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Proteolysis of Some Synthetic Poly-α-amino Acids

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The hydrolysis of poly-L-glutamic acid (PLG) and poly-L-lysine (PLL) by a number of commercial proteolytic enzymes was followed by thin layer chromatography. On the basis of these chromatograms, pH activity curves were made by a viscosimetric method. The chromatograms as well as the pH curves showed significant variations between the different enzymes although their pH optima were around 5 for PLG and between 6.7 and 10.1 for PLL. The degradation products were analyzed by thin layer chromatography at pH 5, 6.5, and 8 for PLG and at pH 5, 7, and 8.5 for PLL. The chromatograms from the actual enzymes were different. Among the enzymes tested, thermolysin and proteinase Novo did not hydrolyse poly-L-glutamic acid.

Poly-L-ornithine was split by pronase and subtilisin with pH optima at 10.6 and 10.2, respectively.

Streptomyces protease fixed to agarose or to carboxymethyl cellulose showed weak hydrolysis of poly-L-glutamic acid around pH 5 and of poly-L-lysine at pH 8.3 – 8.7. Insolubilized agarose trypsin hydrolysed poly-L-lysine more rapidly, but more than 50 % of the hydrolysis was due to autolysis of the enzyme conjugate.

Carboxymethyl cellulose trypsin was stabile and hydrolysed poly-L-lysine at pH 7.75 – 8.75.

Some bacterial enzymes and pancreas extracts tested for poly-D-glutamic acid hydrolysis were found to be inactive.

Very weak hydrolysis of poly-D-lysine around pH 8 was found in extracts of pancreas protease Merck and pancreatin Sigma, Grade III.

Some years ago one of the present writers (G.L.) studied the hydrolysis of poly-α-L-lysine and poly-α-L-glutamic acid.[1] The enzymes trypsin, chymotrypsin, ficin, pronase, and pepsin were investigated as well as laboratory extracts from thyroid glands, sea urchin eggs, the fungus *Aspergillus fumigates* and the culture medium of *Staphylococcus aureus*. The hydrolysis at different pH was studied by a viscosimetric method, and the breakdown was followed by means of gel chromatography.

In the present work the investigation of the influence of pH on the hydrolysis of some poly-α-L-amino acids by commercial enzymes was extended and the split products were followed by means of thin layer chromatography.

The hydrolysis of poly-L-glutamic acid and poly-L-lysine by insolubilized trypsin, *i.e.* trypsin bound to agarose or carboxymethyl cellulose and by *S. griseus* protease bound to agarose, was also studied.

In 1956 Tsuyuki, Tsuyuki and Stahmann [2,3] reported the breakdown of poly-α-D-lysine by strong extracts of pancreas powder. These results have not been repeated — as far as the present authors know — and therefore the susceptibility to hydrolysis of poly-D-lysine and poly-D-glutamic acid by different proteolytic enzyme preparations was investigated in the present paper. A number of proteinases such as subtilisin, pronase (*S. griseus* protease) and Nagarse (subtilisin BPN'), extracts of fresh bovine and swine pancreas and of different commercial pancreas powders were tested as well as *Helix pomatia* digestive juice, *Myxococcus virescens* culture medium, and amnion from hen's egg.

MATERIAL AND METHODS

Substrates. Poly-L-lysine hydrobromide (mol. wt. 200 000), poly-D-lysine (mol. wt. 60 000), poly-L-glutamic acid sodium salt (mol. wt. 148 000), poly-D-glutamic acid sodium salt (mol. wt. 94 000), poly-L-arginine (mol. wt. 60 000), and poly-L-ornithine hydrobromide (mol. wt. 70 000) were purchased from Pilot Chemicals Inc., Watertown, Mass. USA. Poly-L-lysine hydrobromide (mol. wt. 104 000, lot

Ly 172) was from Miles Lab. Ltd., Slough, England.

Commercial enzymes. Acid protease (*Rhizopus chinensis* Batch 7015, Miles Lab. Ltd., England); Alcalase (Subtilisin Carlsberg, cryst. 27.5 A.U./g, batch No. 1 x 16, Novo Industri A/S, Copenhagen); *Aspergillus* protease I (*A. oryzae* Astra, Sweden); *Aspergillus* protease II (*A. oryzae* type II, Crude, 0.15 μmol tyrosine/(min mg) pH 7.5 and 37 °C from casein, Sigma Chem. Co., St. Louis); *Bacillus subtilis* protease (*B. subtilis* type VIII, Sigma); Bacterial proteinase Novo (cryst. 25.9 A.U./g, batch No. 58, Novo Ind.); Bromelain (Mann Research Lab., Inc. USA); Crystalline subtilisin (27.5 A.U./g, batch No. 1 x 16, Novo Ind.); Nagarse (Subtilisin BPN′, lyophilized, cryst. bacterial proteinase, potency 150 000 P. U. N. in vial, batch DAF 0406, Teikoku Chem. Ind., Osaka, Japan); Pronase B grade (*Streptomyces griseus* 45 000 PUK/g, lot 53997, Calbiochem, La Jolla, Calif.); *Streptomyces* protease (*S. griseus*, type V, purified, lot 17B-1740, 1.7 μmol tyrosine/(min mg), Sigma); Thermolysin (*B. thermoproteolyticus* Rokko proteinase 50 PU/mg, lot TA8C91, Daiva Kasei, K.K., Japan). Enzite-agarose-trypsin (3 x cryst. bovine trypsin bound to agarose), Enzite-trypsin (trypsin bound to carboxymethyl cellulose), and Enzite-agarose-protease (*S. griseus* protease bound to agarose) were purchased from Miles-Seravac, Miles Lab. Ltd., England.

Snail digestive juice, batch No. 61310 was from Koch-Light Lab., England.

Crude enzyme preparations

1. *Pancreatin extract.* Pancreatin powder from Weddel Pharmaceuticals Ltd., The British Drug House Ltd., Schwarz-Mann, Merck, Sigma and Koch-Light Laboratories Ltd., was used. A suspension of 5 g in 100 ml dist. water was stirred for 3 h at + 4 °C and then centrifuged at 5000 *g* for 20 min in the cold.

2. *Extract of fresh pancreas.* Fresh pancreas from ox or swine was freed from fat and connective tissue, cut in pieces, ground in a mincing-machine and disintegrated in a "VirTis" blender in 0.9 % NaCl for 90 s. After centrifugation at 10 000 *g* for 30 min, a second centrifugation of the supernatant was made at 20 000 *g* for 30 min. All operations were carried out in the cold. The supernatant was used for proteolytic test.

3. *Amnion* fluid from hen's egg was tested immediately for proteolytic activity.

4. Culture medium "enzyme 1 and 3" from *Myxococcus virescens* was obtained from Dr. G. Haskå, University of Lund, Sweden.

Assay of proteolytic activity

1. *The viscosimetric method* elaborated by Hultin [4-7] was used. The determinations were made in Ostwald viscosimeters at 35.5 °C, and the enzyme activity was calculated according to Hultin's formula [1] and expressed in Hultin Units (HU).

2. *Potentiometric determination.* A continuous potentiometric (pH-stat) titration [8] of the carboxyl groups, liberated by the action of the enzyme at various constant pH values, was made for the determination of the enzyme activity.[9,10] A pH meter model 26 with Titrator 11 and Autoburette ABU12 from Radiometer, Copenhagen, was used for the assay which was carried out in a thermostated vessel under constant magnetic stirring in a nitrogen atmosphere.

3. *Thin layer chromatography.* The hydrolysis of poly-amino-acids was followed by chromatography on DC-Fertigplatten Kieselgel F254 (Merck). Methanol-chloroform-ammoniak 2:2:1 was used as solvent. Each sample was divided into two parts and frozen. One part was incubated at 35.5 °C for 48 or 72 h, and 20 μl was then applied to the plate and the front left to move for 3 h about 15 cm. After drying in air the spots were developed by 0.1 % ninhydrin solution in butanol. The other part used as a control was directly applied to the plate after thawing.

RESULTS

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

1. *Investigation of poly-L-glutamic acid.* The hydrolysis of poly-L-lysine (PLL) and poly-L-glutamic acid (PLG) by some commercial proteolytic enzyms was followed at different pH values by means of thin layer chromatography (TLC). The results of the TLC on the split products of poly-L-glutamic acid are shown in Figs. 1 – 3, and it can be seen that all enzymes, except thermolysin and proteinase Novo, degraded poly-L-glutamic acid.

As the L-glutamic acid oligopeptides were not commercially available the PLG chromatograms were compared with those of other reports.[10,11] As can be seen in Fig. 1, three of the four enzymes tested gave rise to an extensive degradation of the poly-L-amino acid. At pH 5 and 6.5 mono-, di-, tri- and tetra-glutamic acids were formed, at pH 8.0 only pronase formed split products such as tri-, tetra- and pentaglutamic acids. The controls for subtilisin and bromelain (Fig. 1 b and d) show an advanced degradation of the substrate at pH 5, indicating a rapid enzymatic activity to have

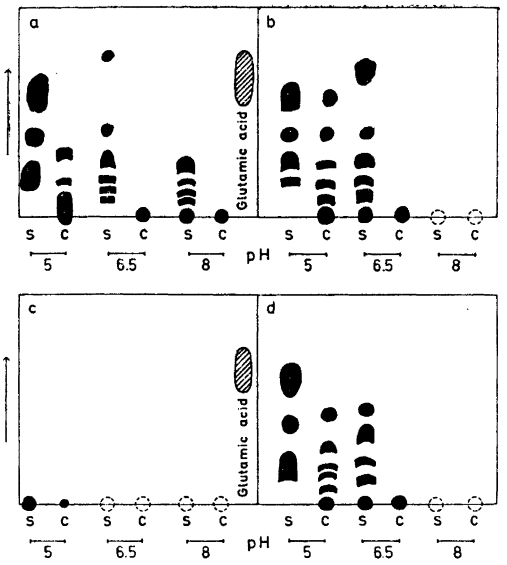

Fig. 1 a–d. Thin layer chromatograms from the action of some proteolytic enzymes on poly-L-glutamic acid (PLG). The reaction mixture contained 0.5 ml enzyme (1 mg/ml), 0.5 ml McIlvaine buffer and 1.0 ml 1 % PLG. Incubation at 35.5 °C: 48 h for a and b; 72 h for c and d. For other data see Methods. a, Pronase B grade; b, cryst. subtilisin (Novo); c, thermolysin, and d, bromelain. S = sample and C = control.

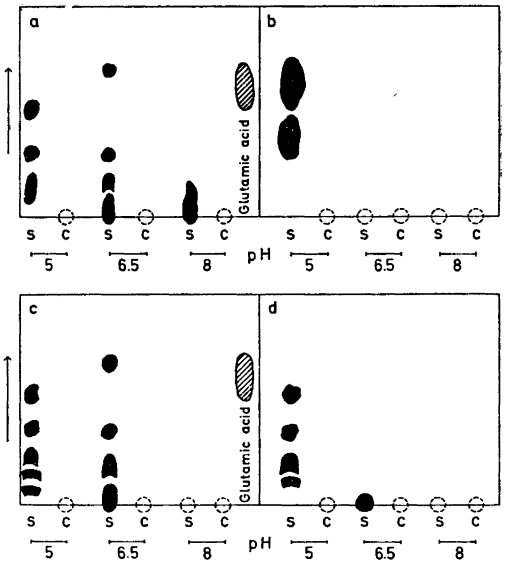

Fig. 2 a–d. Thin layer chromatograms from the action of some proteolytic enzymes on PLG. Incubation 48 h at 35.5 °C. For other data see Fig. 1 and Methods. a, *Streptomyces* protease (Sigma); b, *Aspergillus* protease II (Sigma); c, *B. subtilis* protease (Sigma), and d, *Aspergillus* protease I (Astra).

taken place between the time of mixing and freezing of the control samples. Figs. 2 a, c, and d show the breakdown of PLG to different oligoglutamic acids by protease from *Streptomyces*, *B. subtilis* and *Aspergillus* (protease I, Astra), respectively. *Aspergillus* protease II (Sigma) was more active and degraded the substrate to glutamic and diglutamic acid, as can be seen in Fig. 2 b. The enzyme alcalase (Fig. 3 b) digested PLG so quickly that also the control gave an advanced degradation, whereas Nagarse (Fig. 3 a) and acid protease (Fig. 3 d) gave normal controls. The bacterial proteinase (Novo) gave ninhydrin stainable spots at pH 5 and 6.5 which means that only relatively few peptide bonds were split.

Based on these results the influence of the active enzymes on this substrate was studied viscosimetrically as shown in Figs. 4 and 5. It can be seen that the pH optimum for the proteolytic degradation of poly-L-glutamic acid lies around pH 5 for all the enzymes and for

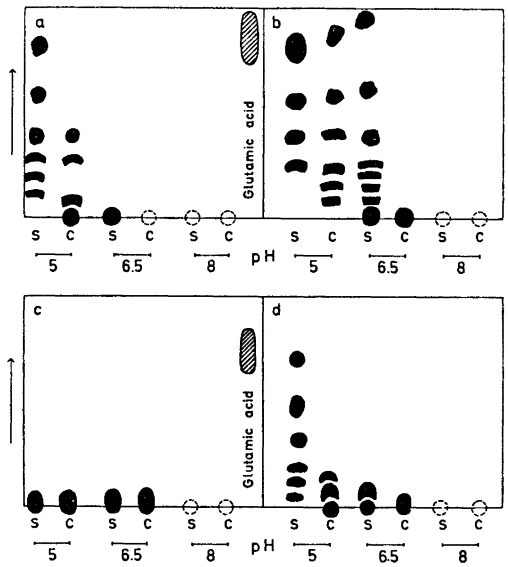

Fig. 3 a–d. Thin layer chromatograms from the action of some proteolytic enzymes on PLG. Incubation 48 h at 35.5 °C. For other data see Fig. 1 and Methods. a, Nagarse (subtilisin BPN′); b, Alcalase; c, bacterial proteinase (Novo), and d, acid protease (Miles).

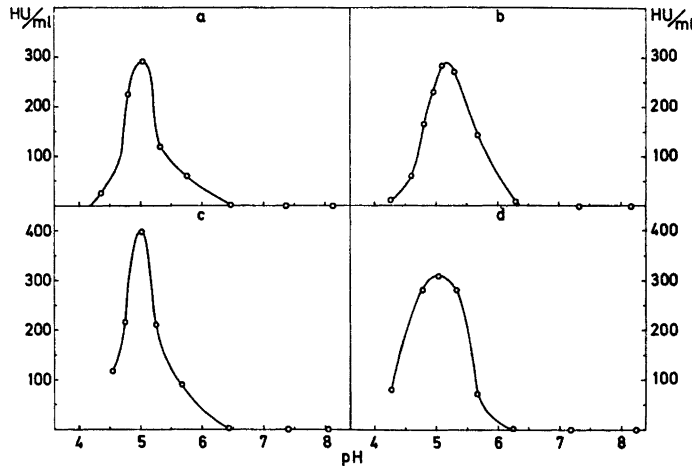

Fig. 4 a–d. Influence of pH on the hydrolysis of poly-L-glutamic acid by: a, Alcalase; b, *Streptomyces* protease (Sigma); c, *B. subtilis* protease (Sigma) and d, *Aspergillus* protease II (Sigma). Incubation mixture: 0.5 ml enzyme (1; 100; 5 and 100 μg/ml, resp.) + 1.0 ml McIlvaine buffer + 1.0 ml 1.5 % substrate. The proteolytic activity was followed viscosimetrically at 35.5 °C and expressed in HU/ml.

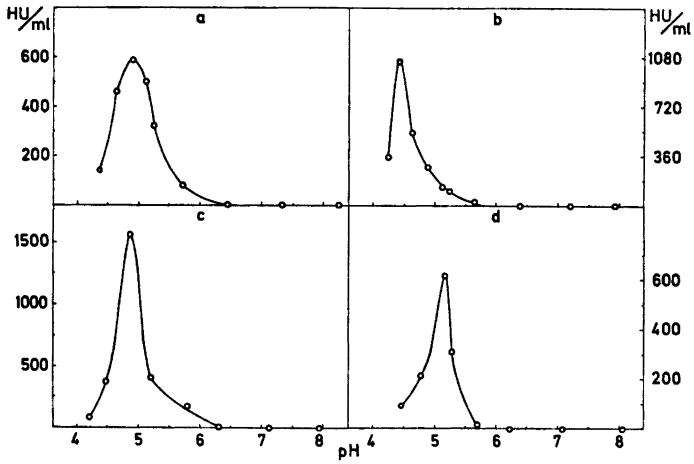

Fig. 5 a–d. Influence of pH on the hydrolysis of poly-L-glutamic acid by: a, *Aspergillus* protease I (Astra); b, acid protease (Miles); c, bromelain and d, Nagarse. Incubation mixture: 0.5 ml enzyme (10; 100; 100 and 10 μg/ml, resp.) + 1.0 ml McIlvaine buffer + 1.0 ml substrate. Enzymatic assay as in Fig. 4.

crystalline subtilisin Novo, not shown in the figures. For acid protease shown in Fig. 5 b, the pH optimum lies at 4,4. The form of the pH activity curves and the extent of the active interval varies for the different enzymes, however.

2. *Investigation of poly-L-lysine and poly-L-ornithine.* The thin layer chromatograms of proteolytically hydrolyzed poly-L-lysine (PLL) are shown in Figs. 6 and 7. Identification of the split products by control samples was made but is not shown here. The results agree with those of other authors.[10,12,13] *Aspergillus* protease II degraded PLL completely at the three pH values investigated: 5, 7 and 8.5 (Fig. 6 a), whereas *Aspergillus* protease I (Fig. 6 e) had

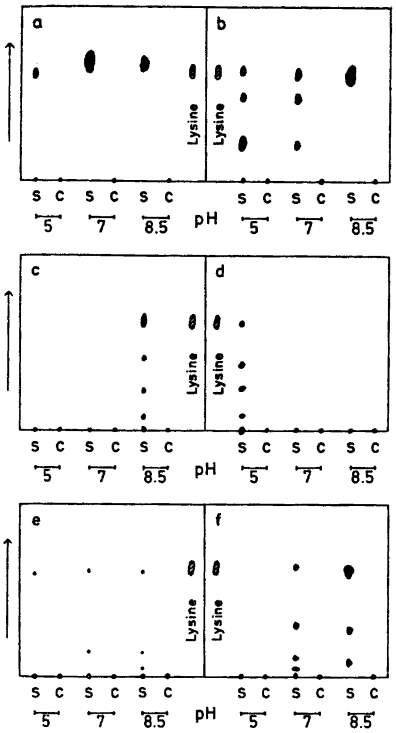

Fig. 6 a–f. Thin layer chromatograms from the action of some proteolytic enzymes on poly-L-lysine (PLL). The reaction mixture contained 0.5 ml enzyme (1 mg/ml), 0.5 ml McIlvaine buffer and 1.0 ml PLL. Incubation time 48 h at 35.5 °C. For other data see Methods. a, *Aspergillus* protease II (Sigma); b, *Streptomyces* protease (Sigma); c, *B. subtilis* protease (Sigma); d, acid protease (Miles); e, *Aspergillus* protease I (Astra); f, Nagarse. S = sample and C = control.

a less strong effect. *Streptomyces* protease split PLL to lysine at pH 8.5 and partly to di- and tetralysine at pH 5 and 7 (Fig. 6 b). *B. subtilis* protease hydrolysed the substrate at pH 8.5 (Fig. 6 c) and acid protease at pH 5 (Fig. 6 d), both enzymes giving the same split products. Nagarse exerted degradation at pH 8.5 and less at 7, the hydrolysis resulting in lysine, trilysine, and oligolysines but no dilysine. Alcalase degraded PLL to lysine and tetra- and pentalysine at pH 8 (Fig. 7 a), and bromelain split the substrate to lysine, trilysine and pentalysine (Fig. 7 d). Bacterial proteinase (Fig. 7 b) and crystalline subtilisin (Fig. 7 c)

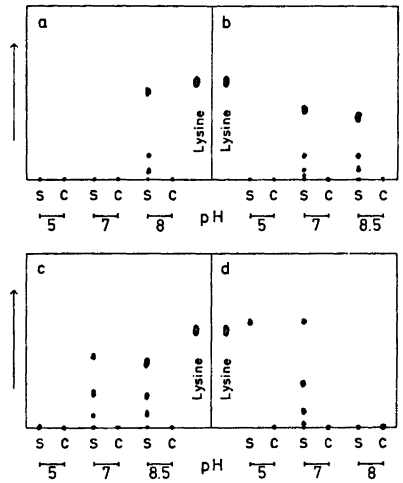

Fig. 7 a–d. Thin layer chromatograms from the action of some proteolytic enzymes on PLL. a, Alcalase; b, Bacterial proteinase (Novo); c, cryst. subtilisin (Novo); d, bromelain. For data see Fig. 6 and Methods.

hydrolysed PLL to di-, tri- and tetralysine at pH 7 and 8.5.

The pH optima for the hydrolysis of PLL by the investigated enzymes varied between pH 6.7 and 10.1 as shown in Figs. 8–9. *Aspergillus* protease II gave an optimum around 9.2 (Fig. 8 a), whereas the other three enzymes shown in Fig. 8 b–d had optima around pH 9.7. *Streptomyces* protease had its optimum around pH 9.5 (Fig. 9 a), bromelain around 6.7 and *B. subtilis* protease and alcalase around pH 10 (Fig. 9 c–d).

Poly-L-ornithine which is not degraded by trypsin [14] can be split by pronase and subtilisin as is seen in Fig. 10 a–b. The pH optima are around 10.6 and 10.15, respectively.

II. Effect of insolubilized trypsin and *S. griseus* protease on the hydrolysis of poly-L-glutamic acid and poly-L-lysine

Suspensions of insolubilized enzyme conjugate were mixed with the actual poly-amino acids and buffer, and the consumption of NaOH was assayed by pH-stat titration during magnetic stirring at 30 °C in a nitrogen-atmosphere. *Streptomyces* protease attached to

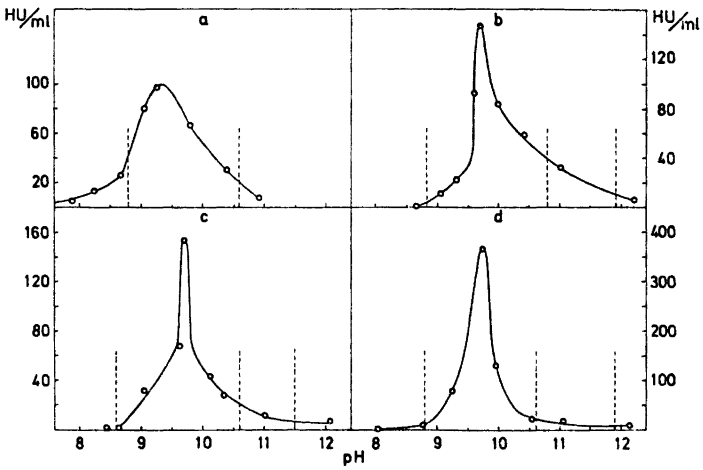

Fig. 8 a – d. Influence of pH on the hydrolysis of poly-L-lysine by: a, *Aspergillus* protease II (Sigma); b, cryst. subtilisin (Novo); c, bacterial proteinase (Novo); d, Nagarse. Incubation mixture: 0.5 ml enzyme (50; 0.13; 0.05 and 12.5 μg/ml, resp.) + 1.0 ml buffer (below pH 5.6 acetate; pH 5.6 – 7.8 phosphate; pH 7.6 – 8.8 Tris-HCl; pH 8.8 – 10.6 glycine-NaOH; pH 10.6 – 11.9 phosphate-NaOH, and higher than pH 11.9 NaOH) + 1.0 ml 0.5 % poly-L-lysine. Enzymatic assay as in Fig. 4. The dotted lines indicate the intervals of the buffers.

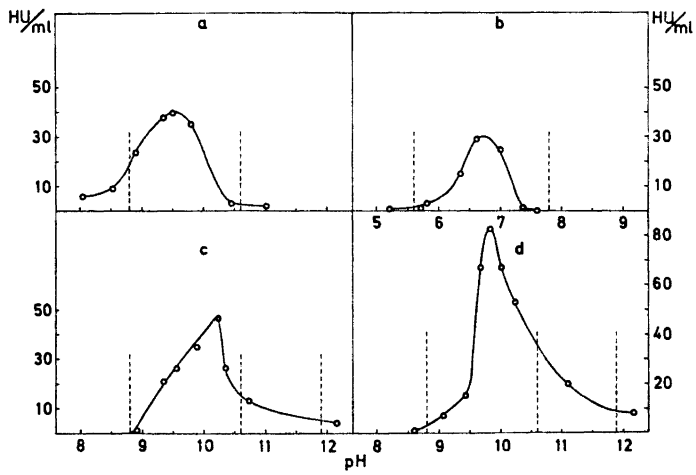

Fig. 9 a – d. Influence of pH on the hydrolysis of poly-L-lysine by: a, *Streptomyces* protease (Sigma); b, bromelain; c, *B. subtilis* protease (Sigma); d, Alcalase. Incubation mixture: 0.5 ml enzyme (20; 500; 0.25 and 0.25 μg/ml, resp.) + 1.0 ml buffer + 1.0 ml 0.5 % poly-L-lysine. Enzymatic assay as in Fig. 4. The dotted lines indicate the intervals of buffers described in Fig. 8.

agarose exerted very low hydrolysis of poly-L-glutamic acid at pH 4.9 and 5.2. The autolysis of the enzyme conjugates could be left out of consideration in the actual pH interval. Poly-L-lysine was weakly hydrolyzed at pH 8.3 – 8.7 by the insolubilized enzyme. At this pH interval, however, an equally strong autolysis of the enzyme conjugate occurred.

The Enzite-agarose-trypsin conjugate hydrolyzed poly-L-lysine to a higher degree than the insolubilized *Streptomyces* protease. The optimal splitting of this substrate was

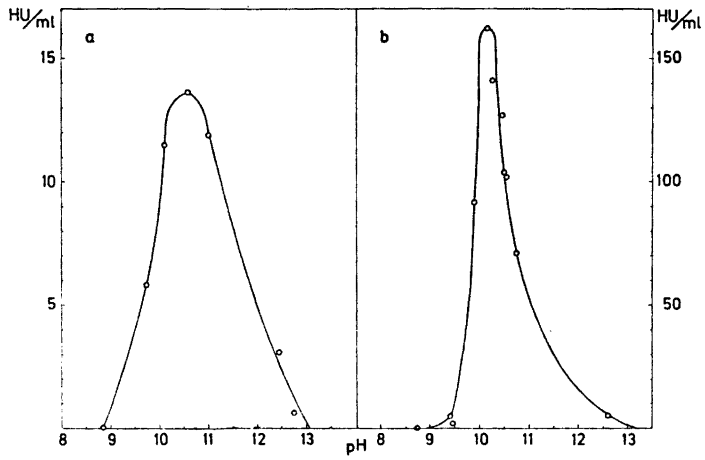

Fig. 10 a–b. Influence of pH on the hydrolysis of poly-L-ornithine by: a, Pronase; b, cryst. subtilisin (Novo). Incubation mixture: 0.25 ml enzyme (50, resp. 10 μg/ml) + 1.00 ml buffer (0.1 – 1 M glycine-NaOH, Sørensen) + 0.25 ml 5.0 % poly-L-ornithine. The proteolytic activity was followed viscosimetrically at 30.0 °C and expressed in HU/ml.

around pH 9 as shown in Fig. 11. However, the autolysis of the Enzite-agarose-trypsin in a system free from substrate was between 50 and 65 % of the degradation of the complete enzyme-conjugate-substrate-buffer system.

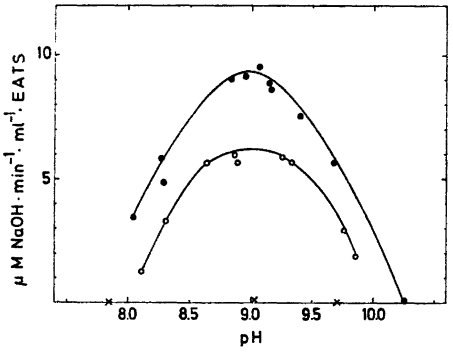

Fig. 11. Influence of pH on the hydrolysis of poly-L-lysine by Enzite-agarose-trypsin suspension (EATS). Incubation mixture: 0.50 ml EATS (diluted 1:6) + 3.00 ml 0.01 M glycine-NaOH buffer (Sørensen) + 1.00 ml poly-L-lysine (2 % in 0.45 % NaCl, pH 7.85) + 1.00 ml 0.5 mM $CaCl_2$. pH-stat titration under constant stirring at 30 °C in N_2-atmosphere. Alkali concentration 0.0445 M. For control of autolysis of enzyme conjugate and spontaneous hydrolysis of the substrate the excluded component was replaced by 0.45 % NaCl. For other data see Methods. ●, hydrolysis of poly-L-lysine by EATS; ○, autolysis of EATS; ×, autolysis of poly-L-lysine.

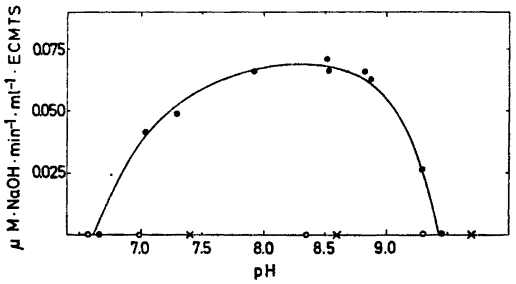

Fig. 12. Influence of pH on the hydrolysis of poly-L-lysine by Enzite carboxymethyl cellulose trypsin (ECMTS). Incubation mixture: 0.50 ml ECMTS (10.4 mg/ml) + 3.00 ml buffer (0.01 M McIlvaine for pH 6.67 – 7.29; 0.01 M collidine-HCl for pH 7.00 and 0.01 M glycine-NaOH for pH 7.90 – 9.80) + 1.00 ml poly-L-lysine (2 % in 0.45 % NaCl, pH 7.77) + 1.00 ml 0.5 mM $CaCl_2$. pH-stat titration under constant stirring at 30 °C in N_2-atmosphere. For control of autolysis of enzyme conjugate and spontaneous hydrolysis of the substrate the excluded component was replaced by 0.45 % NaCl. For other data see Methods. ●, hydrolysis of poly-L-lysine by ECMTS; ○, autolysis of ECMTS; ×, autolysis of poly-L-lysine.

The Enzite carboxymethyl cellulose trypsin was stable in the pH interval 6.7 – 9.8 as can be seen in Fig. 12. The optimal hydrolysis of poly-L-lysine was at a broader pH interval, 7.75 – 8.75, than the agarose-trypsin shown in Fig. 11.

Table 1. Enzyme preparations tested for hydrolysis on poly-D-glutamic acid. Reaction mixture: 1.00 ml enzyme + 0.50 ml 2.0 % poly-D-glutamic acid + 1.50 ml 10 mM McIlvaine buffer. pH-stat titration in N_2-atmosphere with 5 mM NaOH at 23 °C under constant stirring.

Enzyme preparation	Manufacturer	Concentration mg/ml	Concentration ml/ml	Poly-D-glutamic acid Manufacturer	Poly-D-glutamic acid Mol.wt.	Endpoint pH
Pancreas, swine	Fresh extract	14.8		Pilot	94.000	5.52
Pancreatin	Schwarz/Mann	6.6		Pilot	94.000	5.83
Pancreatin	Sigma, Grade III	7.0		Miles	29.000	5.47
Nagarse (subtilisin, BPN)	Teikoku, Japan	1.67		Pilot	94.000	5.45
Nagarse	Teikoku, Japan	3.34		Pilot	94.000	5.49
Snail digestive juice	Koch-Light		0.17	Pilot	94.000	5.47
Pronase	Calbiochem, B-grade	0.8		Pilot	94.000	5.30
Myxococcus vir. cultivation medium	University, Lund		0.08	Sigma	60.000	5.00

Table 2. Enzyme preparations tested for hydrolysis of poly-L-lysine (PLL) and poly-D-lysine (PDL) by pH-stat titration. Reaction mixture: 1.00 ml enzyme + 0.50 ml 2 % substrate + 1.50 ml 10 mM glycine-NaOH buffer.

Enzyme preparation	Enzyme mg/ml	Substrate		Endpoint pH	Consumption of NaOH nmol/(mg/min)
Extract from calf pancreas	0.13	PLL		8.43	156
	0.26	PLL		8.38	250
	0.13	–		8.41	11
	3.3		PDL	8.40	6.1
	3.3		–	8.30	14.1
Nagarse (subtilisin, BPN)	3.3	PLL		8.26	26.8
„	3.3		PDL	8.33	0
Pancreatin Sigma	0.14	PLL		8.60	432
	6.7		PDL	8.22	16.7
	6.7		PDL	8.22	18.3
	6.7		PDL	8.32	23.3
	6.7		–	8.29	13.3
	6.7		–	8.17	13.5
Pronase, Calbiochem, B-grade	0.17	PLL		8.11	31
	1.67		PDL	8.10	0 (pH-
	0.8		PDL	8.60	0 in-
	0.8		PDL	7.60	0 crease)

III. Effect of some proteolytic enzymes and extracts on the hydrolysis of poly-α-D-amino acids

The enzymatic hydrolysis of poly-D-glutamic acid and poly-D-lysine was investigated by pH-stat titration (regulated 'endpoint') or by incubation for 24 h at buffered pH and assay of the decrease in pH after incubation. Before the enzyme preparations were tested on their ability to hydrolyse poly-D-amino acids, the poly-L-amino acid degradation was always controlled.

In Table 1 there are data for some bacterial enzymes and digestive extracts tested on poly-D-glutamic acid under pH regulated conditions. None of these enzyme samples hydrolysed the

D-form. That means that the hydrolysis estimated as NaOH consumption of a complete enzyme substrate sample was not greater than the one without enzyme under in other respects identical conditions. The L-form was enzymatically split at the corresponding pH.

The hydrolysis of poly-D-lysine assayed by pH-stat titration resulted in a significant difference for pancreatin Sigma Grade III as shown in Table 2. For the other preparations tested no hydrolysis of poly-D-lysine occurred. Neither was this the case by viscosimetric assay on pancreatin extract (Weddel, 10 mg/ml reaction mixture) which strongly hydrolysed poly-L-lysine with a maximum around pH 7.

Studies of prolonged hydrolysis, *i.e.* 24 h incubation of pancreatic enzyme and poly-D-lysine, were also performed. After incubation the samples were titrated back to the starting pH under controlled conditions. Pancreatin Sigma did not hydrolyse poly-D-lysine, *i.e.* the decrease in pH was about the same also in the absence of substrate, which means that the autolysis of the pancreatin was high. Pancreas protease Merck seemed to split poly-D-lysine, the difference in NaOH consumption being 4 – 5 μM.

DISCUSSION

Hydrolysis of poly-L-amino acids. The action of some bacterial and fungal enzymes and of bromelain on poly-L-lysine and poly-L-glutamic acid has been studied. The influence of pronase and subtilisin on the hydrolysis of poly-L-ornithine was also investigated. Thermolysin did not split poly-L-lysine or poly-L-glutamic acid, and bacterial proteinase Novo had no effect on poly-L-glutamic acid. For the other proteinases tested the pH optimum for splitting of poly-L-glutamic acid was around pH 5. Miller [15] has shown that, at this pH, the substrate undergoes a sharp helix-random coil transition at which approximately half of the sidechain carboxyl groups are ionized. At pepsin hydrolysis of poly-L-glutamic acid at least some α-helix form seems to be necessary.[16]

The thin layer chromatograms (Figs. 1 – 3) show that there are characteristic differences in the formation of split products of PLG by the different enzymes investigated. Also the affinity to the substrate, *i.e.* the concentration of enzyme necessary for an appropriate time for viscosimetric assay (20 – 30 min), varied considerably between the enzymes tested (Figs. 4 – 5).

The proteolytic hydrolysis of poly-L-lysine (PLL) by the enzymes tested also resulted in different patterns of split products (Figs. 6 – 7) and in different shapes of the pH-activity curves (Figs. 8 – 10). Also the affinity for PLL among the enzymes tested varied.

Hydrolysis of poly-L-amino acids by insolubilized trypsin and a bacterial protease. Several investigation have been published on proteolytic activity of insolubilized proteinases. Insolubilized trypsin bound to different carriers with use of appropriate techniques has been prepared and studied for several years.[17–25] Stability, influence of pH on the enzyme activity as well as different physico-chemical constants have been determined and compared with those of soluble trypsin. No reports on the pH dependent autolysis of the enzyme-conjugate have been found, however. As described above the Enzite-agarose-trypsin conjugate, when assayed as control without substrate, showed a pH dependent autolysis as did Enzite-agarose protease. The pH optima of the enzyme conjugates were the same as those of complete enzyme-poly-L-lysine systems. Carboxymethyl cellulose-trypsin showed, however, no autolysis in the actual pH intervals. It seems as if just agarose bound enzymes are instable. As the titration of enzymic activity in the present study is based on decrease in pH, *i.e.*, liberation of protons, an enzymic assay based on spectrophotometric, viscosimetric or other methods might not reveal autolysis.

Contrary to the autolysis of the insolubilized agarose-trypsin, as demonstrated in the present study, Bar-Eli and Katchalski [17,18] reported that the relatively high enzymic stability of the water-insoluble polytyrosyl trypsin may be attributed mainly to the prevention of autodigestion as a result of fixation of the enzyme to the insoluble carrier. Porath *et al.*[26] who introduced the fixation of enzymes to agarose found good stability for chymotrypsin-agarose gel, but agarose-rennin was reported to release active enzyme in solution.[27] *Arthrobacter* proteinase fixed to agarose was studied by Gable and v. Hofsten [28] who found good stability and no leakage of enzyme from the gel into the

solution under various conditions. As the pH dependent autolysis curve is determined by the consumption of hydroxyl ions, *i.e.* caused by the liberation of protons, it cannot be caused by small amounts of negatively charged groups from the gel reported by Carlsson *et al.*[29]

Hydrolysis of poly-D-amino acids. In nature no proteins or polypeptides entirely built up by D-amino acids have been found. Different D-amino acids occur, however, not only in bacteria[30] but also in several insect species and even in higher animals, *e.g.* in guinea pig blood and shark liver.[31] The existence of D-peptides has been reported by Hanson and others.[32,33]

Due to lack of D-forms of proteins and polypeptides, the possibility to find D-peptide bond splitting enzymes in nature could not be demonstrated until the synthesis of poly-D-amino acids. The reports by Tsuyuki, Tsuyuki and Stahmann [2,3] seem to be the first in this field and the only published so far.

The present work, being a continued report on proteolytic hydrolysis of some polyamino acids, gave room only for a limited study on poly-D-amino acid hydrolysis. The enzyme preparations were tested on poly-D-glutamic acid and poly-D-lysine near the pH optimum for proteolytic splitting of the corresponding L-forms. No indications for enzymatic splitting of poly-D-glutamic acid were found (Table 1). The results seem, however, to indicate the existence of poly-D-lysine splitting enzyme(s). Pancreatin (Sigma Grade III) showed a weak hydrolysis of poly-D-lysine assayed by pH-stat titration (Table 2).

Evidences in the present paper for the existence of proteolytic enzyme(s) that split poly-D-amino acid are not strong. However, Gill, Papermaster and Mowbray [34] demonstrated that poly-D-Glu 55, D-Lys 39, D-Tyr 6 is degraded by rabbits very slowly, *i.e.* to about 25 to 30 % of the injected D-polymer over 15 to 20 days. The amount of degraded D-polymer was a constant fraction of the injected dose. The authors propose that constitutive D-proteases and D-peptides in the rabbit are involved in the metabolism and are not induced by the injection of polypeptide. The L-polymer, on the other hand, is completely degraded within 3 to 4 days. Other experiments in this field [35] showed that a D-polymer [247, p(D-Tyr, D-Glu, D-Ala)] breaks down twenty-two times more slowly than the L-polymer when injected in mice. The complicated nature of the metabolic mechanism is apparent from the fact that the D-polymer is broken down slowly and retained in large quantities in the tissues, especially in the kidneys.[36,37] This persistence of the D-polymer indicates that the enzymes necessary for its degradation are lacking in the kidneys. The L-polymer is not retained in the liver or kidneys and is thus rapidly broken down.

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