Limited proteolysis of porcine pancreatic lipase

Lability of the Phe 335-Ala 336 bond towards chymotrypsin

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Mild chymotrypsin digestion of native lipase (449 amino acids) preferentially cleaved the Phe 335-Ala 336 bond. On SDS-gel electrophoresis, 3 major bands were observed: band 1 (52 kDa) representing native lipase, bands 2 and 3 (40 and 12 kDa) representing the two lipase fragments A and B. Fragment A does not retain lipase activity but maintains its ability to adsorb to interfaces. Fragment B was identified with the lipase C-terminal region (336–449). It does not exhibit any activity towards tributyrylglycerol emulsions and any ability to adsorb to interfaces.

Limited proteolysis *Protease susceptibility* *Chymotrypsin digestion* *Domain structure* *Interface adsorption* *Triacylglycerol lipase* *(Porcine pancreas)*

1. INTRODUCTION

Porcine pancreatic lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) is composed of a single polypeptide chain of 449 amino acid residues [1] connected to a glycan chain (2 kDa) by Asn 166 [2,3]. During the in vivo digestion, lipase catalyzes the hydrolysis of natural water-insoluble triacylglycerols. To perform this heterogeneous catalysis in the presence of bile salts, two recognition sites, one for the water-lipid interface, the other for colipase, are required for the lipase molecule in addition to the active site [4–6].

2. MATERIALS AND METHODS

The lipase used was purified from 100 g of defatted porcine pancreas powder prepared in the laboratory [7]. The preparation procedure [8] was followed up to the filtration on Sephadex. Then, each half of the enzyme preparation, after concentration to 70 ml, was again filtered on Sephadex (same column size as in the first filtration). The buffer (5 mM Tris-HCl, 3.3 mM $CaCl_2$, 0.4 M NaCl, 0.77 mM NaN_3 and 1 mM benzamidine) was adjusted to pH 9.2 for all the Sephadex filtrations. These modifications are performed to remove colipase impurity as much as possible. The separation of the lipase isomers was not carried out. Lipase activity was measured against tributyrylglycerol emulsion [9] in the presence of bile salt and an excess of colipase (A_s = 1200 (μmol $min^{-1} \cdot mg^{-1}$)). The suppliers of the proteolytic enzymes have been mentioned [3]. The dansyl method and the determination of the amino acid composition have been described [3]. The 7 steps scheduled on the Socosi automatic sequencer [10] were performed on 30 nmol of B fragment with a repetitive yield of about 92%. Analytical electrophoresis on polyacrylamide-SDS slab gel (10% acrylamide) was performed according to [11]. Prior to electrophoresis, samples were treated with SDS, dithioerythritol and iodoacetate [12]. Adsorption assays on siliconized glass beads (1 g beads for 50 nmol protein) were carried out according to [13].

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3. RESULTS AND DISCUSSION

3.1. *Selection of chymotrypsin as proteolytic enzyme*

Native lipase (1 mg per ml of 0.06 M ammonium bicarbonate, pH 8.5) was incubated with proteolytic enzymes in various conditions: temperature 0, 8 and 25°C; incubation times varying from 30 min to 72 h, and enzyme-substrate ratio from 0.01 to 0.25 (w/w). The extent of the proteolysis was followed by SDS-gel electrophoresis. Out of all the enzymes used, only chymotrypsin was capable of attacking native lipase. Trypsin, elastase, bromelain, papain, thermolysin and subtilisin left the lipase molecule intact even after prolonged incubations. The best conditions for the chymotrypsin proteolysis appeared to be: 25°C with a chymotrypsin-lipase ratio 0.05. The SDS-electrophoresis patterns of the chymotrypsin proteolysates (fig.1) showed the major cleavage fragments A and B of lipase to be about 40 and 12 kDa (bands 2 and 3). Band 1 represents the remaining lipase (52 kDa) and the front band contains all the small peptides resulting from a high hydrolysis. Up to an incubation time of 18 h, bands 2 and 3 increased. When incubation was prolonged, the uncleaved lipase slowly disappeared and the amount of A and B fragments decreased as they were gradually converted into much smaller components.

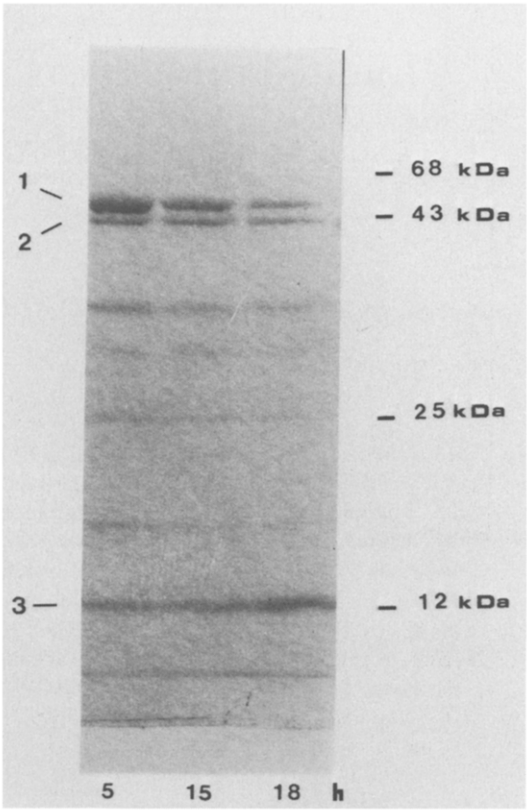

Fig.1. Polyacrylamide-SDS slab gel electrophoresis of lipase digested with chymotrypsin for 5, 15 and 18 h incubation. Before electrophoresis the digests were denatured, reduced and S-carboxymethylated. Standard polypeptides used: bovine serum albumin (68 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and cytochrome *c* (12.3 kDa).

3.2. *Studies on the two major fragments obtained*

As these investigations required a much greater amount of lipase, the proteolysis conditions were: lipase concentration, 10 mg per ml; chymotrypsin-lipase ratio, 0.01; incubation time, 15 h, temperature, 25°C. The specific activity of the lipase proteolysate was half of that of native lipase. The enzyme, incubated in the same conditions but without chymotrypsin, retained its full activity. The elution profile on Sephadex G-100 of the proteolysate (fig.2) shows 3 peaks. The first peak eluted was a mixture of lipase and A fragment; the second peak was identified as the B fragment and the third peak was a mixture of numerous small peptides. It is interesting to note that fragment B is detached from the rest of the molecule whether the proteolysate is reduced-carboxymethylated (fig.1) or not treated (fig.2).

If the mixture (lipase and A fragment) was easily separated on SDS-gel electrophoresis, up to now, it has not been possible to separate these components in the native state in spite of the numerous attempts (ion-exchange chromatography on DE- or CM-cellulose; size exclusion chromatography on Biogel or on HPLC columns; non-denaturing gel electrophoresis and gel isoelectrofocusing; affinity chromatography on decyl agarose). Consequently, the lipase activity was measured on the mixture after gel filtration (fig.2, peak 1). Several assays were performed with variations of the proteolysis times. The different proportions of the two components were roughly evaluated by the intensity of each electrophoresis band. The lipase activity sharply decreased as the amount of A fragment in-

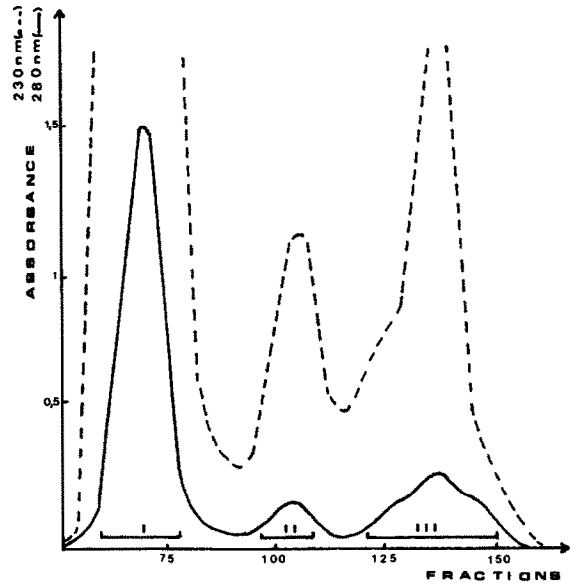

Fig.2. Gel filtration through Sephadex G-100 of the chymotrypsin digestion of native lipase (600 nmol, 31 mg) at 5°C. Two columns (1 × 100 cm) were connected in series and were equilibrated and eluted with 60 mM ammonium bicarbonate buffer (pH 8.5). Flow rate 8 ml/h; 1.2-ml fraction.

creased. Seemingly, the A fragment has no lipase activity. On the other hand, the mixture displayed the same ability as lipase alone to adsorb to siliconized glass bead interface [13]. When fragment B was added to the mixture (lipase + A fragment) no increase in the lipase activity was observed. In conclusion, the A fragment does not retain lipase activity but maintains its ability to adsorb to interfaces. It is interesting to note that Ser 152, which was reported to be essential for the lipase interfacial adsorption [14,15], is included in the sequence of A fragment (see later).

The B fragment was further purified by filtration on Sephadex G-50 and its identification as the segment (336–449) of the C-terminal sequence of lipase was deduced from the following results on its structure: a single N-terminal residue, alanine; the first 7 residues of its N-terminal sequence, Ala-Arg-Trp-Arg-Tyr-Lys-Val and its amino acid composition (table 1) [1]. Of the two half-cystines, Cys 433 and Cys 449, one represents the C-terminal residue of lipase. These two amino acid residues

Table 1

Amino acid composition of B fragment

Amino acid	B fragment	Lipase C-terminal sequence (336–449)
	Number of residues (experimental)	Number of residues
Cys[a]	2.3	2
Asx	14.8	16
Thr	7.6	8
Ser	7.5	8
Glx	12.5	12
Pro	4.0	4
Gly	8.2	8
Ala	2.8	2
Val	12.7	13
Ile	4.6	5
Leu	8.4	9
Tyr	3.9	5
Phe	3.7	4
Lys	8.0	8
His	1.7	2
Arg	5.6	6
Trp	nd[b]	2
Total residues		114
Molecular mass (Da)		12978

[a] Cys was determined as $CySO_3H$
[b] nd, not determined

have been found to be connected by an S-S bridge in a previous study on lipase disulfide determination [16]. The yield of the pure peptide (336–449) was 14% of the initial amount of lipase (mol per mol). This peptide did not exhibit any activity toward tributyrylglycerol emulsion or any ability to adsorb to interfaces.

The Phe 335-Ala 336 bond is then preferentially cleaved by chymotrypsin in native lipase. The two fragments obtained might represent two domains of lipase. Such a possibility would agree with Busetta (personal communication) who, considering the energy of secondary structure formation, predicted the existence of two separated domains. The zone separating the domains was supposed to be between residues 240 and 320.

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