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Human milk bile-salt stimulated lipase

Sequence similarity with rat lysophospholipase and homology with the active site region of cholinesterases

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To determine the active site residue, human milk bile-salt stimulated lipase (BSSL) was labelled with [^{3}H]diisopropyl fluorophosphate (DFP) Partial sequence analysis of cyanogen bromide fragments (a total of 146 residues from 6 peptides) revealed 84% sequence identity with a putative rat lysophospholipase Sequence analysis of a [^{3}H]DFP-labelled peptide indicated that the active site serine was contained in the sequence Gly-Glu-Ser Ala-Gly. In addition to similarity with rat lysophospholipase, this sequence showed homology with regions of human butyrylcholinesterase and electric ray acetylcholinesterase (68% identity). It is concluded that these proteins are members of a new supergene family

Human milk bile-salt stimulated lipase, Lysophospholipase, Active site Acetylcholinesterase

1. INTRODUCTION

The major lipase activity in human milk is stimulated by bile-salts (bile-salt stimulated lipase; BSSL). This lipase is activated by bile salts containing a 7-hydroxyl group [1,2] and the enzyme contributes significantly to the utilization of milk lipids in the newborn [3].

The BSSL enzyme is very non-specific, hydrolysing a wide range of triglycerides as well as cholesterol and aryl and alkyl esters [2,4]. It represents 1% of human milk protein and has been purified using several procedures [5,6]. The reported molecular masses of the two preparations of BSSL are 125 000 and 90 000. This difference is likely to be due to variations in the mobility of this glycoprotein in different gel electrophoresis systems. Blackberg and Hernell [6] showed that treatment with DFP resulted in inhibition of activity and incorporation of radiolabelled reagent into the protein.

The properties of BSSL are very similar to a bile salt-stimulated lipase, carboxyl-ester hydrolase (CEH) which is present in human pancreatic secretions [7,8]. The amino acid content was shown to be similar to rat cholesterol esterase and bovine lysophospholipase and these authors suggested that the three activities may belong to the same protein. Immunological and kinetic comparisons indicated that the bile-salt stimulated lipases in human milk and pancreatic juice were very similar [9].

The NH_2-terminal 22 residues of the milk enzyme have been reported [5]. Recently the NH_2-terminal 30 residues of human pancreatic carboxylesterase and milk bile-salt stimulated lipase were found to be identical and to show a high degree of homology with rat and dog pancreatic carboxylesterases [10].

In the present study we have obtained partial amino acid sequence data for the human milk enzyme and found a high degree of sequence similarity with a putative rat lysophospholipase, deduced from a cloned cDNA corresponding to the largest mRNA species isolated from adult rat pancreas [11]. In addition, the sequence containing a serine residue reactive with DFP was determined and found to be homologous with active site sequences of cholinesterases.

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Abbreviations: BSSL, human milk bile-salt stimulated lipase, DFP, diisopropylfluorophosphate, CEH, pancreatic carboxyl-ester hydrolase; CB, CNBr-cleavage peptide

2. MATERIALS AND METHODS

2.1 *Preparation of BSSL*

The enzyme was purified from fresh human milk using the procedure described [6] The enzyme was dialysed extensively, freeze-dried and stored at 4°C prior to use

2.2 *Reaction of BSSL with [^{3}H]DFP*

An aliquot (100 µl, 100 µCi) of radioactively labelled DFP [1,3-^{3}H]DFP (NEN, Dupont, 3 Ci/mmol) was combined with 10 µl of unlabelled DFP (10 86 µM in propan 2-ol) and 78 µl (70 9 µCi/100 nmol) added to the BSSL enzyme (~2 mg in 1 ml of 5 mM sodium barbitone/0 1 M NaCl, pH 7 4). The enzyme was incubated (final concentration 0 1 mM DFP) for 30 min at 37°C and applied to a column (1 6×22 cm) of Sephadex G-25 equilibrated with 0 1 M

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NH_4HCO_3. Fractions (0.75 ml) were collected and aliquots taken for radioactive determination. A total of 10.89 μCi of [^{3}H]DFP radioactivity was incorporated into protein. This corresponded to an incorporation of 15.35 nmol DFP/25 nmol BSSL (0.61 mol/mol), assuming a molecular mass of 65 000 and $E_{1\%}$ = 13.6 for the BSSL enzyme [10]. An aliquot of the labelled preparation was analysed by SDS-polyacrylamide gel electrophoresis on a 10% acrylamide gel [12]. Protein was detected by Coomassie blue staining and radioactive bands detected by fluorography using sodium salicylate [13]. The radioactivity of [^{3}H]DFP-containing fractions was determined by scintillation counting (LKB Wallac Betarac). The efficiency of counting was 52%.

2.3. *Preparation of CNBr-cleavage of peptides of [^{3}H]DFP-labelled BSSL*

Approximately 15 nmol of [^{3}H]DFP-labelled BSSL was dissolved in 100 μl of 70% formic acid containing 10 mg CNBr and incubated for 6 h at room temperature and then overnight at 4°C followed by freeze drying after dilution with water. The sample was redissolved in 600 μl of 6 M guanine-HCl in 0.1% aqueous trifluoroacetic acid and injected onto a Vydac C4 (0.46 × 25 cm) column equilibrated with 90% solvent A (0.1% aqueous trifluoroacetic acid) and 10% solvent B (80% acetonitrile/0.1% trifluoroacetic acid). The column was eluted with a linear gradient of 10% to 70% solvent B over 80 min, at a 1 ml/min flow rate. Peptides were detected by absorbance at 214 nm. Aliquots of fractions were taken for scintillation counting. Peptides recovered from this column were further purified using similar conditions on a Vydac Phenyl column (0.46 × 25 cm). In some cases, additional purification was carried out by microbore HPLC using an Aquapore RP300 C8 column (0.2 × 22 cm), a 140A solvent delivery system and 1000S diode array detector (Applied Biosystems).

2.4. *Protein sequence analysis*

Sequence analysis was performed using a gas phase sequencer (model 470A) equipped with an on-line phenylthiohydantoin amino acid analyser (120A) with chemicals and the program (03RPTH) supplied by the manufacturer (Applied Biosystems). Protein sequence data was used to search the NBRF database (release 28.0) using the University of Wisconsin software [14].

3. RESULTS

Preliminary kinetic studies showed that treatment with a 2–10-fold molar excess of DFP resulted in complete inhibition of human milk lipase activity. Incubation with [^{3}H]DFP resulted in the labelling of the enzyme to a specific activity of 0.43 μCi/nmol (0.61 mol DFP/mol of enzyme). Analysis by SDS-polyacrylamide gel electrophoresis and fluorography showed that the radioactivity was incorporated into a single component running as a broad band, M_r 120 000 (Fig. 1) which corresponded to the major protein detected by Coomassie blue staining in the preparation.

3.1. *Sequence similarity of BSSL and rat lysophospholipase*

Cleavage of the protein at methionine residues with CNBr resulted in the conversion of all of the [^{3}H]DFP-labelled material to low molecular weight material as indicated by SDS-polyacrylamide gel electrophoresis (data not shown). The CNBr fragments were separated by reverse phase HPLC (Fig. 2). A number of the CNBr peptides from pools denoted C, D, and E were further purified by HPLC (data not shown) and subjected to

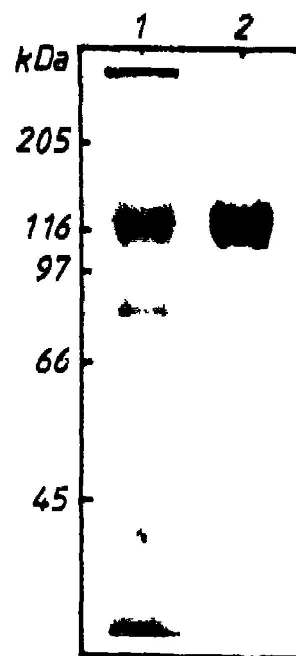

Fig. 1. Analysis of BSSL by SDS-polyacrylamide gel electrophoresis. A sample of [^{3}H]DFP-labelled BSSL was run on a 10% polyacrylamide gel in the presence of SDS under reducing conditions. (1) Staining with Coomassie blue, (2) detection of radioactively labelled material by fluorography.

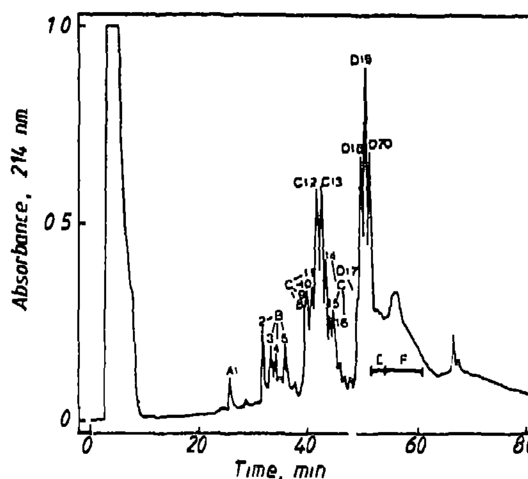

Fig. 2. Separation of CNBr-cleavage peptides from [^{3}H]DFP-labelled BSSL by reverse phase HPLC. The CNBr-digested material was dissolved in 600 μl of 6 M guanidine-HCl/0.1% trifluoroacetic acid and injected onto a Vydac C4 column (0.46 × 25 cm) equilibrated with 90% solvent A (0.1% aqueous trifluoroacetic acid) and 10% solvent B (80% acetonitrile/0.1% trifluoroacetic acid). The column was eluted with a linear gradient of 10% to 70% solvent B over 80 min at 1 ml/min flow rate. Peptides in pools C-13, D20 and E-1 were further purified and subjected to sequence analysis (Fig. 3A). Pools E and F contained all of the [^{3}H]DFP-labelled material which was recovered from the column (62% of radioactivity injected onto the column). The majority of this was recovered in pool F (76%). A peptide CB, F7-9A was purified and subjected to sequence analysis (Table I, Fig. 3B).

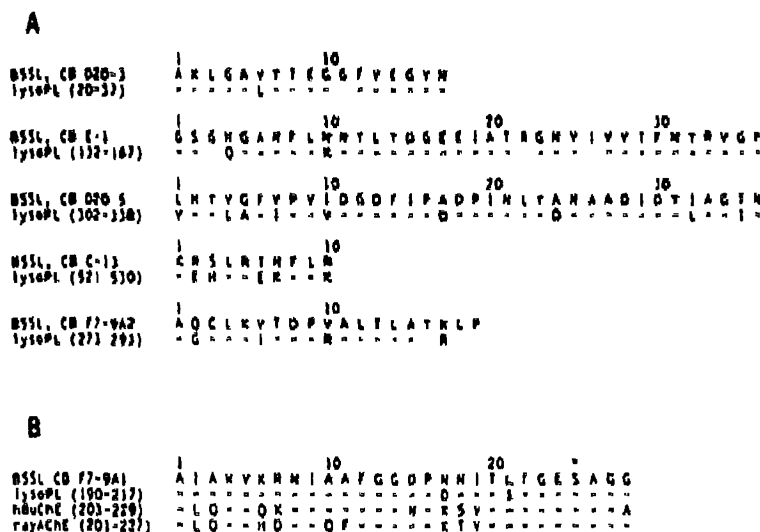

Fig. 3 NH_2-terminal sequences of CNBr-cleavage peptides from [^{3}H]DFP-labelled BSSL and comparison with segments of rat lysophospholipase and cholinesterases. (A) Alignment of NH_2-terminal sequences of CNBr peptides D20-3, E-1, D20-5, C-13 and F7-9A2 with rat lysophospholipase, lysoPL [13]. The recovery of phenylthiohydantoin amino acids at cycle 1 of Edman degradation corresponded to 38, 81, 90 and 6 pmol for peptides D20-3, E-1, D20-5, and C-13, respectively. The average repetitive yields for those corresponded to 91 6, 92 5, 95 0 and 97 4%, respectively (B) Alignment of the NH_2-terminal sequence of the [^{3}H]DFP labelled peptide, CB F7-9A1 with regions of lysoPL, human butyrylcholinesterase hBuChE [18] and marbled ray acetylcholinesterase, rayAChE [19] Identical residues are indicated (–) * Indicates the position corresponding to the release of radioactivity.

automated sequence analysis. A search of the NBRF protein sequence database with each of these sequences revealed sequence similarity with a putative rat lysophospholipase (Fig. 3A). The observed sequence identity for these alignments corresponded to 82 out of 99 residues (83% identity). No sequence similarity was identified with other lipases.

3.2. *Characterization of [^{3}H]DFP-labelled material from the CNBr digest of BSSL*

All of the [^{3}H]DFP-labelled material following HPLC was recovered in pools E and F (Fig. 2). The overall recovery from the column was 62%, 76% of which was recovered in pool F. This fraction contained a number of peptides which were very difficult to separate. This necessitated repeated reverse phase HPLC, using a variety of columns and conditions and resulted in high adsorptive losses of the labelled material. Fractions containing peptides of highest specific activity were pooled from each HPLC run. A fraction, designated F7-9A was obtained which containing 9.8% of the initial pool F radioactivity. An aliquot of this fraction (containing 99 000 cpm equivalent to 120 pmol [^{3}H]DFP-containing material) was subjected to automated sequence analysis. This gave two sequences in approximately equal amounts. However, one of the sequences, designated CB F7-9A1 was equivalent to residues 190–217 of rat lysophospholipase (93% identity). The NH_2-terminal 19 residues of the other peptide CB F7-9A2 was similar to residues 275–293 of rat lysophospholipase (Table I, Fig. 3A). The sequencing efficiency of the two peptides differed and residues 12–28 of peptide F7-9A1 could be identified without reference to the rat sequence The sequence of peptide CB F7-9A1 corresponded to Ala-Ile-Ala-Trp-Val-Lys-Arg-Asn-Ile-Ala-Ala-Phe-Gly-Gly-Asp-Pro-Asn-Asn-Ile-Thr-Leu-Phe-Gly-Glu-(Ser)-Ala-Gly-Gly and peptide CB F7-9A2, Ala-Gln-(Cys)-Leu-Lys-Val-Thr-Asp-Pro-Val-Ala-Leu-Thr-Leu-Ala-Tyr-Lys-Leu-Pro. The release of radioactivity at each cycle of Edman degradation is also shown in Table I. It can be seen that radioactivity was released at cycle number 25 indicating that the sequence Gly-Glu-Ser-Ala-Gly in peptide CB F7-9A1 contains a serine which is reactive with DFP. In a separate experiment the remainder of the material from pool F7-9A was reduced and alkylated and two peptides F7-9A1 and F7-92 were separated by HPLC Automated sequence analysis verified the NH_2-terminal sequences of each of these peptides and indicated that the two peptides are linked by a disulphide bond (data not shown).

3.3. *Homology of human milk BSSL and rat pancreatic lipase active site sequences with cholinesterase*

The N-terminal 28 residues of peptide CB F7-9A1 containing the DFP reactive site had a high degree of sequence identity with residues 190–217 of rat lysophospholipase. Both of these sequences showed sequence homology with active site regions of human butyrylcholinesterase and electric ray acetylcholinesterase (Fig. 3B).

Table I

Sequence analysis of the [^{3}H]DFP-labelled CNBr-peptide F7-9A from BSSL

Cycle	Amino acid (Yield pmol)	[^{3}H]DFP (cpm)	rlysoPL (190-217)	rlysoPL (275-301)
	Ala(112)	180	Ala	Ala
2	Ile(46) Gln(37)	73	Ile	Gly
3	Ala(21)	53	Ala	Cys
4	Trp(16) Leu(26)	65	Trp	Leu
5	Val(31) Lys(23)	71	Val	Lys
6	Lys(24) Val(28)	82	Lys	Ile
7	Arg(18) Thr(10)	54	Arg	Thr
8	Asn(14) Asp(21)	62	Asn	Asp
9	Ile(28) Pro(19)	66	Ile	Pro
10	Ala(32) Val(8)	218	Ala	Arg
11	Ala(54)	107	Ala	Ala
12	Phe(30) Leu(18)	106	Phe	Leu
13	Gly(24) Thr(5)	94	Gly	Thr
14	Gly(21) Leu(3)	102	Gly	Leu
15	Asp(19) Ala(9)	116	Asp	Ala
16	Pro(20) Tyr(10)	136	Pro	Tyr
17	Asn(8) Lys(5)	185	Asp	Arg
18	Asn(18) Leu(4)	184	Asn	Leu
19	Ile(16) Pro(3)	177	Ile	Pro
20	Thr(6)	200	Thr	Leu
21	Leu(9)	195	Ile	Lys
22	Phe(13)	200	Phe	Ser
23	Gly(8)	191	Gly	Gln
24	Glu(5)	196	Glu	Glu
25	(Ser)*	1420	Ser	Tyr
26	Ala(5)	804	Ala	Pro
27	Gly(4)	390	Gly	Ile
28	Gly(4)	220	Gly	

The [^{3}H]DFP peptide subjected to sequence analysis contained 99 000 cpm This is equivalent to 120 pmol based on the specific activity (0 43 μCi/nmol) following reaction of the BSSL enzyme with [^{3}H]DFP The sample was not reduced and alkylated so Cys at position 3 would not have been detected. (Ser)* This residue was inferred from the release of radioactivity corresponding to this cycle The sequences of residues 190-217 and 275-301 of rat lysophospholipase (rlysoPL) as reported [11] are shown for comparison

4. DISCUSSION

Human milk BSSL has many properties in common with a carboxyl-ester hydrolase (CEH) found in the pancreatic juice from a number of species. It appears that the carboxylesterase is responsible for activities known as cholesterol esterase, non-specific lipase and lysophospholipase [15]. In the present study, the relationship of BSSL to the pancreatic enzymes has been clarified by partial amino acid sequence analysis. A high degree of sequence similarity was observed between BSSL and a putative rat lysophospholipase [11]

The sequence of peptide CB D20-3 corresponded to the NH_2-terminal sequence of BSSL (data not shown, [5]). Recently, the NH_2-terminal sequences of pancreatic CEHs were reported and compared to BSSL [10]. The NH_2-terminal 30 residues of human CEH and BSSL were identical and a high degree of homology was seen between these sequences and those of dog, rat and pig CEH. We note that the reported NH_2-terminal sequence of rat CEH [10] corresponds exactly with residues 21-36 of rat lyosophospholipase, indicating that this protein contains a 20 residue leader sequence [11].

The activity of BSSL is very sensitive to the action of DFP [6,16]. Incorporation of [^{3}H]DFP resulted in the labelling of a 110-120 kDa band which is in agreement with the relative molecular masses reported for BSSL in human pancreatic CEH [5,6,10]. The human enzyme appears to be extensively glycosylated compared with rat CEH [10]. Automated Edman degradation of a [^{3}H]DFP-labelled peptide revealed sequences corresponding to two peptides (CB F7-9A1 and CB F7-9A2) which appear to be linked by a disulphide bond. The two sequences were aligned with sequences commencing at residues 190 and 275 of the rat sequence. It is significant that in the rat sequence both sequences are preceded by a methionine residue and cysteine residues are found at positions 266 and 277. In addition, Cys residues corresponding to these positions have been shown to form a disulphide bridge in *Torpedo* acetylcholinesterase [17]. The NH_2-terminal sequences of CB F7-9A1 and CB F7-9A2 together with data obtained from the four other CNBr-peptides (Fig. 3A) provide a total of 146 residues. This is equivalent to approximately 25% of the BSSL sequence, assuming the same number of residues as reported for rat lysophospholipase [11]. It is important to note that the six CNBr peptides revealed sequence similarity with segments found throughout the rat lysophospholipase sequence [11]. Furthermore, all of the BSSL CNBr peptides start at positions preceded by Met in the rat sequence, except CBD20-5 where the preceding residue was Ile. With the exception of peptide CB F7-9A1 (discussed below) search of the NBRF data base revealed no obvious homologies other than with rat lysophospholipase It is likely, therefore, that BSSL represents the species counterpart of rat lysophospholipase CEH and that it represents a pancreatic protein which is also secreted by the lactating mammary glands in humans.

Sequence analysis of peptide CB F7-9A1 resulted in the release of radioactivity at position 25, indicating that this position contains the serine which reacts with DFP. This residue is contained within a sequence -Gly-Xaa-Ser-Xaa-Gly- which is a consensus sequence found in the active site of pancreatic lipase, serine proteases and cholinesterases Search of the NBRF database with the NH_2-terminal sequence of CB, F7-9A1 revealed additional homology (16/28 identities) with human butyrylcholinesterase and marbled ray acetylcholinesterase [18,19]. This homology is consistent with the esterase activity and DFP sensitivity of BSSL. It was of interest to compare the entire rat lysophospholipase sequence with human butyrylcholinesterase [18]. The two proteins were found to be 32% identical using the program GAP (data not shown).

In conclusion, it appears that BSSL and human pancreatic CEH are likely to be identical, representing the species counterparts of rat lysophospholipase (CEH). BSSL is a member of a supergene family including cholinesterases.

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REFERENCES

[1] Hernell, O and Olivecrona, T. (1974) Biochim Biophys Acta 369, 234-244
[2] Wang, C.-S. (1981) J. Biol. Chem 256, 10198-10202.
[3] Fredrikzon, B., Hernell, O., Blackberg, L and Olivecrona, T (1978) Pediatr. Res. 12, 1048-1052
[4] O'Connor, C J and Wallace, R.G. (1985) J. Pediatr Gastroenterol Nutr. 4, 587-590
[5] Wang, C -S and Johnson, K. (1983) Anal Biochem. 133, 457-461
[6] Blackberg, L. and Hernell, O. (1981) Eur. J Biochem. 116, 221-225
[7] Lombardo, D., Guy, O and Figarella, C. (1978) Biochim Biophys. Acta 527, 142-149.
[8] Guy, O., Lombardo, D and Brahms, J.G. (1981) Eur. J. Biochem 117, 457-460b.
[9] Blackberg, L., Lombardo, D., Hernell, O, Guy, O and Olivecrona, T. (1981) FEBS Lett. 136, 284-288.
[10] Abouakil, N, Rogalska, E, Bonicel, J. and Lombardo, D. (1988) Biochim Biophys Acta 961, 299-308.
[11] Han, J H, Stratowa, C. and Rutter, W J (1987) Biochemistry 26, 1617-1625.
[12] Laemmli, U K (1970) Nature 227, 680-685
[13] Chamberlin, J.P (1979) Anal. Biochem 98, 132-135
[14] Devereux, J., Haeberli, P. and Smithies, O (1984) Nucleic Acids Res. 12, 387-395
[15] Lombardo, D, Fauvel, J. and Guy, O (1980) Biochem Biophys Acta 611, 136-146.
[16] Abouakil, N., Rogalska, E and Lombardo, D (1989) Biochim Biophys Acta 1002, 225-230
[17] MacPhee Quigley, K, Taylor, P and Taylor, S (1985) J Biol Chem. 260, 12185-12189.
[18] Prody, C A, Zevin-Sonkin, D, Gnatt, A., Goldberg, O and Soreq, H. (1987) Proc. Natl Acad Sci. USA 84, 3555-3559
[19] Sikorav, J -L, Krejci, E. and Massoulie, J (1987) EMBO J. 6, 1865-1873