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The Characterization of Proteolytic Enzymes with Synthetic Poly-α-amino Acids

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Poly-amino acids with very high molecular weight (> 150 000) were found to be well suited for rapid viscosimetrical determinations.

The two water soluble poly-amino acids, poly-L-lysine and poly-L-glutamic acid, were suitable substrates for trypsin, ficin, *Aspergillus* proteinase, and *Paracentrotus* egg cathepsin D 2. On the other hand these substrates were not hydrolyzed by pepsin, thyroid proteinases, or cathepsin D 1.

Aspergillus proteinase hydrolyzed both substrates optimally in the pH interval 4.6–5.3. Cathepsin D 2 had pH optimum for poly-L-lysine at 4.5–4.8 and for poly-L-glutamic acid at 4.4–4.5.

Judging from the gel filtration patterns of hydrolysis trypsin and crude ficin randomly degraded the substrate into products of varying magnitude whereas cathepsin D 2 split the substrate into products of a determined pattern. Purified ficin and *Aspergillus* extract acted primarily as exopeptidases giving low molecular components.

In presence of poly-D-glutamic acid which was not hydrolyzed by cathepsin D 2, the hydrolysis of poly-L-glutamic acid was reduced by 75 % at the optimal pH 4.45.

For more than 15 years synthetic poly-α-amino acids have been used as models for proteins. They are polymers composed of α-amino acid residues linked by peptide bonds. The water soluble poly-amino acids poly-L-lysine and poly-L-glutamic acid have been used as substrates for proteolytic enzymes in several investigations. The influence of trypsin, pepsin, papain, and ficin upon these poly-amino acids have been studied by Katchalski *et al.*[1,2] Waley and Watson,[3,4] Miller,[5] and others. Ankel and Martin[6] investigated the hydrolysis of poly-lysine, poly-glutamic acid, and poly-aspartic acid by *Penicillium* protease. Martin and Jönsson[7] studied the hydrolysis of poly-lysine and poly-glutamic acid by an extra cellular extract from *Aspergillus fumigatus*. The proteolytic activity in these cases was followed by means of paper chromatography and the ninhydrin method of Matheson *et al.*[8] The more convenient viscosimetric method of Hultin[9–12] was used by Söder,[13] who studied the hydrolysis of poly-lysine and poly-glutamic acid by proteinases in dental plaque material.

In the present work the influence of pH upon the hydrolysis of poly-lysine and poly-glutamic acid by different proteolytic enzymes was investigated. The hydrolysis, performed at the optimal pH of the enzymes tested, was followed by means of gel filtration on Sephadex gels. The activity was estimated according to the viscosimetrical method of Hultin. Two groups of proteolytic enzymes were investigated. The first group consisted of the commercial enzymes trypsin, ficin, pronase, chymotrypsin, and pepsin, and the second group of extracts from thyroid glands, sea urchin eggs and oocytes as well as culture medium from *Staphylococcus aureus* and fungal extract of *Aspergillus fumigatus*, the whole group constituting a part of material investigated at the authors' laboratory.

MATERIALS AND METHODS

Substrates. Poly-L-lysine, HBr (mol. wt. 90 000 and 225 000), poly-D-glutamic acid (mol. wt. 94 000), and poly-L-glutamic acid, Na salt (mol. wt. 75 000), from Pilot Chemicals Inc., Watertown, Mass., USA. Human haemoglobin (AB Kabi, Stockholm, Sweden), Gelatin (Fisher Scientific Company, Pittsburgh, Penn., USA).

Commercial enzymes. Trypsin (cryst. lyophilized, Wortington Biochemical Corp.); Ficin (Mann Research Lab.); Pronase (B. grade; Calbiochem.); Chymotrypsin (Cryst. Sigma Chem. Co.); Pepsin (Cryst. Nutritional Biochem. Corp.); Soy bean trypsin inhibitor (Sigma).

Enzymes prepared at the laboratory

1. Thyroid extract. Human thyroid tissue was promptly frozen and freed from fat and connective tissue in the frozen states, cut into thin slices and suspended in 3 ml/g tissue of 0.9 % NaCl containing 1 % butanol. The mixture was stirred for 20 h at + 4°C, filtered through nylon cloth, and centrifuged at 6000 *g* for 20 min. at + 2°C.

*2. Aspergillus fumigatus extract.** The strain *A. fumigatus* was cultivated in Roux-flasks in a medium containing yeast extract bouillon, protease and peptone for 3 days at 37°C. After that the fungi were collected, deep-frozen (−22°C), homogenized in aq. dest. (Warren blendor), and extracted for 5 days in the cold. After centrifugation at 5000 *g* for 15 min the supernatant was Seitz filtered and kept at pH 6.7 in the cold. In some experiments the extract was dialyzed against 0.02 M phosphate buffer at pH 6.8. A slight precipitate was formed which was centrifuged down at 1000 *g* for 10 min.

3. Cathepsins from Paracentrotus eggs. Lyophilized, unfertilized egg material from the sea urchin species *Paracentrotus lividus* was suspended in 0.14 M LiCl in 0.02 M Na-citrate and 0.01 M cysteine, pH 6.5. The mixture was homogenized in a Dounce homogenizer and extracted with stirring for 17 h in the cold. The suspension was centrifuged at 15 000 *g* for 15 min with subsequent centrifugation of the supernatant at 105 000 *g* for 60 min in the cold. A part of this supernatant was gel filtered and the two cathepsins D 1 and D 2 separated. The enzymatic assay was made according to the method of Anson-Kunitz,[14] with haemoglobin as substrate. The top fractions from D 1 and D 2 were pooled,[15] concentrated by means of sucrose, and dialyzed against 0.05 M Tris—HCl buffer, pH 7.2.

4. Culture medium from Staphylococcus aureus. S. aureus, strain Wood-46, variant NCTC 10344 (National collection of type cultures, England) was stored in the lyophilized state. The organism was grown for three days at 37°C on a casein hydrolysate medium with the continual passage of 25 % CO_2 and 75 % air as described earlier by Tirunarayanan,[16] for α-haemolysin production. The culture was centrifuged at 30 000 *g* and the supernatant was Seitz filtered.

* The extract was a gift from Dr. Rutqvist, National Veterinary Institution, who is hereby gratefully acknowledged.

Assay of proteolytic activity

The viscosimetrical method elaborated by Hultin [9-13] was used. The determinations were performed in Ostwald viscosimeters at 35.5°C and the enzymic activity was calculated according to Hultin's formula:

$$A = \frac{a+b}{a} \times C_s^2 \times \frac{d(1/\eta_{sp})}{dt} = \frac{a+b}{a} C_s^2 \alpha$$

Where a = enzyme solution (ml)
b = substrate solution (ml)
C_s = the concentration of the substrate in the reaction mixture (g substrate/g solution)
η_{sp} = specific viscosity
t = time in seconds
α = the slope of the plotted values of $1/\eta_{sp}$ and t
η_{sp} = the flow time of mixture − flow time of water/flow time of water = $(\tau - \tau_{H_2O})/\tau_{H_2O}$,
thus $1/\eta_{sp} = \tau_{H_2O}/(\tau - \tau_{H_2O})$

The values for activity (A) obtained were multiplied by 10^9 and called Hultin Units (H.U.).

Gel filtration. The Sephadex gels (AB Pharmacia Fine Chemicals, Uppsala, Sweden) were treated according to the manufacturer's instructions, equilibrated with the eluting medium and poured into columns. The eluting medium which always contained 2 % butanol as sterilizing agent was collected in a fraction collector furnished with a special device * for obtaining constant fraction volumes. The columns were always run at + 6°C and the optical density was measured at 240, 260, and 280 mμ in a Beckman DU spectrophotometer.

Procedure for the hydrolysis of the poly-amino acids. Immediately after mixing of the incubation components the zero time sample was taken, mixed with an inhibitor when one was available and applied to the Sephadex column. The rest of the mixture was kept in a shake incubator at 35.5°C. At appropriate time intervals samples were taken and kept in ice-water until applied to the column.

RESULTS

I. Hydrolysis of poly-amino acids by some commercial enzymes

1. Trypsin. The sequential change of degradative components upon hydrolysis of poly-L-lysine by trypsin at pH 7.3 is shown by gel filtration of samples taken immediately after mixing the reaction components (zero time), 30 min, and 2 1/2 h (Fig. 1). The successive decrease of the high molecular weight (90 000) substrate peak is quite evident; the initial low molecular weight products of the hydrolysis, visible in the diagram after 30 min, disappeared later. This disappearance may be explained by a continued break-down of split products to small peptides which are adsorbed by the column. The final concentration (2.5 μg/ml) of trypsin used had an activity of 750 H.U. Addition of 0.4 mg soy bean trypsin inhibitor caused complete inhibition. Therefore, an excess of 0.5 mg was added to the samples taken after different incubation periods before gel filtration. Trypsin was unable to split poly-L-glutamic acid.

* Constructed by Eng. L-G. Falksveden at the Chemistry Dept., Natl. Bact. Lab., Stockholm.

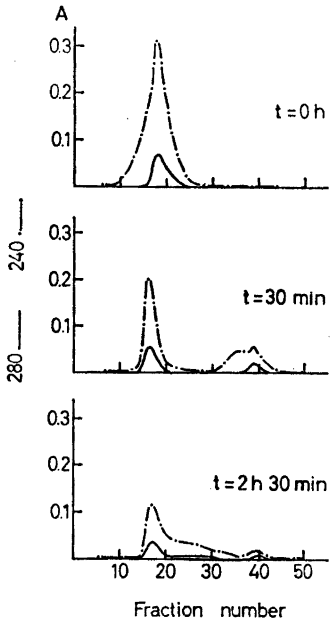

Fig. 1. Fractionation by gel filtration of the products from poly-L-lysine (Mw = 90 000) hydrolyzed by trypsin at 25°C and pH 7.3. The incubation mixture consisted of 1.0 ml trypsin (10 μg/ml) + 1.5 ml Tris—HCl buffer pH 7.3 + 1.5 ml 4 % poly-L-lysine. Samples of 0.5 ml taken at 0.30 min and 2.5 h after mixing were applied separately on the Sephadex G-25 fine column after addition of 0.5 mg soy bean trypsin inhibitor (SBI). Column: 1.2 × 49 cm; eluting medium: 0.05 M Tris—HCl, pH 7.5 + 0.2 M NaCl. Flow rate 22 ml/h at + 6°C. Fractions 1.5 ml.

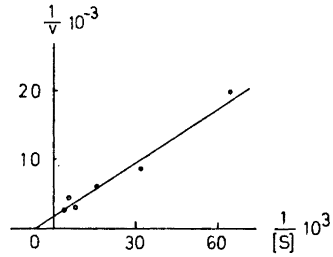

Fig. 2. Determination of Michaelis constant for the hydrolysis of poly-L-lysine by trypsin. Incubation mixture: 0.5 ml trypsin (1 μg/ml) + 1.0 ml Tris—HCl buffer pH 7.4. Temperature 35.5°C.

Fig. 2 demonstrates the curve obtained by plotting the values found in the experiment. The slope of this line determines the Michaelis constant, K_m, which was found to be $= 0.16 \times 10^{-3}$ mole/l.

2. Ficin. The hydrolysis of poly-L-glutamic acid by a crude ficin solution (100 μg/ml final conc.) with subsequent gel filtration is shown in Fig. 3. The elution pattern of the substrate and a 100 times more concentrated ficin solution are given separately at the top of the figure. For the incubation a mixture of 0.5 ml 0.06 % ficin, 1.0 ml McIlvaine buffer pH 6.0 and 1.5 ml 4 % poly-L-glutamic acid was used. The hydrolysis proceeded with a decrease of the substrate peak and an increase of products of a lower molecular character.

Since the chromatogram of ficin demonstrated a heterogeneous composition the enzymatic activity in the various fractions was investigated. The gelatinase activity values at pH 6.0 and 35.5°C are given in Table 1.

Table 1. The gelatinase activity in Sephadex G-50 fine fractions of crude ficin (see Fig. 3). Reaction mixture: 0.50 ml fraction + 0.5 ml 0.04 M cysteine + 3.0 ml 4 % gelatin incubated at 35.5°C, pH 6.0.

Fraction	Activity in H.U./ml	Fraction	Activity in H.U./ml
23	9 600	52	0
26	11 550	57	0
30	260	62	0
37	10	67	0
40	2	70	0

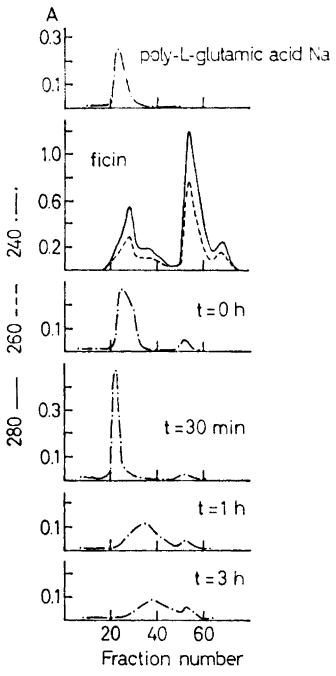

Fig. 3. Fractionation by gel filtration of the products from poly-L-glutamic acid (Mw = 75 000) hydrolyzed by crude ficin at 35.5°C and pH 6.0. Starting with the top figure the fractionations represent samples of (1) 20 mg poly-L-glutamic acid in 0.9 % NaCl; (2) 10 mg ficin; (3—6) 0.1 mg ficin + 20 mg poly-L-glutamic acid after different incubation times. A Sephadex G-50 fine column of 1.4 × 56 cm was eluted with 0.05 M Tris—HCl, pH 7.5 + 0.2 M NaCl. The flow rate was 22 ml/h at 6°C and 1.5 ml fractions were collected.

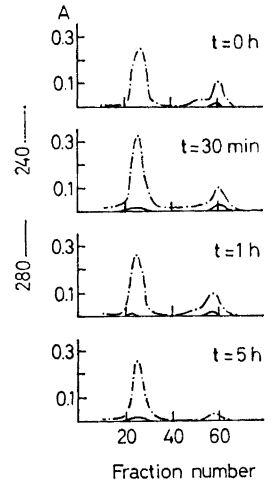

Fig. 4. Fractionation by gel filtration of the products from poly-L-glutamic acid hydrolyzed by partially purified ficin at 35.5°C and pH 6.0. The incubation mixture consisted of 0.5 ml ficin (1:100 of the partially purified pool) + 0.2 ml 0.1 M cysteine + 1.3 ml McIlvaine buffer + 2.0 ml 4 % poly-L-glutamic acid. After 0, 30 min, 1 h, and 5 h 1.0 ml of the mixture was taken, 50 μl 2 × 10^{-3} M iodoacetamide was added and the sample was applied to the column. For column and elution procedure see Fig. 3.

The fractions 25—32 were pooled and the resulting gelatinase activity was 8250 H.U./ml. The hydrolysis products of poly-L-glutamic acid by this partially purified ficin was fractionated by gel filtration from samples taken at different times after mixing (Fig. 4). It was necessary to use cysteine as an activator for the partially purified ficin. The ficin pool was diluted 1:100 for this hydrolysis experiment since the activity of the undiluted pool against poly-L-glutamic acid as substrate was 17 700 H.U./ml at pH 6.0. The gel filtration diagram of the hydrolysis of poly-L-glutamic acid by partially purified ficin was different from that obtained with crude ficin. The material in the interval between the high and low molecular components found in Fig. 3 is missing in Fig. 4. A concentration of 10^{-4} M iodoacetamide in the samples from the incubation mixture inhibited the reaction 25 %. Higher concentrations interferred with the absorbance of the split products.

3. Pronase. The effect of pH upon the hydrolysis of the two poly-amino acids used in this paper is shown in Fig. 5. The splitting of poly-L-lysine was very rapid and had an optimum on the acid side at pH 6.5. After a minimum at pH 9 there was a strong increase in hydrolysis, being twice as high at pH 10 as at 6.5. The real optimum could not be found since spontaneous hydrolysis gave erroneous values at pH over 10 (the dotted line). The hydrolysis of poly-L-glutamic acid had an optimum at pH 5. No activity could be found at pH

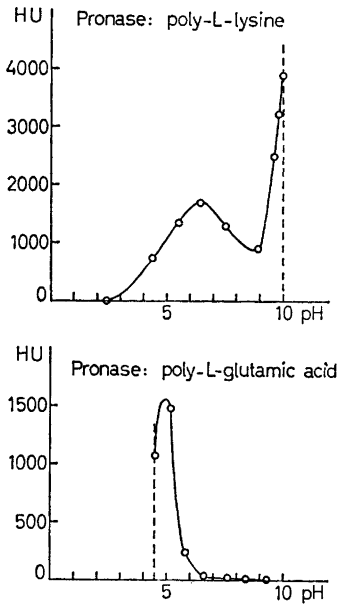

Fig. 5. Influence of pH upon the hydrolysis of poly-L-lysine and poly-L-glutamic acid by pronase. Incubation mixture: 0.5 ml (100 μg/ml) pronase + 1.0 ml buffer + 1.0 ml 4 % substrate. Temperature 35.5°C.

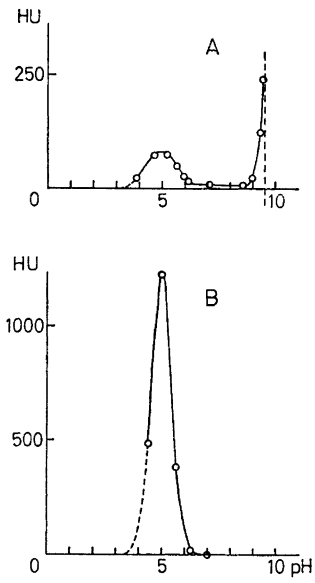

Fig. 6. Influence of pH upon the hydrolysis of poly-L-lysine (A) and poly-L-glutamic acid (B) by *Aspergillus fumigatus* extract. Incubation mixture: 0.5 ml extract + 1.0 ml buffer + 1.0 ml 4 % poly-amino acid. Temperature 35.5°C.

higher than 6.5. At pH lower than 4.2 the poly-L-glutamic acid precipitated (the dotted line being the border for accurate values).

4. Chymotrypsin and pepsin. Chymotrypsin at a concentration of 100 μg/ml had no effect on poly-L-glutamic acid but hydrolyzed poly-L-lysine at this concentration at pH 7.4 with an activity of 80 H.U./ml.

Pepsin at a concentration of 100 μg/ml possessed no hydrolytic activity against poly-L-lysine when tested at pH 1.96—5.40. However, a very weak activity was found at pH 4.5 when poly-L-glutamic acid was used as substrate.

II. Enzymes prepared at the laboratory

1. Extract from human thyroid gland.[17] The proteolytic activity of the thyroid extract was measured according to the method of Anson-Kunitz. A 16 h incubation at 37°C hydrolyzed the equivalent of 900 μg tyrosine per ml extract (225 μg/0.25 ml). The activity also was measured viscosimetrically on a 3 % gelatin solution, containing 0.01 M cysteine at a pH 3.5 and gave

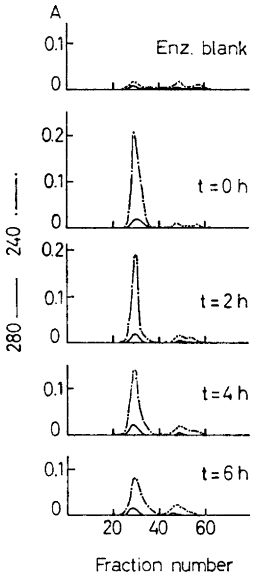

Fig. 7. Fractionation by gel filtration of the products from poly-L-glutamic acid hydrolyzed by an extract of *Aspergillus fumigatus*. Incubation mixture: 1.30 ml extract + 2.70 ml McIlvaine buffer pH 5.0 + 2.0 ml 4 % poly-L-glutamic acid. Temperature 35.5°C. Samples of 1.0 ml were taken at different times, applied to a Sephadex G-25 fine column (1.1 × 163 cm) and eluted in 0.05 M Tris—HCl buffer, pH 7.5 + 0.2 M NaCl at 6°C. Flow rate 8 ml/h. Fractions 2.5 ml.

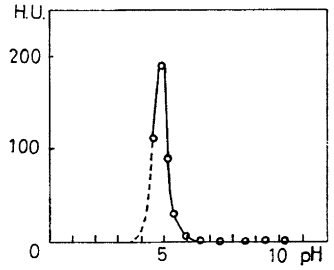

Fig. 8. Influence of pH upon the hydrolysis of poly-L-glutamic acid by *Staphylococcus aureus* medium. Incubation mixture: 0.5 ml enzyme + 1.0 ml buffer + 1.0 ml 4 % substrate. Temperature 35.5°C.

120 H.U. per ml extract. Before measuring at this low pH the extract was acidified to pH 2.8, kept with stirring at 4°C for 16 h and then centrifuged at 15 000 *g* for 20 min. This procedure lowered the enzyme activity about 20 %. For viscosimetric estimation of the thyroid activity against the two poly-L-amino acids a reaction mixture of 1.0 ml 4 % substrate + 0.75 ml buffer + 0.25 ml 0.1 M cysteine + 0.5 ml enzyme was used. Neither of the two poly-L-amino acids were broken down by this enzyme. Poly-L-lysine was investigated from pH 2.4 to 8.7 and poly-L-glutamic acid from pH 4.3 to 7.7. Only traces of activity were found, the values being within the experimental range of error.

2. Extract from Aspergillus fumigatus. The effect of pH upon the hydrolysis of poly-L-lysine and poly-L-glutamic acid by *Aspergillus* extract is shown in Fig. 6. Optimum activity against both substrates occurred at pH 5. For poly-L-lysine there was also a hydrolytic activity starting at pH 9; the optimum, however, could not be estimated because of the spontaneous hydrolysis of this substrate at pH's higher than 10.

The gel filtration fractionation of poly-L-glutamic acid after hydrolysis by the *Aspergillus* extract is shown in Fig. 7. Since the crude extract showed a very high absorption at 240 and 280 mμ it was dialyzed before use in the experiment. The fractionation pattern of the extract at the concentration used in the incubation mixture is shown at the top of Fig. 7. The activity of the extract at this concentration was 2990 H.U./ml. The decrease of the substrate upon prolonged incubation time and the increase of low molecular weight split products can be seen from the chromatograms.

3. Culture medium from Staphylococcus aureus. The influence of pH on the hydrolysis of poly-L-glutamic acid by an extracellular protease present in *Staphylococcus* culture medium is shown in Fig. 8. The pH optimum is localized at pH 5.0. No hydrolysis of poly-L-lysine in the pH interval between 3.4 and 9.8 was observed. When tested on gelatin as substrate at pH 8.2 an activity of 445 H.U./ml was obtained.[16]

The hydrolysis of poly-L-glutamic acid after 0, 2, and 16 h was investigated first by gel filtration through Sephadex G-25 fine. No reduction of the first peak was found which means that the split products had molecular weights greater than 5000. Therefore, Sephadex G-50 fine with an exclusion limit of 30 000 was used and the result is shown in Fig. 9. As can be seen the enzyme blank is fairly complex, but since dialysis destroyed most of the activity the *Staphylococcus* culture medium was used without dialysis.

Gel filtration of the enzyme-substrate mixture immediately after the start showed the UV pattern of the high molecular substrate peak and the *Staphylococcus* medium. Three hours incubation before gel filtration resulted in small amounts of split products in the fraction interval 40—60 and changes in the medium pattern at the interval 75—90. After 16 h incubation the substrate peak had decreased greatly at the same time as an increase in material with molecular weights just below 30 000 was found in the interval 40—65. A double peak of split products of a few thousand in molecular weights was found also (interval 75—85). Amino acids and di- to tetra-peptides could not be found even after 16 h hydrolysis. The initial activity of the samples used for these experiments was 420 H.U./ml at pH 4.85.

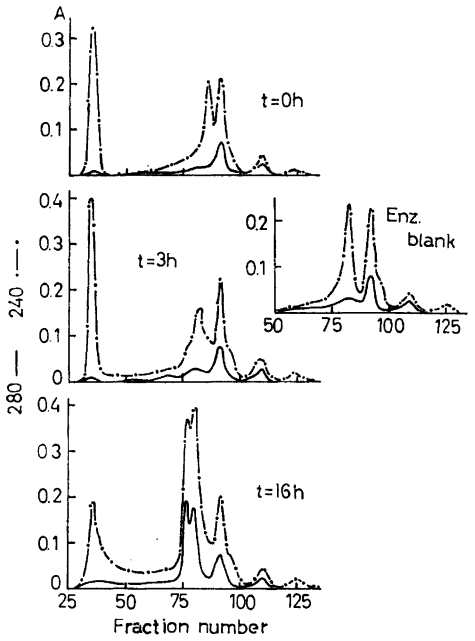

Fig. 9. Fractionation by gel filtration of the products from poly-L-glutamic acid hydrolyzed by *Staphylococcus aureus* culture medium. Incubation mixture: 1.5 ml *Staph.* medium + 3.0 ml McIlvaine buffer pH 4.85 + 3.0 ml 4 % poly-L-glutamic acid. Temperature 35.5°C. Samples of 2.0 ml were taken at different times, applied to a Sephadex G-50 fine column (1.6 × 156 cm) eluted in 0.05 M Tris—HCl buffer, pH 7.5 at 6°C. Flow rate 9 ml/h. Fractions 2.5 ml.

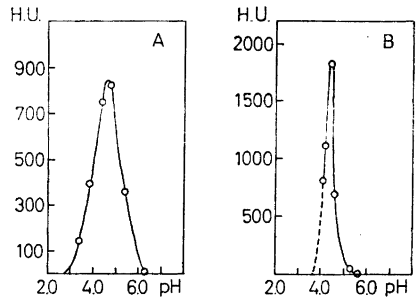

Fig. 10. Influence of pH upon the hydrolysis of poly-L-lysine (A) and poly-L-glutamic acid (B) by cathepsin D 2 from sea urchin eggs. Incubation mixture: 0.25 ml D 2 + 0.25 ml 0.1 M cysteine + 1.0 ml buffer + 1.0 ml 4 % poly-amino acid. Temperature 35.5°C.

4. Sea urchin egg cathepsin. In the unfertilized egg of the sea urchin *Paracentrotus lividus* two proteolytic enzymes have been separated by means of gel filtration.[15] Both have been characterized with haemoglobin as substrate as being of cathepsin D type. However, the enzymes are different in many respects. They are called D 1 and D 2.

D 1 was inactive against the two poly-L-amino acids when tested at different pH's. The same was true when poly-D-glutamic acid or gelatin was used as substrate.* The influence of pH on the hydrolysis of the two poly-amino acids by cathepsin D 2 can be seen in Fig. 10. The optimum for poly-L-lysine was at pH 4.5—4.8 and for poly-L-glutamic acid at pH 4.4—4.5. The presence

* A comparison of initial activities of cathepsin D 2 with different substrates gave the following values: 1 μg Tyrosine liberated per milliliter and minute from bovine haemoglobin (Anson's method) corresponds to 1.6 H.U. with gelatin, 4.7 H.U. with poly-L-lysine, and 10.3 H.U. with poly-L-glutamic acid.

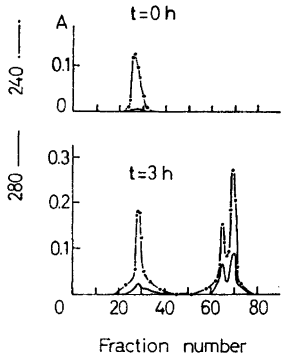

Fig. 11. Fractionation by gel filtration of the products from poly-L-lysine (Mw = 90 000) hydrolyzed by cathepsin D 2. The reaction mixture was the same as that used in Fig. 10 and was incubated at 35.5°C and pH 4.9. Samples of 1.0 ml were applied to a Sephadex G-75 superfine column (1.1 × 166 cm) and eluted in 0.05 M Tris—HCl buffer pH 7.5 + 0.5 M NaCl at 6°C. Flow rate 4.2 ml/h. Fractions 2.5 ml.

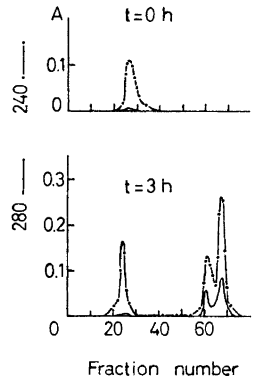

Fig. 12. Fractionation by gel filtration of the products from poly-L-lysine (Mw = 225 000) hydrolyzed by cathepsin D 2. The incubation and fractionation conditions were similar to those in Figs. 10 and 11.

of cysteine was necessary for full activity. Only 8 % of poly-L-lysine was split in the absence of cysteine.

Cathepsin D 2 was unable to hydrolyse poly-D-glutamic acid. It was tested against poly-L-glutamic acid in the presence of poly-D-glutamic acid by incubating 0.25 ml D 2 + 0.1 ml 0.25 M cysteine + 0.25 ml 4 % poly-D-glutamic acid + 1.0 ml citrate buffer + 1.0 ml 4 % poly-L-glutamic acid at 35.5°C and pH 4.45. The activity was 40 H.U./ml. When tested with water added instead of poly-D-glutamic acid the activity was 160 H.U./ml. Thus, an inhibition of 75 % was obtained.

A series of gel filtration analyses were made with cathepsin D 2 under varying conditions. The D 2 solution used for the hydrolysis had an activity of 120 H.U./ml on poly-L-lysine. The hydrolysis of this substrate with a mol. weight of 90 000 is shown in Fig. 11. A distinct double peak appeared after 3 h incubation at 35.5°C and pH 4.9.

The experiment was repeated under identical conditions, with the exception that the poly-L-lysine used had the mol. weight 225 000 . The same double peak also was obtained with this substrate as shown in Fig. 12. However, the enzyme activity was quantitatively different when these two substrates were used at the same concentrations. The undiluted D 2 solution had an activity of 340 H.U./ml against the 90 000 poly-L-lysine whereas an activity of 640 H.U./ml was obtained against the 225 000 substrate. When the latter substrate, possessing a relative viscosity higher than that of the 90 000 poly-L-lysine,

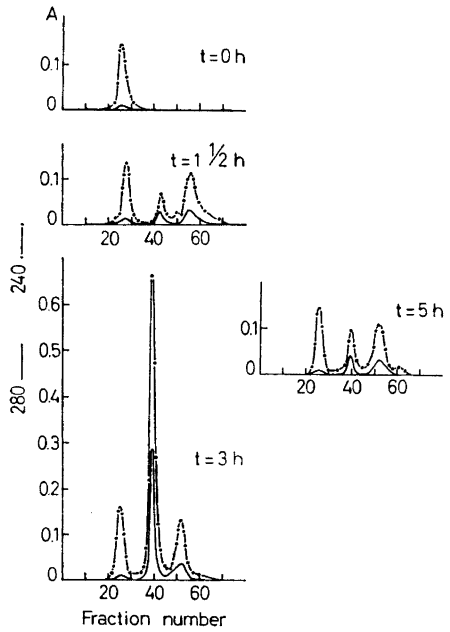

Fig. 13. Fractionation by gel filtration of the products from poly-L-lysine (Mw = 90 000) hydrolyzed by cathepsin D 2. A Sephadex G-25 fine column (1.1 × 163 cm) was used and the flow rate was 8 ml/h at 6°C. For other data, see Figs. 10 and 11.

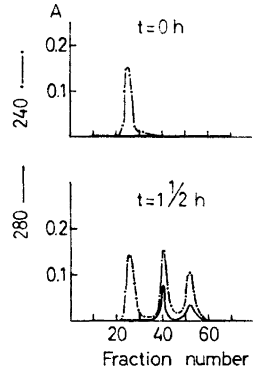

Fig. 14. Fractionation by gel filtration of the products from poly-L-glutamic acid hydrolyzed by cathepsin D 2. The Sephadex column was similar to that used in Fig. 11 and the incubation conditions similar to those in Figs. 10 and 11.

was diluted to the same relative viscosity, the same enzyme activity was obtained. Under these conditions, however, the reaction rate was 3 times higher against the 225 000 poly-L-lysine.

The hydrolysis products of poly-L-lysine of mol. weight 90 000 was fractionated with G-25 fine instead of G-75 superfine Sephadex. The results can be seen in Fig. 13. The double peak was obtained after 1 1/2 h incubation, reached a maximum after 3 h and then decreased by 5 h. The left peak of the two resulting from the hydrolysis had moved towards the middle part of the fraction series compared with the chromatograms shown in Figs. 11 and 12. The relative position of peaks is dependent on the Sephadex gel used. The following rough estimate of the mol. weights in this chromatogram suggests that the mol. weight of the first peak from left to right was greater than 5000, that of the second around 3400, and that of the third around 1700.

The results of fractionating the products from poly-L-glutamic acid hydrolyzed by cathepsin D 2 through G-25 fine is shown in Fig. 14. The distinct double peaks formed by the split products manifest the same order of distribution as the poly-L-lysine degradation products.

Table 2. Comparison of the hydrolysis of poly-L-glutamic acid and poly-L-lysine by some proteolytic enzymes. The activity is determined by means of the viscosimetrical method.

Enzyme	I poly-L-glutamic acid		II poly-L-lysine		Activity of I / Activity of II
	pH	activity HU/ml	pH	activity HU/ml	
Ficin	6.1	3072	8.9	166	19
Chymotrypsin	7.5	0	7.4	81	—
Pepsin	4.5	10	2.0—5.4	0	—
Pronase	5.0	1540	6.5	1707	0.90
Trypsin	7.5	0	7.5	269	—
Aspergillus fumigatus	5.1	932	5.0	24	39
Cathepsin D 2	4.45	1830	4.7	840	2.2
Oocyte cathepsin	4.45	4184	4.8	3405	1.2
Staphylococcus aureus	4.85	420	3.2—6.1	0	—
—»—	8.5	0	7.1—9.5	0	—
Human thyroid proteinase	4.3—7.7	0	2.4—8.7	0	—

The quotients of the activities of the enzymes studied against the two substrates at the pH optimum for each substrate are listed in Table 2. This ratio is suggested as a criterion for classification of such proteolytic enzymes as endopeptidases.

DISCUSSION

A comparison of the different split products found by gel filtration of poly-L-glutamic acid hydrolyzed by crude ficin and partially purified ficin (Figs. 3 and 4, respectively) show that no products of medium mol. weights, *i.e.* in the interval 10 000—20 000, are formed by the latter enzyme preparation. This difference must be a result of the purification, suggesting that the amount of endopeptidase had decreased. As a consequence of the reduced or inactivated endopeptidase the degradation of the high molecular substrate proceeded slower. In fact, it can be seen in Fig. 4 that even after 5 h incubation most of the substrate (the first peak) was undegraded whereas in Fig. 3 it had disappeared after 1 h. Purified ficin was different from crystalline trypsin which also acted as an endopeptidase as seen in Fig. 1. *Aspergillus* proteinase acted as an endopeptidase with a distinct decrease of the substrate peak and visible increase of split products.

Quite another type of UV pattern was obtained by the hydrolysis of poly-L-lysine and poly-L-glutamic acid by the sea urchin egg cathepsin D 2. A double peak of split products was obtained with mol. weights of approximately 1700 and 3400. There was another striking difference also. The UV patterns of the hydrolysis of the two poly-amino acids with the other enzymes studied in this work showed that the split products were obtained in concentrations corresponding to the quantity of substrates hydrolyzed. Since the same sub-

strates and identical or corresponding Sephadex gels were used for the non catheptic enzymes, it does not seem likely that most of the split products were absorbed by the gel. It seems more reasonable to assume that after hydrolysis by these enzymes a certain amount of split products are bound to the undegraded substrate. On the other hand, the visible results of the D 2 hydrolysis suggests that the amount of split products was greater than that of the substrate hydrolyzed.

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