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Synthesis and degradation of nonpeptidic α-amino acid-containing polyamides

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Summary
This paper describes the synthesis of new water-soluble nonpeptidic α-amino acid-containing polyamides. Preliminary results of hydrolytic and enzymatic degradations of L-lysine and L-cystine-based polyamides are also presented. Most of these polymers revealed to be stable towards hydrolytic degradation. Only poly(L-cystyl-L-cystine) PCC IV was rapidly degraded in Tris buffer pH 7,4. Insoluble poly(adipoyl-L-lysine benzyl ester) could be degraded by papain and pepsin. Polyamides from L-cystine were shown to be more susceptible to enzymatic degradation. Trypsin, papain and glutathione reductase degraded PCC IV much more rapidly than Tris buffer 7,4 alone.

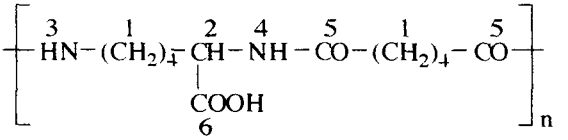
Introduction

During the last two or three decades, there has been a growing interest for preparing biodegradable and biocompatible polymers. Many studies have been devoted to the synthesis of polymers containing natural α-amino acids, their degradation providing units that can be metabolized. The main potential medical applications for synthetic α-amino acid-based polymers are bioresorbable sutures, screws or plates, and drug delivery systems (controlled release and targeting). Until now, most of the polymers studied for this goal are polyesters. But some uses may require other or improved mechanical or chemical properties. Some polyamides could be suitable for special purposes if their biodegradability could be improved. These nonpeptidic α-amino acid-based polyamides have recently been reviewed (1).
After use, these polymers have to be degraded in nontoxic units for elimination. Into the human body, two kinds of degradation can occur: an hydrolytic one, that is generally slow and an enzymatic one, that is more rapid.
We recently published the preparation of two families of diamine-diacid type polyamides based on natural multifunctional α-amino acids: polyamides derived from L-cystine: poly(L-cystyl-L-cystine) (2,3) and polyamides derived from L-lysine-adipic acid (polyadipamide type) and glutaric acid (polyglutaramide type) (4).
The present paper reports the different methods to obtain water-soluble polyadipamides and the first results of the hydrolytic and enzymatic degradations of these two families of polyamides derived from L-lysine and L-cystine.

Experimental

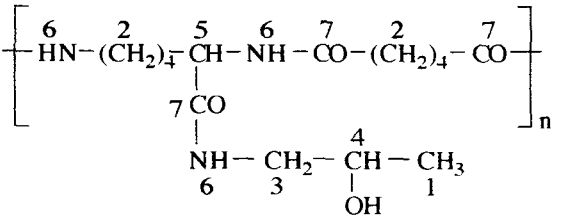
Deprotection of the poly(adipoyl-L-lysine benzyl ester): 2,18 g of poly(adipoyl-L-lysine benzyl ester) ($6{,}30\ 10^{-3}$ mol) were dissolved at room temperature (about 15 min) in 30 mL of trifluoroacetic acid (TFA). The mixture was brought to 15°C, 6,14 mL of methanesulfonic acid (MSA) and 1,38 mL of anisole were added at once. The polymer

was precipitated with ether. After several washings with ether, it was filtered and dried. Yield: 1,5 g (93%).



M = 256,12 g/mol. IR (KBr, cm^{-1}): 3442 (NH, OH); 2947 (CH, CH_2); 1736 (CO acid); 1655 (Amide I); 1560 (Amide II). ^1H NMR (DMSO d6): 1,2-3,0 (H1); 4,1 (H2); 7,7 (H3); 8,0 (H4). ^{13}C NMR (DMSO d6): 23-40 (C1); 52 (C2); 172 (C5); 174 (C6). $[\alpha]_{25}^{578}$ = -5,7° (c = 1%; dichloroacetic acid). Anal.: % calcul.: C: 56,3; H: 7,8; N: 10,9; % found: C: 59,4; H: 8,1; N: 11,1. The difference noted between the two values is attributed to the presence of 5% of benzyl ester groups.

Fixation of a solubilizing group: 500 mg (1,44 10^{-3} mol) of PAL I was dissolved in 5 mL of 1-methyl-2 aminoethanol. The reaction mixture was maintained at 60°C for 24 hours. The excess of reactant was removed under vacuum, and the residue was treated with dichloromethane and filtered. Yield: 450 mg (99%)



M = 313,15g/mol. IR (KBr, cm^{-1}): 3300 (NH, OH); 2937-2886 (CH, CH_2); 1637 (Amide I); 1547 (Amide II); ^1H NMR (DMSO d6): 1,0 (H1); 1,3-3,0 (H2, H3); 3,6 (H4); 4,2 (H5); ,7-7,8 (H6). ^{13}C NMR (DMSO d6): 21 (C1); 23-40 (C2); 46 (C3); 52 (C5); 65 (C4); 12 (C7). mp: 136°C. $[\alpha]_{25}^{578}$ = -20,9° (c = 1%; dichloroacetic acid). Anal.: % calcul.: C: 57,53; H: 8,62; N: 13,41; % found: C: 56,73; H: 8,82; N: 12,92.

Degradation tests

- The enzymes were provided by Sigma (carboxylesterase 6800 U/mL, carboxypeptidase A 1092 U/mL, trypsin 1,44 U/mg, glutathione reductase 0,08 U/mg) and Fluka (Papain 3,18 U/mg, pepsin 37,1 U/g)
- Insoluble polymers were tested as hot-melt pressed pellets or as a powder when pellets cannot be obtained. 30-40 mg of the polymers were placed in 5 mL of Tris buffer pH 7,4 (0,2 M) containing 5 mg of the enzyme (trypsin, papain, α-chymotrypsin) or 100 µL of carboxylesterase. The mixture was incubated at 37°C. Every week, a pellet was washed and analyzed (loss of mass, viscosity).
- Soluble polymers: 30 mg of polymer were dissolved in 2 mL of Tris buffer pH 7,4 (0,2 M) (for PCC IV: (0,3 M)) containing 2,4-4,8 10^{-5} mol/L of enzyme. This solution was incubated at 37°C and degradation was characterized by viscometry of the solution.

Results and discussion

Synthesis of water-soluble poly(adipoyl-L-lysine)s.

The diamine-diacid type polyamide was prepared by the activated ester method as described earlier (4). Adipic acid was polycondensed with L-lysine, a natural trifunctional α-amino acid. For the necessity of the reaction under mild conditions, the

acid group had to be protected as a benzyl ester, usually easily removable. The repetitive unit is represented in Scheme 1 (PAL I). The covalent immobilization of biologically active substances requires the regeneration of the carboxylic group. Several methods were tested for the deprotection. Vert et al. (5) obtained an entirely deprotected poly(L-lysine citramide) when the well-known catalytic hydrogenolysis was carried out in N-methylpyrrolidone. We attempted this hydrogenolysis in trifluoroethanol (TFE); the polymer recovered (PAL II, see Scheme1) was totally deprotected but even after several extractions of the catalyst, the yield did not exceed 50%.
The total deprotection of the polymer by a concentrated HBr/acetic acid solution required a long time (3 days). We noted for the polyadipamide, a decrease of the viscosity (0,78 dL/g versus 0,92 dL/g), which can be attributed to the deprotection. Moreover, the polymer obtained was yellow. Idelson and Blout (6) studied the deprotection reaction of poly(γ-benzyl-L-glutamate) and showed that chain scissions reactions occurred under these conditions. That is the reason why we used a method recently reported for the deprotection of polyamides (3,7) and that revealed to provide the best results for poly(L-cystyl-L-cystine). In this method, the polymer was dissolved in trifluoroacetic acid (TFA), using methanesulfonic acid (MSA) as the deprotecting reagent and anisole as a scavenger. Feijen et al. (7) showed the importance of the reaction temperature on the chain scission reactions: at room temperature, amide bond cleavage occurred. So we preferred to carry out the reaction at 15°C. The results are collected in Table 1. The rate of deprotection was monitored by NMR ^1H.

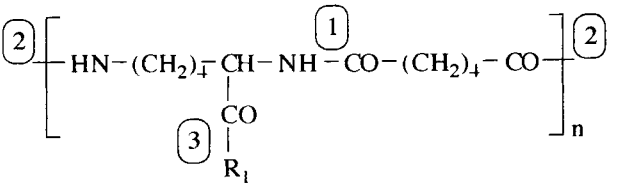
Time (min)	% Bz	η_{inh} (dL/g)
0	100	1,02
45	60	0,74
90	20	0,73
180	6	0,75
390	5	0,71

Table 1: Deprotection of PAL I by TFA/MSA/Anisole

The deprotection of poly(L-cystyl-L-cystine benzyl ester) was described elsewhere (3). The deprotection of the benzyl ester groups systematically entailed a decrease of the inherent viscosity (0,75 dL/g versus 1,02 dL/g). This value remained constant whatever was the ratio of deprotection. The last result showing another drop of the viscosity could be interpreted as the cleavage of some amide bonds. After 3 hours, the polymer was 94% deprotected, this polymer was not water-soluble but was soluble in Tris buffer pH 7,4; so, it could be used to test the degradability.

Fixation of a solubilizing group
Solubilization of poly(adipoyl-L-lysine) in pure water could be obtained by linking an adequat group on the carboxylic side group. Several authors (8, 9, 10, 11) already used 1-methyl-2-amino ethanol. Direct reaction of PAL I (η_{inh} = 0,31 dL/g, TFE, c = 1%) with this compound provided a PAL III (η_{inh} = 0,41 dL/g, TFE, c = 1% ; η_{inh} = 0,21 dL/g, Tris buffer 7,4, c = 1%). ^{13}C and ^1H NMR showed the total disparition of benzyl groups.
Similar treatment of solubilization was not necessary for poly(L-cystyl-L-cystine) PCC IV which , under the deprotected form, was naturally hydrosoluble.

Degradation of polyamides derived from L-lysine and L-cystine
For most medical applications, the polymer used must be eliminated from the human body either by the natural pathways or by metabolization after degradation. The polymer synthesized from L-lysine and L-cystine contain various potentially cleavable bonds.
The general formula of poly(adipoyl-L-lysine)s is



with $R_1 = O\text{-}CH_2\text{-}C_6H_5$ PAL I

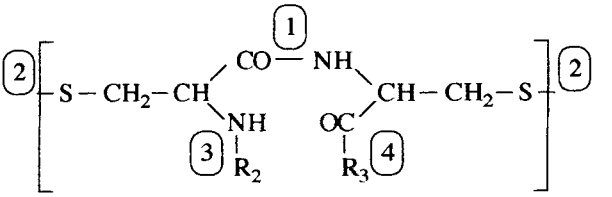
$R_1 = OH$ PAL II

$R_1 = NH\text{-}CH_2\text{-}CHOH\text{-}CH_3$ PAL III

Scheme 1

These polymers contain:
- one semi-peptidic amide bond (1) in α position of a carboxylic group
- one amide bond (2)
- one ester bond for PAL I (3)
- another amide bond for PAL III.(3)

The general formula of poly(L-cystyl-L-cystine)s is:



with $R_2 = CO\text{-}O\text{-}CH_2\text{-}C_6H_5$, $R_3 = O\text{-}CH_2\text{-}C_6H_5$ PCC I

$R_2 = H$, $R_3 = O\text{-}CH_2\text{-}C_6H_5$ PCC II

$R_2 = CO\text{-}O\text{-}CH_2\text{-}C_6H_5$, $R_3 = OH$ PCC III

$R_2 = H$, $R_3 = OH$ PCC IV

Scheme 2

These polymers contain:
- one peptidic amide bond (1)
- one disulfide bridge (2)
- one carbamate bond (3) for PCC I, PCC III
- one ester bond (4) for PCC I, PCC II

In the human body, degradation can occur through two different pathways: hydrolytic and enzymatic. These two reactions were tested on the two preceding families.

Hydrolytic degradation in Tris buffer 7,4

The 0,2M Tris-buffer (pH 7,4) insoluble polymers were studied in heterogeneous phase as melt-pressed pellets (PAL I, PCC I) or as a powder when pellets could not be obtained (PCC II). Degradation could be characterized by a loss of mass or by decrease of the molar mass (viscometry) of the insoluble polymer, or by an increase of the viscosity of the solution (due, for example, to the dissolution of modified chains). These three insoluble polymers showed no degradation or solubilization over 6 weeks.

The other polymers, PAL II, PAL III, PCC III revealed to be soluble in 0,2M Tris buffer (pH 7,4). Total deprotection of PCC I, by HBr, for example, provides a polymer PCC IV

with side NH_3^+-Br^- groups. This acidic polymer was soluble in pure water, but its dissolution in Tris buffer 7,4 required a higher concentration of buffer (0,3M). PAL II, PAL III and PCC III revealed to be stable under these conditions over 6 weeks in spite of an improved hydrophilicity compared to the insoluble polymers studied above. Only polymer PCC IV (a polyampholyte) showed a rapid degradation in 0,3M Tris buffer. The decrease of the viscosity of the solution is represented on Fig. 2 (upper curve): the degradation is complete within 50 hours and is only due to the cleavage of peptidic bonds, disulfide bridges revealed to be stable under these conditions. Many authors who previously used Tris buffer did not mention amide bond cleavage. Only Asano (12) reported a 40% degradation of a copoly(γ-L-benzyl glutamate-L-glutamic acid-L-leucin), within 10 days. This degradation cannot simply be explained by an autocatalysis, since PCC II and PCC III were not degraded under the same conditions.

Enzymatic degradation
The same polymers (30-40 mg) were submitted to the action of 5 mL solutions of various enzymes (1 mg/mL) in the solid state or in solution. The degradation was monitored by the methods above mentionned. All the enzymes are proteases except glutathione reductase.

Insoluble polymers: PCC I and PAL I studied as pellets revealed to be very stable. PCC I was not degraded over 12 weeks by papain, trypsin, α-chymotrypsin (known to degrade peptide bonds of hydrophobic α-amino acids (13)), carboxylesterase (endopeptidase) and carboxypeptidase A (exopeptidase). Even the very specific glutathione reductase (which quantitatively cleaves disulfide bridges) had no effect. This can be attributed to the high hydrophobicity of PCC I, preventing the enzyme to form a complex. PCC II (powder) was submitted to the action of carboxylesterase in Tris buffer pH 7,4. The degradation was monitored by viscometry and by loss of mass. The results (Fig. 1) showed a decrease of the viscosity of this polymer (from 0,19 to 0,07 dL/g) much higher than the one obtained by simple debenzylation (from 0,18 to 0,15 dL/g). The loss of mass was 50% after 7 weeks. Besides, the U.V. study of the solution showed the presence of aromatic compounds between 250 and 300 nm. So, the degradation of PCC II by carboxylesterase can be interpreted as being due to semi-peptidic and ester bond breaking.

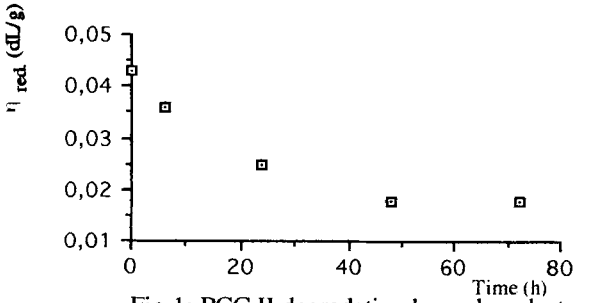


Fig 1: PCC II degradation by carboxylesterase

No degradation of PAL I occurred by trypsin, which is known to break peptide or ester bonds containing carbonyl groups of L-lysyl. This could be due to the absence of cation - NH_3^+ present in L-lysyl residues of peptides. When submitted to the action of papain (that has a very broad specificity), a pellet of PAL I showed a slight decrease of the viscosity (from 0,44 dL/g to 0,39 dL/g within 6 days) and a loss of mass of 95% within 12 days, without any increase of the viscosity of the solution. ^{13}C NMR did not show the presence of carboxylic group in the pellet residue. The degradation seemed to take place by dissolution of oligomers.
A pellet (40 mg) of PAL I submitted to pepsin (pH 2) showed a loss of mass of 15% after 6 weeks and a decrease of its viscosity from 0,59 dL/g to 0,29 dL/g, without any increase

of the viscosity of the solution. ^{13}C NMR spectrum showed the presence of a carboxylic group at 174 ppm, due to a partial debenzylation of PAL I. However, a simple debenzylation could not explain such an important decrease of the viscosity (see table 1). Cleavage of some -CO-NH- bonds to give shorter insoluble chains at pH 2 must take place. (A parallel test carried out at pH 2 without pepsin showed a slow decrease of viscosity from 0,59 to 0,46 dL/g).

Soluble polymers: PAL II and PAL III revealed to be quite stable when incubated at 37°C with trypsin, papain, carboxylesterase and carboxypeptidase A over 6 weeks. This means that the semi-peptidic bonds coud not be cleaved, probably because the enzyme-substrate complex could not be realized. These results are quite comparable to those obtained by Drobnik about polyaspartamides (14). The highest degradability was noted for poly(L-cystyl-L-cystine). Three enzymes were tested: trypsin, papain and glutathione reductase. The results are shown on figure 2.

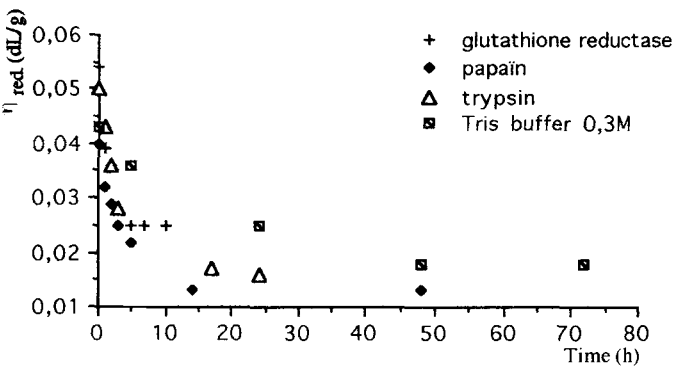


Fig. 2: Hydrolytic and enzymatic degradations of PCC IV

For these three enzymes, we noted an increase of the rate of degradation compared to the degradation by Tris buffer alone. A total degradation required about 50 hours with the buffer and only 15-20 hours were sufficient for trypsin and papain. This rate of degradation probably means that the enzyme-substrate complexes could be formed. In particular the presence of NH_3^+ is well known to favor the trypsin-substrate complex. With glutathione reductase (cleavage of disulfide bonds), the degradation was total in about 5 hours.

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