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Enzymatic Degradation of Side Chains of Soluble Polymers. Rate of Release of 4-Methyl-2-Nitroaniline as a Function of the Side Chain Length

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Summary

A series of water-soluble polymers was prepared, formed in the copolymerization of 4-nitrophenol esters of methacryloylated ω-amino acids with N-(2-hydroxypropyl)methacrylamide and containing c. 3 mol.% of reactive groups. Groups degradable with chymotrypsin were bound to ends of variously long side chains by a polymeranalogous reaction with L-phenylalanine-4´-methyl-2´-nitroanilide. The dependence of the rate of degradation of 4-methyl-2-nitroaniline on the length of the side chain was investigated.

Introduction

Soluble polymers are widely used in medicine; among other applications, they are employed as blood plasma expanders, in diagnostics, as carriers of biologically active compounds (drugs, hormones, inhibitors, enzymes etc.). In most cases, these groups of compounds are bound to the polymer carrier through a covalent bond which may be degradable or stable under physiological conditions.

Selective degradation of the chemical bond can be readily achieved with enzymes. A number of polymer systems have been described where a readily detectable group was split off from the polymer carrier (e.g., KOPEČEK et al. 1981, FU and MORAWETZ 1976, LABSKÝ et al. 1979).

The rate of enzymatic degradation of side chains of soluble polymers is affected by many factors, such as the specific amino acid characteristic of the given enzyme, length and structure of the spacer which separates the enzymatically degradable group from the main polymer chain, character of the polymer carrier or character of the degraded group.

In this study we used L-phenylalanine-4´-methyl-2´-nitroanilide bound to side chains arising from ω-amino acids and studied the effect of length a spacer consisting of various numbers of methylene groups on the rate of release of 4-methyl-2-nitroaniline (MNA).

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Experimental

Preparation of 4-nitrophenol esters of methacryloylated ω-amino acids has been described (LABSKÝ and KÁLAL, 1979, REJMANOVÁ et al. 1977).

L-Phenylalanine-4´-methyl-2´-nitroaniline (LPMNA): 15.2 g 4-methyl-2-nitroaniline (0.1 mole) is dissolved in a mixture of 100 ml dioxane and 15 ml pyridine. 15 g L-phenylalanylchloride hydrochloride (0.07 mole) is added in parts within 30 min with stirring (25°C) (ERLANGER et al. 1966). The reaction mixture was stirred at room temperature for 3 h, 400 ml water was added, and the mixture was acidified with conc. HCl to pH 2. The precipitate was removed, the reaction mixture was evaporated to some 400 ml and alkalized with a sodium carbonate solution to pH 9. The product thus obtained was recrystallized from methanol. M.p.: 80-1°C, yield 8 g (27%). Analysis for $C_{16}H_{17}N_3O_3$ (433.46): calculated C 64.21, H 5.65, N 14.05; found C 64.05, H 5.85, N 13.97.

Copolymers: Copolymers of 4-nitrophenol esters of methacryloylated ω-amino acids with N-(2-hydroxypropyl)methacrylamide (HPMA) were prepared by precipitation radical polymerization in acetone (REJMANOVÁ et al. 1977, LABSKÝ and KÁLAL 1971). The polymeranalogous reaction with LPMNA and purification of polymers thus formed were carried out according to (LABSKÝ et al. 1979). Characteristics of the polymers are given in Table 1.

TABLE 1

Characteristics of polymers and kinetic constants for polymeric substrates degraded with chymotrypsin

Side chain (n)	LPMNA content (mol.%)	Molecular weight (M_W)	$K_M.10^3$ (M)	k_{cat}/K_M ($M^{-1}.s^{-1}$)
1	3.1	42 000	-[a]	-[a]
2	3.1	37 000	0.97	9.4
3	2.8	40 000	1.26	25
4	3.0	34 000	0.318	96
5	3.0	37 000	4.99	75
6	3.1	41 000	0.715	129
7	3.0	42 000	0.168	115
11	3.1	37 000	0.300	51
Gly-Gly	3.0	27 000	0.032	14

[a]Does not degrade within 10 min.

Molecular weights of the polymers were determined by the light scattering method with a Photo Gonio Diffusometer Sofica 42 000 apparatus.

Enzyme: α-chymotrypsin, crystallized three times, was used in the degradation (Organochema, Czechoslovakia). The enzyme activity determined by titration with sultone of 2-hydroxy-5-nitro-α-toluenesulphonic acid (KÉZDY and KAISER 1970) was 51.7%. The concentration of the enzyme was determined spectrometrically, $\varepsilon = 5 \times 10^4$ ($1\ cm^{-1}\ mole^{-1}$) at 282 nm. The molecular weight of chymotrypsin was 25 000. A stock solution of enzyme in 10^{-3} M HCl was used in the measurement, the concentration of enzyme was 4.64×10^{-5} mole l^{-1}.

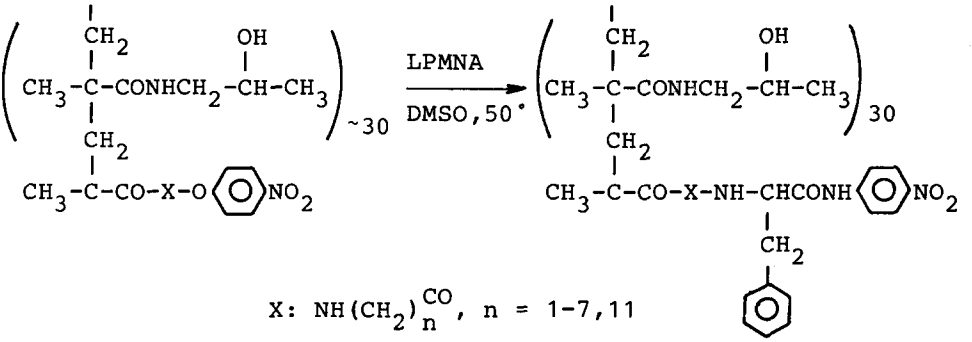
The concentration of bound and degraded 4-methyl-2-nitroaniline was determined spectrometrically, the range of measurements was $0.25\text{-}1.1 \times 10^{-4}$ M LPMNA. 4-Methyl-2-nitroaniline (MNA): bound $\varepsilon = 4.9 \times 10^3$ ($1\ cm^{-1}\ mole^{-1}$) (at 353 nm), free $\varepsilon = 5.1 \times 10^3$ ($1\ cm^{-1}\ mole^{-1}$) (at 430 nm). The measurement was carried out with a Specord UV-VIS spectrophotometer in the range 0.1-1.4 A (absorbancy). A UNICAM SP 1750 Ultraviolet Spectrometer was used for measurements in the range 0.2 A.

Buffer: 0.08 M TRIS (2-amino-2-hydroxymethyl-1,3-propanediol) and 0.1 M calcium chloride, pH = 8 (adjusted with conc. HCl).

Kinetic measurements: The measurement was performed in a thermostated cell (1 cm) at 25°C. On mixing, the cell contained 2.5 ml of the mixture which also included 0.2 ml of enzyme solution. The kinetic constants K_M, k_{cat} and k_{cat}/K_M were determined. The Michaelis constant K_M was determined using a Lineweaver-Burke relation.

Results and Discussion

The copolymerization of N-(2-hydroxypropyl)methacrylamide with 4-nitrophenol esters of methacryloylated amino acids and methacryloylated diglycine yielded polymers which by a polymeranalogous reaction with LPMNA gave water-soluble polymers with an enzymatically degradable bond formed at the end of the side chain. The polymers had the following composition:



The rate of chymotrypsin catalyzed hydrolysis of L-phenylalanine-4´-methyl-2´-nitroanilide bound at the ends of variously long side chains was expressed through k_{cat}/K_M (BROT and BENDER, 1969). For short side chains (n = 1,2), when the degradable bond lies in the immediate vicinity of the main polymer chain, the rate of degradation is low or almost unmeasurable (for n=1). Steric hindrances produced by the main polymer chain, along with the bulky side substituents of the polymer carrier, impede the formation of the enzyme-substrate complex which is a necessary condition for enzymatic degradation.

The extension of the side chain by including further methylene groups removes the degradable bond from the main polymer chain and increases the rate of enzymatic reaction. The rate of hydrolysis reaches its maximum for the side chain consisting of 7-aminoheptanic acid (n=6). A further extension of the side chain (n=7,11) leads to a decrease in enzymatic hydrolysis. With increasing length of the side chain, its hydrophobic character becomes more pronounced, and it is very probable that the side chain collapses in the polar medium of the aqueous buffer in which the hydrolysis takes place. This assumption is in accordance with the increaseing rotational relaxation times of dansyl fluorochrome bound on the same polymers, when on the chain with six methylene groups the dependence under investigation has a maximum (MIKEŠ and LABSKÝ).

The necessary condition for enzymatic degradation of the chemical bond is that along with other conditions (pH, temperature etc.), there should also be a perfect contact between the substrate and enzyme. Low-molecular weight substrates for chymotrypsin have been investigated in a number of papers which concentrated on the analysis and optimization of the structure of the oligopeptidic chain on which the degradable group is bound. For the description of interactions between the substrate and enzyme, SCHECHTER and BERGER 1967 suggested symbolics where the individual α-amino acids of the substrate are denoted with P_1, P_2 etc. (numbered starting from the amino acid whose amide bond is degraded in the direction of the end of the substrate bearing a free amino group). The corresponding subsites of the enzyme are denoted with S_1, S_2 etc. The resulting energetic balance of the individual S-P interactions consists of two contributions: formation of hydrogen bonds between the amide bonds of the enzyme and substrate, and hydrophobic interactions of hydrophobic groups of the individual amino acids of the substrate with the enzyme.

The basic interaction in the polymeric substrates investigated in this study is due to the S_1-P_1 interaction of L-phenylalanine. If (formally) the three methylene groups of the side chain of the substrate are regarded as an α-amino acid sequence, then the acceleration of enzymatic hydrolysis for n=6 (cf. Table 1) may be attributed to the S_4-P_4 interaction between the enzyme and amide bond of the substrate (scheme 1):

n	S_5	S_4	S_3	S_2	S_1	S_1'
6	Polymer — $CONHCH_2$ —		$CH_2CH_2CH_2$ —		CH_2CH_2CO — L-Phe — MNA	

If the side chain is extended by adding another methylene group, this interaction also disappears, and the rate of degradation is slowed down.

The oligopeptidic side chain formed from diglycine and LPMNA is degraded very slowly, even if in this case hydrogen bonds may be assumed to exist in the individual S-P interactions. The lack of hydrophobic interactions on P_2 and P_3 subsites of the substrate reduces the rate of enzymatic degradation (cf. KOPEČEK et al. 1981).

It may be said, on the whole, that the rate of degradation of MNA from side chains consisting of a series of $-CH_2-$ groups is very small. It seems to be due to the hindrance of the degradable bond caused by a bulky nitro group in the ortho position of the aromatic ring.

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