Two forms of human milk bile-salt-stimulated lipase

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This study provides the first direct evidence for the existence of two active forms of the human milk enzyme bile-salt-stimulated lipase. Heparin-based affinity chromatography and size-exclusion chromatography were used to obtain two active forms of this enzyme with molecular masses of 97 and 120 kDa.

INTRODUCTION

Bile-salt-stimulated lipase (BSSL; EC 3.1.1.3) is an important digestive enzyme originally found in the milk of humans and certain other primates [1,2]. In 1986 this lipase was also discovered in dog and cat milk [3]. The milk from many other animals, including the rat, guinea pig, rabbit, goat, cow, pig, horse and Rhesus monkey, have all tested negative for the presence of BSSL [2,4]. It was postulated by Freed *et al.* [3] that the exclusive occurrence of BSSL in milk from higher primates and carnivores might be related to the composition of milk fat in the various species.

Several groups have reported BSSL to be a glycoprotein with a single polypeptide chain. The molecular mass of the enzyme has been determined by SDS/PAGE to be 125 kDa [5,6], 112 kDa [7], 100 kDa [8] and 90 kDa [9]. In addition, the molecular mass of BSSL was determined to be 107 kDa by sedimentation-equilibrium centrifugation [10]. It has been suggested that differences in the reported molecular mass of BSSL are due to variations in the mobility of this glycoprotein in different electrophoresis systems [11].

Baba *et al.* [12] have very recently reported possible evidence for the existence of two variants of the cDNA for human BSSL, although they did not find any evidence for the expression of the second cDNA. The two variants were reported to be identical except for the deletion of 198 bases in the smaller form. Because the nucleotide sequences of the long and short cDNAs were otherwise identical, it was suggested that they may be products of the same gene, and could have been produced by alternative splicing of the BSSL mRNA precursor. No protein corresponding to the smaller cDNA was found.

We report here the separation of two active forms of human milk BSSL, demonstrating that some individuals produce two forms of this enzyme in approximately equal amounts.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. or Fisher Scientific. The TSK-GEL Heparin-Toyopearl 650M affinity packing was from Supelco (Bellefonte, PA, U.S.A.). Separations were done by f.p.l.c. with Pharmacia equipment, with peak detection by u.v. absorption at 280 nm. Chromatograms were recorded on a Hewlett-Packard model 3396A computing integrator. Human milk samples (40 ml) were manually expressed by volunteer donors. The samples were immediately frozen at -20°C and were transferred to a -70°C freezer as soon as possible.

Protein concentration

The protein content of various fractions was determined by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, U.S.A.), by either the standard or the enhanced protocol as described in the Pierce literature, with BSA as the standard protein.

Enzyme activity

The esterase activity of BSSL was assayed with 4-nitrophenyl acetate as substrate [6,13]. A stock solution of 100 mM substrate in h.p.l.c.-grade acetonitrile was stored at 4°C when not in use. The buffer used for the assays and the stock solution of bile salt activator (100 mM-sodium taurocholate) was 50 mM-Tris/HCl/0.40 M-NaCl (pH 7.5). Buffer was equilibrated at 25°C and then mixed with taurocholate (when used) and 20–100 μl of enzyme solution. After incubation of this mixture for 2 min at 25°C , the reaction was initiated by addition of 10 μl of substrate. The final assay volume was 1 ml and contained 1% (v/v) acetonitrile, 2 mM-taurocholate and 1 mM substrate. The initial rate of 4-nitrophenol production was determined by monitoring the increase in A_{418} with a spectrophotometer interfaced to a Hewlett-Packard Vectra computer. The molar absorption coefficient of 4-nitrophenol is $11\,500\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 418 nm and pH 7.5 [14]. One unit of enzyme is defined as the activity producing 1 μmol of product/min. The purified enzyme had a specific activity of 100–150 units/mg of protein.

PAGE

SDS/PAGE was carried out in 7%-acrylamide gels as described by Laemmli [15]. The molecular-mass markers were carbonic anhydrase (29 kDa), egg albumin (45 kDa), BSA (66 kDa), rabbit muscle phosphorylase *b* (97.4 kDa) and *Escherichia coli* β -galactosidase (116 kDa). The gel was stained with Coomassie Blue.

Enzyme purification

A 35 ml sample of human milk was centrifuged at 29000 *g* for 45 min in a Sorvall RC-2B refrigerated centrifuge and a model SS-34 rotor. After removal of the cream, the pH of the skim milk was adjusted to 4.6 with HCl. The milk was then heated at 40°C for 30 min to precipitate the caseins. The milk whey was obtained by centrifuging as above to remove the precipitate and then decanting the whey. Milk whey contained approx. 15 mg of protein/ml.

A 15 ml sample loop constructed of Teflon tubing (1.5 mm internal diam.) was filled with milk whey, and the whey was injected on to the heparin-Toyopearl affinity column as described by Gissh *et al.* [16]. The affinity column was a 30 cm $\times$ 2.5 cm

Abbreviations used: BSSL, bile-salt-stimulated lipase; CEH, carboxylic-ester hydrolase.

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glass column containing 100 ml of the heparin-Toyopearl resin. A linear gradient was run at 2.0 ml/min with 20 mM-Tris/HCl (pH 7.5) as buffer A and 20 mM-Tris/HCl/1.0 M-NaCl (pH 7.5) as buffer B. The gradient program was as follows: 0% B for the first 20 min, then to 100% B in 65 min, hold at 100% B for 85 min, and then return to 0% B in 1 min. Peaks were assayed for protein and BSSL activity as described above. The peak with BSSL activity contained 15–20 ml at approx. 0.3 mg of protein/ml, and was termed crude BSSL.

After a 5-fold concentration of the crude BSSL, 100 μ l portions of it were injected on to a Pharmacia Superose 12 size-exclusion column (10 mm $\times$ 300 mm). Proteins were eluted from the column with 50 mM-Tris/HCl/0.40 M-NaCl (pH 7.5) at a flow rate of 0.15 ml/min. After 85 min the flow was increased to 0.40 ml/min to elute the last peak more quickly. Peaks with BSSL activity were subjected to SDS/PAGE as described above.

In one case the above purification scheme was carried out in the presence of proteinase inhibitors. The following inhibitors were added to a 40 ml milk sample from donor A: pepstatin (1 mg), phenylmethanesulphonyl fluoride (7 mg) and hydrocinnamic acid (12 mg). After the first centrifugation and removal of cream, the same amounts of inhibitors were again added to the milk sample. The buffers used for the heparin-Toyopearl affinity chromatography contained 7 μ M-pepstatin, 1 mM-phenylmethanesulphonyl fluoride and 2 mM-hydrocinnamic acid.

Fig. 1. Heparin affinity chromatography of human milk whey

Milk whey proteins were eluted from the affinity column with an NaCl gradient in 20 mM-Tris/HCl (pH 7.5) as described in the text. Detector was set at 2.0 A_{280} units full scale. Arrows mark BSSL-containing peaks. (a) Sample from donor A. (b) Sample from donor B.

The treatment of this milk sample was otherwise identical with that described above.

RESULTS

Human milk samples from five different donors gave very similar separations on the heparin affinity column. Fig. 1 shows two of these separations. BSSL is contained in a peak (marked with an arrow) at approx. 104 min.

Further processing of the crude BSSL by size-exclusion chromatography gave three major peaks in samples from donor A (Fig. 2a). Two of these peaks (marked with arrows) showed high BSSL activity, with the first peak (at approx. 65 min) giving specific activities of 15 units/mg in the absence of bile-salt stimulation and 110 units/mg with 2 mM-taurocholate present, whereas the second peak (at approx. 72 min) gave 14 units/mg and 120 units/mg respectively. Preliminary studies indicate that the two forms are in approximately equal amounts in milk from donor A. The molecular masses of these two active forms of BSSL were determined to be 97 and 120 kDa by SDS/PAGE (Fig. 3). The electrophoretic bands were rather broad, and in that respect were similar to the one band reported by Abouakil *et al.* [7] for immunoaffinity-purified BSSL.

Fig. 2. Size-exclusion chromatography of the crude BSSL from the heparin affinity separation

Crude BSSL was further purified with a Pharmacia Superose 12 size-exclusion column. Proteins were eluted with 50 mM-Tris/HCl/0.4 M-NaCl (pH 7.5) as described in the text. Detector was set at 0.05 A_{280} unit full scale. Arrows mark BSSL peaks. (a) Sample from donor A. (b) Sample from donor B.

Fig. 3. SDS/PAGE of BSSL

Lanes 1 and 2 are the crude BSSL from the heparin affinity column (Fig. 1a). Lanes 3 and 4 are the first BSSL peak (65 min) from the size-exclusion column (Fig. 2a). Lanes 5 and 6 are the second BSSL peak (72 min) from the size-exclusion column (Fig. 2a). Conditions as stated in the text.

When a milk sample from donor A was treated with proteinase inhibitors during the BSSL purification, the same BSSL peaks were found in both the affinity chromatography and the size-exclusion chromatography. Neither of the two peaks (65 min and 72 min) from the size-exclusion column had enzyme activity when assayed in the absence of bile-salt stimulation, and each showed less than 25% of normal activity in the presence of 2 mM-taurocholate. This decreased activity was not unexpected, since BSSL is known to be inhibited by phenylmethanesulphonyl fluoride [7].

The samples from the other four donors, as represented by donor B, each yielded two peaks by size-exclusion chromatography (Fig. 2b), and only one peak (at approx. 66 min) had BSSL activity. This peak had specific activities of 9.3 units/mg without bile-salt stimulation and 150 units/mg in the presence of 2 mM-taurocholate.

DISCUSSION

Two active forms of human BSSL were found in the milk from donor A. This is the first reported separation of more than one form of BSSL, and these two forms have been observed in every sample analysed from donor A, including milk from 3, 4, 5, 6, 8 and 21 months *post partum*. The two forms have very similar activities with 4-nitrophenyl acetate as substrate, in both the presence and the absence of bile-salt stimulation. Since only one form of the enzyme was found in milk from the other donors, the finding of two forms in donor A is not likely to be an artifact of the procedures used.

One potential explanation for the presence of two forms of BSSL in donor A would be partial degradation of the larger

enzyme form to produce a smaller (but still active) form. This is unlikely to be the case, since the presence of proteinase inhibitors during the purification did not affect the results.

The possibility of differences in the extent or type of post-translational glycosylation as an explanation for the two forms of BSSL cannot be ruled out at present, but this would require different reactions in donor A as compared with the other donors.

Alternative splicing of the BSSL mRNA precursor could also explain the existence of two forms of BSSL. Baba *et al.* [12] reported finding two versions of BSSL cDNA, although they only detected the expression of one BSSL protein. We, however, have found approximately equal amounts of two enzyme forms in one subject, and only one detectable form in the other four subjects. It is unclear whether the two forms of BSSL that we have separated are related to the cDNAs reported by Baba *et al.* [12], even though it is conceivable that individual differences in the regulation of the alternative splicing could account for different yields of the two enzyme forms [17]. The molecular mass difference of our two enzyme forms is approx. 23 kDa, which is more than the difference of 198 bases (66 amino acids) reported for the cDNAs by Baba *et al.* [12] would account for. It is possible that glycosylation could amplify the apparent molecular-mass loss, although no glycosylation sites were specifically identified among the deleted 198 bases [12].

Another plausible explanation for the two forms of BSSL would be co-dominant alleles, such as occurs in the amylase system of *Drosophila* [18]. If donor A was heterozygous and donor B was homozygous, co-dominant alleles would explain the observed results.

An alternative explanation for our findings would be post-transcriptional editing of the mRNA for BSSL, as in the case of apolipoprotein B [19].

BSSL has many properties in common with pancreatic carboxylic-ester hydrolase (CEH; EC 3.1.1.1), and because of their great similarity it has been suggested that BSSL and CEH may be identical, and that BSSL may represent a pancreatic protein which is also secreted by lactating mammary glands [7,11]. The main reported difference between the two enzymes is in molecular mass, with that of BSSL being about 10% higher than that of CEH, although the wide variability in the reported molecular mass of BSSL overlaps the range for CEH, allowing the possibility of the two enzymes being identical. The question of whether BSSL and CEH are identical is now complicated by the fact that BSSL can exist in two different forms.

Note added in proof (received 3 February 1992)

Milk from a sixth donor has yielded two forms of BSSL. From a total of six subjects, two donors have had the two forms of BSSL and the other four have each had only the larger form of the enzyme.

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