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EPR Study of Enzymatic Cleavage of Side Chains of Water Soluble Polymers Using Spin-Label as a Leaving Group

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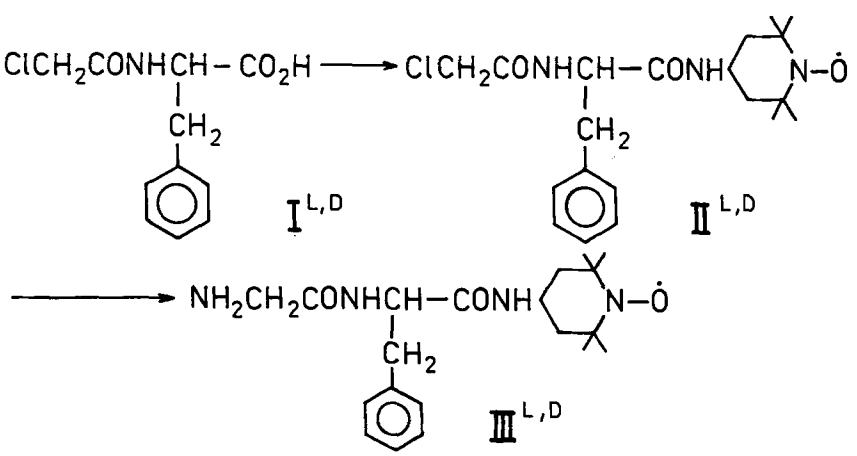
Synopsis

It was confirmed by EPR that 4-amino-2,2,6,6--tetramethylpiperidine-N-oxyl (TEMP-NH_2) is cleaved by chymotrypsin from 4-(N-glycyl-L-phenylalanyl)amino -2,2,6,6-tetramethylpiperidine-N-oxyl attached as a substrate with paramagnetic leaving group to the end of side chains of water soluble hydroxypropyl methacrylamide (HPMA) compolymers.

Introduction

TIEH-YIN FU and MORAWETZ (1976) and also DROBNÍK et al.(1976) described the enzymatic cleavage at the ends of side chains of soluble polymers with L-phenylalanine-4'-nitroanilide attached as a substrate; as free and bonded 4-nitroaniline absorb at different wavelengths, released nitroaniline was followed spectroscopically.

We attempted to utilize EPR in an analogous study of substrate cleavage. For this purpose a substrate with a paramagnetic leaving group, 4-(N-glycyl-L-phenylalanyl)amino-2,2,6,6-tetramethylpiperidine-N-oxyl (III^L) was prepared by the following reaction sequence:



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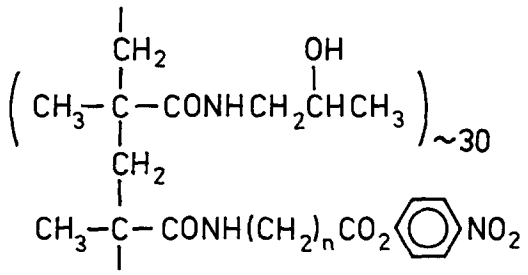
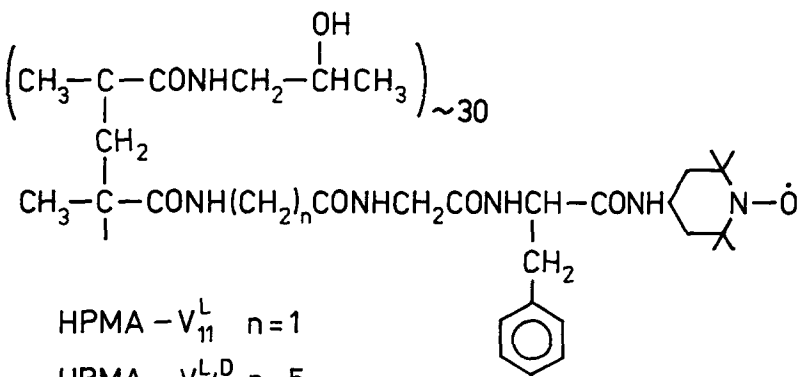
By a polymeranalogous reaction of substrate III^L with reactive copolymers (scheme A, table 1) described previously (LABSKÝ et al. 1977)



n = 1,5,11

Scheme A

spin-labelled copolymers HPMA-$V^L_{11,15,21}$ were prepared, where the spin-label acts also as the paramagnetic leaving group (scheme B). The lower index in the symbol of the copolymer denotes the number of atoms in the side chain, the upper index (L or D) indicates the presence of L- or D-phenylalanine derivative at the end of side chains.



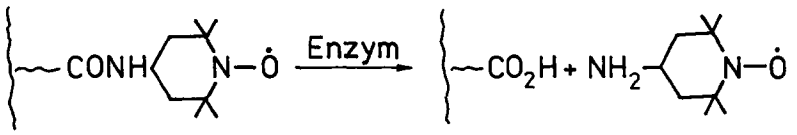
HPMA – V^L_{11} n = 1

HPMA – $V^{L,D}_{15}$ n = 5

HPMA – $V^{L,D}_{21}$ n = 11

Scheme B

Enzymatic hydrolysis by chymotrypsin of these copolymers should lead to a cleavage of free spin-label, 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMP-NH_2) (scheme C).



HPMA-V TEMP-NH_2

Scheme C

Accordingly, a decrease in the intensity of EPR spectrum of the bonded, partially immobilized spin-label can be expected during the enzymatic cleavage, accompanied by a superposition of the spectrum of free, non-immobilized TEMP-NH_2. It follows from our previous results that the correlation times characterizing the rotational reorientation of the spin-label are nearly ten-times higher (even in the case of the longest side chain) compared to the free spin-label in the same medium (PILAŘ et al., 1979). The above-mentioned superposition in the motional-narrowing region should manifest itself by a change in the ratio of first derivative peak-to-peak amplitudes of the components of nitrogen splitting in the EPR spectra of the spin-label during the enzymatic cleavage. The copolymers HPMA--$V^D_{15,21}$ were prepared with the aim of ascertaining whether the formation of a complex between D-phenylalanine (which acts as a competitive inhibitor of chymotrypsin) and the enzyme would lead to enhanced immobilization of the spin-label bonded at the ends of side chains in substrate III^D.

TABLE 1

Characteristics of reactive copolymers (scheme A)

Side chain (n)	Content of 4-Np[a] (mole percent)	Molecular weight M_w
1	3.45	42 000
5	3.2	37 000
11	2.9	37 000

[a]4-nitrophenyl esters; measured by spectroscopy (274 nm) in methanol at 22°C. Extinction coefficients taken from (LABSKÝ et al., 1977).

Experimental

Preparation of Substrates with Paramagnetic Leaving Group

Chloracetyl-L(D)-phenylalanine (I^L or I^D) were prepared according to RAO et al. (1952) from L- or D-phenylalanine (Fluka).

4-(N-chloroacetyl-L-phenylalanyl)amino-2,2,6,6-

-tetramethylpiperidine-N-oxyl (II^L): 20 mmole (4.8 g) of chloroacetyl-L-phenylalanine (I^L) and 20 mmole (2 g) of triethylamine were dissolved in 100 ml of methylene chloride and cooled to -5°C; 20 mmole (2.16 g) of ethyl chloroformiate was then added. After 60 minutes 20 mmole (3.4 g) of 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMP-NH_2) was added. After 24 hours the reaction mixture was extracted consecutively with 3% HCl, 5% $NaHCO_3$ and water. The product was crystallized from a mixture of methylene chloride and heptane. M.p. 189-91°C, yield 6.1 g (78%).

4-(N-glycyl-L-phenylalanyl)amino-2,2,6,6-piperidine-N-oxyl (III^L): 10 mmole (3.9 g) of 4-(N-chloroacetyl-L-phenylalanyl)amino-2,2,6,6-tetramethylpiperidine-N-oxyl (II^L) was dissolved in 50 ml of ethanol and saturated with ammonia. After 2 days the reaction mixture was evaporated; the remaining oil crystallized gradually. The product was crystallized from a mixture of ethyl acetate and hexane. M.p. 110-11°C, yield 1.8 g (53%).

Derivatives of D-phenylalanine (II^D and III^D) were prepared in an analogous manner. The physical constants of new substrates are summarized in Table 2.

TABLE 2

Characteristics of prepared substrates with paramagnetic leaving group

Substrate[a]	M.p. (°C)	Yield (%)	Formula (mol.weight)	Analysis % (calc./found) C	H	N	Cl
II^L	189-90	60	$C_{20}H_{29}N_3O_3Cl$	60.81	7.40	10.64	8.98
			394,97	60.71	7.21	10.69	8.71
II^D	189-91	78	$C_{20}H_{29}N_3O_3Cl$	60.81	7.40	10.64	8.98
			394.97	60.71	7.21	10.44	8.35
III^L	109-10	62	$C_{20}H_{31}N_4O_3$	63.97	8.33	14.92	
			375.50	63.29	8.80	14.25	
III^D	110-11	53	$C_{20}H_{31}N_4O_3$	63.97	8.33	14.92	
			375.50	63.75	8.14	14.81	

[a] Indices L and D denote derivatives of L- and D-phenylalanine, respectively.

Preparation of spin-labelled copolymers

The preparation of reactive copolymers (scheme A and Table 1) has been described elsewhere (LABSKÝ et al. 1977). The preparation of spin-labelled copolymers (scheme B) by a polymeranalogous reaction of the paramagnetic substrate, and their purification by dialysis were performed by analogy to procedures described in the above paper for similar paramagnetic substrates

Sample preparation

A stock solution of chymotrypsin (Enzyme Limited 2-times crystallized) in 10^{-3} M HCl was stored at 2°C not longer than 6 hours. Aqueous solutions of copolymers, pH 8.2 (0.01 M tris(hydroxymethyl)aminomethane partially neutralized by hydrochloric acid) contained 1.10^{-4} M of the spin-label. Samples for measurements were prepared by mixing the stock chymotrypsin solution with a solution of the studied copolymer in such a manner that the concentration for enzymatic cleavage was $4.3.10^{-6}$ M (molecular weight of chymotrypsin is 25,000). Precision syringes were used for handling the enzyme solution; the recording of the spectrum was started not later than 2 minutes after the reaction components came into contact.

Measurement of EPR spectra

Spectra were recorded at 25°C with JES-PE-3X JEOL spectrometer using microwave power 1 mW and 100 kHz modulation. The temperature of the sample in the cavity was stabilized by means of JES-VT-3A Temperature Controller and measured by a platinum resistance thermometer.

Evaluation of free spin-label concentration

The free spin-label concentration in studied samples was evaluated by means of a calibration procedure. Solutions in the given solvent with known ratio of free and bonded spin-label and having concentrations corresponding to those observed during the enzymatic cleavage were first prepared. Calibration lines were then obtained by evaluating the ratio of first derivative peak-to-peak amplitudes of central and high-field lines in spectra of these solutions in dependence on the content of free spin-label; these calibration dependences were subsequently used in evaluating the relative concentration of free spin-label from ratios obtained by analysing spectra measured during the enzymatic cleavage. It is imperative to determine calibration lines for each type of copolymer and for each temperature separately in view of the dependence of correlation time characterizing the rotational reorientation of the spin-label (and, accordingly, also of the first derivative peak-to-peak line widths and amplitudes, respectively) upon the length and type of side chains as well

as on temperature.

Results and Discussion

A typical time dependence of EPR spectra of a sample containing HPMA-V^L_{15} copolymer is shown in Fig. 1;

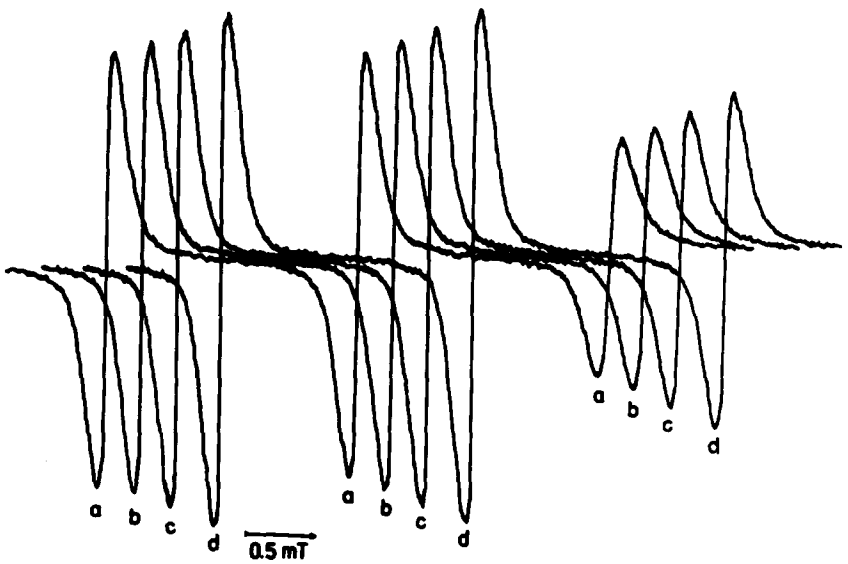


Fig.1 Time dependence of EPR spectra of a sample containing the copolymer HPMA-V^L_{15}. Measured at 25°C, interval between the starts of spectra recordings 10 minutes, pH 8.2. Concentration of paramagnetic substrate attached to the end of the side chain in the copolymer $[S_0] = 1.10^{-4}$ M, chymotrypsin concentration [E] = $= 4.3 \cdot 10^{-6}$ M.

similar dependences were observed also for copolymers HPMA-V^L_{11} and HPMA-V^L_{21}. Evaluation of these data leads to an unequivocal qualitative conclusion that during enzymatic hydrolysis free TEMP-NH_2 is cleaved and that the rate depends on the length of the side chain (about three-times higher reaction rate was observed with the copolymers HPMA-V^L_{15} and HPMA-V^L_{21} compared to the sample HPMA-V^L_{11}). However, in view of a considerable error involved in the calibration procedure necessary for evaluation of relative concentration of free TEMP-NH_2, it was unfortunately not possible to obtain accurate quantitative data required for a description of the kinetics of enzymatic cleavage. Therefore we decided to abandon this time-consuming approach for the time being.

By studying samples containing the copolymer HPMA-V^D_{15} we were not able to validate the assumption

that the formation of a complex between D-phenylalanine and the enzyme would enhance the immobilization of the spin-label. Under similar experimental conditions no changes of the shape and/or intensity of EPR spectra were observed during 60 minutes at different concentrations of chymotrypsin (up to 150 per cent excess). The reason probably lies in low concentration and/or short life of the complex formed, or the complex is too weak and cannot bring about increased immobilization of the spin-label.

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