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# Hydrolysis of Polyamino Acids by an Extracellular Protease from *Penicillium cyaneo-fulvum*

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*Penicillium cyaneo-fulvum*, a member of the *P. chrysogenum* group, produces an extracellular protease when grown in an ox-heart infusion–peptone–glucose medium (Singh & Martin, 1960). Highly purified preparations of the enzyme did not appear to have peptidase activity, being unable to hydrolyse a wide variety of short-chain peptides (Martin, Singh, Ankel & Khan, 1962) or various substituted amino acids and peptides, such as have been used to characterize the better-known proteases. However, the enzyme was able to cleave the isolated peptide chains of bovine insulin (Martin *et al.* 1962). Examination of the reaction products suggested that the enzyme was an endopeptidase capable of cleaving a wide range of inner peptide bonds.

To investigate the mode of action of this protease we have studied the hydrolysis of several synthetic water-soluble polyamino acids. This paper reports on properties of polyaspartic acid, polyglutamic acid, polylysine and polyproline as substrates, the pH optima, evidence for the random nature of cleavage and some aspects of the kinetics of the hydrolysis. Also undertaken was a more detailed study of the hydrolysis of polylysine: the final products of the reaction were identified, the accumulation and subsequent disappearance of intermediate peptides was observed and the rates of hydrolysis of some of these intermediates were estimated.

## EXPERIMENTAL

### *Materials*

Diethylaminoethylcellulose, type 40 (DEAE-cellulose), was obtained from Carl Schleicher and Schull Co., Keene, N.H., U.S.A., and Sephadex G-25 from Pharmacia Co., Uppsala, Sweden. Di-isopropyl phosphorofluoridate (DFP) was from Aldrich Chemical Co., Milwaukee, Wis., U.S.A., ethylenediaminetetra-acetic acid (disodium salt) from

Bersworth Chemical Co., Framingham, Mass., U.S.A., and tris from Sigma Chemical Co., St Louis, Mo., U.S.A. Polyamino acids were obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Poly-L-aspartic acid, mol.wt. 15000, poly-L-glutamic acid (sodium salt), mol.wt. 50000, poly-L-lysine hydrochloride, mol.wt. 182000, and poly-L-proline, mol.wt. 68000, were used in the first portion of this study (molecular-weight estimates were supplied by the manufacturer). A second sample of poly-L-lysine hydrochloride, mol.wt. 100000–200000, was used in the investigation of the products of digestion of poly-L-lysine, and poly-L-lysine hydrobromide, mol.wt. 283000, was used in the preparation of lysine oligopeptides.

*Buffers.* The following buffers were used: sodium acetate–acetic acid, pH 3·5–5·5; disodium hydrogen phosphate–monosodium dihydrogen phosphate, pH 6·0–8·0; sodium carbonate–sodium hydrogen carbonate, pH 9·1–10·7; sodium phosphate–sodium hydroxide, pH 11·0–11·5. Buffer concentrations were as indicated in the text.

## *Analytical procedures*

Protease activity was determined by a modification of the method of Anson (1938) with casein as the substrate, a unit of protease being defined as that amount of enzyme which liberated 1 m-equiv. of tyrosine in 1 min. under the conditions of test (Singh & Martin, 1960).

Protein concentration was determined by the method of Gornall, Bardawill & David (1949).

Quantitative estimations of hydrolysis were made by the colorimetric ninhydrin method of Matheson, Tigane & Hanes (1961). Colour yields of 2·0 and 2·2 were used for poly-L-aspartic acid and poly-L-glutamic acid respectively, these being the reported values (Matheson *et al.* 1961) for the respective amino acids. The lysine colour yield was listed as 2·4. However, a part of this colour is contributed by the $\epsilon$-amino group. Since monoamino-monocarboxylic amino acids have colour yields between 2·0 and 2·2, we elected to use a factor of 2·0 as an approximation for the ninhydrin colour value corresponding to the $\alpha$-amino group of lysine. The use of these values tended to underestimate the extent of hydrolysis since it is known that colour yield falls off with increasing chain length.

Viscosity was measured in an Oswald viscometer at 38°.

*Chromatography.* Hydrolysis of polyamino acids was detected by paper chromatography. Butan-1-ol–pyridine–acetic acid–water (15:10:3:12, by vol.) (Waley & Watson, 1953*a*) was used as the solvent system when poly-L-lysine was the substrate, whereas butan-1-ol–acetic acid–water (12:3:5, by vol.) (Smith, 1958) was used with the other substrates. Samples for chromatography were spotted on Whatman 3MM paper, to which had been stitched a wick of Whatman no. 1 paper so as to control the rate of flow of solvent. To stop enzyme action, a drop of 0·1M-acetic acid was immediately placed on top of each sample spot. Chromatograms were irrigated for periods from 1 to 7 days depending on the substrate, dried, re-irrigated with the same solvent for the same time, dried and stained with ninhydrin (0·1% in chloroform). Rerunning the chromatograms 'tightened' the spots and reduced streaking. In experiments in which the digestion of poly-L-lysine was followed chromatographically, ninhydrin-stained chromatograms were cut into strips corresponding to each sample and scanned with a recording/integrating densitometer (Spinco Analytrol, Beckman Instruments Inc., Belmont, Calif., U.S.A.), unfiltered tungsten light being used. The area under each peak was used as an index of the amount of the individual peptides present in the sample.

*Preparation of lysine oligopeptides.* Lysine oligopeptides were prepared by a procedure essentially similar to that of Waley & Watson (1953*b*). Poly-L-lysine (100 mg.) was hydrolysed with 1 ml. of conc. hydrobromic acid at 28° for 7 days. The mixture was then evaporated to dryness, taken up in 2 ml. of water, re-evaporated to remove excess of hydrobromic acid and then taken up in 2 ml. of water. The entire sample was streaked on to eight 46 cm. × 57 cm. sheets of Whatman 3MM paper (Whatman no. 1 wicks). The chromatograms were developed for 5 days, dried and run again. Strips containing the peptides were cut out and the peptides eluted with a total of 50 ml. of 0·1M-acetic acid. Di-, tri-, tetra-, penta-, hexa- and a small amount of hepta-lysine were separated in this way. Each solution was evaporated to dryness, taken up in a small amount of water and rechromatographed. The samples of di-, tri- and tetra lysine were each streaked on two sheets of paper and chromatographed for 5 days, whereas the penta- and hexa-lysine samples were each streaked on a single sheet and chromatographed for 7 days. All chromatograms were run again as described above. The trace of heptalysine was not repurified. The yield of each peptide, from dimer to hexamer, was approximately 0·5–0·2 $\mu$mole.

*Purification of the enzyme. Penicillium cyaneo-fulvum* was grown in static culture in an ox-heart infusion–peptone–glucose medium as reported by Singh & Martin (1960). Filtrate (9 l.) from a single batch of cultures was concentrated approximately sixfold in a glass circulating evaporator at 30°. The total protease activity, as estimated by caseolytic activity, amounted to 0·49 unit. The concentrated culture filtrate was then saturated with solid ammonium sulphate. The resulting precipitate was dissolved in 400 ml. of water, dialysed for 5 hr. against running tap water, then against 2 l. of cold distilled water for 3 hr. more and then freeze-dried. The freeze-dried sample was taken up in 24 ml. of distilled water, and the solution chilled and fractionated by the addition of 1·2 vol. of cold 95% ethanol. The precipitate, collected by centrifugation, was dissolved in water and assayed for protease activity (0·46 unit total).

To this point the procedure was similar to that employed by Martin *et al.* (1962). Instead of continuing with ammonium sulphate and ethanol precipitations, we used chromatographic techniques to effect further purification. The whole sample (22 ml.) was put on a DEAE-cellulose column (80 cm. × 4 cm.) and eluted at 3° with 20mM-disodium hydrogen phosphate–35 mM-sodium chloride, adjusted to pH 8·0 with hydrochloric acid. Of the protein peaks eluted, only one showed protease activity. When pooled and freeze-dried, the fractions comprising this protease peak yielded 174 mg. of protein, having 0·45 unit of protease activity. The product was dissolved in 30 ml. of water and eluted through a column (85 cm. × 2 cm.) of Sephadex G-25 with 5 mM-tris, adjusted to pH 7·8 with hydrochloric acid. Fractions from the protease peak were pooled and freeze-dried; 100 mg. of protein, having 0·39 unit of protease activity, was obtained. The sample was dissolved in 10·5 ml. of water, rechromatographed on DEAE-cellulose as above and freeze-dried. By this procedure 86·5 mg. of protein having 0·36 unit of protease

activity (74% of the original total activity) was obtained. Rechromatography on DEAE-cellulose or Sephadex did not result in further purification of the enzyme. The final product was dissolved in water (7 mg. of protein/ml.) and stored at −10° until required for use.

A portion of the preparation was subjected to electrophoresis at 3–4 v/cm. for 7½ hr. in tris–EDTA–borate buffer (Aronsson & Gronwall, 1958), pH 9·2, *I* 0·075, with cellulose acetate as the supporting medium. On staining with Amido Black a single sharply defined band was observed. No peptidase activity could be detected toward Leu-Gly-Gly, Ala-Gly-Gly, Leu-Tyr, Leu-Phe, Leu-Ala, Leu-Leu or Leu-Gly; peptidases toward these substrates might have been expected in the original culture filtrate (Martin *et al.* 1962). Hydrolysis of both poly-L-lysine and poly-L-glutamic acid was completely inhibited by 2 mM-DFP. Also, the enzyme preparation showed the same specific activity toward casein as the apparently homogeneous preparation obtained earlier (Martin *et al.* 1962). These observations suggest that the present preparation is a single homogeneous protein. We have assumed a mol.wt. 44000 for the enzyme (Martin *et al.* 1962).

## RESULTS

Under appropriate conditions poly-L-lysine, poly-L-glutamic acid and poly-L-aspartic acid were hydrolysed by the enzyme. As will be seen in Fig. 1, the pH curve for the hydrolysis of poly-L-lysine showed a very sharp peak at pH 10·7, whereas with poly-L-glutamic acid and poly-L-aspartic acid the peaks occurred at pH 4·5 and 4·3 respectively. Previous work indicated that the enzyme is irreversibly inactivated at pH values above 12 and below 3, a fact which may account, in part at least, for the rapid decreases of activity at the ends of the range.

The rapid decrease in viscosity which was observed during the digestion of poly-L-lysine and poly-L-glutamic acid (Fig. 2), relative to the number of bonds cleaved, suggests that cleavage is essentially random in nature. (Viscosity changes during the hydrolysis of poly-L-aspartic acid were not measured because of the low initial viscosity of the reaction mixture.) The data show that the viscosity of poly-L-lysine dropped 37% when the extent of hydrolysis, calculated from the initial rate of reaction, was only one peptide bond/mole. The corresponding value with poly-L-glutamic acid was 57%. As further evidence of random cleavage, paper chromatograms of reaction mixtures showed an array of split products when poly-L-lysine, poly-L-glutamic acid and poly-L-aspartic acid were used as substrates (cf. Waley & Watson, 1953*b*). With digestion of poly-L-lysine, a homologous series of oligopeptides with degrees of polymerization from 2 up to at least 9 was observed.

From the data plotted in Fig. 3, initial rates of cleavage of 680, 400 and 50 mole bonds cleaved/mole of enzyme/min. were calculated for poly-L-

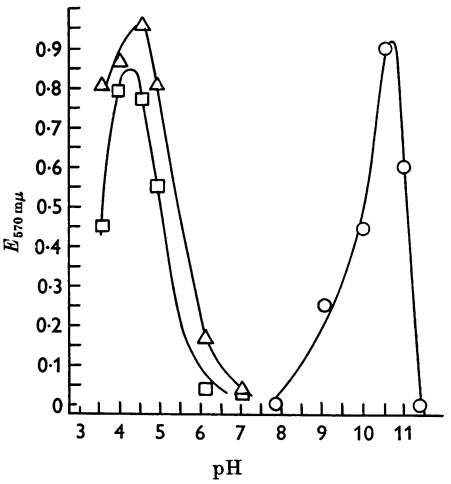


Fig. 1. pH optima with polylysine, polyglutamic acid and polyaspartic acid as substrates. Amounts given were in a final volume of 0·27 ml. of 70 mM buffer. ○, 0·7 mg. of poly-L-lysine and 1·4 μg. of enzyme; △, 0·44 mg. of poly-L-glutamic acid and 14 μg. of enzyme; □, 0·6 mg. of poly-L-aspartic acid and 14 μg. of enzyme. Incubation was for 50 min. at 38°.

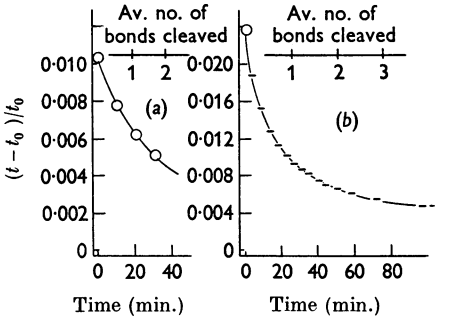


Fig. 2. Changes in viscosity during hydrolysis of polylysine and polyglutamic acid. (*a*) Because of the low viscosity of solutions of poly-L-lysine at pH 10·7, viscosities were measured at pH 4·5. The digestion mixture contained 10 mg. of poly-L-lysine and 0·2 μg. of enzyme/ml. of 20 mM buffer, pH 10·7. At the times indicated, 0·8 ml. of digestion mixture was added to 3·5 ml. of 1·0 M buffer, pH 4·5, and the viscosity determined. The zero-time value was obtained before the addition of enzyme and $t_0$ was determined with a sample containing 0·8 ml. of 20 mM buffer, pH 10·7, in 3·5 ml. of 1·0 M buffer, pH 4·5. The average number of bonds cleaved was calculated on the basis of a turnover number of 680 for poly-L-lysine. (*b*) The digestion (4·0 mg. of poly-L-glutamic acid and 0·35 μg. of enzyme/ml. of 0·1 M buffer, pH 4·5) took place in the viscometer. Since digestion continued during the time measurements were taken, values of $(t-t_0)/t_0$ are plotted against the appropriate time-range. The average number of bonds cleaved was calculated from a turnover number of 400 for poly-L-glutamic acid.

lysine, poly-L-glutamic acid and poly-L-aspartic acid respectively. The initial rates would suggest that hydrolysis of these polyamino acids is comparatively little influenced by the sign of the electric charge on the substrate, since the initial rate of cleavage of poly-L-lysine differs relatively little from that of poly-L-glutamic acid. However, the nature of the amino acid residue is clearly significant as the initial rates of cleavage of the two acidic polyamino acids differ by a factor of about 10.

Hydrolysis of poly-L-proline could not be demonstrated by any of several methods. Split products could not be detected by paper chromatography with isatin used as the detecting agent when poly-L-proline was incubated with the enzyme over the range pH 4·0–10·7. The viscosity of reaction mixtures at pH 4·5, 7·0 and 10·7 remained constant over a 20 hr. period. Also, trials made in the ultracentrifuge failed to show cleavage of the substrate after long periods of incubation.

When the digestion of poly-L-lysine was followed chromatographically, it was apparent that there was a build-up of intermediate-length peptides (from pentamer upwards) and that these were subsequently broken down to yield the final products of the reaction, di-, tri- and tetra-lysine. No evidence could be found for the presence of monomer among the products. Fig. 4 shows tracings of representative chromatograms accompanied by their corresponding densitometer records. In each case the intensities of the well-defined spots are compared with the intensity of the corresponding dimer spot (see Table 1 for identification of peptides). The accumulation and subsequent utilization of intermediates was most clearly seen when the enzyme was allowed to act at a pH value below the optimum (e.g. pH 9·1).

From the data plotted in Fig. 5 it was estimated that the initial rate of cleavage of pentalysine was about 5 mole bonds cleaved/mole of enzyme/min. and for hexalysine the corresponding value was about 50. This 1:10 ratio corresponds quite closely to the relative rates of disappearance of penta- and hexa-lysine which were calculated from the densitometer data obtained in the preceding set of experiments. Also, since chromatographic evidence showed that the heptamer accumulated and then disappeared before the hexamer did, it may be assumed that its rate of cleavage was less than that of the polymer but greater than that of the hexamer. Di-, tri- and tetra-lysine were unreactive even when tested under conditions of much higher enzyme concentration. This is in accord with the observations reported above that these peptides were the final products of enzymic digestion of poly-L-lysine.

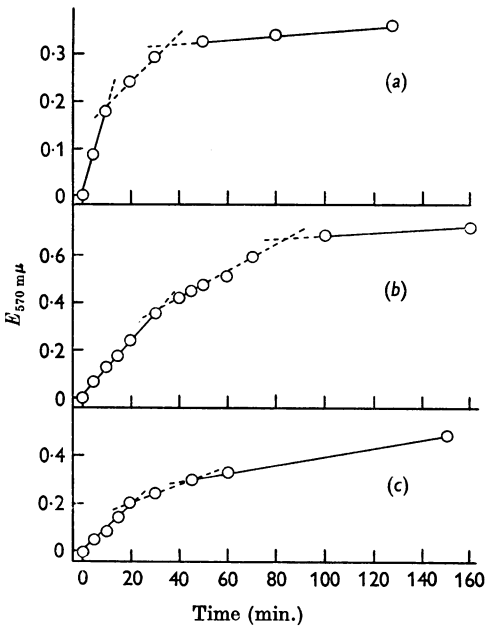


Fig. 3. Rates of hydrolysis of polylysine, polyglutamic acid and polyaspartic acid. (*a*) 0·82 mg. of poly-L-lysine and 4·0 μg. of protease/ml. of 90 mM buffer, pH 10·7; temperature, 38°; sample size, 220 μl. (*b*) 2·4 mg. of poly-L-glutamic acid and 4·0 μg. of protease/ml. of 90 mM buffer, pH 4·5. (*c*) 1·3 mg. of poly-L-aspartic acid and 22 μg. of protease/ml. of 90 mM buffer, pH 4·3.

## DISCUSSION

In a comprehensive study of the action of trypsin on poly-L-lysine at pH 7·6 Waley & Watson (1953*b*) obtained evidence which suggested selective fission of the polymer. They showed that end bonds were not split but that those near the end were split preferentially. Thus the main reaction was a stepwise formation of lower peptides. They were able to divide the reaction into two phases. In the first, the polymer was rapidly broken down, with the principal products being di-, tri- and tetra-lysine. Pentalysine was formed and then cleaved. In the second phase, tetralysine was slowly broken down, the main product being dilysine. They proposed that there were two opposing factors governing rates of hydrolysis: (*a*) the closer the bond to the end of the chain the lower the rate of attack; (*b*) the farther the bond from the middle of the chain the higher the rate of attack.

Miller (1961) showed that, during the hydrolysis of poly-L-glutamic acid by papain, attack was random except for discrimination near the ends of the chain. A very sharp pH optimum was found at

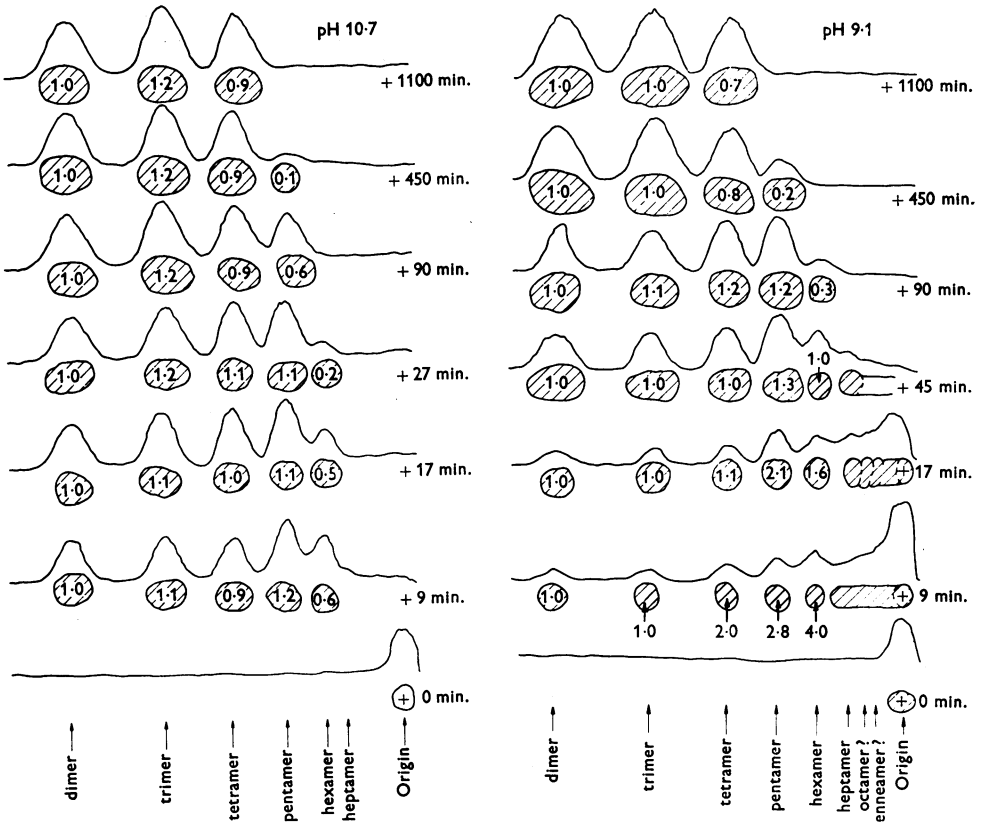


Fig. 4. Action of protease on polylysine. 5·2 mg. of poly-L-lysine and 73 $\mu$g. of protease/ml. of 80 mM buffer, pH 9·1 and 10·7; temperature, 38°.

Table 1. *Lysine peptides*

| Spot no. | $R_{Lys}$ obs. | $R_{Lys}$ calc.* | Peptide |
|---|---|---|---|
| 1 | 0·72 | 0·71 | Dilysine |
| 2 | 0·51 | 0·50 | Trilysine |
| 3 | 0·35 | 0·37 | Tetralysine |
| 4 | 0·24 | 0·25 | Pentalysine |
| 5 | 0·17 | Not reported | Hexalysine |

* Calculated from the data of Waley & Watson (1953*a*).

pH 4·85 and on the alkaline side of optimum the rate of hydrolysis was thought to parallel closely the α-helix content of the polymer. However, since the pH curve was very sharp on both sides of the optimum, it was difficult to attribute the shape of the curve to cleavage of helical regions and Miller interpreted the data as being most consistent with the assumption that cleavage occurs at the helix-coil junction.

We have interpreted our results as suggesting that cleavage of polyamino acid substrates proceeds in a random manner except near the ends of the peptide chains. The refractility of tetralysine and the low rates of hydrolysis of penta- and hexalysine would suggest that the terminal reactive groups (α-amino and carboxyl) exert a long-range effect on the susceptibility of interior bonds to attack. These rather strong end-group effects might reasonably be expected to 'steer' cleavage toward the centre of peptide chains, thereby influencing the degrees of polymerization of the oligopeptides which accumulate.

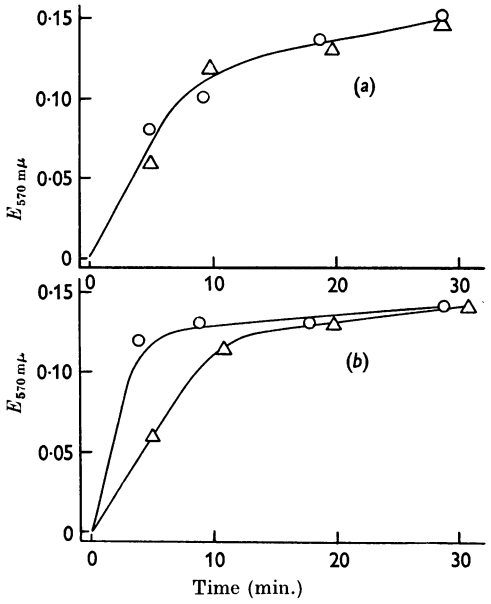


Fig. 5. Hydrolysis of penta- and hexa-lysine. (*a*) Pentalysine: ○, Expt. 1 (100 μg. of pentalysine and 44 μg. of protease in 60 μl. of 0·1 M buffer, pH 10·7; temperature 38°; sample size, 10 μl.); △, Expt. 2 (100 μg. of pentalysine and 40 μg. of protease in 110 μl. of buffer; sample size, 20 μl.). (*b*) Hexalysine: ○, Expt. 1 (160 μg. of hexalysine and 8·8 μg. of protease in 120 μl. of 80 mM buffer, pH 10·7; sample size, 20 μl.); △, Expt. 2 (160 μg. of hexalysine and 4·4 μg. of protease in 100 μl. of buffer; sample size, 20 μl.).

The course of hydrolysis appears to assume three rather distinct phases. The first consists, in the main, of the rapid hydrolysis of long-chain polymers and the accumulation of oligopeptides having considerably lower susceptibility to attack. The second reflects the cleavage of these intermediates to yield either refractile peptides (final products of digestion) or a peptide(s) which is cleaved very slowly. The third phase reflects the cleavage of this peptide(s).


## SUMMARY

1. The hydrolysis of polyamino acids by an extracellular protease from *P. cyaneo-fulvum* was investigated. Polyaspartic acid, polyglutamic acid and polylysine were substrates for the enzyme, with hydrolysis occurring optimally at pH 4·3, 4·5 and 10·7 respectively. Polyproline was not a substrate.

2. Cleavage was found to be essentially random in nature.

3. The initial rates of cleavage at the pH optima and 38° were estimated to be 50, 400 and 680 mole bonds cleaved/mole of enzyme/min. for polyaspartic acid, polyglutamic acid and polylysine respectively.

4. Di-, tri- and tetra-lysine are the final products of the enzymic digestion of polylysine.

5. During hydrolysis of polylysine there is an accumulation of intermediate peptides, from the pentamer upwards.

6. The relative rates of cleavage of penta-, hexa- and poly-lysine were found to be approximately 1:10:140.


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