

9. Letsinger, R. L., Fontaine, J., Mahadevan, V., Schexnayder, D. A. and Leone, R. E. *J. Org. Chem.* **29** (1964) 2615.
10. Boxer, G. E. and Everett, P. M. *Anal. Chem.* **21** (1949) 670.

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Reaction of Two Enzyme Fractions from *Streptomyces griseus* Protease with Diisopropylphosphorofluoridate

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Several proteolytic and other enzymes are inactivated by DFP* (see Ref. 1). All these enzymes have esterase activity, and are phosphorylated on a serine residue at the active site during inactivation. It has also been reported that rat-liver tissue contains many proteins that are phosphorylated on a serine residue by DFP (see Ref. 2). These proteins seem to have esterase activity.³

Since Pronase (Calbiochem, U.S.A.), a new commercial protease preparation obtained from *Streptomyces griseus*, also has esterase activity,⁴ a preliminary experiment was made to ascertain whether this preparation would be phosphorylated on a serine residue by DF³²P. 10 mg of Pronase were dissolved in 0.5 ml of 0.1 M Tris-acetic acid buffer (pH 7.5) and incubated with 5×10^{-4} M DF³²P (Radiochemical Centre, Amersham, England) for 10 min at 20°C. The enzyme was then precipitated by trichloroacetic acid at a final concentration of 10% (w/v). The precipitate was washed twice with acetone and dried *in vacuo*. After acid hydrolysis, Ser³²P was isolated from the hydrolysate, using the same methods as previously described.⁵

Hiramatsu and Ouchi⁶ have shown by starch zone electrophoresis that Pronase

contains four different fractions with proteolytic activity. The activity of one of these fractions was found to be inhibited by DFP. It was therefore of interest to see whether this fraction would have esterase activity and — in this event — could be phosphorylated on a serine residue by DFP. For this reason, Pronase was chromatographed on CM-cellulose, and the fractions containing esterase activity were used for an experiment with DF³²P.

300 mg of Pronase (Calbiochem, Lot no. 42834) in 15 ml of 0.01 M calcium acetate-acetic acid buffer (pH 5.0), was purified with respect to esterase activity (measured with N-benzoyl-L-arginine ethyl ester as substrate)⁷ by adding solid ammonium sulphate until 0.7 saturation at 4°C. The precipitate was dissolved in 9 ml of the buffer, and dialyzed overnight against the same buffer. The enzyme solution was then chromatographed on a 2 × 6.5 cm CM-cellulose column, equilibrated with the aforementioned buffer. Elution was performed with a linear gradient of 0.01 M to 0.20 M calcium acetate-acetic acid buffer, pH 5.0. Two main protein peaks were eluted, one appearing with the front of the effluent and one at a buffer concentration of about 0.03 M. The latter peak contained all the esterase activity. The fractions of the second peak were pooled and, after adjustment of the pH to 7.4 with Tris, incubated at 20°C with DF³²P (3500 counts/min/ μ mmole) at a final concentration of 10^{-3} M. After 2 h, when all the esterase activity had disappeared, the incubation mixture was filtered at 4°C through a Sephadex G 50 column with 0.001 M Tris acetate-acetic acid buffer, pH 5.0, to remove excess DF³²P.

About one-third of the labelled protein was used for isolation of Ser³²P after acid hydrolysis, as described earlier.⁵ The recovery of Ser³²P was 28%, calculated from the total radioactivity eluted from the Dowex 50 column. This value indicates that all the labelled phosphorus had been bound to serine in the protein.⁵

Half the total labelled enzyme solution was titrated to pH 8.6 with Tris, and then applied to a 1.2 × 22 cm TEAE-cellulose column equilibrated with 0.001 M Tris-acetic acid buffer, pH 8.6. The column was eluted with a linear gradient of the same buffer to 0.20 M Tris-acetic acid buffer, pH 8.6, also containing 0.1 M calcium acetate. Two labelled protein

* Abbreviations: DFP, diisopropylphosphorofluoridate. DF³²P, radioactive diisopropylphosphorofluoridate. SerP, phosphorylserine. Ser³²P, radioactive phosphorylserine.

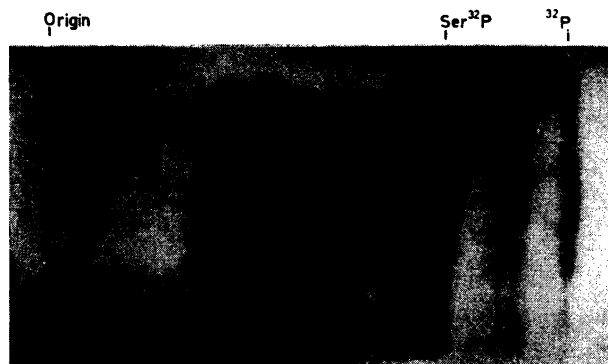


Fig. 1. Radioautograph of electropherogram of partial acid hydrolysates of DF³²P-incubated enzyme fractions from Pronase (A and C) and chymotrypsin (B). Amounts corresponding to (A) 0.4 mg, (B) 1.3 mg and (C) 0.2 mg applied to Whatman No. 3 paper on a continuous line at 5-cm intervals. Buffer: 0.05 M pyridine-acetic acid, pH 3.5. Voltage: 66 V/cm; electrophoresis time: 1.75 h. Most of the inorganic phosphate was allowed to run off the anode end of the paper. X-Ray film (Ilford, Goldseal) exposed for 14 days.

peaks were eluted, with a constant radioactivity/protein ratio in each fraction of the peaks. The molar incorporation was roughly estimated to be one mole of phosphorus per mole of enzyme, assuming the enzyme to have a molecular weight of 20 000 (see Ref. 8). The fractions of each peak were pooled separately, and the protein precipitated by adding trichloroacetic acid and silicotungstic acid to the final concentration of 5% and 0.02 M, respectively. The two precipitates were washed twice with acetone and dried *in vacuo*. Their weight was 2.5 mg and 1.3 mg.

As a first attempt to investigate the amino acid sequence around the phosphorylated serine residue in the two DFP-inhibited enzyme fractions, the dry precipitates were hydrolyzed with 5.7 N HCl at 100°C for 30 min. After removal of the HCl, the hydrolysates were subjected to high-voltage electrophoresis at pH 3.5, together with unlabelled SerP as reference. A hydrolysate of DF³²P-inactivated bovine chymotrypsin, which had been treated in the same way, was run in parallel for comparison.

It is seen from Fig. 1 that the electrophoretic pattern of the hydrolysates of the two Pronase enzyme fractions and chymotrypsin were apparently identical, with the possible exception of the region next to the starting point. This indicates that the different hydrolysates contain identical

³²P-labelled peptides. In such a case, the amino acid sequence near the reactive serine residue in the *Streptomyces griseus* enzyme fractions, is, in all probability, identical with that of the active site of chymotrypsin.

With regard to the probable similarities between the active sites of bovine chymotrypsin and the *Streptomyces* enzymes, further studies on the subject seem to be of interest.

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1. Boyer, P. D. *Ann. Rev. Biochem.* **29** (1960) 15.
2. Ramachandran, B. V., Engström, L. and Ågren, G. *Biochem. Pharmacol.* **12** (1963) 167.
3. Ramachandran, B. V. and Ågren, G. *Biochem. Pharmacol.* **12** (1963) 981.
4. Nomoto, M., Narahashi, Y. and Murakami, M. *J. Biochem. (Tokyo)* **48** (1960) 906.
5. Engström, L. *Biochim. Biophys. Acta* **52** (1961) 49.
6. Hiramatsu, A. and Ouchi, T. *J. Biochem. (Tokyo)* **54** (1963) 462.
7. Schwert, G. W. and Takenaka, Y. *Biochim. Biophys. Acta* **16** (1955) 570.
8. Nomoto, M. and Narashi, Y. *J. Biochem. (Tokyo)* **46** (1959) 1645.

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