PRELIMINARY NOTES

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Action of organophosphates on pancreatic lipase

Lipases (EC 3.1.1.3) represent a special group of esterases preferentially acting on emulsified[1] and micellar[2] ester substrates. Preliminary evidence is presented below showing that porcine pancreatic lipase is inhibited by relatively high concentrations of two organophosphates (diethyl *p*-nitrophenyl phosphate (E 600) and diisopropyl-fluorophosphate (DFP)) and that a phosphoryl radical is attached to a tyrosine residue in the enzyme molecule during the reaction with DFP.

Lipase was purified, either from fresh pancreas[3], or from a fully defatted pancreas powder[4]. These latter preparations were free of lipids and of any protein impurities detectable by disc electrophoresis. But they still contained the two active forms of lipase known to exist in porcine pancreas[4]. Activities were measured titrimetrically against an emulsion of long-chain triglycerides in the presence of an optimal concentration of bile salts. An extinction coefficient ($E^{1\%}_{1\,cm}$) of 17.3 at 280 mμ and an approximate molecular weight value of 45 000 (ref. 4) were used for the evaluation of the molarity of the enzyme solutions.

Fig. 1a shows that lipase is inhibited, not only, as believed earlier[5], by emulsions

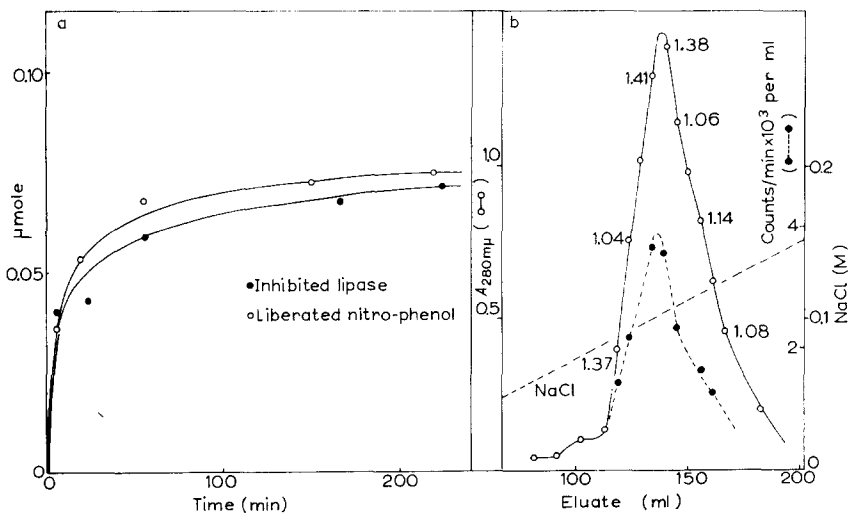

Fig. 1. Action of diethyl *p*-nitrophenyl phosphate and DFP on lipase. a. Incubation at 0° of 0.097 μmole of lipase dissolved in 2 ml of 0.1 M acetate buffer (pH 6.0) containing NaCl (0.1 M) and $CaCl_2$ (50 mM) with a solution of diethyl *p*-nitrophenyl phosphate in bovine bile salts (final concn., 4.6 mM and 0.1%, respectively). Appropriate blank assays showed that the spontaneous inactivation of lipase and hydrolysis of diethyl *p*-nitrophenyl phosphate were very slow under these conditions. *p*-Nitrophenol was determined by spectrophotometry at 400 mμ of aliquots brought to pH 9. b. Chromatography of 38 mg DFP-inhibited lipase (see text) on a 0.9 cm × 35 cm DEAE-cellulose column equilibrated with a 5 mM Tris buffer (pH 8.0) 3 mM in $CaCl_2$. Elution with a NaCl concentration gradient. The figures indicate the number of phosphorus atoms per mole of enzyme. The radioactivities were measured in a Packard Tricarb Liquid Scintillation Spectrometer after dissolving the samples in 10 ml Bray's mixture.

of diethyl *p*-nitrophenyl phosphate, but also by aqueous solutions of this inhibitor in the presence of bile salts. In the course of the reaction, about 1 mole of *p*-nitrophenol was liberated per mole of inhibited enzyme.

Lipase was also found to be inhibited by aqueous solutions of DFP. In a typical experiment, an 80% inhibition was attained by a 4-h incubation of the enzyme (0.02 μmole in 1 ml of a 0.5 M Tris–HCl buffer (pH 8.2) containing NaCl (0.1 M) and $CaCl_2$ (50 mM)) with DFP at a concentration of 50 mM. The same results were obtained with a commercial sample of DFP or a sample prepared in the laboratory and carefully distilled twice in a glass microdistillator. For preparative purposes, 7.8 μmoles (350 mg) of lipase in 70 ml of the buffer were incubated for 3 h with 50 mM [^{32}P]DFP. After two additions of DFP and $CaCl_2$ and further incubation, the 96% inhibited solution was freed of low molecular weight radioactive derivatives by dialysis and filtration through Sephadex G-25, and it was submitted to chromatography on DEAE-cellulose as indicated in Fig. 1b. The number of phosphorus atoms in inhibited lipase was calculated by reference to a sample of α-chymotrypsin (Worthington, 3× crystallized) which was inhibited by the same solution of [^{32}P]DFP, dialyzed and filtered through Sephadex G-25. Fig. 1b shows that this number was equal to, or slightly higher, than one in all the fractions of the lipase peak.

Another preparation (6.45 μmoles) of labelled lipase was reduced, S-carboxymethylated[6] and digested for 24 h at pH 7.9 with 10% chymotrypsin. The resulting peptides were passed through a 2 cm × 200 cm Sephadex G-25 column, and the radioactive peak emerging at the end of the second retention volume of the column was submitted to chromatography on a 1 cm × 50 cm DEAE-Sephadex A-50 column equilibrated with 0.1 M acetic acid. About 30% of the radioactivity emerged unretarded in this system. The remainder (about 70%) was eluted as a narrow peak by a linear gradient of the acid concentration. This peak was found by electrophoresis chromatography on paper to contain only one peptide with the following composition (single determination after a 24-h hydrolysis): Asp_{3-4}, Glu_{2-3}, Thr_1, Tyr_1, Leu_1. Five Edman degradations and a digestion with carboxypeptidase A were consistent with the sequence: Thr–Asn–Gln–Asn–Gln–(Asx_{1-2}, Glx_{0-1}, Tyr)–Leu.

The binding of the phosphoryl radical to the tyrosine residue of the peptide rather than to the N-terminal threonine was demonstrated by the following observations: (a) The spectrum of the peptide in the 260–290 mμ region was similar to that of synthetic *o*-diisopropylphosphoryltyrosine[7] and it was not modified by alkalinization to pH 12. (b) The chymotrypsic cleavage liberating the peptide did not occur after the tyrosine, but after the neighbouring leucine. (c) After digestion with subtilopeptidase, all the tyrosine-containing fragments were radioactive, while the others were not radioactive. (d) The bond linking the phosphorus atom to the peptide was unstable in acid, but relatively stable in an alkaline medium.

The observations that concentrated E_{600} and DFP react with lipase in a nearly stoechiometric ratio, that both organophosphates are inhibitory and that DFP is found to bind to tyrosine are not sufficient to prove that this tyrosine is essential for the activity of the enzyme. Although doubly distilled DFP was used in some assays, the possibility exists that inhibition was caused by an impurity, as for bromelain and ficin[7,8]. In any case, the single tyrosine residue binding DFP must be considered as especially reactive in the molecule of lipase. The strong inhibitory effect of iodine[9] on lipase is also being studied.

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