, 100 μg heparin, 745 μg EDTA and 17.4 μg phenylmethylsulfonyl fluoride (Serva, Heidelberg), pH 7.4 (buffer A). 10 μl antiserum (or control serum) was added and samples were incubated for 15–20 h at 4°C. Protein A-coated *Staphylococcus aureus* cells (Pansorbin, Calbiochem) were added to bind the immune complexes.

After 1 h at 4°C precipitates were recovered by centrifugation and washed three times in PBS with 0.5% Triton X-100 and 1 mg/ml bovine serum albumin, PBS with 0.5% Triton X-100 and PBS, respectively. The radioactivity was determined in a gamma-counter and the immune complexes were prepared for gel electrophoresis.

When tissue homogenate was used 200 μl was diluted 5-fold in buffer A followed by the procedure above, omitting the radioactivity counting.

2.4. *Gel electrophoresis*

SDS-PAGE was run in 12.5% gels essentially according to Laemmli [14]. Immunoprecipitates as well as other samples (made 1% in SDS) were boiled for 5 min and applied to the gel. Gels were dried and autoradiograms were obtained by exposure of Kodak X-omat films to gels at −70°C. For quantitation, bands were cut out and either measured directly in a gamma-counter (^{125}I samples) or eluted in a scintillation cocktail (Optiscint/Optisolve/H_2O, 9:1:0.2) (LKB, Sweden) and counted in a liquid scintillation counter.

2.5. *Determination of enzyme activity and protein content*

Enzyme activity was determined with long-chain triacylglycerol as substrate as described [7]. The procedure to extract released free fatty acids was modified [15] to minimize co-extraction of [^{35}S]methionine. As a control enzyme activity was also determined on boiled samples.

Protein was determined according to Lowry et al. [16] with bovine serum albumin as standard.

3. RESULTS

3.1. *Immunoprecipitation*

To evaluate the immunoprecipitation procedure, iodinated purified bile salt-stimulated lipase was used. 10 μl antiserum precipitated 80–85% of the antigen in the range 1–1000 ng. The background (radioactivity precipitated by a control serum) was in all cases below 0.5%. Selectivity was tested by

precipitating bile salt-stimulated lipase from skim milk proteins labelled with ^{125}I. The resulting autoradiogram is shown in fig.1. Except for bile salt-stimulated lipase no skim milk protein was selectively precipitated by the antiserum as judged by counted radioactivity in slices from the original gel. In these experiments the amount precipitated as bile salt-stimulated lipase from the skim milk protein was 1–2% of total radioactivity. This figure should be compared to 1% which is the relative amount of bile salt-stimulated lipase in milk protein as determined by enzyme activity and protein analyses [7].

Fig.2. shows an autoradiogram of the precipitate obtained when the lactating mammary gland tissue homogenate was mixed with antiserum to bile salt-stimulated lipase. A protein with the same mobility as ^{125}I-labelled bile salt-stimulated lipase can be seen together with some minor components with a slightly higher mobility. None of these were seen with the control serum. Moreover, when unlabelled bile salt-stimulated lipase was included prior to the precipitation the major, as well as the minor components, disappeared. This indicates that the minor components are also related to bile salt-stimulated lipase. Whether these are preforms, e.g. incompletely glycosylated protein, or proteolytically degraded enzyme protein cannot be deduced from these experiments. Based on its fraction of trichloroacetic acid precipitable radioactivity bile salt-stimulated lipase accounted for 0.51 and 0.53% of total protein synthesis in two identical experiments.

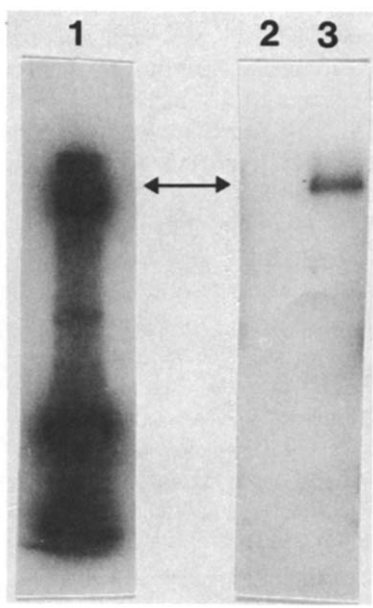

Fig.1. Autoradiogram demonstrating immunoprecipitation of bile salt-stimulated lipase from iodinated skim milk proteins. Electrophoresis and immunoprecipitation were as described in section 2. The samples are skim milk (lane 1) and precipitate from precipitation with a control serum (lane 2) or with antiserum to bile salt-stimulated lipase (lane 3). The migration of purified bile salt-stimulated lipase is indicated by the arrow.

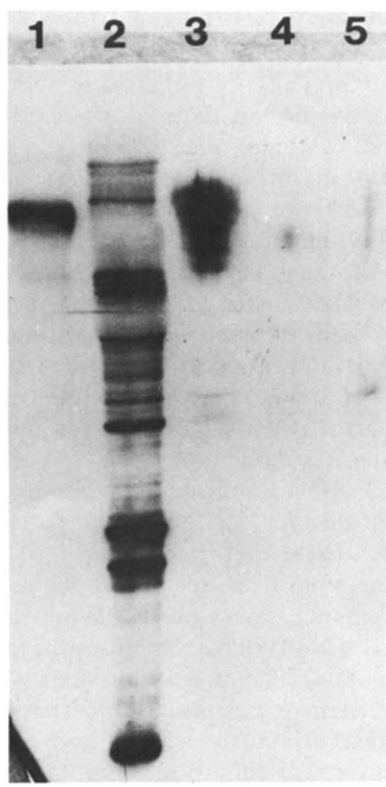

Fig.2. Autoradiogram demonstrating immunoprecipitation of bile salt-stimulated lipase from a homogenate of human lactating mammary gland. The tissue piece was incubated with [^{35}S]methionine and homogenized. Electrophoresis and immunoprecipitation were done as described in section 2. Samples are iodinated purified bile salt-stimulated lipase (lane 1), tissue homogenate (lane 2), precipitate from homogenate using antiserum (lane 3) or control serum (lane 4). In the sample for lane 5, 20 μg purified unlabelled enzyme was added prior to treament with antiserum.

3.2. *Enzyme activity*

Using our routine assay with long-chain triacylglycerol as substrate bile salt-stimulated lipase activity was detected in the tissue homogenate as well as in the medium. The activities were 0.46 and 0.03 μmol fatty acid released per min, respectively. Calculating with a specific activity of 100 μmol/min per mg protein [7] this corresponds to 14 and 1.2 μg enzyme protein. The amount of lipase in the tissue was thus 0.1 μg/mg tissue or 1.3 μg/mg tissue protein.

4. DISCUSSION

Apart from the nutritionally important whey proteins and caseins human milk contains a number of proteins with enzymatic activities [6]. Most of these do not have known specific functions in the milk or in the gastrointestinal tract of the recipient infant, but may be present in milk as a result of leakage from the blood, the mammary gland or cells in milk. To a few distinct physiological functions have been attributed, but, bile salt-stimulated lipase being an exception, evidence is scarce [3,6]. Therefore, it is not surprising that very little is known about the regulatory mechanisms of synthesis and secretion of the enzymes in milk. In fact, the same is also true for human milk proteins in general and other milk constituents. So far, the secretion of milk fat [17], and to some extent casein micelles, has been studied but mainly on a structural level [18].

Since there has been uncertainty with regard to the tissue origin of human milk bile salt-stimulated lipase [9] it was of decisive interest to demonstrate convincingly whether it is synthesized within the lactating mammary gland. From experiments in which a piece of human lactating mammary gland tissue was kept in culture medium it was evident from incorporation of [^{35}S]methionine into proteins of various sizes that protein synthesis took place. By immunoprecipitation techniques we could show that one of the proteins into which the labelled amino acid had been incorporated was indeed bile salt-stimulated lipase. The enzyme accounted for about 0.5% of the total protein synthesis recorded. Since the lipase protein constitutes about 1% of total milk protein [7] this experiment suggests that milk proteins account for half of the total protein synthesis in the lactating mammary gland. From the amount of radioactivity precipitated, and the known number of methionine residues in bile salt-stimulated lipase [7] the total protein synthesis was calculated. During the complete incubation period about 30 ng protein had been synthesized. With the assumption of an average methionine content in the protein approximately the same figure was obtained when trichloroacetic acid-precipitable radioactivity was the basis for the calculation. Moreover, by enzyme activity determinations it was clear that activity enzyme was stored in the tissue (0.1 μg active lipase protein/mg tissue). Since bile salt-stimulated lipase constitutes 1% of the milk protein the amount of stored milk protein was 10 μg/mg tissue or 130 μg/mg tissue protein. Based on these determinations it could be calculated that 10–15% of the protein present in the tissue was milk protein. Obviously, the value for the milk-producing cells should be higher since they make up only part of the tissue.

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REFERENCES

[1] Freudenberg, E. (1953) Die Frauenmilch-Lipase, Karger, Basel.
[2] Hernell, O. (1975) Eur. J. Clin. Invest. 5, 267–272.
[3] Hernell, O., Bernbäck, S. and Bläckberg, L. (1987) in: Perinatal Nutrition (Lindblad, B. and Weaver, R.D. eds) Bristol-Myers Symposia Series, vol. 6, in press.
[4] Hernell, O., Bläckberg, L. Fredrikzon, B. and Olivecrona, T. (1981) in: Gastroentereology and Nutrition in Infancy (Lebenthal, E. ed.) pp. 465–472 Raven, New York.
[5] Bläckberg, L., Hernell, O., Olivecrona, T., Domellöf, L. and Malinov, M.R. (1980) FEBS Lett. 112, 51–55.

[6] Hernell, O. and Lönnerdahl, B. (1987) in: Proteins and Non-Protein Nitrogen in Human Milk (Atkinson, S.A. and Lönnerdahl, B. eds) CRC Press, Boca Raton, in press.
[7] Bläckberg, L. and Hernell, O. (1981) Eur. J. Biochem. 116, 221–225.
[8] Bläckberg, L., Lombardo, D., Hernell, O., Guy, O. and Olivecrona, T. (1981) FEBS Lett. 136, 284–288.
[9] Carey, M.C., Small, D.M. and Bliss, C.M. (1983) Annu. Rev. Physiol. 45, 651–677.
[10] Bläckberg, L., Blind, P.J., Ljungberg, B. and Hernell, O. (1985) J. Pediatr. Gastroenterol. Nutr. 4, 441–445.
[11] Hamosh, M. (1982) Adv. Pediatr. 29, 33–49.
[12] Hunkapiller, M.W., Lujan, E., Ostrander, F. and Hood, L.E. (1983) Methods Enzymol. 91, 227–236.
[13] Peterson, J., Olivecrona, T. and Bengtsson-Olivecrona, G. (1985) Biochim. Biophys. Acta 837, 262–270.
[14] Laemmli, U.K. (1970) Nature 227, 680–683.
[15] Spooner, P.M., Chernick, S.S., Garrison, M.M. and Scow, R.O. (1979) J. Biol. Chem. 254, 10021–10029.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Thompson, B.J. and Smith, S. (1985) Pediatr. Res. 19, 139–143.
[18] Ferguson, D.J.P. and Anderson, T.J. (1985) Anat. Embryol. 168, 349–359.