Biochimica et Biophysica Acta, 660 (1981) 148–150
Elsevier/North-Holland Biomedical Press

BBA Report

BBA 61395

CHARACTERIZATION OF THE SERINE REACTING WITH DIETHYL *p*-NITROPHENYL PHOSPHATE IN PORCINE PANCREATIC LIPASE

A. GUIDONI, F. BENKOUKA, J. DE CARO and M. ROVERY *

Centre de Biochimie et de Biologie Moléculaire du Centre National de la Recherche Scientifique, 31, Chemin Joseph-Aiguier, 13274 Marseille Cedex 2 (France)

(Received January 27th, 1981)

Key words: Triacylglycerol lipase; Serine residue; Active site; Diethyl p-nitrophenyl phosphate (Porcine pancreas)

The position in porcine pancreatic lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) of the serine reacting specifically with emulsified or micellar diethyl *p*-nitrophenyl phosphate has been investigated. This serine which appears to be involved in lipase adsorption to insoluble triglyceride interfaces, is at position 152 in the enzyme chain. The sequence around this amino acid is: His-Val-Ile-Gly-His-Ser-Leu-Gly.

Diethyl *p*-nitrophenyl phosphate, a general inhibitor for serine enzymes, has been reported to inhibit pancreatic lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) only when used in the form of emulsified particles [1] or mixed micelles with bile salt in the presence of colipase [2,3]. This inhibition is due to a stoicheiometric reaction between the organophosphate and a serine tentatively localized by Maylié et al. [2] in a Leu-Ser-Gly-His sequence. However, unlike authentic serine enzymes, this amino acid is not involved in the catalytic site but in the binding site [4]. Actually after reaction, the lipase retains its low catalytic activity on monomeric substrates and completely loses its capacity to recognize hydrophobic interfaces. In other words, emulsified or micellar diethyl *p*-nitrophenyl phosphate appears to be a specific marker of the site normally responsible for one of the typical steps of heterogeneous biocatalysis, i.e., lipase adsorption to insoluble substrate interfaces [4,5].

Having found that the above arrangement does not exist in the now fully elucidated sequence of porcine pancreatic lipase (Refs. 6 and 7 and Bianchetta, J., Guidoni, A., Bonicel, J. and Rovery, M., unpublished data), it was advisable to re-investigate the position of the reactive serine. Evidence is presented here of this serine being at position 152 in the enzyme chain.

Since [^{32}P]diethyl *p*-nitrophenyl phosphate was not available on the market at the time this work was undertaken, lipase prepared as indicated [6] was incubated with non-radioactive inhibitor according to Rouard et al. [3]. Methods previously published for phosphorus mineralization [8] and colorimetric determination [9] were modified as follows: mineralization was carried out on 4–10 nmol peptide for 2 h at 200°C and the volume of solutions used for colorimetry was reduced 20-fold.

The purified diethylphosphoryl lipase derivative (2.5 μmol) resulting from action of the organophosphase on the native enzyme was dissolved in 13 ml 5% formic acid and incubated with pepsin at 37°C (enzyme/substrate ratio, 1 : 10, w/w). After 24 h an equal amount of enzyme was added and digestion was continued for another 24 h. The digest was filtered on Sephadex G50 (Fig. 1a). Phosphorus-containing fractions (pool a) were passed through Bio-gel P 10

* To whom correspondence should be addressed.

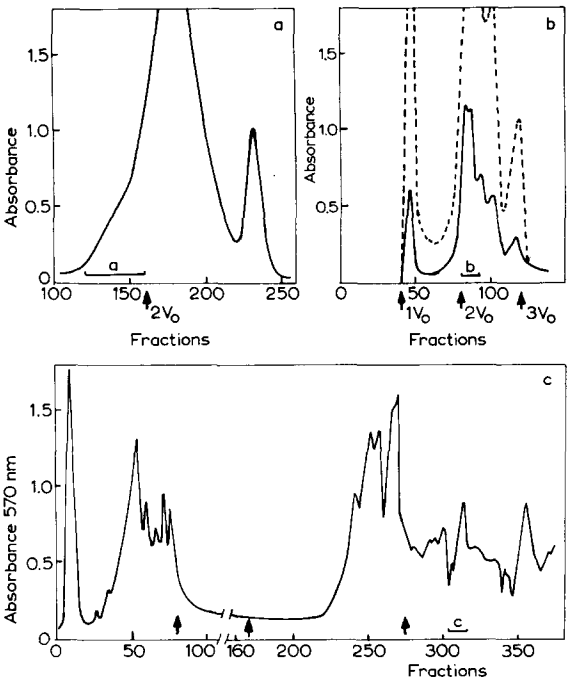

Fig. 1. Purification of the peptide containing phosphorus after peptic digestion of diethylphosphoryl lipase. a: Gel filtration on Sephadex G-50 fine of the digest. Three columns (1.5 × 90 cm) connected in series were equilibrated and eluted with 1% acetic acid at room temperature, 1.5 ml fraction volume, flow rate 5 ml/h. V_0, retention volume. Absorbance was measured at 280 nm. Pool a (horizontal bar), phosphorus-containing fractions. b: Gel filtration on Bio-gel P 10 of pool a. Two columns (1 × 100 cm) connected in series were equilibrated and eluted with 1% acetic acid at room temperature, 1.3 ml fraction volume, flow rate 2.5 ml/h. V_0, retention volume. Absorbances were measured at 230 nm (------) and 280 nm (———). Pool b, phosphorus-containing fractions. c: Chromatography on SP Sephadex C-25 of pool b. The column (0.6 × 35 cm) was equilibrated with pyridine/acetic acid (50 mM) buffer, pH 2.55, at room temperature. After elution with 70 ml buffer, three concentrations and pH gradients were successively applied. 1st gradient 50 mM, pH 2.55–100 mM, pH 3.20; 2nd gradient 100 mM pH 3.20 –250 mM, pH 3.90; 3rd gradient 250 mM, pH 3.90–500 mM, pH 4.90. The arrows indicate the starting point of the gradients. Volume of each of the two chambers was 70 ml. Fractions were monitored by ninhydrin assay after alkaline hydrolysis. Pool c, phosphorus-containing fractions.

(Fig. 1b). The resulting pool b chromatographed on SP-Sephadex (Fig. 1c) yielded pool c. Amino acid analysis and NH_2-terminal determination showed that pool c contained several peptides but that the peptide in position (147–160) of the lipase chain [6] was its main constituent. This peptide had already been characterized from a peptic digestion of native lipase (peptide P II 2, Fig. 2 of Ref. 6):

147 152 155 160
His-Val-Ile-Gly-His-Ser-Leu-Gly-Ser-His-Ala-Ala-Gly-Glu

Since this peptide contains two serines, it was preferable instead to further purify it, to cleave the chain between these two serines and purify the new peptides obtained. The pool c was digested by thermolysin. About 400 nmol of the main peptide in 100 µl *N*-ethylmorpholineacetic acid (0.1 M) buffer, pH 6.2/20 mM $CaCl_2$, were incubated for 18 h, at 37°C, with thermolysin (enzyme/substrate ratio 1 : 50, w/w). The digest was purified by electrophoresis and chromatography on thin layer cellulose [10]. Phosphorus was detected in one spot only. The latter contained the following peptide in pure form:

147 152
His-Val-Ile-Gly-His-Ser (yield 13%)

The peptide was characterized by its amino acid composition and the Edman-dansyl analyses.

Another spot having a slower chromatographic migration and a negative reaction to the phosphorus reagent was characterized as:

153 155
Leu-Gly-Ser (yield 13%)

The indicated yields for the two peptides were based on total phosphoryl lipase.

These results demonstrate that the serine in porcine pancreatic lipase reacting in a highly specific way with diethyl *p*-nitrophenyl phosphate is Ser_{152}. The sequence around this serine contains a second serine at position 155, three histidines at position 147, 151 and 156 and three short hydrophobic sequences Val-Ile-Gly, Leu-Gly and Ala-Ala-Gly linked by His-Ser or Ser-His. An explanation of the results published by Maylié et al. [2] might be that the two peptides Leu-Gly-Ser-His and His-Ser-Leu-Gly were present together in the authors' experfiment and that the identical amino acid compositions of these peptides gave rise to a misinterpretation.

Acknowledgements

We owe our thanks to Professor P. Desnuelle for his interest in our work and the supervision of the manuscript.

References

1 Desnuelle, P., Sarda, L. and Ailhaud, G. (1960) Biochim. Biophys. Acta 276, 570–571
2 Maylié, M.F., Charles, M. and Desnuelle, P. (1972) Biochim. Biophys. Acta 276, 162–175
3 Rouard, M., Sari, H., Nurit, S., Entressangles, B. and Desnuelle, P. (1978) Biochim. Biophys. Acta 530, 227–235
4 Chapus, C. and Sémériva,, M. (1976) Biochemistry 15, 4988–4991
5 Sémériva, M. and Desnuelle, P. (1979) Adv. Enzymol. 48, 319–370
6 Bianchetta, J.D., Bidaud, J., Guidoni, A.A., Bonicel, J.J. and Rovery, M. (1979) Eur. J. Biochem. 97, 395–405
7 Guidoni, A., Bonicel, J., Bianchetta, J. and Rovery, M. (1979) Biochimie 61, 841–845
8 Hess, H.H. and Derr, J.E. (1975) Anal. Biochem. 63, 607–613
9 Chen, P.S. Jr., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758
10 Lindeman, H. and Wittman-Liebold, B. (1977) J. Physiol. Chem. 358, 843–863