THE REACTION OF CHYMOTRYPSIN AND DIISOPROPYLPHOSPHOROFLUORIDATE

I. ISOLATION AND ANALYSIS OF DIISOPROPYLPHOSPHORYL-PEPTIDES

R. A. OOSTERBAAN, P. KUNST, J. VAN ROTTERDAM AND J. A. COHEN

Medical Biological Laboratory of the National Defence Research Council, T.N.O., Rijswijk, Z.H. (The Netherlands)

INTRODUCTION

It is well known that the reaction of chymotrypsin with diisopropylphosphorofluoridate (DFP) results in the formation of a diisopropylphosphoryl-derivative of the enzyme (chymotrypsin-DP)[1] leading to the complete inhibition of its esteratic and proteolytic activity. Analysis of chymotrypsin-DP has enabled a number of investigators[2-5] to collect information on the chemical nature of the DFP-binding site.

According to at present generally accepted views a close relationship exists between the DFP-binding structure and the esteratic site of esterases; this relationship is relevant to the biochemical significance of these investigations.

The elucidation of the structure of the DFP-binding site of a number of esterases was attempted in this laboratory by a direct chemical approach. In preliminary papers[6-8] we reported the identification of some diisopropylphosphoryl-substituted peptides from proteolytic digests of chymotrypsin-DP, horse liver aliesterase-DP and trypsin-DP.

In recent work the findings on chymotrypsin-DP could be confirmed and extended. In the present paper the isolation of two diisopropylphosphoryl-substituted peptides from a proteolytic digest of chymotrypsin-DP and their composition are described in detail.

EXPERIMENTAL

Preparation of chymotrypsin-DP

α-Chymotrypsin was prepared according to the procedure of KUNITZ AND NORTHROP[9]. The reaction of $DF^{32}P$ with a four-times crystallized sample of chymotrypsin (1 g) and the isolation of the reaction product (chymotrypsin-DP; yield 70%) was carried out as detailed by JANSEN *et al.*[10]. The $DF^{32}P$ used (20 μc/mg) was a ten-times diluted sample of $DF^{32}P$ of high specific radioactivity (200 μc/mg)[11].

Proteolytic digestion of chymotrypsin-DP

A commercially available pancreatic extract (Cotazym*) was used as a proteolytic agent. The Cotazym (15 tablets) was suspended in 60 ml of water and dialyzed for 48 h against distilled water

* N.V. Organon, Oss, The Netherlands.

References p. 555.

at 4°C. After centrifugation, the supernatant was preliminarily tested for its proteolytic activity. Increasing quantities of the supernatant (0.1, 0.2 and 0.4 ml) were added to 0.1 ml samples of 2% chymotrypsin-DP in 0.2 *M* sodium phosphate buffer (pH 7.5). Water was added to a final volume of 0.5 ml. After incubation for 4 h at 37°C the undigested protein was precipitated with 2 ml of 10% trichloroacetic acid. After centrifugation, the fraction of the total radioactivity released in the supernatant was determined. Tentatively a Cotazym concentration causing 50% of the initial protein-bound radioactivity to appear in the supernatant after a 4-h incubation period was taken as the standard concentration.

1 g of chymotrypsin-DP was dissolved in 100 ml of water and digested by the standard concentration of Cotazym in a final volume of 250 ml. During the digestion at 37°C and pH 7.5, the solution was agitated. The pH was kept constant by the continuous supply of 0.2 *N* ammonia by means of an apparatus for automatic titration. After digestion (15–24 h), the incubation mixture was concentrated *in vacuo* to 50 ml. Residual proteins were precipitated by the addition of 400 ml of ethanol. The precipitate was removed by centrifugation and discarded. The supernatant was concentrated *in vacuo* and lyophilized.

Dowex-50 chromatography

The column used was prepared from Dowex-50x4 (250–400 mesh). No preservatives or detergents were added and sodium acetate buffers were used throughout. The column was equilibrated with 0.1 *M* sodium acetate buffer (pH 3.60) and the proteolytic digest subjected to chromatography as detailed in Fig. 3. Eluate fractions were pooled and desalted on a Dowex-50x4 (H^+) column (1 ml resin/mequiv. Na^+). The radioactive components were concentrated in a narrow zone at the boundary of the H^+ and Na^+ resin by percolating 1 ml of 0.5 *M* sodium acetate buffer (pH 5.6). Following a washing with 5 ml of water the resin containing the radioactive zone was cut from the column. The resin was suspended in 5 ml of water and the material eluted at pH 8.0 with 0.2 *N* ammonia. The elution was repeated twice.

Paper chromatography

For qualitative purposes Schleicher-Schüll 2043 b paper was used. For preparative purposes Whatman No. 1 paper was pre-washed with 2 *N* HCl followed by an excessive amount of water. Solvents used were: phenol–ammonia; butanol–acetic acid–water (BAW) (4:1:5) pyridine–water (PW) (4:1), and butanol–water (BW).

Phenol–water distribution

One of the components eluted from the Dowex-50x4 column was purified by a distribution between water and phenol. For this purpose the desalted and freeze-dried material was dissolved in 5 ml of phenol (saturated with water). The contaminating constituents were removed by a five-times repeated extraction of the phenol solution with 5 ml portions of water (saturated with phenol). The upper layers were discarded while the resulting phenol solution was treated in high vacuo (10^{-3} mm Hg) to remove the phenol.

High-voltage electrophoresis

This technique was performed according to RYLE *et al.*[12] with some modifications. The material was applied to a strip of filter paper (Whatman No. 1 measuring 42 × 14 cm, see Fig. 4). The paper was clamped in a horizontal position in an all-glass frame and wetted with the buffer solution by dipping the free hanging ends of the horizontal paper strip in the buffer solution. Pyridine–acetic acid buffers[12] were used. The current at 2500V amounted to 30 and 20 mA at pH 3.6 and pH 6.7, respectively.

Estimation of terminal NH_2

The method developed by SANGER was used. To the peptide (1 μmole) dissolved in 0.1 ml of a $NaHCO_3$ solution, a solution of 4 mg dinitro-fluorobenzene (DNFB) in 0.2 ml of ethanol was added. After standing for 2 h at 37°C the ethanol was evaporated, and the excess of DNFB was extracted three times with ether. The resulting solution was acidified and the extraction was repeated. Finally, the dinitro-phenylated peptide was recovered by an extraction with methyl acetate.

Quantitative determination of amino acids

The determination of the amino acids was performed by paper chromatography essentially according to the technique outlined by PERNIS *et al.*[13]. After development of the chromatogram (Whatman No. 1) the amino acids were partially stained by spraying the paper with the diluted ninhydrin reagent (1 vol. diluted with 3 vol. of cellosolve–sodium acetate buffer) and heating at 60°C for 3 min. After the staining the zone of each visualized spot was outlined. Absorbed ammonia was eliminated by spraying the paper with a 0.5% solution of KOH in ethanol (1 ml/25 cm^2).

References p. 555.

The paper was dried by a warm air stream and the spraying with the KOH solution repeated on the other side. The paper was then heated for 15 min at 60°C. Without delay the spots were cut in small pieces and placed in carefully cleaned test tubes. Following the addition of 5 ml of ninhydrin reagent the test tubes were covered with aluminium caps and placed for 20 min in a boiling water bath. After cooling, the colour was eluted from the paper by the addition of 5 ml of propanol–water (1 : 1). After centrifugation the spectrophotometric aborption is measured at 570 mμ (light path 1 cm). Under the conditions of the experiment, a density of 0.190–0.200 corresponds to the presence of 0.1 μmole of amino acid on the original paper chromatogram. On the average the paper blank readings (0.050–0.070 against water) were equivalent to about 0.03 μmole amino acid. Chemicals of highest grade purity are needed to guarantee low blank values.

Reagents

Acetic acid (A.R.): distilled from CrO_3. Methyl cellosolve (BDH): after boiling 500 ml under reflux at 20 mm Hg with 1 g of $SnCl_2$ for 15 min. 2 ml H_3PO_4 are added and the middle fraction of the distillate at 20 mm Hg is recovered. Propanol (BDH): redistilled monthly. The ninhydrin reagent is prepared by dissolving 500 mg of ninhydrin (Hoffman–La Roche) and 75 mg of $SnCl_2$ (A.R.) in a mixture of 50 ml of methyl cellosolve and 50 ml of sodium acetate buffer (pH 4.80). The buffer solution was prepared by mixing equal volumes of 1 *N* NaOH and 2 *N* acetic acid, and adjusting to pH 4.80.

RESULTS

Chymotrypsin-DP was subjected to proteolytic digestion by Cotazym as indicated in the experimental section. During the digestion aliquots containing 15 μg of nitrogen were taken at the intervals indicated in the first column of Table I. Paper chromatography of these samples in BAW and staining by ninhydrin resulted in the picture presented in Fig. 1 which clearly indicates that the mixture of proteolytic enzymes (Cotazym) had split the chymotrypsin-DP in a mixture of products consisting mostly of amino acids. This greatly facilitated the purification of the desired radioactive larger fragments. The positions of the latter are revealed in Fig. 2, which illustrates the autoradiogram of the paper chromatogram of Fig. 1. The complete course of the proteolytic digestion may be judged from Figs. 1 and 2 and from the compiled data of Table I. It will be seen that five radioactive fractions are formed in the course of the proteolysis; the fractions are indicated as A, B, C, D and E (Fig. 2).

TABLE I

THE PRODUCTION OF ^{32}P-CONTAINING COMPONENTS BY PROTEOLYTIC DIGESTION OF CHYMOTRYPSIN-DP

Time (h)	*Yield of component**					
	A	*B*	*C*	*D*	*E*	*Total*
0					100.0	100.0
3	2.6	23.6	3.9	15.3	39.3	84.7
6	4.5	44.7	8.9	11.0	16.5	85.6
12	6.3	63.3	14.3	4.7	0.7	89.3
18	11.4	61.4	18.5	2.3	0.3	93.9

* Yields accounted for as % of total ^{32}P applied to the paper. The letters correspond to those indicating the spots in Fig. 2.

As shown by the data of Table I both stability and yield of component B are very remarkable. Continuation of the digestion period after 18 h caused only minor changes of the distribution of the radioactivity on the chromatogram. Because of its high yield, the isolation of the ^{32}P-containing fraction B, which was soon found to

References p. 555.

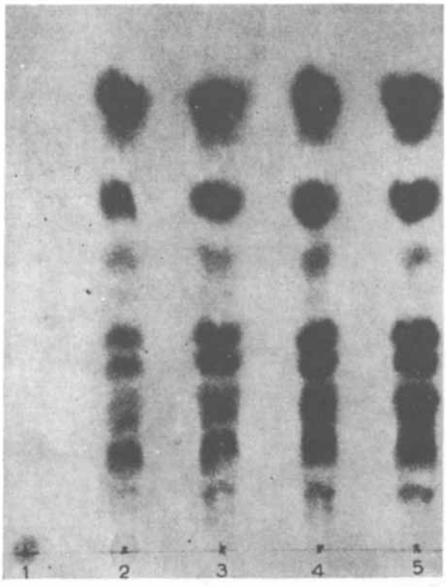

Fig. 1. Paper chromatogram (BAW) of aliquots (1–5) corresponding to 0.2 mg of chymotrypsin-DP after 0, 3, 6, 12 and 18 h incubation with Cotazym respectively.

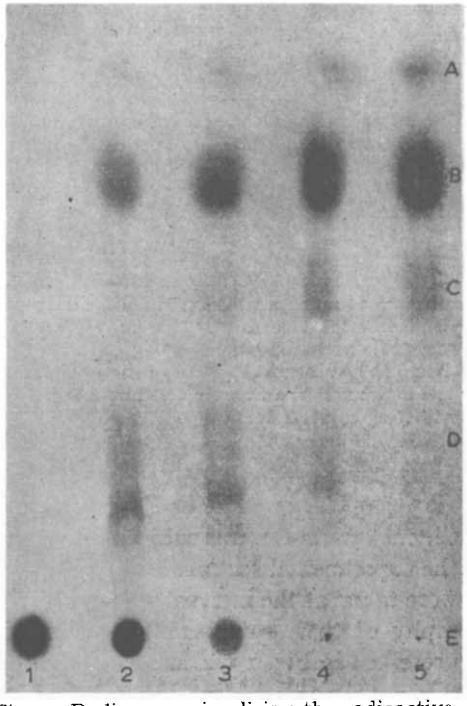

Fig. 2. Radiogram visualizing the radioactive spots on the paper chromatogram of Fig. 1.

contain a peptide, seemed most promising. To that end the freeze-dried material resulting from the digestion was chromatographed on a Dowex-50x4 column as detailed in Fig. 3.

The figure indicates the presence of a number of radioactive components. The fractions B and C (Fig. 3), which correspond to the spots B and C of Fig. 2, contained peptides and were pooled. The main purpose of this chromatographic procedure was the recovery of large quantities of peptide B. Therefore, the column was heavily loaded at the price of a relatively poor resolution.

In contrast to the material of peak B containing only minor quantities of contaminating constituents large quantities of the short-chain neutral amino acids could be demonstrated in the pooled eluates of peak C (Fig. 3). The radioactive component of peak A was identified as diisopropylphosphate by comparing its R_F value in BAW with an added authentic sample of diisopropylphosphate (R_F = 0.84). The blackened area on an autoradiogram of this chromatogram fully covered the blue coloured area of the added marker substance after staining by the phosphate reagent of HANES *et al.*[14].

The ultimate purification of peptide B was performed by distribution of the material in the phenol–water system. Following this procedure 8 μmoles of peptide B were recovered from 500 mg of chymotrypsin-DP.

The desalted and lyophilized material containing peptide C was subjected to a high-voltage paper electrophoresis at pH 3.60 (2500 V for 3 h). The radioactive zone was eluted from the paper (Fig. 4) and further purified by paper chromatography in BAW (R_F = 0.53). Peptide B and C proved to be homogeneous on paper electro-

References p. 555.

phoresis (pH 3.60 and pH 6.7) and paper chromatography in several solvents. Further proof of the absence of contaminating non-radioactive peptides and amino acids was supplied by the finding that the molar ratio free-NH–P was unity. The number of free NH_2 groups was estimated from the extinction at 361 mμ of the dinitrophenyl derivatives of peptides B and C*. The number of bound P-containing groups was derived from radioactivity measurements.

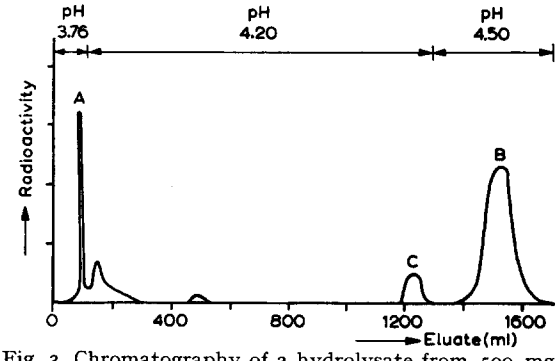

Fig. 3. Chromatography of a hydrolysate from 500 mg of chymotrypsin-DP on a 95 × 1.4 cm column of Dowex-50x4. The eluent was collected in 3 ml fractions. As eluents 0.1 *M* acetic acid–sodium acetate buffers were used. The ordinate refers to the radioactivity in arbitrary units.

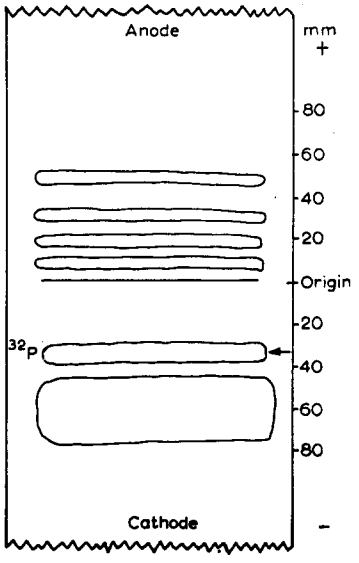

Fig. 4. High voltage electrophoresis at pH 3.6 (2500 V for 3 h) of the material contained in peak C from the Dowex-50x4 column. From the ninhydrin-positive zones only the radioactive one (at →) was eluted.

The isolated peptides B and C were hydrolyzed in 6 *N* HCl (105°C for 20 h) and the hydrolysates were subjected to two-dimensional chromatography in phenol–ammonia and BAW. On the stained chromatogram of the hydrolysate of peptide B, aspartic acid, serine, glycine, proline and leucine could be demonstrated while in the hydrolysate of peptide C, aspartic acid, serine, glycine and proline could be identified. The presence of leucine rather than isoleucine in peptide B was demonstrated by paper chromatography with the solvent system *sec.*-butanol–3% ammonia (1:1)[16]. Moreover, in alkaline hydrolysates tryptophane could not be detected excluding the presence of this amino acid. To determine the number of the individual amino acids present in peptide B and peptide C, quantitative paper chromatography of their acid hydrolysates was performed as described in the experimental section. For the determination of serine, glycine, and aspartic acid, an amount of hydrolysate (6 *N* HCl at 105°C for 20 h) equivalent to 0.04 μmole ^{32}P was chromatographed in phenol–ammonia, while leucine and proline were estimated on separate chromatograms developed with *sec.*-butanol–3% ammonia (1:1). Proline was estimated according to the procedure of SCHWEET[17]. The molar values were found by comparison of the strength of the ninhydrin colours obtained with values resulting from the parallel processing of known amounts of amino acids (0.04 μmole each of aspartic acid, serine, leucine, and proline, and 0.12 μmole of glycine).

* On the basis of an E_{361} of 17,200 in 1% $NaHCO_3$ reported for DNP-glycine[15]. It will be shown in a forthcoming paper that glycine is indeed the amino-terminal amino acid of both peptides.

References p. 555.

The mean values of four estimations with their standard error are recorded in Table II. The values given have not been corrected for losses due to acid hydrolysis. As may be concluded from the literature a recovery of serine after hydrolysis of only 80–85% is not abnormal. Therefore, the figures found for serine are consistent with an equimolar serine/phosphorus relationship. No explanation can be offered for the fact that the glycine values found do not conform satisfactorily to stoichiometric requirements. As will be shown in a forthcoming paper the results of subsequent studies on the structure of peptide B strongly indicate the presence of only three glycine residues.

TABLE II

AMINO ACID COMPOSITION OF PEPTIDE B AND PEPTIDE C

Amino acid	*Peptide B*	*Peptide C*
Aspartic acid (asparagine)	1.01 ± 0.06	0.95 ± 0.08
Serine	0.87 ± 0.08	0.82 ± 0.06
Glycine	3.20 ± 0.14	3.30 ± 0.16
Leucine	0.94 ± 0.04	
Proline	0.90 ± 0.05	0.96 ± 0.09

The figures refer to the mean values of 4 estimations of the number of moles of amino acid per gram atom of P with the corresponding standard errors.

Heating of the peptides in 0.01 *N* NaOH at 100°C for 4 min resulted in the release of the ^{32}P-containing group. Paper chromatography of the heated solution in BAW and BW showed that the radioactive component travelled at the same speed as added authentic samples of diisopropylphosphate (R_F = 0.82 and 0.32 in BAW and BW, respectively). This result established the identity of the ^{32}P-containing group as diisopropylphosphate.

Comparison of the composition of the peptides B and C suggests that peptide C resulted from further breakdown of peptide B and that the diisopropylphosphoryl-group is localized on a peptide containing aspartic acid or asparagine (1), serine (1), glycine (3), proline (1), and leucine (1). The techniques employed did not allow differentiation between aspartyl and asparaginyl residues. Comparison of the composition of the peptides suggests that leucine occupies a terminal position.

DISCUSSION

Two DP-substituted peptides were obtained by enzymic hydrolysis of chymotrypsin-DP. It has been shown that one of these peptides contains the following amino acids: glycine (3), serine (1), aspartic acid or asparagine (1), and proline (1). In addition to these amino acids the second peptide (peptide B) contains leucine. The related composition and the amino-terminal position of glycine in both peptides (to be demonstrated in the next paper) suggests that leucine occupies a carboxyl-terminal position. These peptides carry the radioactive diisopropylphosphoryl group, which was introduced into the intact protein as a result of the incubation of the enzyme with DF^{32}P. The presence of one free amino group per atom of bound phosphorus and the homogeneity of the peptides as demonstrated by paper chromatography and electrophoresis are ample evidence for their purity.

References p. 555.

It may be asked whether the position of the DP-group in the peptides corresponds to the primary site of attack of DFP on the enzyme. The first possibility to be considered is a migration of the DP-group during the hydrolysis of the enzyme-DP or the isolation of the peptides. Thus, it has been shown[18] that the DP-group of N-diisopropyl phosphorylserine ethylester is transferred to the oxygen atom of the hydroxyl group on acid hydrolysis.

To minimize the chance of transfer an enzymic rather than an acidic hydrolysis of chymotrypsin-DP was preferred. However, by this procedure side reactions caused by transpeptidation may be introduced. In our opinion, the high yield and the fast formation of the investigated peptides (especially peptide B) are indicative that transpeptidation did not interfere to any extent and that the peptides resulted from a straightforward degradation of the parent chymotrypsin-DP. The possibility of transformations in the peptides due to the isolation techniques may be dismissed on account of the mild conditions used. Thus it is very likely that the isolated peptides are integral parts of the chymotrypsin-DP molecule as subjected to enzymic attack. On the other hand, one may not claim that a corresponding peptide in the intact protein molecule is the site that primarily combines with DFP; migration of the DP-group in the period preceding the moment at which enzymic hydrolysis is started has not been excluded. This problem and the significance of our findings in relation to the results of other investigators will be discussed in the next paper.

SUMMARY

1. After reaction with $DF^{32}P$, α-chymotrypsin was subjected to a proteolytic digestion. From the digest two related peptides containing the radioactive diisopropylphosphoryl-group were isolated.

2. The amino acid composition of one peptide was established as aspartic acid or asparagine (1), serine (1), glycine (3), and proline (1). In addition to these amino acids, the second peptide contained a leucine residue.

REFERENCES

1 E. F. JANSEN, M. D. F. NUTTING, R. JANG AND A. K. BALLS, *J. Biol. Chem.*, 179 (1949) 189.
2 N. K. SCHAFFER, C. S. MAY AND W. H. SUMMERSON, *J. Biol. Chem.*, 202 (1953) 67.
3 N. K. SCHAFFER, S. HARSHMAN, R. A. ENGLE AND R. DRISKO, *Federation Proc.*, 14 (1955) 275.
4 N. K. SCHAFFER, R. A. ENGLE, L. SIMET, R. W. DRISKO AND S. HARSHMAN, *Federation Proc.*, 15 (1956) 347.
5 F. TURBA AND G. GUNDLACH, *Biochem. Z.*, 327 (1955) 186.
6 R. A. OOSTERBAAN, P. KUNST AND J. A. COHEN, *Biochim. Biophys, Acta*, 16 (1955) 299.
7 R. A. OOSTERBAAN, H. S. JANSZ AND J. A. COHEN, *Biochim. Biophys. Acta*, 20 (1956) 402.
8 J. A. COHEN, R. A. OOSTERBAAN, M. G. P. J. WARRINGA AND H. S. JANSZ, *Discussions Farad. Soc.*, 20 (1955) 114.
9 M. KUNITZ AND H. J. NORTHROP, *J. Gen. Physiol.*, 18 (1935) 433.
10 E. F. JANSEN, M. D. F. NUTTING AND A. K. BALLS, *J. Biol. Chem.*, 179 (1949) 189.
11 R. A. OOSTERBAAN AND J. VAN ROTTERDAM, *J. Am. Chem. Soc.*, 78 (1956) 5641.
12 A. P. RYLE, F. SANGER, L. P. SMITH AND R. KITAI, *Biochem. J.*, 60 (1955) 541.
13 B. PERNIS AND C. H. WUNDERLY, *Biochim. Biophys. Acta*, 11 (1953) 209.
14 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
15 H. FRAENKEL-CONRAT, J. S. HARRIS AND A. L. LEVY, in DAVID GLICK, *Methods of Biochemical Analysis*, Vol. 2, Interscience Publ., New York, 1955, p. 374.
16 J. F. ROLAND AND A. M. GROSS, *Anal. Chem.*, 26 (1954) 502.
17 R. S. SCHWEET, *J. Biol. Chem.*, 208 (1954) 603.
18 R. E. PLAPINGER AND T. WAGNER-JAUREGG, *J. Am. Chem. Soc.*, 75 (1953) 5757.

Received August 12th, 1957