BBA 61131

On the homology of the active-site peptides of liver carboxylesterases

Diisopropylphosphorofluoridate (DFP) reacts stoichiometrically with a number of enzymes to produce derivatives which are completely enzymatically inactive and in which one serine is phosphorylated. The sequence of amino acids adjacent to this serine has been determined using [^{32}P]DFP (ref. 1). In several peptidases, the sequence has been found to be

–Gly–Asp–$\overset{*}{\text{Ser}}$–Gly–,

while for cholinesterases and horse-liver carboxylesterase, the reported sequence is

–Glu–$\overset{*}{\text{Ser}}$–Ala–.

It is of interest, therefore, to compare the sequences around the labelled serine in a series of esterases to see what relationship these residues may have to the physical properties and high catalytic efficiency of the esterases (J. K. STOOPS AND B. ZERNER, unpublished results).

The liver carboxylesterases from horse, ox ("slow" and "fast"), sheep and chicken were purified in a manner similar to that described for pig-liver carboxylesterase[2]. Starch- or polyacrylamide-gel electrophoresis indicates that the liver enzymes are in a high state of purity. The orange-skin esterase was purified approx. 250-fold from an acetone powder extract but is still grossly impure and contains an amylase and another esterase. The enzymes (1–2 mg/ml, in 0.1 M phosphate buffer, pH 7) were treated with [^{32}P]DFP† (10^{-4} M) and assays of enzyme activity were

TABLE I

RESIDUAL ENZYMIC ACTIVITIES AFTER TREATMENT WITH DFP

All reactions were run in 0.1 M phosphate buffer (pH 7.0) and 25°.

Enzyme	*Time of reaction with DFP (min)*	*Per cent original activity*
Liver carboxylesterases*		
Pig	20	1
Horse	20	0.1
Chicken	40	0.1
Ox ("fast")	40	0.2
Ox ("slow")	40	<0.1
Sheep	40	0.2
Orange-skin esterase**	600	9
Trypsin***	280	2.5
α-Chymotrypsin***	20	<1

* Liver esterases were purified by the following workers: pig, D. J. HORGAN; ox and sheep, M. T. C. RUNNEGAR; horse, E. A. BENNETT; chicken, P. A. INKERMAN.

** Orange-skin esterase was prepared by E. M. SAMPEY.

*** Twice- or thrice-crystallized preparations from the Worthington Biochemical Corporation.

† From the Radiochemical Centre, Amersham, England as a solution in propylene glycol (0.466 mg/ml; 200 μC/ml).

carried out using the substrates *p*-nitrophenyl acetate and *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester for the esterases and peptidases respectively (Table I).

The solutions were dialysed exhaustively against distilled water at room temperature (23°), and passed through a column of Sephadex G-25 in 1% formic acid. The ratio $^{32}P:A_{280\ m\mu}$ for the solutions of inhibited enzymes was determined. For α-chymotrypsin and pig-liver carboxylesterase this corresponded to 0.79 and 1.03 moles respectively of ^{32}P per equiv of enzyme.

The enzyme solutions were lyophilized and hydrolysed with 5.7 M HCl for 20 min at 100°, according to the procedure of NAUGHTON *et al.*[3]. The hydrochloric acid was removed and the partial acid hydrolysates dissolved in water. Suitable aliquots (approx. 10 mμC) were subjected to high-voltage electrophoresis on Whatman 3 MM paper in pyridine–acetic acid–water (1:10:189, by vol., pH 3.6) at 40 V·

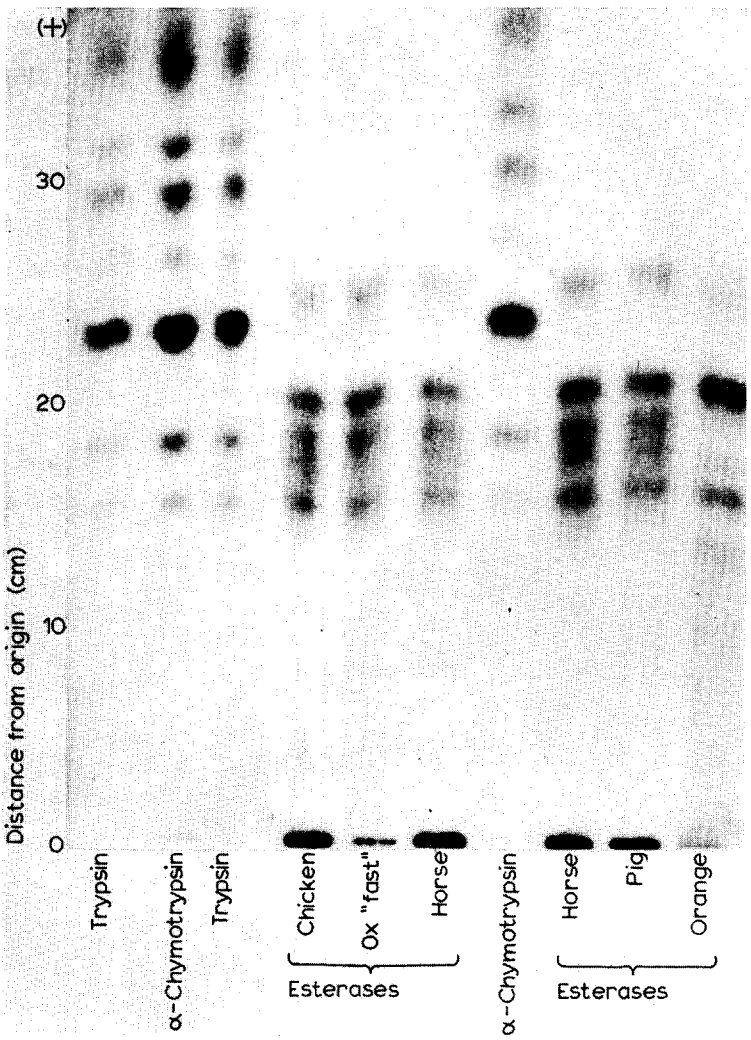

Fig. 1. Radioautograph of ionogram of partial acid hydrolysates of [^{32}P]DIP-enzymes.

cm^{-1} for 2–2.5 h. A 4-day radioautograph of this ionogram is shown in Fig. 1. The four liver esterases shown have the same pattern of peptides which differs markedly from the pattern found for α-chymotrypsin and trypsin and also from that of the orange-skin esterase . The sheep and "slow" ox enzymes show a pattern apparently identical with that of the other liver carboxylesterases.

Two-dimensional electrophoresis (pyridine–acetic acid–water, pH 3.6; then pyridine–acetic acid–water (10:0.4:90, by vol., pH 6.5)) of the horse hydrolysate alone gave an identical ionogram to that obtained from the two-dimensional electrophoresis of the horse, ox ("fast"), pig and chicken enzyme hydrolysates, aliquots of each being placed on a single spot.

A peptic digest of the [^{32}P]DFP-inhibited enzymes was carried out at pH 2 and 37° using approx. 0.3 mg/ml pepsin with approx. 3 mg/ml enzyme. Under similar conditions, JANSZ, POSTHUMUS AND COHEN[4,5] found that the horse-liver esterase gave predominantly one radioactive peptide,

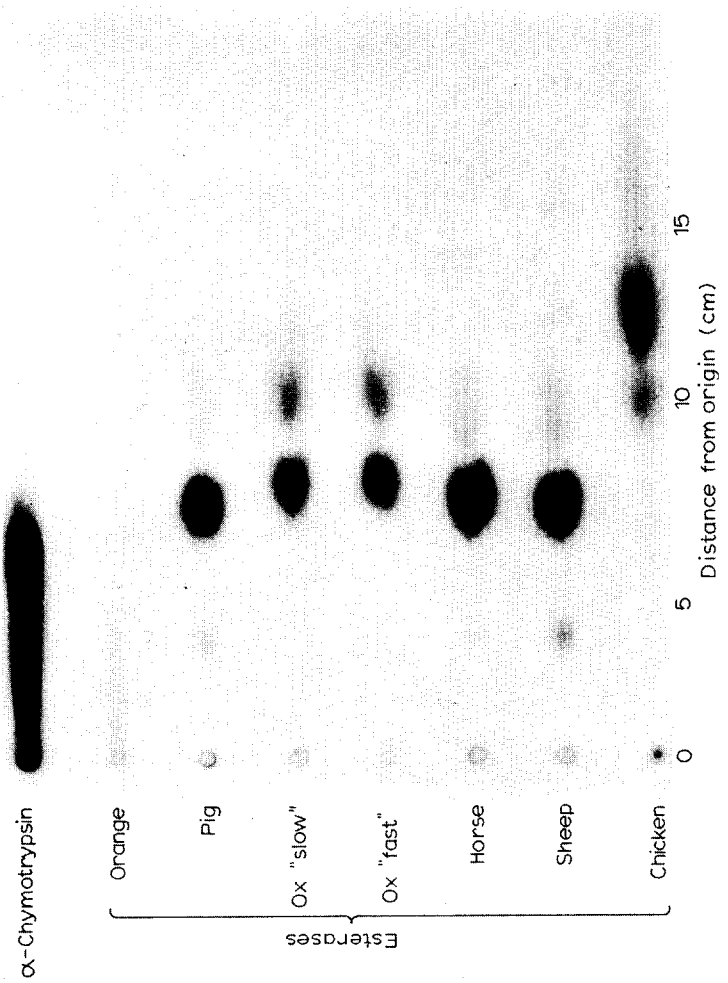

Fig. 2. Radioautograph of chromatogram of peptic hydrolysates of [^{32}P]DIP-enzymes.

Gly–Glu–Ser–Ala–Gly–Gly–(Glu, Ser)
|
DIP–O

Chromatography in butanol–acetic acid–water (4:1:5, by vol., upper phase) was carried out after peptic digestion for 1, 3 and 24 h. Fig. 2 shows a radioautograph of a chromatogram of a 3-h digest. Peptic action on the esterase peptides shown appears to be complete after 1 h. The probable identity of the major peptides from the sheep, horse and pig enzyme was substantiated by two-dimensional paper chromatography (butanol–acetic acid–water; then butanol–pyridine–acetic acid–water, 15:10:3:12, by vol.). Similarly, the major peptides from the two ox enzymes were shown to be identical. From Fig. 2, it is clear that the peptic peptides of the two ox enzymes are different from those of the chicken and also from those of the sheep, horse and pig enzymes.

Partial acid hydrolysis of the [^{32}P]DFP-labelled liver carboxylesterases produces the same set of radioactive peptides. Therefore, the sequences close to the labelled serine are the same in each case. The orange-skin esterase, however, while probably a "serine" enzyme (Fig. 1), clearly gives a slightly different pattern. Differences in amino acid sequence near the labelled serine of the liver carboxylesterases are revealed by the differences in chromatographic behaviour of the peptic peptides. These sequences are under active investigation in this laboratory.

We acknowledge with thanks the indefinite loan of a Cary 14 spectrophotometer from the Wellcome Trust, London. This work was supported in part by the N.H.M.R.C. (Australia) and in part by U.S. Public Health Service Research Grant GM 13759 from the National Institute of General Medical Sciences. Two of us (R.L.B. and J. De J.) acknowledge a National Institutes of Health Postdoctoral Fellowship and a General Motors Holden Scholarship respectively.

Department of Biochemistry,
University of Queensland,
Brisbane, Queensland (Australia)

Robert L. Blakeley
John De Jersey
Edwin C. Webb
Burt Zerner

1 F. Sanger, *Proc. Chem. Soc.*, (1963) 76 and references therein.
2 D. J. Horgan, E. C. Webb and B. Zerner, *Biochem. Biophys. Res. Commun.*, 23 (1966) 18.
3 M. A. Naughton, F. Sanger, B. S. Hartley and D. C. Shaw, *Biochem. J.*, 77 (1960) 149.
4 H. S. Jansz, C. H. Posthumus and J. A. Cohen, *Biochim. Biophys. Acta*, 33 (1959) 387.
5 H. S. Jansz, C. H. Posthumus and J. A. Cohen, *Biochim. Biophys. Acta*, 33 (1959) 396.

Received January 31st, 1967

Biochim. Biophys. Acta, 139 (1967) 208–211

BBA 61133

Chemical derivatives of subtilisin Carlsberg with increased proteolytic activity

During investigations of the significance of the functional side-chain groups for the stability and activity of the subtilisins (subtilopeptidase A, EC 3.4.4.16) it has been found that carbamylation of subtilisin Carlsberg and subtilisin Novo reduces the