

# Electron paramagnetic resonance (EPR) study of the short-living radicals generated thermally in phosphorylated maize starch with different amounts of amylose

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**Abstract.** Quantitative electron paramagnetic resonance (EPR) spectroscopy was used for the determination of a number and properties of short-living radicals generated thermally in maize starch with various amounts of amylose. The EPR spectra of radical adducts with N-tert-butyl- $\alpha$ -phenylnitron (PBN) spin trap reveal the presence of three types of radicals of mobility related with their localization in the zones of various degree of crystallinity. Hylon VII, which represents a matrix with a high content of amylose, offers better conditions for free rotation of radical species, whereas waxy maize, containing almost exclusively amylopectin, exhibits more restricted dynamics.

**Key words:** carbon centred radicals • electron paramagnetic resonance (EPR) spectroscopy • PBN spin trap • starch phosphates • thermal treatment

## Introduction

Modification of the starch by incorporation of phosphorus into its structure as terminal or bridging  $\text{PO}_4^{3-}$  groups changes functional properties of this biopolymer [10]. The positive consequences of the chemical modification consist on alteration of gelatinization and pasting behaviour, better stability during freeze-thaw cycles and increasing content of resistant starch exhibiting anti-diabetic and anti-cancer properties. It was shown by us that besides these improvements, important for the application of starch in the food industry, phosphorylation causes weakening of the starch structure which results in an increasing tendency to thermal degradation with the formation of radicals [2, 4, 5]. Two types of radicals – stable and short-living ones, with unpaired electron localized at one of the carbon atoms of the glucose unit are generated in native and phosphorylated starch by heating in the temperature range commonly used for processing food. We have recently shown that the short-living radicals, stabilized by a N-tert-butyl- $\alpha$ -phenylnitron (PBN) spin trap, generated in the native and phosphorylated maize starch with natural content of amylopectin (75%) and amylose (25%), reveal dynamic properties depending on localization in zones of different crystallinity [2]. Therefore, EPR spectra of PBN adducts with short-living radicals may be used as sensitive indicators of the structure ordering.

The aim of the present work was to study, by quantitative EPR spectroscopy, the effect of different content of particular components of the maize starch (amylose and amylopectin) on a number and properties

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of short-living radicals generated thermally in Hylon VII (enriched in amylose) and waxy maize starch (containing almost 100% of amylopectin). Modification of the starch by hydrolysis and phosphorylation as well as by pretreatment with a high hydrostatic pressure was also considered.

## Materials and methods

The experimental material used for phosphorylation was Hylon VII (containing 68% of amylose) refined from high amylose maize produced by National Starch and Chemical, Food Starch, Poland and waxy maize starch (amylopectin with trace amounts of amylose) purchased from Sigma (S-9679). Phosphorylation was performed according to Lim and Seib [10] and Sang and Seib [12]. Modifications of the samples before EPR measurements by chemical (hydrolysis, phosphorylation) and physical (thermal, high pressure) methods are described in papers listed in Refs. [2, 4, 5].

EPR measurements were performed with a Bruker ELEXSYS 500 spectrometer (Karlsruhe, Germany) operating in X-band (9.5 GHz) at modulation frequency 100 kHz, modulation amplitude 0.3 mT and microwave power 0.3–3.0 mW. The EPR spectra were recorded at room temperature. The number of spins was determined by comparison of the integral signal intensity of the investigated samples with that of the standard containing the known amount of paramagnetic centres.  $\text{VO}_2^+ \cdot 5\text{H}_2\text{O}$  diluted with diamagnetic  $\text{K}_2\text{SO}_4$  ( $5 \times 10^{19}$  spins/g) was used as a primary standard. All necessary precautions were followed in order to assure good precision of the quantitative EPR measurements [8]. Generation of radicals was investigated on starch samples (native and modified) before and after chemical and physical treatments. For stabilization of the short-living radicals, PBN was used because of its relatively high thermal stability compared to other spin traps. EPR parameters of the radicals were determined by a simulation procedure using the program EPR SIM 32 [14]. The EPR SIM 32 program allows simulation of the powder multicomponent continuous wave electron paramagnetic resonance (CW-EPR) spectra with  $S = 1/2$  and  $I \geq 0$ . The second-order perturbation formula was used to calculate resonance fields. The line shape can be either Lorentzian, Gaussian, or mixed, and the line width dependence of  $m_l$  can be calculated. Powell and genetic algorithm are used for optimization of the EPR parameters. The accuracy of determinations of  $g$  factor values was equal to  $\pm 0.001$  and that of hyperfine splitting (hfs) constant  $A$  amounted to  $\pm 0.05$  mT.

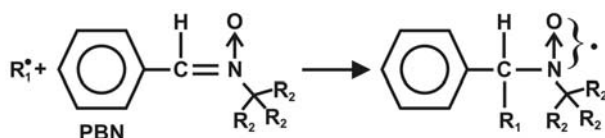
All measurements were performed simultaneously on reference samples (denoted: “Reference Mono” or “Reference Di”), obtained by the same procedure as phosphorylated starches but without using the phosphorylating agents, what enabled extraction of the effect of phosphorus from the experimental data.

## Results and discussion

Native and phosphorylated maize starch did not give any EPR signal before treatment at elevated temperature.

**Table 1.** EPR parameters of short-living radicals generated at 180°C in native and phosphorylated Hylon VII and waxy maize (AP) starch and in corresponding reference samples, before treatment with high hydrostatic pressure

Sample	Total number of spins $N \times 10^{-16}$ (spins $\times$ g $^{-1}$ )	Center 1 (FAST)				Center 2 (MEDIUM)				Center 3 (SLOW)			
		Content (%)	$A_N$ (mT)	$A_H$ (mT)	$\Delta B$ (mT)	$g$ factor	Content (%)	$A_N$ (mT)	$\Delta B$ (mT)	$g$ factor	Content (%)	$A_N$ (mT)	$\Delta B$ (mT)
Native Hylon VII	4.30	1.5	1.48	0.28	0.21	2.006	51.4	1.01	0.40	2.005	47.1	2.42	5.20
Reference Mono Hylon VII	5.67	0.6	1.49	0.28	0.21	2.006	53.4	1.01	0.41	2.005	46.0	2.44	0.55
Mono Hylon VII	3.93	1.0	1.45	0.28	0.24	2.006	52.7	0.98	0.70	2.005	46.3	2.45	0.57
Reference Di Hylon VII	4.95	0.7	1.46	0.28	0.22	2.006	54.5	1.01	0.46	2.005	44.8	2.43	0.59
Di Hylon VII	3.92	0.5	1.43	0.28	0.17	2.006	53.7	1.01	0.68	2.005	45.8	2.44	0.58
Native AP	3.96	2.2	1.46	0.27	0.21	2.006	48.9	0.93	0.69	2.006	48.9	2.34	0.92
Reference Mono AP	3.81	1.1	1.48	0.28	0.21	2.006	48.9	0.90	0.53	2.005	50.0	2.35	0.94
Mono AP	3.51	1.8	1.48	0.28	0.22	2.006	41.3	0.85	0.52	2.006	56.9	2.19	1.13
Reference Di AP	4.51	0.7	1.48	0.27	0.21	2.006	47.0	0.91	0.54	2.005	52.3	2.36	0.95
Di AP	3.64	2.1	1.50	0.27	0.26	2.006	46.2	0.89	0.50	2.005	51.7	2.38	0.97



**Scheme 1.** PBN-adduct formation.

After heating at 150°C or at higher temperatures, the EPR signals appear related with generation of stable radicals typical for thermally treated starch [6, 7]. The number of these radicals created in native and phosphorylated maize starch heated for 30 min at 180°C amounts to about  $2 \times 10^{14}$  spins/g [2]. Similar number of radicals were generated in the same experimental conditions in the native and phosphorylated Hylon VII and waxy maize samples (this work). When heating is performed in the presence of PBN spin trap, the number of detected radicals increases by two orders of magnitude indicating that short-living radicals ( $R_1^\bullet$ ) are created and stabilized as adducts with the spin trap, according to Scheme 1.

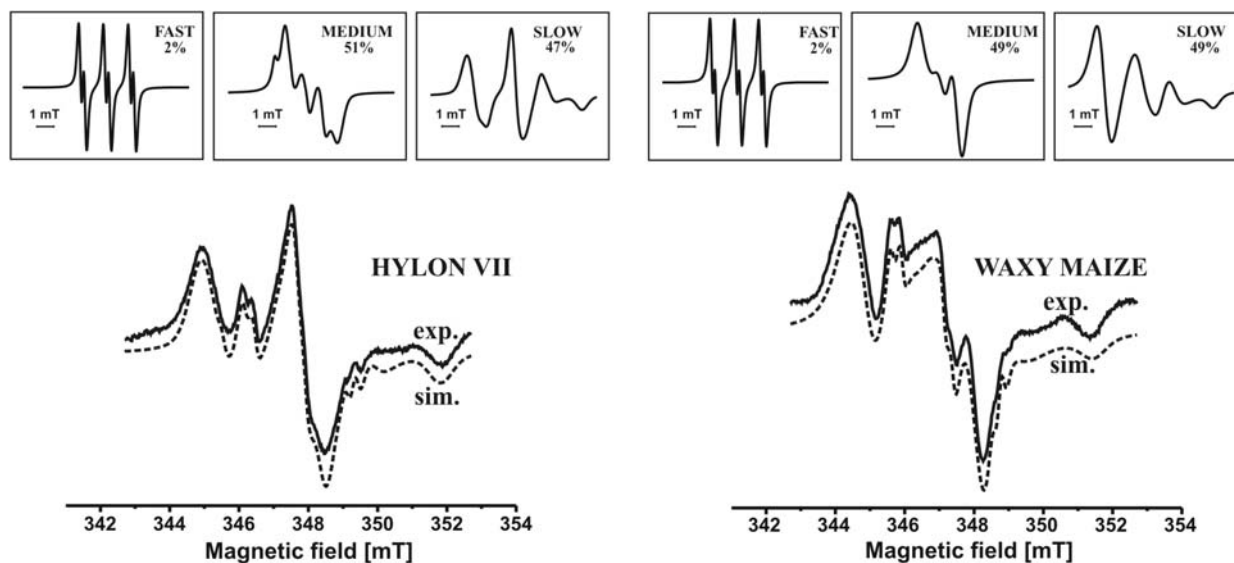
Significant increase in the number of radicals detected in the presence of PBN spin trap after thermal treatment of the starch samples at 180°C was observed previously for native and phosphorylated maize starch [2] and in the present work for native and phosphorylated Hylon VII and waxy maize (Table 1). The adducts were stable at room temperature for several days.

The EPR spectra of the PBN-adducts are complex (Fig. 1). To check if they origin from only one type of radicals or from several radical species, registration of the spectra at different frequency bands (Q or X), various temperatures and different power levels was performed for native thermally treated maize starch [2]. The simulation revealed the presence of three components differing in EPR parameters and denoted – fast, medium and slow. These notation was related with the mobility of particular radical adducts reflected by the shape of their EPR spectra. The rotational correlation time was estimated as being equal to picoseconds for the fast component and to nanoseconds for the slow

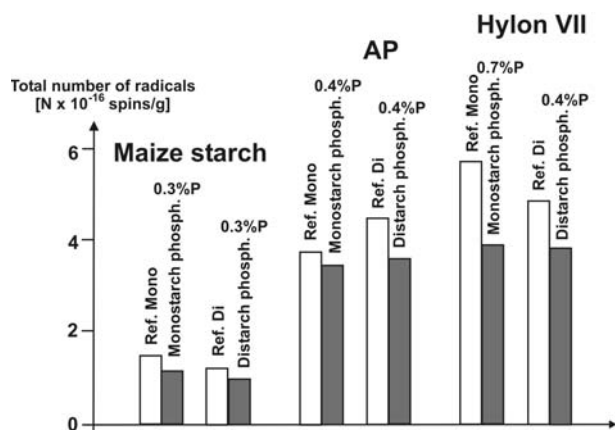
one [13]. Similar components were found by simulation of the EPR spectra of short-living radicals generated in native and phosphorylated Hylon VII and waxy maize. Following EPR parameters determined by simulation of corresponding PBN-adducts are presented in Tables 1 and 2 –  $g$  factor, hyperfine constants  $A_N$  and  $A_H$  (resulting from the interaction of unpaired electron with the nuclear spins of nitrogen and hydrogen, respectively), width of the signals  $\Delta B$ .

We postulated previously [2] that the mobility of radical adducts related with particular components is strongly related with their localization in the starch structure of various degree of crystallinity [9]. Sharp signals, with well resolved hfs from nitrogen and hydrogen nuclei, are related with very mobile species, denoted as fast. These radicals, most probably formed by spin trap and very small fragments detached from polymer, are freely rotating in fully amorphous zones of the starch structure. More spatial components, denoted medium, are likely formed from various fractions of the polymer chains attached to PBN spin trap. They are also located in amorphous layers, but their motion is controlled by the adduct dimensions. The slow components, strongly limited in movement, are most probably situated in semi-crystalline layers, in a close proximity to crystalline lamellae, where the motion is controlled by the interaction with ordered phase and dimensions of the adducts.

The number of short-living radicals generated in Hylon VII and waxy maize diminishes after incorporation of phosphorus as mono- or distarch phosphates (Fig. 2, Table 1). Similar decrease was observed for native and phosphorylated maize starch [2]. It is noticeable that in the case of stable radicals opposite effect was found. It was namely shown that introduction of phosphorus into the starch structure facilitates abstraction of hydrogen atoms from the glucose unit which enhances generation of stable carbon radicals. The controversy concerning stable and short-living radicals may be explained assuming that though formation of both the types of radicals is facilitated in phosphorylated starch, but radical an-



**Fig. 1.** Experimental and simulated EPR spectra of the PBN-adducts of short-living radicals generated at 180°C in native Hylon VII and waxy maize. Upper inserts represent simulated components of the spectra and their contribution (in %) in the overall spectra.



**Fig. 2.** Effect of phosphorylation on the number of short-living radicals generated at 180°C in maize starch of various content of amylase.

nihilation occurs differently. Possibly, the short-living radicals recombine more rapidly in phosphorylated than in native samples whereas stable radicals do not exhibit such behaviour. This may be related with the fact that phosphorylated samples usually retain moisture [3] and it is well known that in the presence of water annihilation of radicals occurs more rapidly [1]. Evidently, higher mobility of short-living radicals compared to the stable ones causes more effective scavenging of the former in a humid medium.

Another interesting effect observed in the case of short-living radicals consists on differences in the values of the hyperfine structure (hfs) constants  $A$  for Hylon VII and waxy maize. The values of  $A$  for medium and slow components of the PBN-adducts are higher for Hylon VII than for waxy maize. Evidently, the interactions between unpaired electron and the nuclear magnetic moment of nitrogen atom are stronger in native and phosphorylated samples of Hylon VII than those of waxy maize, which may be caused by shorter distances between the carbon atom of glucose unit at which the unpaired electron is localized and the nitrogen atom of the spin trap. Such differences between starch containing mostly amylose with unbranched chains or amylopectin with branched skeleton may arise from steric reasons.

EPR parameters presented in Table 1 evidence also differences in dynamic properties of short-living radicals stabilized in Hylon VII or waxy maize matrices. The content of medium component is higher and the width of EPR signals (especially for medium and slow components) smaller for Hylon VII than for waxy maize, which indicates a higher mobility of PBN adducts in the Hylon matrix. This fact is in line with a lower value of degree of crystallinity (CD) calculated for Hylon VII as compared to that of waxy maize [4]. This parameter, measured as the ratio of the area of the crystalline reflections to the overall area of the X-ray diffraction patterns [11] amounts to about 18% for Hylon VII and its derivatives, whereas for waxy maize is about twofold higher.

It should be noticed, that all the observed effects became more pronounced after pretreatment of Hylon VII and maize starch with a high hydrostatic pressure (Table 2). This concerns differences in values of hfs constants as well as EPR parameters reflecting mobility of PBN adducts in appropriate matrices.

**Table 2.** EPR parameters of short-living radicals generated at 180°C in native and phosphorylated Hylon VII and waxy maize (AP) starch and in corresponding reference samples, after treatment with high hydrostatic pressure (650 MPa)

Sample	Total number of spins $N \times 10^{-16}$ (spins $\times$ g $^{-1}$ )	Center 1 (FAST)			Center 2 (MEDIUM)			Center 3 (SLOW)					
		Content (%)	$A_N$ (mT)	$A_H$ (mT)	$\Delta B$ (mT)	g factor	Content (%)	$A_N$ (mT)	$\Delta B$ (mT)	g factor	Content (%)	$A_N$ (mT)	$\Delta B$ (mT)
Native Hylon VII	5.15	2.1	1.48	0.28	0.20	2.008	56.8	1.03	0.42	2.005	41.1	2.50	0.63
Reference Mono Hylon VII	5.24	0.6	1.47	0.28	0.20	2.008	54.4	1.04	0.44	2.005	45.0	2.49	0.59
Mono Hylon VII	4.05	0.4	1.48	0.28	0.20	2.007	53.0	1.03	0.53	2.005	46.6	2.45	0.54
Reference Di Hylon VII	5.84	0.7	1.47	0.28	0.20	2.008	55.5	1.01	0.55	2.004	43.8	2.46	0.60
Di Hylon VII	3.92	1.1	1.47	0.28	0.20	2.008	53.8	1.03	0.47	2.006	45.2	2.45	0.57
Native AP	4.18	0.1	1.47	0.28	0.15	2.007	41.7	0.86	0.64	2.006	58.2	2.13	1.16
Reference Mono AP	3.54	0.3	1.47	0.27	0.18	2.007	37.3	0.79	0.65	2.006	62.4	2.08	1.11
Mono AP	3.88	0.4	1.50	0.28	0.20	2.007	38.3	0.85	0.51	2.006	61.3	2.17	1.18
Reference Di AP	3.70	0.4	1.47	0.28	0.25	2.007	39.2	0.87	0.66	2.006	60.4	2.15	1.19
Di AP	3.22	0.2	1.50	0.28	0.18	2.007	39.6	0.89	0.59	2.006	60.2	2.14	1.20

## Conclusions

The presence of three components in the EPR spectra of short-living radicals reflects correctly the “architecture” of the starch grains containing amorphous and semi-crystalline shells. Hylon VII composed mainly of simple, unbranched amylose chains represents a dynamic medium, easily infiltrated by the PBN spin trap. On the other hand, waxy maize – containing almost exclusively ramified amylopectin chains – offers more resistance against free rotation of radical adducts. Both types of radicals – the stable and short-living ones may be used as sensitive indicators of the changes occurring in the starch structure upon various chemical and physical treatments.

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