Enzymatic Hydrolysis of Poly-DL-Lysine

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(Z. Naturforsch. 28 c, 116–119 [1973]; received November 20, 1972/January 4, 1973)

Racemic polypeptide, pronase, proteolytic enzyme, enantiomeric block copolymer, isotactic polymer

The condensation of racemic lysine to poly-DL-lysine produces a great number of distinguishable molecules. Following merely statistical reasoning the number of "pure peptides" — consisting of only one sort of enantiomers — should be extremely low. The hydrolysis of poly-DL-lysine by pronase and taka-diastase shows that a content of about 3% "pure" poly-peptide is found in the product, which emphasises the preferential sythesis of identical enantiomers within the polymer chain.

1. Introduction

From synthetizing poly-lysine by condensation of racemic DL-lysine, where the therm "racemic" shall be defined as a mixture of D- and L-lysine at an exact molar ratio of 1:1, one may expect a tremendous number of different macromolecular products, due to the large number of ways in which D- and L-monomers can be lined up in chains. The relative abundance of specific groups of combinations depends on the size of the macromolecule. The statistical expectable maximum of a D:L-lysine-combination of 1:1 will be very sharp for long chains, rather flat for short chains. An example may illustrate this: Di-lysine synthetized from racemic lysine contains the following molecules: LL, LD, DL, and DD. Neglecting specifically differring bond strengths within the enantiomeric chain units (*i. e.* different free energies) the probability for the appearance of each molecule is equal to 1/4. If one distinguishes the molecules LD and DL as "mixed peptides" from the molecules LL and DD as "pure peptides" (isotactic), the relative concentrations of both molecular groups are equal to 1/2. Concerning the abundance of "pure peptides" it is quite clear that their concentrations fall off to small values as the molecular weight increases, *i. e.* to $2/2^{10} = 1/512 \approx 0.2\%$ for the case of deca-lysine and to $2/2^{1000} \approx 10^{-298}\%$ for kilo-lysine.

If one were to find measurable amounts of such "pure peptides" in a preparation of poly-lysine of high molecular weight, it would prove, that statistical calculations alone are of no value. The mixing entropy that would be gained in the "mixed peptide" would be offset by other thermodynamic advantages gained by lining-up only one sort of enantiomeric units.

The experimental problem was to find a suitable method that allowed the determination of even small concentrations of "pure peptides" in the presence of an excess of "mixed peptides". Neither Optical Rotation Dispersion nor Infrared Analysis could help solve the problem. ORD gives no clue at all because any optical activity of one component would be exactly cancelled to zero by an equivalent activity of opposite sign of the antipode. Infrared spectrometry proves not sensitive enough in the desired range of detection. An enzymatic analysis seemed to be best-suited: a stereospecific exopeptidase should be able to hydrolyze poly-lysines containing only L-lysine while the poly-lysines containing only D-lysine as well as the "mixed peptides" should resist enzymatic attack.

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2. Reagents and Experimental Details

Taka-diastase was obtained from Serva-Entwicklungslabor, Heidelberg (Clarase 300).

Carboxypeptidase B from Merck Darmstadt, origin pankreas of pig, activity 60 U/mg, phenylmethanesulfonyl-fluoride-treated. Pankreas-protease from Merck Darmstadt, origin beef, activity 8 mAnson/mg, lyophilized. α-Chymotrypsin from Merck Darmstadt, origin beef, activity 45 mU/mg, cryst., lyophilized. Ficin from Merck Darmstadt, activity 30.000 U/g. Pronase E was given to us, generously free of charge, from Kaken Chemical Company, Tokyo, activity 70.000 PUK/g.

The poly-L- and poly-D-lysine were synthetized in our laboratory by classical procedures from the *N*-carboxyanhydrides of the corresponding ε-carbobenzoxy-lysines[1]. Racemic poly-DL-lysine was synthetized by the same method, using lysine that was carefully racemized before under controlled conditions at elevated temperature in 18% HCl[2]. The racemization step was necessary because commercially available DL-lysine (Merck, EGA) was rarely racemic in a strict sense, but contained up to 2% excess L-lysine. The molecular weight of the poly-DL-lysine was estimated from viscosimetric analysis of the ε-CbO-lysine in dimethylformamide, which were compared with the values of APPLEQUIST and DOTY[3], to be about 200,000.

The reaction scheme was standardized as follows: 1 ml of enzyme solution was mixed with 1 ml of poly-lysine substrate and incubated in closed vials at 37 °C. Concentrations of enzyme solutions and pH of the reaction mixtures, which were adjusted by phosphate or citrate buffers, are given in Table I.

Table I. Experimental conditions for the enzymatic hydrolysis of poly-lysines.

Peptidase that reacted with poly-lysine	Concentration of enzyme in mg/ml H_2O, if not given otherwise	pH
taka-diastase	0.50	4.85
carboxypeptidase B	0.01 ml/ml	7.65
pankreas-protease	0.20 in 0.001 N HCl	7.65
α-chymotrypsin	0.71 in 0.001 N HCl	7.65
ficin	a 0.55	6.00
ficin	b 0.43	8.00
pronase E	a 0.50	7.00
pronase E	b 0.50	8.30

Concentrations of substrate and content of lysine units per polypeptide are seen from Table II.

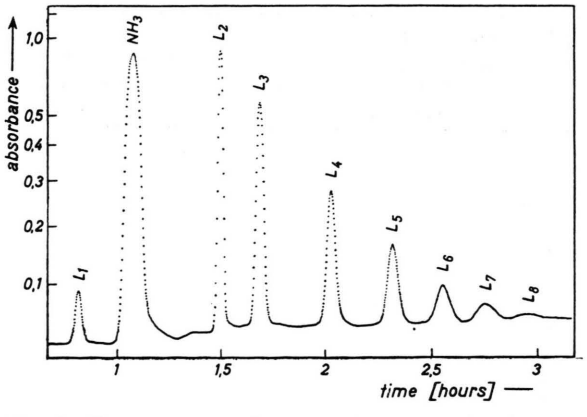

Fig. 1. Chromatogram of a test mixture of oligo-lysines; L_1, . . . 8 = mono-, . . . octa-L-lysine. Details in the text.

Table II. Concentration of substrates and content of lysine.

Substrate	Concentration [mg/ml H_2O]	Amino-acid units [mg/ml H_2O]*
poly-L-lysine.HCl	1.08	0.386 L-lysine
poly-D-lysine.HCl	1.18	0.961 D-lysine
poly-DL-lysine.HCl	1.32	0.384 L-lysine + 384 D-lysine

Analysis

Figure 1 shows a typical analysis of the oligo-lysines up to octa-lysine by ion exchange chromatography. Complete separation of the components was achieved by gradient elution in an automatic amino acid analyzer. The column of size 12.2 · 0.8 cm was filled with "Aminex A 5" resin; temperature was 80 °C. Three different eluents were used as shown in Table III.

Table III. Buffers used in the chromatographic elution of oligo-lysines.

Buffer No.	pH (Adjusted by Na-citrate and HCl)	Concentration of Na^+ eq/l (adjusted by NaCl)
1	5.28	0.35
2	5.62	2.00
3	5.62	~ 6.70 (saturated)

The buffer solutions were prepared according to published procedures[4]. The rate of elution was 80 ml/hour. Buffer 1 was applied for 50 min, buffer 2 thereafter for 10 min, then the sodium concentration in buffer 2 was slowly raised by continuous displacement with buffer 3 until the octa-lysine appeared after about 3 hours. The oligo-lysines were identified by the method of internal standardization. The concentration of components was determined by the usual ninhydrin reaction.

Mono-L-lysine could be clearly identified as the end product of enzymatic hydrolysis by its retention time and its reaction with L-lysine-decarboxylase (Fluka). Figure 2 shows a test of this method: L-lysine-decarboxylase was added to racemic lysine, the mixture incubated at 37 °C for a period of half an hour, and a chromatogram run thereafter. The area of the lysine peak (in this case at a retention time of 0.8 hour) decreased to half of its original value while at the same time the cadaverine peak (at a retention time of 1.7 hours) increased to a value that corresponded to the equivalent concentration lost in the lysine peak.

* Difference to the figure given in the left column corresponds to some water or ammonia content in the polymer, left-over from the polymerization reaction.

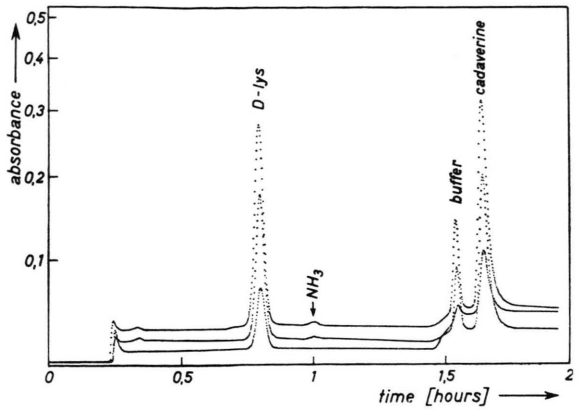

Fig. 2. Chromatogram showing the products of the action of L-lysine-decarboxylase on racemic DL-lysine.

3. The Choice of Enzymes

We tested six commercially available peptidases: taka-diastase, carboxypeptidase B, pankreas, protease, α-chymotrypsin, ficin, and pronase E. The rate as well as completeness of hydrolysis was studied for poly-L-lysine, poly-D-lysine, equimolar mixtures of both, and racemic poly-DL-lysine. While there was no reaction at all with α-chymotrypsin and ficin without BAL-activation[5] and incomplete reaction with pankreas-protease, the poly-L-lysine was completely hydrolyzed by taka-diastase, carboxypeptidase B, and pronase E. However, the rates of the overall reactions and the yields of intermediate oligo- lysines differed greatly. Also, no inhibition of enzyme activity due to poly-D-lysine was observed nor was the poly-D-lysine decomposed (cf. Table IV, column 5 and 6). The efficiency of the enzymatic hydrolysis of poly-L-lysine in the presence of poly-D-lysine was somewhat surprising since TSUYUKI[6] reported that with trypsin the poly-D-lysine inhibits the enzyme.

4. Kinetics of Hydrolysis

Enzymatic attack of pronase E and carboxypeptidase B on poly-L-lysine produced oligomers as intermediates. Figures 3 and 4 give the results for two

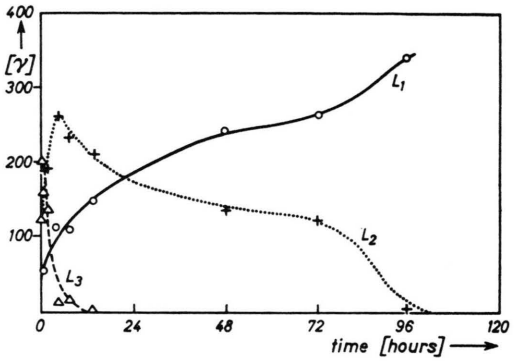

Fig. 3. Hydrolysis of poly-L-lysine by pronase E at pH 7.00; $L_{1,2,3}$ = mono-, di-, tri-L-lysine.

runs under different conditions. The rate and mechanism of the reaction change considerably with the acidity of the solution. Pronase E combines obviously endo- and exopeptidatic activity within itself, a fact that has been confirmed by other authors[7]. Further

Table IV. Summary of experimental results, where the maximum reaction time and the yields of intermediate and end products of hydrolytic attack can be seen.

Enzyme	pH	Maximum reaction time [hours]	Yield of mono-lysine in %**. as end product starting from poly-L-lys	poly-D-lys	poly-L-lys + poly-D-lys 1:1	poly-DL-lys	Fission products* (i = intermediate, e = end product)
carboxy-peptidase B	7.65	12	100	0	50	—	L_1^e, L_2^i
pankreas-protease	7.65	48	16	0	—	0	L_1^e, L_2^e, L_3^e
α-chymotrypsin	7.65	69	0	0	—	—	0
ficin a	6.00	48	0	0	—	0	0
ficin b	8.00	48	0	0	—	0	0
takadiastase	4.85	63	100	0	50	2.9	L_1^e
pronase E a	7.00	120	100	<0.2	50	3.2	L_1^e, L_2^i, L_3^i
pronase E b	8.30	120	100	<0.2	50	—	L_1^e, L_2^i, L_3^i

* $L_{1,2,3}$ = mono-, di-, tri-l-lysine.

** Statistical relative error of figures given about ±2%.

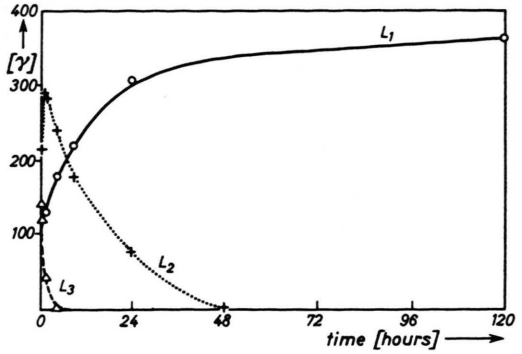

Fig. 4. Hydrolysis of poly-L-lysine by pronase E at pH 8.30; $L_{1,2,3}$ = mono-, di-, tri-L-lysine.

experiments on the possible elucidation of the pronase reaction are published elsewhere[8].

5. Results and Discussion

From Table IV it is seen that pronase E as well as taka-diastase are able to hydrolyze racemic poly-DL-lysine up to an extent of 2.9 to 3.2%. Since poly-L-lysine is hydrolyzed in 100% yield and poly-D-lysine only in 0.2% yield (0.2% is about the limit of detection) the 2.9 to 3.2% is expected to represent blocks of (isotactic) poly-L-lysine within the poly-DL-lysine.

It is interesting to note which types of molecules were hydrolyzed by our method in the racemic poly-lysine case. Let us suppose that the enzyme can cleave only L-monomers from the C-terminal end. Thus the enzyme produces L-lysine not only from the above defined "pure peptides", but also from all such "mixed" molecules that contain "pure blocks" of L-monomers at the C-terminal end. This may be illustrated by the formula

$$(\mathrm{LD})_{n-x}\mathrm{L}_x \rightarrow (\mathrm{LD})_{n-x} + x\,\mathrm{L}$$

The number of L-monomers arising from this general type of reaction is naturally much higher than that arising from the "pure peptides" alone. It can be shown — by listing all the possible molecules, counting the L-lysines produced by the above hydrolysis reaction, and dividing this number by the number of total monomers that could be hydrolyzed by reaction with hydrochloric acid — that the statistical abundance of enzymatically produced L-lysine is given by the formula

$$\frac{2^n - 1}{n \cdot 2^n}$$

In our case of poly-DL-lysine with a molecular weight of 200,000 one would expect an abundance of L-lysine, that is produced by the enzymatic hydrolysis, of about 0.05%, if there were only statistical lining-up of D,L-monomers during the synthesis of poly-lysine. Although this number is much higher than the abundance of the "pure L-peptides" calculated on mere statistical reasons above, it is still by a factor of 60 smaller than the figure found experimentally. From this it may be concluded that there is a strong kinetic preference for sequences of identical enantiomers in the peptide chain, which again emphasises that statistical considerations are of no use in estimating any relative abundances of stereospecific molecules in polymerization experiments.

Other authors came to similar conclusions, among them LUNDBERG and DOTY[9], who claimed that racemic amino-acids tend to polymerize as block copolymers rather than to randomly mixed polymers. It seems worth noting that they arrived at this result on purely kinetic studies without making use of stereospecific enzymes and giving quantitative figures concerning the relative amounts of such block copolymers.

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