ON PORCINE PANCREATIC COLIPASE : LARGE SCALE PURIFICATION AND SOME PROPERTIES

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SUMMARY : Two colipases (94-95 and 83-84 residues, respectively) preventing lipase inhibition by bile salts have been purified with a 30 % yield from defatted porcine pancreas. In a tributyrin system at pH 6.0, NaTDC[*] was found to inhibit lipase for concentrations (0.20-1.25 mM) below the critical micelle concentration. Ultracentrifugation assays on pure products were consistent with a bile salt-mediated conversion of colipase into another form possessing a higher MW and the ability to give with lipase a bile salt-resistant complex.

A new pancreatic cofactor designated colipase (1) preventing lipase inhibition by bile salts (2) has been fully purified for the first time in this Laboratory from crude porcine lipase preparations and found to be a small protein (MW, about 10,000) with 5 disulfide bridges, no methionine and no tryptophan (1). A large-scale purification technique starting from defatted porcine pancreas and some properties of the 2 active proteins obtained (colipase I and II) are now described.

Colipase was routinely measured at 25°C and pH 8.7 through its reactivating effect on colipase-free porcine lipase prepared from the mono TNB-derivative of the enzyme by gel filtration and dithiothreitol treatment (3). A purified olive oil emulsion (0.75 g in 20 ml) stabilized by 0.6 % Methocel 60 (Dow Chemical Co.) and containing 0.15 M NaCl, 0.4 mM $CaCl_2$ and 19 mM sodium deoxycholate was used as the substrate. Strict proportionality between rate increase and added colipase was noted in this system below 60 % saturation. Other measurements were performed with tributyrin or

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[*] Abbreviations : MW, molecular weight ; NaTDC, sodium taurodeoxycholate.

olive oil emulsions under special conditions specified in the text.

The procedure finally adopted for colipase purification was to start from 200 g lots of carefully defatted pancreas (4) which were stirred for 90 min. at 0°C and pH 9.0 in 1800 ml of a solution containing 1 mM DFP and 1 mM benzamidine for zymogen stabilization. The sediment collected at 25,000 g (10 min.) was washed by 800 ml of the above solution and the pooled supernatants (2100 ml) were brought to pH 3 and stirred for 1 h at 4°C. The pH 3 supernatant was further delipidated by addition of n-butanol (420 ml) and ammonium sulfate (630 g). After stirring and centrifugation (25,000g; 10 min.), the sediment was substantially freed from butanol on a Büchner funnel and taken up into 400 ml of a 10 mM Tris-HCl buffer (pH 8.7) 1 mM in benzamidine. The resulting suspension was incubated for 1 h in 5 mM DFP at 0°C and dialyzed against the buffer. The clarified dialyzate was chromatographed in a 3.5 x 40 cm QAE-Sephadex A-25 column equilibrated with the same buffer and eluted by a linear NaCl concentration gradient from 0 to 300 mM in 8 l. The material under the active peak was chromatographed on SP-Sephadex with the results indicated in fig. 1. Disc electrophoresis showed that the first and the third peaks in the diagram contained 2 forms of colipase (colipase I and II), whereas the second consisted of a mixture of both. Substantial fractionation of this mixture was achieved, as shown in fig. 2, by a second chromatography on SP-Sephadex under the same conditions. Finally, traces of strongly bound benzamidine were removed by passage through a 1.5 x 10 cm Dowex 50 x 8 column equilibrated and eluted by 0.5 % acetic acid. In this way, an average of 100 mg of colipase I and 80 mg of colipase II was obtained with an overall yield of 30 %.

The amino acid composition of colipase I and II is summarized in Table I. The composition of another sample separated from lipase by Sephadex G-100 filtration after S-carboxymethylation (a kind gift from Dr. M. Rovery) is also indicated in the Table for comparison. Colipase I is seen to be composed of a single peptide chain with a N-terminal glycine and a total of about 95 residues.

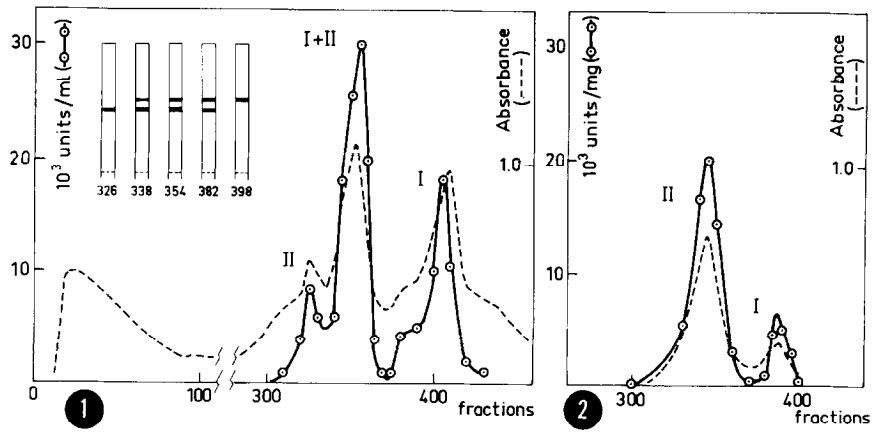

Fig. 1 : Separation of colipase I and II on SP-Sephadex G-25. The 3 x 40 cm column was equilibrated with a 90 mM acetate buffer (pH 4.4) 1 mM in benzamidine and eluted by a linear pH gradient from 4.4 to 5.9 in 4 l. Disc electrophoresis assays were performed at pH 8.6 in 15 % gels.

Fig. 2 : Second chromatography under the same conditions of the materiel under peak (I + II) in fig. 1.

The other two have also a N-terminal glycine, but approximately 10 and 25 residues less, respectively.

Moreover, the N-terminal sequence of colipase I and II was determined on native colipase I and II with the aid of an automatic Sequenator Socosi Model PS 100 (Paris, France) and found to be in both proteins :

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 1                                          10
Gly-Ile-Ile-Ile-Asn-Leu-Asp-Glu-Gly-Glu-Leu-   -Leu-

                                  20
Asn-   -Ala-Gln-   -Lys-   -Asn-
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The presence of 3 adjacent isoleucine residues near the N-terminal end of the chain is noteworthy. The missing residues 12, 15 and 18 are 2 half cystine and 1 serine which have already been identified in the corresponding tryptic peptide 1-19 (C.Erlanson and M.Charles, to be published).

MW determinations by the method of Yphantis on 0.83 mg/ml solutions in 0.1 M NaCl at 20°C gave values of 9870 ± 100 for colipase I and 8720 ± 260 for colipase II ($\bar{v}$ (calculated from the amino

TABLE I

Amino acid composition of colipase

Residue	Number of residues in					
	Colipase I		Colipase II		Colipase from lipase	
	Analytical	Next Integer	Analytical	Next Integer	Analytical	Next Integer
Ala	5.25	5	3.96	4	4.3	4
Arg	4.32	4	3.07	3	3.2	3
Asx	10.67	11	10.25	10	9.1	9
Cys	9.94	10	9.25	10	10.3	10
Glx	9.15	9	8.35	8	5.3	5
Gly	9.34	9	7.97	8	6.1	6
His	2.12	2	1.78	2	1.8	2
Ile	6.95	7	5.75	6	3.0	3
Leu	10.79	11	8.84	9	8.2	8
Lys	4.47	4-5	3.83	4	3.5	3-4
Met	0.00	0	0.00	0	0.0	0
Phe	2.11	2	2.02	2	1.1	1
Pro	1.70	2	1.96	2	0.7	1
Ser	7.06	7	6.54	6-7	7.1	7
Thr	4.83	5	4.20	4	3.5	3-4
Trp	0.00	0	0.00	0	0.0	0
Tyr	2.81	3	2.87	3	2.1	2
Val	3.12	3	3.22	3	1.1	1
Total number of residues		94-95		84-85		68-70
Molecular weight		10,157-10,285		9103-9190		7303-7533
N-terminal		Glycine		Glycine		Glycine

acid composition), 0.72 ; ρ_o (experimental), 1.0033). The observed difference (1150) was considered to be significant and to confirm that colipase II is distinctly smaller than colipase I. It is not yet known whether the 2 colipases are present in fresh pancreas or whether colipase II is formed from colipase I by limited proteolysis during purification. The third colipase with only 68-70 residues is likely to result from an extensive proteolytic degradation occuring during lipase purification performed in the absence of trypsin inhibitors. It is of interest that several proteins of distinctly different sizes can display colipase activity.

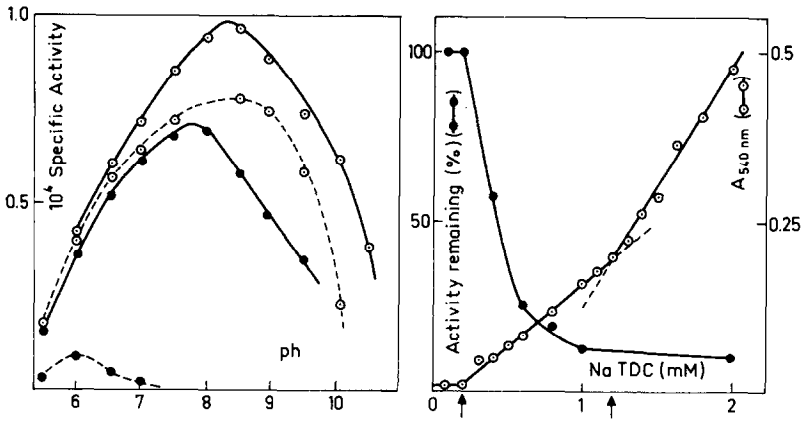

Fig. 3 : pH-activity curves of colipase-free lipase (....) and of lipase saturated with 2.5 moles/mole of colipase (——) in the presence of 2 mM NaTDC (●) or in its absence (O). In all assays the substrate (300 mg of pure tributyrin) was stirred at 25°C with 15 ml of a solution 0.1 M in NaCl and 1 mM in $CaCl_2$. Maximal velocities were measured in all cases.

Fig. 4 : Correlation between lipase inhibition and formation of "pre-micelles" by NaTDC. Activities were measured at pH 6.0 in the above system. Micelle formation in NaTDC solutions was followed (7) with the aid of Rhodamine 6B in the NaCl-$CaCl_2$ mixture buffered at pH 6.0 by 0.1 M Tris-HCl. Arrows correspond to NaTDC concentrations of 0.20 and 1.25 mM, respectively. This latter value (critical micelle concentration) is not modified by the presence of colipase.

Colipase I is probably similar to the protein recently isolated from acid extracts of porcine pancreas (5) and reported to possess 95 residues, a MW of 9640-12,000 and a N-terminal isoleucine residue. Colipase II is similar to that previously separating by us from lipase by gel filtration (1).

Some preliminary assays were also carried out, using pure porcine lipase and colipase, in order to elucidate the mechanism of the colipase effect. The pH-activity curves of colipase-free lipase and of enzyme samples saturated by 2.5 moles/mole of colipase are reproduced in fig. 3. These curves confirm in the first place that the main action of the cofactor is to protect lipase from inhibition by bile salts (2,6). They also reveal a distinct activation even in the absence of bile salts. They further show, in contrast with a recent report (6), that addition of the cofactor al-

most completely abolishes the acid shift of the optimal pH of lipase occurring in a system containing bile salts and no colipase. The origin of this discrepancy is not known.

Impure rat (6) and human (8) lipase preparations as well as a pure fungal lipase (9) have recently been reported to be inhibited exclusively by concentrations of bile salts higher than the critical micelle concentration. Fig. 4 shows that, in our assays, inhibition occurs before NaTDC micelle formation, in a region (0.20-1.25 mM) where slight spectral variations of the dye suggest the appearance of "pre-micelles".

Moreover, results given by a conventional kinetic investigation of the colipase effect are summarized in Table II. In spite of unavoidable difficulties due to the complexity of the systems used, colipase appears to oppose the sharp V_m fall induced by NaTDC when tributyrin is the substrate, and the K_m increase occurring with long-chain triglycerides.

Finally, ultracentrifugation assays in the presence (4mM) or absence of NaTDC were performed with pure colipase, pure lipase and a mixture of both (molar ratio, 3). The proteins were dissolved in the $NaCl$-$CaCl_2$ solution buffered at pH 8.0 by 0.1 M Tris-HCl. Under these conditions, NaTDC was found to increase the sedimentation coefficient ($s_{20,w}$) of colipase from 1.35 S (concentration, 3.55 and 6.30 mg/ml) to 3.10 or 3.50 S (4.6 or 6.0 mg/ml), whereas the MW of the protein (Yphantis) was raised from 9870 (see above) to 20,700. A similar variation of the migration rate of rat colipase activity through Sephadex (2,6) has been attributed to dimerization (6).

By contrast, the sedimentation coefficient of lipase was not affected by NaTDC (3.69 and 3.75 S without NaTDC (concentration, 6 and 8 mg/ml ; 3.50 S in 4 mM NaTC (concentration, 4 mg/ml)). With the lipase-colipase mixture referred to above (lipase concentration, 4.25 mg/ml), 60 % of the material migrated with a coefficient as high as 4.95 S. Although incomplete and preliminary, these results suggest a bile salt-mediated conversion of colipase into another form possessing a higher MW and the ability to give with lipase a bile salt-resistant complex.

TABLE II

Effect of NaTDC and colipase on the kinetic parameters of lipase-catalyzed hydrolysis of short and long-chain triglycerides.

Preformed stock long-chain triglyceride emulsions were prepared by 3 x 20 s. sonications of 3 g of purified olive oil with 40 ml of a solution 0.1 M in NaCl and 1 mM in $CaCl_2$, containing 2.5 ml 2 mM sodium oleate as stabilizer. Volumes of this emulsion were diluted to 15 ml with the NaCl-$CaCl_2$ mixture. Tributyrin was stirred with 15 ml of the NaCl-$CaCl_2$ mixture in the course of the automatic titration.

Substrate used	pH	NaTDC (mM)	Colipase			
			With		Without	
			V_m(units)	K_m(g/l)	V_m(units)	K_m(g/l)
Tributyrin	8.0	0.0	10,000	2.8	6,750	1.4
		0.4	6,200	3.4	1,620	2.5
		4.0	4,800	1.2	120	1.2
Long-chain triglycerides	9.0	0.0	1,700	0.3	950	0.6
		0.4	2,000	0.3	3,000	3.0
		0.8	1,800	0,2	3,300	14
		1.6	2,100	1.5	-	-

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