

Electron Paramagnetic Resonance as a tool for detection changes in the starch polymer occurring upon phosphorylation and high pressure treatment: A review

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Abstract

Modification by chemical and physical methods is widely used to improve features of the starch polymer important for industrial applications. In this review it was shown, that thermally generated radicals studied by Electron Paramagnetic Resonance (EPR) spectroscopy may serve as sensitive detectors of the changes occurring in the polymer structure and properties upon modification. Results obtained by using two types of radicals, relatively stable and short-lived ones, and two methods of modification, phosphorylation and high pressure treatment, were presented. Significance of the following experimental factors: optimal heating parameters, correctly prepared reference samples, sufficient amount of modifying agent, controlled moisture on the effectiveness of this method was evidenced. The proposed approach enables distinction between two types of phosphorus bonded as terminating or bridging polymer chains. Moreover, EPR data of the short-lived radicals reflect correctly the starch granule architecture and its changes caused by applied treatment.

Key words: Electron paramagnetic resonance, modified starch, stable radicals, short-lived radicals, phosphorylation, high pressure treatment, thermal treatment

1. Introduction

Starch is, after cellulose, the second most abundant carbohydrate polymer in the biosphere. Starch is composed of α -D-glucose units forming highly branched chains of amylopectin and linear, non-ramified chains of amylose. These two types of chains are arranged in the starch granule in form of concentric amorphous and semicrystalline layers [1]. Starch is produced by plants as a bio-renewable and bio-degradable raw material for numerous types of industry. It is consumed mainly by food industry, but finds also application in pharmaceutical, paper, textile, foundry and cosmetic industries. Starch as well as cellulose are widely applied to purify the industrial sewages.

Specific starch functionalities important for a given type of industry may be improved by modification of the native starches with chemical and physical methods [2]. Chemical modification consists on hydrolysis, oxidation and esterification. Physical modification is realized by heating at elevated temperatures, freezing, irradiation, high-pressure and mechanical treatment. These processing improves certain features of the starch important for a given type of the industry, e.g. gelatinization, pasting behavior and rheological properties of the food products or ability to bio-degradation of package materials. Such treatments cause partial depolymerization of the starch and diminishing of its thermal stability. In consequence, modified starches may easier undergo thermal destruction occurring with formation of radicals [3].

At the beginning of this century the team at the Faculty of Chemistry, Jagiellonian University, started a research program based on using thermally generated radicals as indicators of the changes occurring in the starch structure and properties upon modification. Two types of radicals were studied: relatively stable [3] and recently discovered in starch

short-lived ones [4-6]. EPR spectroscopy, a very selective and highly sensitive technique, suitable for identification of various types of radicals and quantitative determination of their number [7, 8], was used as a basic method of investigation.

Results obtained for starches treated by a variety of modification methods were concisely presented in a review [9]. In the present work the attention was focused only on phosphorylation and high pressure treatment as representatives of chemical and physical methods of modification. Both of the mentioned treatments have a significant influence on starch functionality. Incorporation of phosphate groups into the starch matrix increases the hydration capacity of the starch [10] and improves its gelatinization and pasting properties [11]. It is also beneficial for human diet because of increasing content of resistant starch [12]. Pretreatment with high hydrostatic pressure may evoke significant changes in the starch crystallinity [13, 14]. The results concerning relatively stable and short-lived radicals, summarized in the present review, provide valuable information about response of the starch matrix to both applied treatments.

Native starches of various botanical origin (potato and maize starch), maize starch with different amount of amylopectin and amylose and starches modified by a chemical (phosphorylation) or physical (high pressure) method were investigated.

The performed studies provided information concerning mechanism of thermal generation and features of the radicals: their number, properties, lifetime and reactivity, strongly related with the thermal stability of the starch. Data on zones of various ordering degree were also available.

2. Experimental

2.1. Materials

Maize starches with various contents of amylose and potato starch were investigated. Normal maize contains 75% of amylopectin, waxy maize 98% of amylopectin, while Hylon VII 32% of amylopectin and rest of amylose. Native potato and maize starches, as well as waxy maize were purchased from Sigma. Hylon VII was supplied by National Starch & Chemical, Food Starch, Poland. The native starches were modified by phosphorylation and high pressure treatment.

Phosphorylation was performed according to Lim & Seib [15] and Sang & Seib [16]. To obtain monostarch phosphates the native starches were added gradually to the aqueous solution containing trimetaphosphate/ sodium tripolyphosphate (STMP/STPP) and Na_2SO_4 at initial $\text{pH} = 6$ (obtained with 0.2 M HCl). The suspension was then mixed at room temperature for 1 hour and afterwards evaporated at temperature $45 - 50^\circ \text{C}$ until content of water was equal $15 - 20\%$. The obtained material was heated at 130°C for 2 h. After cooling the cake was grounded, mixed with distilled water and pH was measured. Then the suspension was centrifuged and distilled water was added to the solid residue. After mixing the pH value was adjusted to 6.5 with 0.2 M solution of NaOH. Washing and centrifugation was repeated three times and finally the product of phosphorylation was dried at 50°C during 20 h.

Similar procedure [15, 16] was applied to obtain distarch phosphates with the only difference consisting on the initial value of $\text{pH} = 11$ (obtained with 0.2 M NaOH) of the STMP/STPP solution.

Reference samples for phosphorylated starches were obtained by the same method as applied for starch transformed into mono- and distarch phosphates but without adding the phosphorylating agent STMP/STPP. Reference samples were used to extract from the experimental data

the influence of modification agent itself on starch structure and properties.

2.2. Methods

2.2.1. Determination of the phosphorus content

Phosphorus content was determined according to Polish norm PN-EN ISO 3946. Samples of phosphorylated starch were mineralized with the concentrated nitric (V) and sulphuric(VI) acids. Orthophosphoric acid formed during mineralization reacted with ammonium molybdate $[(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}]$ giving a complex of molybdenum(VI) $([\text{P}^{\text{V}}\text{Mo}^{\text{VI}}_{12}\text{O}_{40}]^{3-})$, which afterwards was reduced with ascorbic acid to a deep blue complex of Mo(V). The absorbance of the blue solution was measured at wavelength 680 nm with a Spektralfotometer SPECOL 11 produced by C. Zeiss (Jena, Germany). The content of phosphorus was determined by comparing the absorbance values of the investigated solutions with calibration curves. The maximum error of determination, equal to 10%, was calculated as a standard deviation from 3 to 4 independent analytical results.

2.2.2. Pressure treatment

Pressure treatment was performed using 30% (w/v) starch-water suspensions closed in teflon tubes, precisely mixed, deaerated and sealed [13]. For pressurization a press type LV30/16, produced by The Centre of High Pressure Analysis, Polish Academy of Sciences, Warsaw, Poland was used. The teflon tubes were put into a high pressure chamber filled with pressure-transmitting medium, which also minimized adiabatic heating. The samples were treated with pressure at 650 MPa for 9 min. The time of reaching the working pressure was 120 s. The temperature inside the pressure chamber averaged $20 \pm 2^\circ \text{C}$. Pressurized starch pastes were dried at 50°C to constant weight. The dry samples were pulverized with an agate mortar. The non-phosphorylated starch samples, used as

references, and phosphorylated ones were treated with pressure in the same way.

2.1.3. Thermal treatment

EPR spectra were recorded on native and modified starch samples before and after thermal treatment. Starch samples of about 30 mg were placed in EPR quartz tubes of inner diameter equal to 3 mm. The heating parameters for generation of relatively stable radicals were: temperatures 210° C and 230° C and time equal to 30 min. The short-lived radicals were generated by heating of the starch at 180° C for 30 min. The number of thermally generated radicals in modified starches was compared to that obtained for appropriate reference samples.

2.1.4. EPR technique

EPR spectra were measured in X-band (at about 9.5 GHz) with an ELEXSYS 500 spectrometer and in Q-band (33 GHz) with an ELEXSYS E 580 FT spectrometer. Both spectrometers produced by Bruker (Karlsruhe, Germany) were operating at modulation frequency 100 kHz, modulation amplitude 0.3 mT and microwave power 0.3 – 30 mW. The EPR spectra were recorded at temperatures from -196° C to +87° C. The number of spins was determined by comparison of the integral signal intensity of the investigated samples with that of the standard containing known amount of paramagnetic centres. $\text{VO}(\text{SO}_4)_2 \cdot 5\text{H}_2\text{O}$ diluted with diamagnetic K_2SO_4 (5×10^{19} spins/g) was used as a primary standard. All necessary precautions, discussed in papers [7, 8] were followed in order to assure good precision of the quantitative EPR measurements. EPR parameters of the radicals were determined by a simulation procedure using the program EPR SIM 32 [17]. The accuracy of

determination of g factor values was equal to ± 0.001 and that of hyperfine structure (HFS) constant A amounted ± 0.05 mT.

Complementary methods of the study were: X-ray diffraction, differential scanning calorimetry, optical microscopy and measurements of the molecular weight distribution. The experimental details concerning these methods were described in the cited references.

3. Generation of relatively stable radicals

The native non-processed and non-modified starches did not exhibit any EPR signal. In this work thermal treatment of the starch was used to generate radicals. An important condition for successful application of the proposed approach was determination of the optimum heating parameters for the polymer, assuring creation of the significant number of radicals registered by EPR spectroscopy, but not evoking considerable damage of the starch structure. Three methods were applied to accomplish this aim: EPR spectroscopy, X-ray diffraction and measurements of the molecular weight distribution [18]. Native potato starch was heated at four sets of heating parameters (210° C, 30 min.; 210° C, 2 h; 230° C, 30 min.; 230° C, 2 h).

The X-ray diffraction data revealed that the relative crystallinity of native starch was equal to 25%. After thermal treatment at three first sets of the heating parameters moderate lowering of the crystallinity to 16% was observed. However, prolongation of the heating time at 230° C to two hours led to the drastic decrease of the crystallinity value, i.e. to the considerable damage of the starch structure.

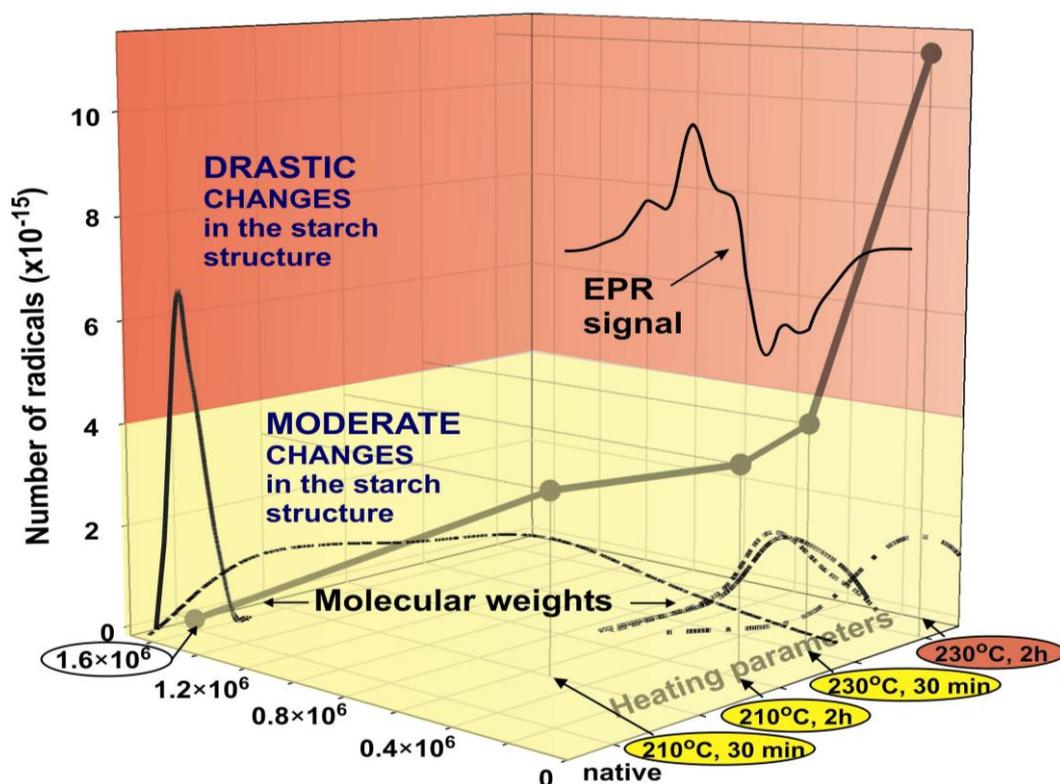


Fig. 1. Effect of thermal treatment on molecular mass distribution and the number of relatively stable radicals generated in potato starch.

Results of EPR spectroscopy and measurements of molecular weights are presented in Figure 1. Distribution of molecular weights in the native starch represents a sharp maximum at about 1.6×10^6 g/mole. At the lowest heating parameters flattening of the peak occurred indicating fragmentation of the polymer. The mean value of molecular weights was still of the order 10^6 g/mole. After longer time of heating (2 h) at 210°C or higher temperature (230°C , 30 min.) two overlapping maxima appeared at 4×10^5 g/mole. On the other hand, after 2 hours of heating at 230°C maximum at value of the order 10^4 g/mole evidenced significant lowering of the molecular weights.

Total number of radicals increased with increasing temperature and prolongation of the heating time (Fig. 1). The increase was moderate for the samples treated by three first sets of heating, however, it was dramatic in the case of the highest heating parameters, for which XRD data evidenced drastic decrease in

crystallinity while the measurements of weights distribution shown significant diminishing of the molecular size. This means, that optimum thermal parameters, sufficient for obtaining measurable effects in the EPR spectroscopy without excessive damage of the starch structure are: temperature not exceeding 230°C and heating time not longer than 30 min [18].

After heating of the starch in the range of parameters determined as optimum a complex anisotropic EPR spectrum appeared with $g_{av} = 2.006$. The presence of two components related with radical I and radical II was evidenced by simulation of the spectrum [17] registered at different frequencies (band X and Q) and various microwave power levels [19]. One of the components (radical I) exhibits two lines of hyperfine structure (HFS), another one (radical II) represents a single isotropic line. Mechanism of thermal generation of the radicals I and II was proposed (Fig. 2) [19].

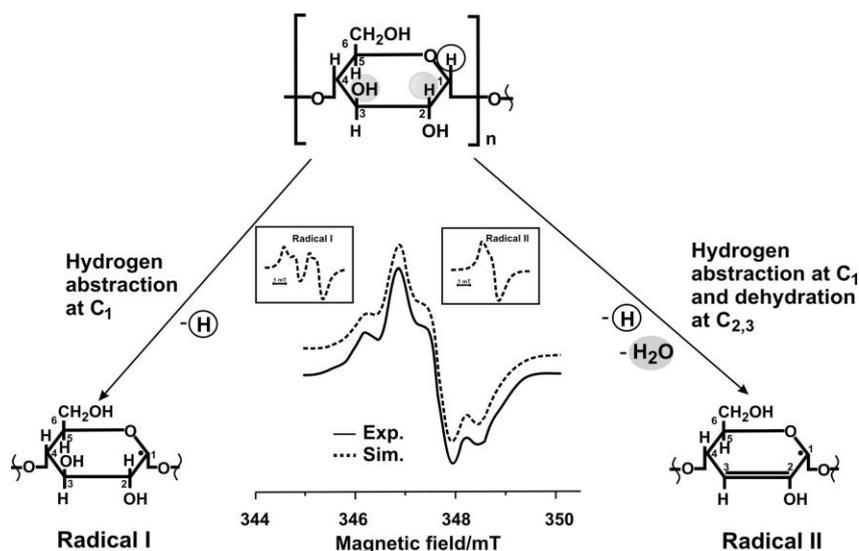


Fig. 2. Mechanism of the thermal generation of relatively stable radicals in starch.

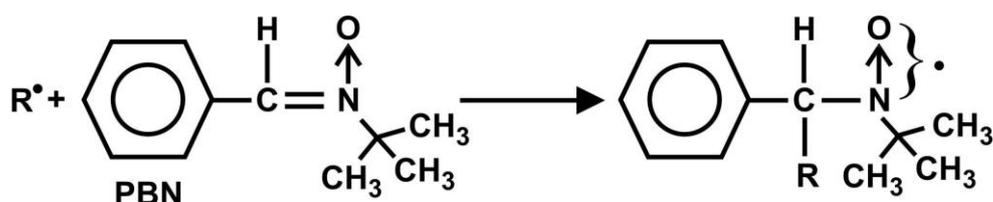
Radical I is formed by dehydrogenation of glucose unit at C₁ atom. EPR signal of this component exhibits hyperfine structure due to the interaction of unpaired electron localized at C₁ with nuclear spin of hydrogen ($I = \frac{1}{2}$) bonded to carbon atom C₂. Generation of radical II requires simultaneous dehydrogenation at C₁ and dehydration. The abstracted water molecule may originate e.g. from the hydrogen atom at C₂ and OH group at C₃. This process eliminates from the structure hydrogen atom responsible for appearance of HFS in signal of component I. Total number of radicals informs about the ability of the starch to dehydrogenate, whereas ratio of the amounts of particular components (I : II) about dehydration properties of the polymer.

Existence of two-component spectra of thermally generated relatively stable radicals was confirmed by Krupska et al. [20]. These authors report also that one of the components reveals hyperfine structure. Łabanowska et al. [21] postulated the presence of three additional components in the EPR spectrum of thermally treated starch, two of them centered at the carbon atom C₆. However, attribution of these additional constituents

– not exceeding 10% of the total amount of radicals – was not confirmed by measuring of the EPR spectra at various frequencies and power levels. Another attempt to interpret the complex EPR spectrum of radicals generated thermally in bread produced from rye and wheat starches was proposed by Yordanov and Mladenova [22]. The authors claim that the overall EPR signal is composed of three different lines but they did not ascribe them to concrete paramagnetic species.

4. Generation of short-lived radicals

To check, if besides relatively stable radicals the short-lived ones may be generated thermally in starch the experiments with a spin trap, i.e. *N-tert-Butyl- α -phenylenitron* (PBN) were performed on native and phosphorylated maize starch with various content of amylose [4-6]. To avoid thermal decomposition of PBN the samples were heated at relatively low temperature (180° C). The EPR spectra revealed that the short-lived radicals ($R\cdot$) were created and stabilized as adducts with the PBN spin trap according to reaction:



The adducts with PBN were stable at room temperature for several days. Number of these radicals was of the order of 10^{16} spins/g. The EPR spectra of the PBN-adducts were complex (Fig. 3). Their average g factor was equal to 2.006. To check if the spectra originate from only one type of radicals or from several radical species, registration of the spectra was performed at different frequency bands (X and Q), different power levels and various temperatures. The simulation [17] revealed the presence of three components differing in EPR parameters and denoted: fast, medium and slow (Fig. 3) [5, 6]. This notation was related with the mobility of particular radical species reflected by the shape of their EPR signals.

The mobility of the adducts is determined by their spatial dimensions and location in zones of various degree of crystallinity [23]. This feature can be quantified by the rotational correlation time τ , i.e. period of time during which a molecule maintains its spatial orientation [24]. The rotational correlation time was estimated as being equal to picoseconds

for the fast component and to nanoseconds for the slow one. Similar components were found by simulation of the EPR spectra of short-lived radicals generated in native and phosphorylated maize starch, Hylon VII and waxy maize [5, 6].

Sharp signals, with well resolved HFS from nitrogen and hydrogen nuclei, were assigned to very mobile species, denoted fast. These radicals, most probably formed by a molecule of spin trap and very small fragments detached from polymer, were freely rotating in fully amorphous zones of the starch structure. More spatial components, named medium, were probably constructed from bigger fractions of the polymer chains attached to PBN spin trap. They are also located in amorphous regions of the starch structure, but their mobility was restricted by the adduct dimensions. The slow components, most distinctly limited in movement, could be situated in a close proximity to crystalline lamellae, where the motion is controlled by the interaction with ordered phase and considerable dimensions of the adducts.

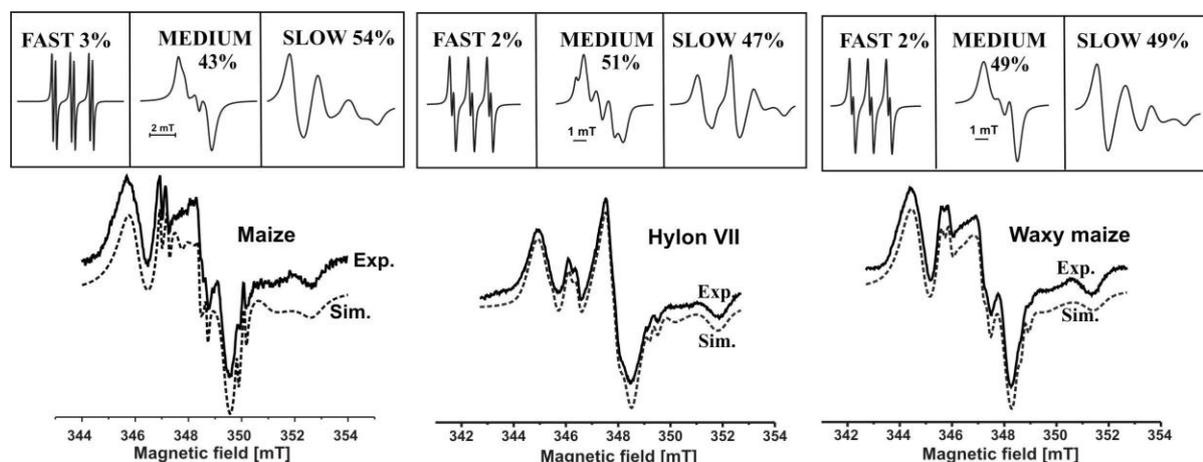


Fig. 3. EPR spectra of the PBN adducts of short-lived radicals generated at 180°C in native maize, Hylon VII and waxy maize. Upper inserts represent particular components [5, 6].

Additional information on the mobility of PBN adducts provided measurements of EPR spectra of maize starch as a function of temperature in the range from -196°C to $+87^{\circ}\text{C}$ [5]. Data in Table 1, obtained by simulation of the EPR spectra [17], and Figure 4 show considerable increase of ΔB values indicating broadening of EPR signals at low temperature. The effect is especially significant for fast component. In this case the splitting caused by HFS interaction with hydrogen disappears and only three lines responsible for HFS splitting by nitrogen are visible in the insert to Fig. 4. Moreover, due to the fact, that ΔB value of fast component augments at -196°C stronger than that of the other constituents, the increase of the relative contribution of fast component to the total content of radicals is also the most significant.

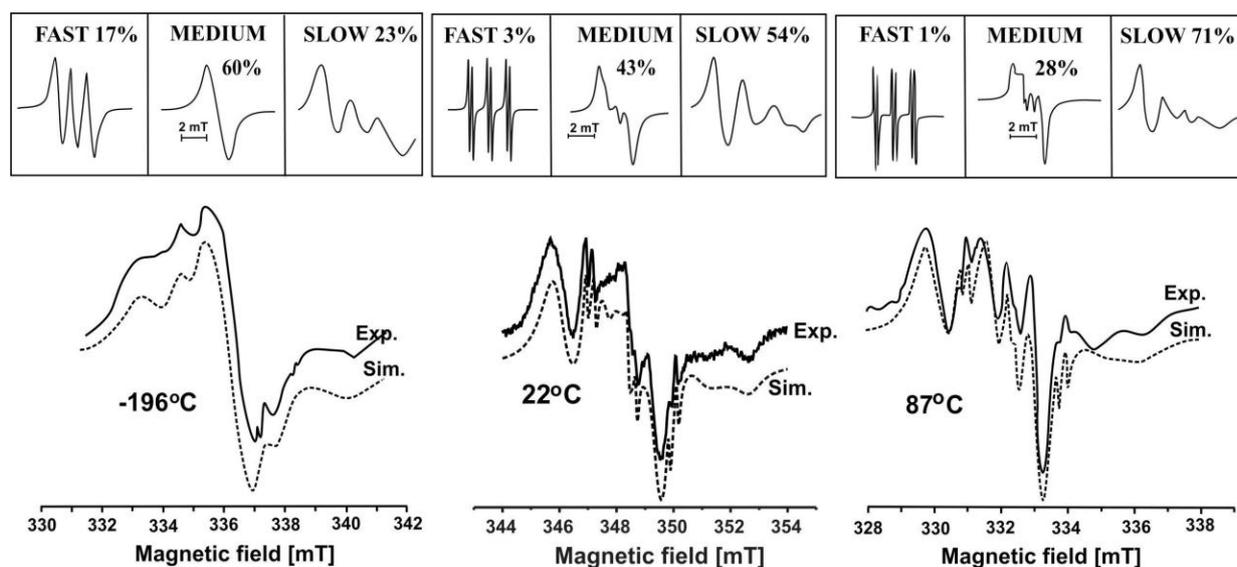


Fig. 4. Temperature dependence of the EPR spectrum of PBN adduct with short-lived radicals generated in native maize starch at 180°C [5].

Increasing temperature from -196°C to $+87^{\circ}\text{C}$ causes sharpening of the signals of all components related with the increase of rotational ability of the adducts. Such changes in mobility at higher temperatures result in diminishing of the adducts anisotropy. Another consequence of the increasing temperature is the fact that the relative content of the slow component increases, at the expense of fast and medium ones (Table 1, Fig. 4). The observed effect may indicate that at higher temperatures semicrystalline zones in the starch matrix became more accessible for radical adducts. Another

explanation is that more mobile fast and medium species annihilate easier at higher temperatures than slow one situated in proximity of better ordered zones which stabilize them.

Different behavior of the EPR signals of the adducts of short-lived radicals with PBN upon changes of temperature firmly supports interpretation of their EPR spectra as an envelope of three different signals. The appearance of the medium and slow components reflects accurately the architecture of the starch granules described in review article by Perez & Bertoft [1].

Table 1. Influence of registration temperature on EPR parameters of PBN adducts with short-lived radicals generated in native maize starch at 180° C [5].

Temperature of registration	Signal components					
	FAST		MEDIUM		SLOW	
	Content [%]	ΔB [G]	Content [%]	ΔB [G]	Content [%]	ΔB [G]
-196° C	17	5.0	60	7.6	23	12.7
+22° C	3	1.7	43	4.9	54	7.3
+37° C	2	1.7	42	4.9	56	7.3
+57° C	2	1.6	33	4.2	65	7.3
+72° C	1	1.3	32	3.4	67	6.8
+87° C	1	1.1	28	3.4	71	6.9

Similar accessibility of the whole starch matrix for the PBN spin trap at room temperature is strongly evidenced by almost equal distribution of the particular components between zones of different degree of crystallinity: 46% of fast & medium adducts occupying in native maize starch amorphous region and 54% of slow adducts are located in semicrystalline zone (Fig. 3) [5]. This situation changes with temperature. At low temperature localization of adducts in amorphous regions is more effective whereas higher temperatures enable easier penetration of the well-ordered zones by radical adducts. Certain role may also pay faster annihilation of mobile radicals at higher temperature.

5. Effect of starch modification studied by relatively stable and short-lived radicals

5.1. Relatively stable radicals as indicators of the phosphorylation

Phosphorylation of the starch with STMP/STPP (sodium trimetaphosphate/sodium tripolyphosphate) [15, 16] leads to the formation of mono- or distarch phosphates with the PO_4^{3-} groups terminating or bridging the polymer chains (Fig. 5A). As mentioned in Section 1, phosphorylation of the starch improves some of its functional properties [10-12].

Influence of temperature on the number of radicals generated thermally in native and phosphorylated starches is

presented in Figure 5. Potato (Fig. 5B) [25] and maize starches with various content of amylose (Fig. 5B, C) [5, 26] were investigated. (Normal maize contains about 25% of amylose, waxy maize only 2 %, while Hylon VII 68%). The results for modified starches are presented jointly with those obtained for reference samples prepared by the same procedure as applied for native starches transformed into mono- and distarch phosphates, but without adding the phosphorylating agent [27].

All reference samples revealed greater number of radicals than the corresponding native starches. Evidently, the basic (pH = 11) or acidic (pH = 6) medium applied during phosphorylation of the starches [15, 16] damaged to certain extend the structure. However, the greatest number of radicals, i.e. the strongest lowering of thermal stability was observed at both temperatures (210° C and 230° C) in phosphates. This effect was in most cases more pronounced for monostarch than for distarch derivatives containing the same amount of phosphorus. The difference in the number of radicals between mono- and distarch phosphates was observed for potato (Fig. 5B) and waxy maize (Fig. 5C) starches. In the case of Hylon VII recalculation of the number of radicals in monostarch phosphate on the same amount of phosphorus as it was in distarch phosphate, i.e. 0.4 weight % gave also higher value for mono derivative.

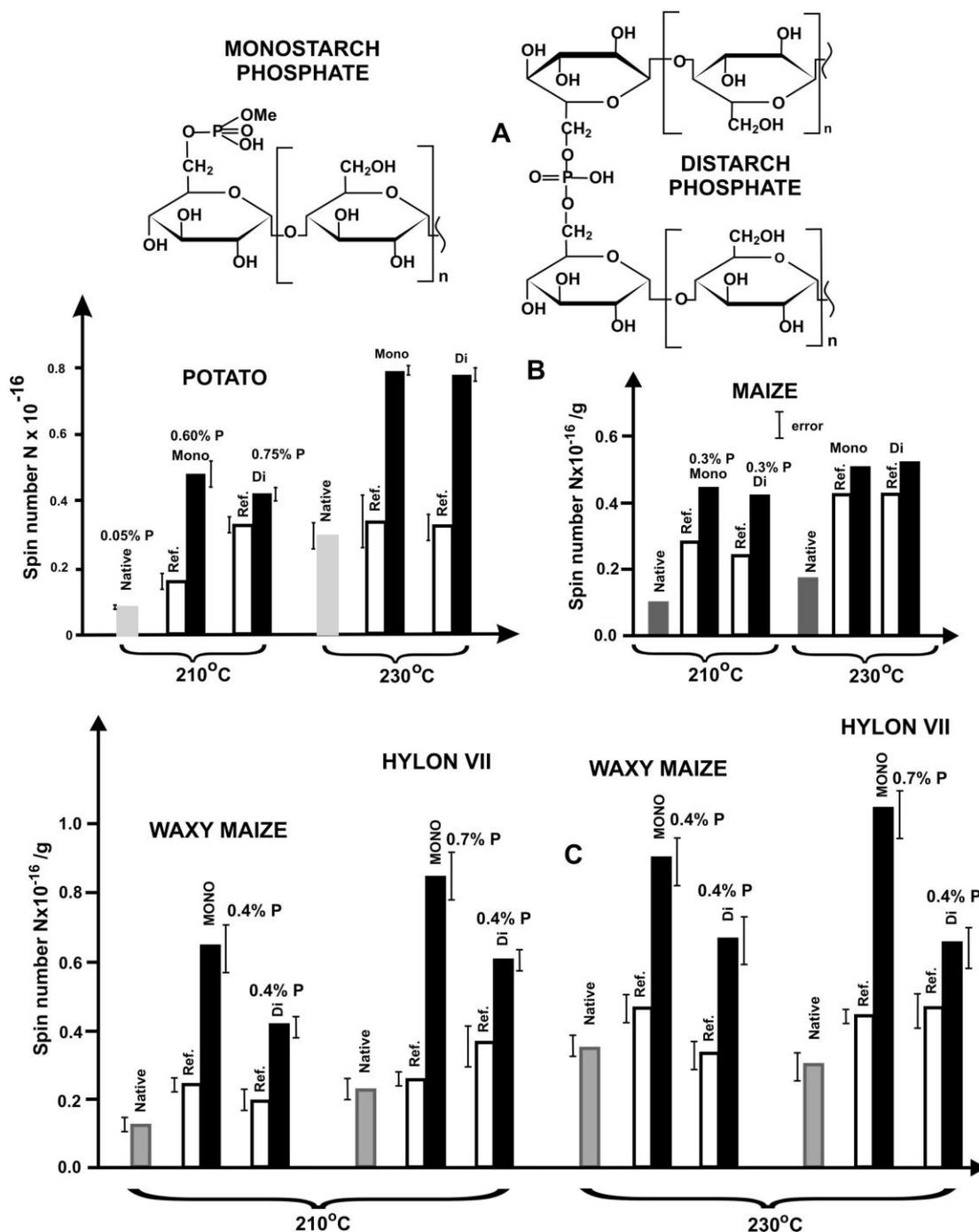


Fig. 5. Effect of phosphorylation on the number of relatively stable radicals generated thermally in starches of various botanic origin and various amylose content [5, 25, 26].

Only maize starch with natural amount of both components (Fig. 5B) did not revealed any difference between two types of phosphates. The lack of the effect may be related with small amount of phosphorus (0.3%) incorporated into the maize structure, in spite of the same for all starches synthesis conditions. The reason of the different behavior of the maize starch may be its dense structure,

typical for representatives of corn starches [1], which accommodate limited amount of phosphorus.

In the case of potato starch and waxy maize the effect was more pronounced for samples heated at lower temperature (Fig. 5B). Equalization of the number of radicals in mono- and distarch phosphates after treatment at 230° C clearly indicates that at this temperature

dominating role in generation of radicals plays thermal activation, whereas the influence of the type of bonding phosphorus became negligible.

It may be thus concluded, that the number of radicals generated thermally in phosphorylated starches is influenced by several factors. The most important is type of bonding the PO_4^{3-} groups: terminating or bridging the polymer chains. Higher thermal stability of the samples containing phosphorus at bridging positions is most probably related with increasing cross-linking of the distarch phosphates, which is in line with the calorimetric data [25]. To significant factors belongs also botanic origin of the starch, evidenced by the behavior of maize starch. On the other hand, similar results obtained for waxy maize and Hylon VII indicate, that relative content of amylose to amylopectin seems to be a less important factor. On the other

hand the role of reference samples should be highly appreciated. The influence of phosphorus itself on thermal stability of the investigated starches could be extract from the experimental data only by comparing results obtained for phosphorylated starches with those of the thoroughly prepared reference samples [27]. Very important is also finding optimal parameters for performing the experiment e.g. temperature of radicals generation, which may decide about possibility to reveal the effect.

5.2. Short-lived radicals as indicators of the phosphorylation

The total number of short-lived radicals generated in native maize starch, Hylon VII and waxy maize diminishes after incorporation of phosphorus as mono- or distarch phosphates (Fig. 6) [5].

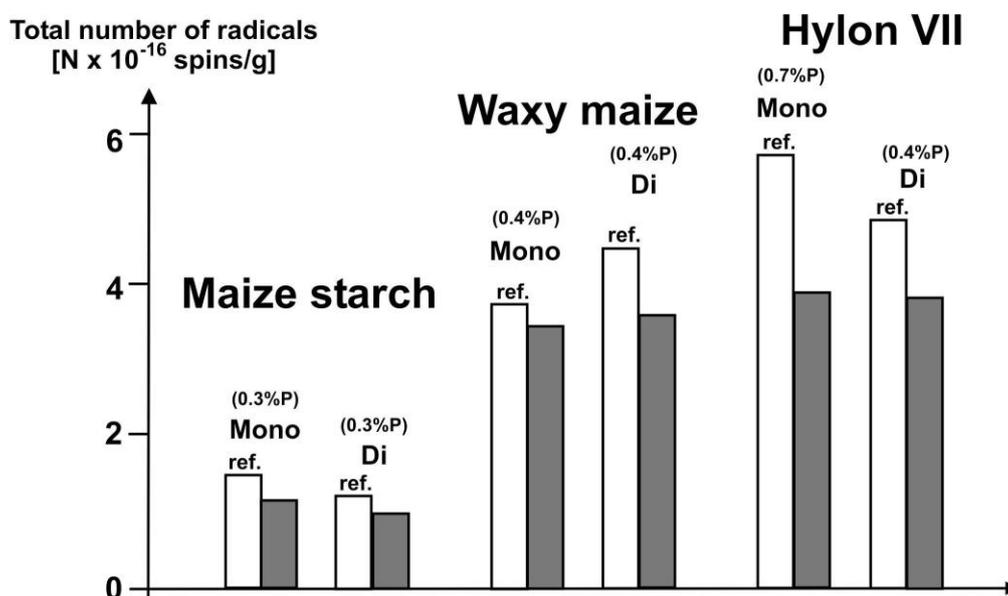


Fig. 6. Effect of phosphorylation on the number of short-lived radicals generated at 180°C in maize starch of various content of amylose [5].

It is noticeable, that in the case of relatively stable radicals opposite effect was observed. Most probably the short-lived radicals recombine more rapidly in phosphorylated than in native samples whereas stable radicals do not exhibit such behavior. The observed effect may be

related with the fact that phosphorylated samples usually retain moisture [10] and it is well known that in the presence of water annihilation of radicals occurs more rapidly [3, 28]. It may be assumed, that higher mobility of short-lived radicals compared to the stable ones facilitates

more effective scavenging of the former in a humid medium.

Interesting effects are related with some parameters of the EPR spectra of the radical adducts with PBN [6]. The following parameters for Hylon VII and waxy maize determined by simulation [17] are presented in Table 2: the hyperfine structure constants A_N and A_H , width of

the EPR signals (ΔB) and relative content of particular components. The values of A_N for medium and slow components of the PBN adducts are higher for Hylon VII than for waxy maize. Evidently, the unpaired electron density at nitrogen atom is more significant in native and phosphorylated samples of Hylon VII than in waxy maize.

Table 2. EPR parameters of short-lived radicals generated in native and phosphorylated Hylon VII and waxy maize [6].

Starches	Signal components									
	FAST				MEDIUM			SLOW		
	Content [%]	A_N [G]	A_H [G]	ΔB [G]	Content [%]	A_N [G]	ΔB [G]	Content [%]	A_N [G]	ΔB [G]
Native Hylon VII	1.5	14.8	2.8	2	51	10.1	4	47	24.2	5
Monostarch phosphate	1.0	14.5	2.8	2	53	9.8	7	46	24.5	6
Distarch phosphate	0.5	14.3	2.8	2	54	10.1	7	46	24.4	6
Native waxy maize	2.2	14.6	2.8	2	49	9.3	7	49	23.4	9
Monostarch phosphate	1.8	14.8	2.8	2	41	8.5	5	57	21.9	11
Distarch phosphate	2.1	15.0	2.7	3	46	8.9	5	52	23.8	10

This effect may be caused by stronger interaction between negatively charged OH groups in starch polymer and the N-O group of the spin trap at which the unpaired electron is localized. Such differences between Hylon VII containing mostly amylose with unbranched chains and waxy maize with branched skeleton may arise from steric reasons. This supposition is confirmed by additional pretreatment of the phosphorylated samples with high pressure what will be discussed in Section 5.6.

EPR parameters presented in Table 2 evidence also differences in dynamic properties of short-lived radicals stabilized in phosphorylated Hylon VII or waxy maize matrices. The relative contents of medium component are higher for Hylon VII than for waxy maize while the contents of slow component higher for waxy maize than those of Hylon VII. Moreover, width of EPR signals (ΔB) for slow component is smaller for Hylon VII which indicates a higher mobility of PBN adducts in the Hylon matrix. These facts are in line with a lower value of degree of

crystallinity (CD) reported for Hylon VII as compared to that of waxy maize [26]. It may be thus concluded that phosphorylation of Hylon VII is more efficient in amorphous zones of the starch granules.

5.3. Relatively stable radicals as indicators of pressurization

Treatment of the starch with high hydrostatic pressure influences the degree of its crystallinity the effect being dependent on relative content of starch components, i.e. on the amylose to amylopectin ratio [13, 14]. Study of pressured Hylon VII and waxy maize revealed that the type of thermally generated relatively stable radicals in previously pressurized samples was the same as in the native ones [4]. Figure 7 presents influence of high hydrostatic pressure on total number of radicals. In non-pressured samples in the temperature range 210°C – 230°C higher number of radicals was generated in waxy maize than in Hylon VII. This suggests that amylopectin is less resistant to thermal

degradation than amylose [4]. The number of radicals drastically diminished after pressurization of both: Hylon VII and

waxy maize, the effect being also more distinct in the case of waxy maize.

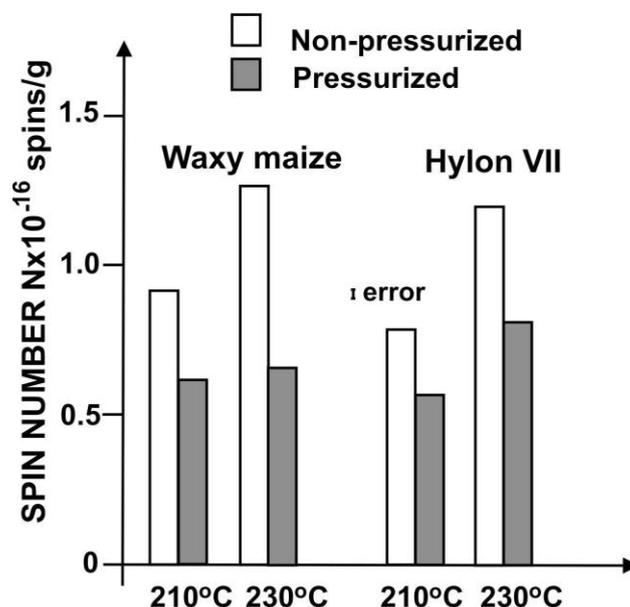


Fig. 7. Influence of high pressure treatment on the number of radicals generated at 210° C and 230° C in waxy maize and Hylon VII.

Most probably the effective reduction of the content of radicals upon pressurization was due to the water molecules present in the starch-water suspension which penetrated interior of the starch granules during treatment with the high hydrostatic pressure. It is known from literature, that presence of water accelerates disappearance of radicals generated in starch by irradiation [28] or by heating at elevated temperature [3]. Effect of the content of moisture in Hylon VII and waxy maize on the number of thermally generated radicals reported in paper [4] speaks in favor of the above presented reasoning.

Pretreatment with high pressure resulted also in diminishing the relative content of the radical I component (see section 3), which indicates easier

abstraction of hydrogen situated at C₂ in pressurized samples.

5.4. Short-lived radicals as indicators of pressurization

Figure 8 shows that after pressurization of waxy maize and Hylon VII the total intensity of the spectra is considerably lower than that observed in the case of starches non-treated with high hydrostatic pressure [4]. This effect is similar to that observed for relatively stable radicals (Fig. 7) but for short-lived ones is more significant. The reduction of the number of radicals is most probably due, similarly as in the case of radicals generated in the absence of PBN, to the water molecules squeezing into the starch granules upon high pressure.

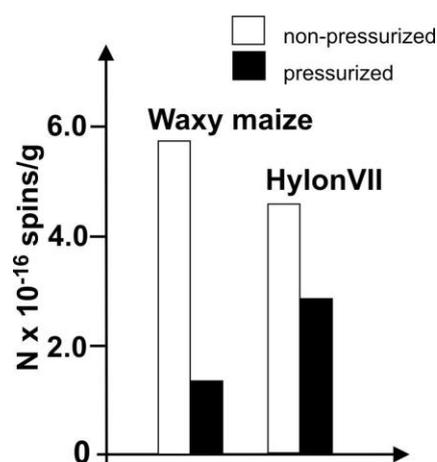


Fig. 8. Effect of high pressure treatment on the number of short-lived radicals generated in waxy maize and Hylon VII by heating at 180° C in the presence of PBN spin trap [4].

5.5. Relatively stable radicals as witnesses of the consorted influence of phosphorylation and high pressure treatment

Very informative was experiment with potato starch and maize starches containing different amount of amylose which were treated by three-step modification: phosphorylation followed by pressurization and heating [25, 26, 4]. Two consecutive modifications—phosphorylation and application of the high pressure – changed in an opposite direction the number of relatively stable thermally generated radicals: phosphorylation led to increasing number of radical species while pressurization caused reduction of this number.

As shown in Section 5.1., effect of phosphorylation was much less significant in distarch than in monostarch phosphates, due to cross-linking stabilizing the structure of distarch phosphates. Effect of both simultaneously applied treatments was also smaller for distarch than for monostarch phosphates.

From the data presented in Figure 9 it results, that in the case of the consorted treatment the most spectacular effects appear in waxy maize and Hylon VII. Decrease in the number of radicals upon pressurization is in monostarch phosphates much more significant (Fig. 9) than in native samples (Fig. 7). Also difference between two types of phosphates is clearly visible. Evidently, cross-linking plays in this case a significant role. Normal maize reveals very effective diminishing of the radical number upon pressurization, but mono- and distarch phosphates behave similarly. It should be remembered that, as noticed in Section 5.1., in maize phosphates the amount of phosphorus is smaller than in other starches presented in Figure 9. In potato starch difference between mono- and distarch phosphates is quite distinct, however, effect of pressurization vanishes. Here again, similarly to waxy maize and Hylon VII, influence of phosphorylation is dominant.

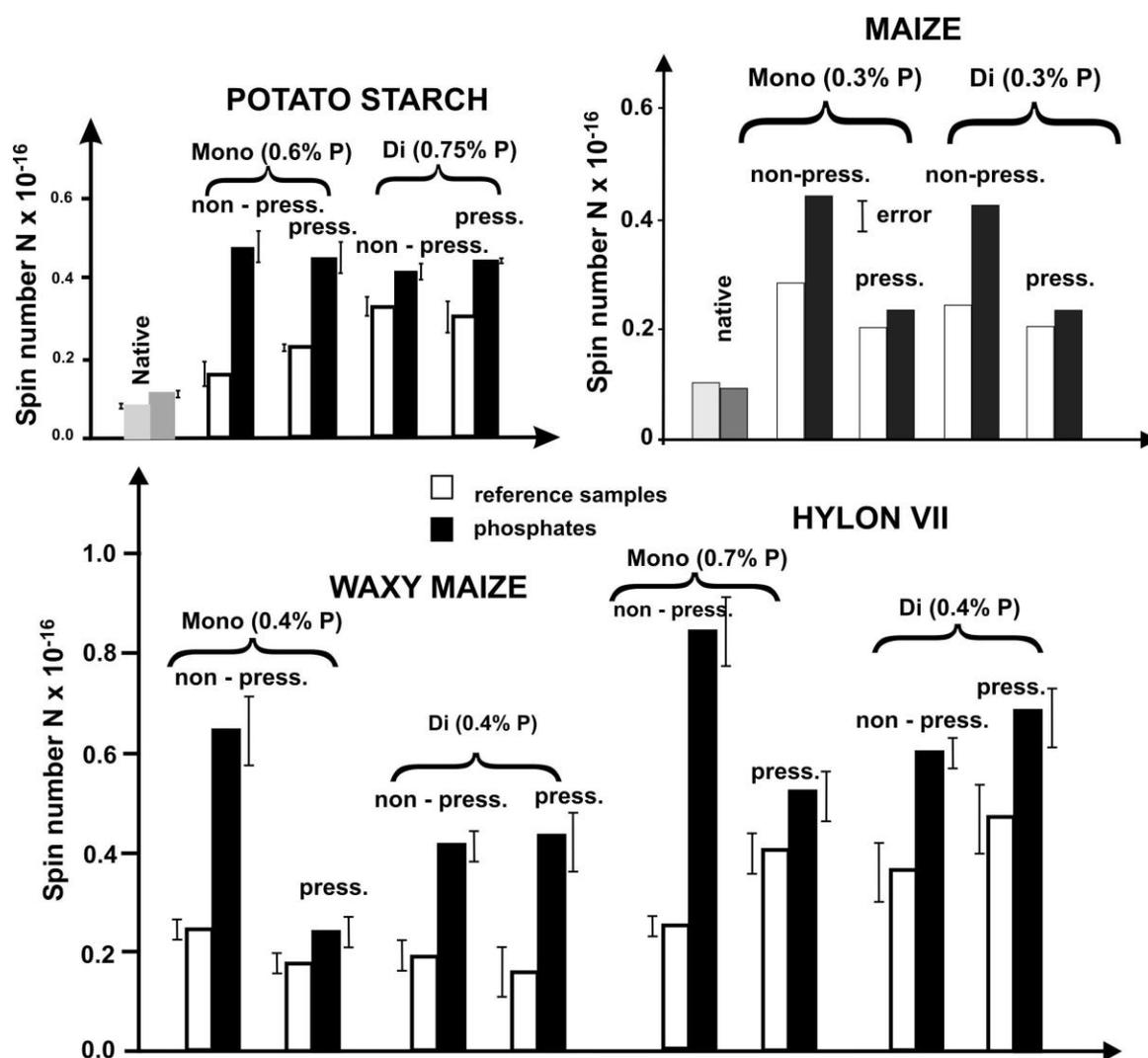


Fig. 9. Effect of multi-step modification (phosphorylation followed by high pressure treatment and heating at 210°C) on the number of radicals generated in starches of various botanic origin (potato [25] and normal maize [5]) and of various content of amylose [26] (normal maize, waxy maize and HylonVII).

It may be thus concluded, that the response of the relatively stable, thermally generated radicals on the applied trifold treatment depends on ability to reinforce crosslinking which most probably is determined by the amount of incorporated phosphorus. Botanic origin and relative content of starch components (amylose: amylopectin) seem to be less important factors. These conclusions are based on the following facts:

- the greatest difference between mono- and distarch phosphates was observed in the case of waxy

- maize, Hylon VII and potato starch, i.e. in samples of various botanic origin and very different amylose to amylopectin ratio,
- normal maize differs in behavior from waxy maize and Hylon VII in spite of the same biological origin and a mean value of the amylose to amylopectin ratio,
- lack of difference between mono- and distarch phosphates occurs in samples containing smallest amount of phosphorus.

5.6. Short-lived radicals as witnesses of the consorted influence of phosphorylation and high pressure treatment

All the effects related with EPR values of short-lived radicals, discussed in Section 5.2. became more pronounced after pretreatment of phosphorylated Hylon VII and maize starch with a high hydrostatic pressure (Table 3) [6]. Electron density at nitrogen atom is after pressurization still more significant in

native and phosphorylated samples of Hylon VII than in waxy maize. The same concerns differences in dynamic properties of short-lived radicals stabilized in Hylon VII or waxy maize matrices. This means that EPR parameters of the short-lived radicals reflect precisely changes in mobility of the PBN adducts and their penetration ability occurring upon phosphorylation and pressurization of the starch matrix.

Table 3. EPR parameters of short-lived radicals generated in phosphorylated HylonVII and waxy maize after treatment with high pressure [6].

Starches	Signal components									
	FAST				MEDIUM			SLOW		
	Content [%]	A _N [G]	A _H [G]	ΔB [G]	Content [%]	A _N [G]	ΔB [G]	Content [%]	A _N [G]	ΔB [G]
Native Hylon VII	2.1	14.8	2.8	2	57	10.3	4	41	25.0	6
Monostarch phosphate	0.4	14.8	2.8	2	53	10.3	5	47	24.5	5
Distarch phosphate	1.1	14.7	2.8	2	54	10.3	5	45	24.5	6
Native waxy maize	0.1	14.7	2.8	2	42	8.6	6	58	21.3	12
Monostarch phosphate	0.4	15.0	2.8	2	38	8.5	5	61	21.7	12
Distarch phosphate	0.2	15.0	2.8	2	40	8.9	6	60	21.4	12

6. Conclusions

It was shown, that both types of thermally generated radicals – the stable and short - lived ones - may be used as indicators of the changes occurring in the starch matrix upon various chemical and physical treatments. The proposed approach provides important information on structure and properties of the native and chemically or physically modified starches. To the most noticeable belongs identification, by using relatively stable radicals, of the type of bonding phosphorus on terminal or bridging positions in the starch. On the other hand, short - lived radicals allow to illustrate correctly the starch granule architecture. Three different adducts of short - lived radicals with PBN spin trap of various mobility were found. Their relative content reflects properly the size of zones in the starch structure of various ordering degree

and accessibility of these zones for reagents.

However, to evaluate properly influence of a given modification on the features of the starch, important from the point of view of its specific application, the following conditions had to be fulfilled:

- Very important is determination of the optimum heating parameters for the polymer, assuring creation of the significant number of radicals registered by EPR spectroscopy, but not evoking considerable damage of the starch structure.
- Reference samples should be prepared considering the greatest possible number of parameters which might influence the modification process.
- Optimal amount of modifying agent assuring significant influence on the change of the starch functionality should be determined.

- Particular attention should be given to the moisture level of the samples. Water plays a special role in numerous processes occurring in starch which determine positively its properties. However, in the presence of water annihilation of radicals occurs very rapidly, which is especially undesirable in the proposed approach.

Using thermally generated radicals as indicators of starch transformation is very advantageous, it is like observation of the changes occurring in starch using magnifying glass. Magnification of the effect is realized thanks to modification of the starch (e.g. by phosphorylation) and radicals playing simultaneously role of witnesses and magnifying lens. The

information about changes occurring in the starch structure upon modification is enclosed in parameters of the EPR spectra. Due to extreme sensitivity of EPR spectroscopy and well established procedure for quantitative EPR measurements the response of the starch to each impulse is very significant.

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8. Conflict of interest

The authors declare no conflict of interest.

9. References

- [1] Perez S., Bertoft E. The molecular structures of starch components and their contribution to the architecture of starch granules: A comprehensive review. *Starch/Stärke*, 2010; 62: 398-420.
- [2] Tomasik P., Schilling C. H. Chemical modification of starch. *Advances in Carbohydrate Chemistry and Biochemistry* 2004; 59: 176-322.
- [3] Ciesielski W., Tomasik P. Starch radicals. Part I. Thermolysis of plain starch. *Carbohydrate Polymers* 1996; 31: 205-210.
- [4] Błaszczak W., Bidzińska E., Dyrek K. et al. Effect of high hydrostatic pressure on the formation of radicals in maize starches with different amylose content. *Carbohydrate Polymers* 2008; 74: 914-921.
- [5] Bidzińska E., Błaszczak W., Dyrek K. et al. Effect of phosphorylation of the maize starch on thermal generation of stable and short-living radicals. *Starch/Stärke* 2012; 64: 729-739.
- [6] Bidzińska E., Dyrek K., Kruczała K. et al. Electron paramagnetic resonance (EPR) study of the short-living radicals generated thermally in phosphorylated maize starch with different amounts of amylose. *Nukleonika* 2013; 58 (3): 429-433.
- [7] Dyrek K., Madej A., Mazur E. et al. Standards for EPR measurements of spin concentration. *Colloids and Surfaces* 1990; 45: 135-144.
- [8] Dyrek K., Rokosz A., Madej A. Spin Dosimetry in Catalysis Research. *Appl. Magn. Reson.* 1994; 6: 309-332.
- [9] Bidzińska E. Thermally Generated Radicals as Indicators of the Starch Modification Studied by EPR Spectroscopy: A Review. *Carbohydrate Polymers* 2015; 124: 139-149.
- [10] Blennow A., Engelsen S. B., Nielsen T. H. et al. Starch phosphorylation: a new front line in starch research. *TRENDS in Plant Science* 2002; 7: 445-450.
- [11] Hemar Y., Hardacre A., Hedderley D. I. et al. Relationship between the pasting behavior and the phosphorus content of different potato starches. *Starch/Stärke* 2007; 59: 149-155.
- [12] Sang Y., Prakash O., Seib P.A. Characterization of phosphorylated cross-linked resistant starch by ^{31}P nuclear magnetic resonance (^{31}P NMR) spectroscopy. *Carbohydrate Polymers* 2007; 67: 201-212.
- [13] Błaszczak W., Valverde S., Fornal J. Effect of high pressure on the structure of potato starch. *Carbohydrate Polymers* 2005; 59: 377-383.
- [14] Błaszczak W., Fornal J., Valverde S. et al. Pressure-induced changes in the structure of corn starches with different amylose content. *Carbohydrate Polymers* 2005; 61: 132-140.
- [15] Lim S., Seib P. A. Preparation and pasting properties of wheat and corn starch phosphates. *Cereal Chem.* 1992; 70: 137-144.
- [16] Sang Y., Seib P.A. Resistant starches from amylose mutants of corn by simultaneous heat-moisture treatment and phosphorylation. *Carbohydrate Polymers* 2006; 63: 167-175.
- [17] Spalek T., Pietrzyk P., Sojka Z. Application of genetic algorithm joint with Powell method to non-linear least-squares fitting of powder EPR spectra. *Journal of Chemical Information and Modeling* 2005; 45: 18-29.

- [18] Bidzińska E., Michalec M., Pawcenis D. Effect of thermal treatment on potato starch evidenced by EPR, XRD and molecular weight distribution. *Magnetic Resonance in Chemistry* 2015; 53: 1051-1056.
- [19] Dyrek K., Bidzińska E., Łabanowska M. et al. EPR study of radicals generated in starch by microwaves or by conventional heating. *Starch/Stärke* 2007; 59: 318-325.
- [20] Krupska A., Więckowski A. B., Słonimska L. et al. Influence of heating time and pressure treatment of potato starch on the generation of radicals: EPR studies. *Carbohydrate Polymers* 2012; 89: 54-60.
- [21] Łabanowska M., Wesełucha-Birczyńska A., Kurdziel M. et al. Thermal effects on the structure of cereal starches. EPR and Raman spectroscopy studies. *Carbohydrate Polymers* 2013; 92: 842-848.
- [22] Yordanov N. D., Lubenova S. Effect of dielectric constants, sample container dimensions and frequency of magnetic field modulation on the quantitative EPR response. *Analytica Chimica Acta* 2000; 403: 305 – 313.
- [23] Kruczala K., Varghese B., Bokria J. G. et al. Thermal aging of heterophasic propylene-ethylene copolymers: Morphological aspects, based on ESR, FTIR and DSC. *Macromolecules* 2003; 36: 1899-1908.
- [24] Schlick S. (Editor) *Advanced ESR methods in polymer research*. New Jersey, USA: Wiley, 2006.
- [25] Błaszczyk W., Bidzińska E., Dyrek K. et al. EPR study of the influence of high hydrostatic pressure on the formation of radicals in phosphorylated potato starch. *Carbohydrate Polymers* 2010; 82: 1256-1263.
- [26] Błaszczyk W., Bidzińska E., Dyrek K. et al. Effect of phosphorylation and pretreatment with high hydrostatic pressure on radical processes in maize starches with different amylose contents. *Carbohydrate Polymers* 2011; 85: 86-96.
- [27] Wenda E., Bidzińska E., Dyrek K. et al. Role of reference samples in studying of the modified starches. *Recent Advances in Biopolymer Science and Technology*, Chapter 8, pp. 103-112. Editors: Fiedorowicz M., Bertoft E., Nova Science Publishers, New York, 2010.
- [28] Bertolini A. C., Mestres C., Colonna P. et al. Free radical formation in UV- and gamma-irradiated cassava starch. *Carbohydrate Polymers* 2001; 44: 269-271.