Carbohydrate Polymers 85 (2011) 86–96

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Effect of phosphorylation and pretreatment with high hydrostatic pressure on radical processes in maize starches with different amylose contents

Wioletta Błaszczak[a], Ewa Bidzińska[b], Krystyna Dyrek[b,*], Józef Fornal[a], Marek Michalec[c], Elżbieta Wenda[b]

[a] Division of Food Science, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, 10-747 Olsztyn, Poland
[b] Department of Inorganic Chemistry, Faculty of Chemistry, Jagiellonian University, Ingardena 3, 30-060 Cracow, Poland
[c] Department of Crystalochemistry and Crystalophysics, Faculty of Chemistry, Jagiellonian University, Ingardena 3, 30-060 Cracow, Poland

ARTICLE INFO

Article history:
Received 17 December 2010
Received in revised form 24 January 2011
Accepted 27 January 2011
Available online 3 February 2011

Keywords:
Starch phosphates
Cross-linking
Amylopectin
Amylose
Thermal treatment
High hydrostatic pressure
Deteriorated structure
Radicals

ABSTRACT

Waxy maize (amylopectin with trace amounts of amylose) and Hylon VII starch containing mostly amylose were phosphorylated in acidic or alkaline medium to produce mono- or distarch phosphates, respectively. The changes in the starch structure and properties occurring upon phosphorylation and high pressure treatment (650 MPa/9 min) were analyzed using X-ray diffraction (XRD), differential scanning calorimetry (DSC), polarized light microscopy (LM) and electron paramagnetic resonance (EPR) spectroscopy. Quantitative EPR measurements revealed that incorporation of phosphorus into the starch structure increased tendency of the starch to be thermally degraded with formation of radicals, the effect being more pronounced in the case of monostarch phosphates than in distarch phosphates. Pretreatment of the starch with high hydrostatic pressure resulted in diminishing of the number of radicals generated thermally in monostarch phosphates.

1. Introduction

Modification of the starch by introduction of phosphorus into its structure as terminal or bridging PO_4^{3-} groups, with the formation of – respectively – monostarch or distarch phosphates, changes functional properties of this biopolymer (Lim & Seib, 1992). The most important modifications consist on alteration of gelatinization and pasting behaviour (Hemar, Hardacre, Hedderley, Clark, Illingworth, Harper, & Boland, 2007; Wongsagonsup, Shopsngob, Oonkhanond, & Varavinit, 2005), better stability during freeze–thaw cycles (Lim & Seib, 1992), increase in the content of resistant starch exhibiting anti-diabetic and anti-cancer properties (Sang & Seib, 2006; Ohr, 2004). However, besides these improvements of the starch functionality caused by phosphorylation some other – not so positive – changes in the starch properties occur, as shown recently for potato starch (Błaszczak, Bidzińska, Dyrek, Fornal, & Wenda, 2010; Błaszczak, Bidzińska, Dyrek, Fornal, Michalec, & Wenda, in press). One of the consequences of incorporation of phosphorus into the potato starch is deterioration of its structure and increasing tendency to be degraded during thermal treatment with formation of radicals. Evidences are available in the literature that free radicals are not indifferent for human health and may be responsible for negative cellular changes associated with aging (Ashok & Ali, 1999) or some diseases (e.g. Steinberg, 1995). Therefore investigation of parameters influencing amount and reactivity of radicals present in food products containing phosphorylated starch is still of interest.

In the case of potato starch, due to the simultaneous presence of amylose and amylopectin, it was not possible to determine the role of the particular components of the starch in the change of its thermal stability occurring upon phosphorylation. Therefore, in the present work the objectives of investigation were: waxy maize starch with almost 100% of amylopectin and Hylon VII containing mostly amylose.

Quantitative EPR measurements were used to determine the influence of the bonding of phosphorus as terminal or bridging PO_4^{3-} groups on the number of thermally generated radicals determined separately for amylopectin and amylose. Moreover, the degree of deterioration of the starch structure upon phosphorylation was controlled by X-ray diffraction (XRD), differential scanning calorimetry (DSC) and polarized light microscopy (LM). The effect of pretreatment of the phosphorylated starches with high hydrostatic

---

* Corresponding author. Tel.: +48 12 663 22 44; fax: +48 12 634 05 15.
E-mail address: dyrek@chemia.uj.edu.pl (K. Dyrek).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.01.055

pressure on their thermal degradation was also investigated. The aim of this treatment was to check if the recently discovered effect of the reduction of the number of radicals generated in pressurized waxy maize and Hylon VII (Błaszczak, Bidzińska, Dyrek, Fornal, & Wenda, 2008) appears also in phosphorylated samples.

To conclude about the effect of phosphorus itself on starch properties the results of all measurements were discussed in relation to the reference samples.

2. Materials and methods

2.1. Materials

The experimental material used for phosphorylation was Hylon VII (containing 68% of amylose) refined from high amylose maize produced by National Starch & 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 (1992) and Sang and Seib (2006).

2.1.1. Monostarch phosphates

Hylon VII or waxy maize (10 g) were added gradually to the aqueous solution (14 mL) containing 10.5% (starch base) of sodium trimetaphosphate/sodium tripolyphosphate (STMP/STPP, 99/1 w/w) and 0.5 g Na_2SO_4 at initial pH = 6 (obtained with 0.2 M HCl). The suspension was then mixed during 60 min at room temperature. Afterwards the slurry was evaporated at temperature 45–50 °C until content of water was equal to 15–20%. The obtained material was heated at 130 °C for 2 h. At these conditions the most effective incorporation of phosphorus into the starch structure took place (Sang & Seib, 2006). After cooling the cake was mixed with distilled water (70 mL) and pH was measured. Then the suspension was centrifuged and 120 mL of 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.

2.1.2. Distarch phosphates

Similar procedure (Lim & Seib, 1992; Sang & Seib, 2006) was applied to obtain distarch phosphate with the only difference consisting on the initial value of pH = 11 (obtained with 0.2 M NaOH) of the STMP/STPP solution used for phosphorylation of the starch.

2.1.3. Reference samples

Reference samples were obtained by the same procedure as applied for starch transformed into mono- and distarch phosphates but without adding the phosphorylating agents (STMP, STPP). In the text and on the figures the reference samples are labeled: “reference Mono” and “reference Di”, respectively.

2.2. Methods

2.2.1. Determination of phosphorus content

Total phosphorus content was determined according to Polish norm PN-EN ISO 3946. Samples of phosphorylated starch were mineralized with the concentrated nitric and sulphuric acids. Orthophosphoric acid formed during mineralization reacted with ammonium molybdate [$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$] giving a complex of molybdenum (VI) ($[P^{V}Mo^{VI}{}_{12}O_{40}]^{3-}$), 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 (10 mL), precisely mixed, deaerated and sealed (Błaszczak, Valverde, & Fornal, 2005). For high pressure treatment 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 (with the capacity of approximately 25 mL), filled with pressure – transmitting medium, which also minimalized adiabatic heating. The samples were pressure – treated at 650 MPa for 9 min. The time for reaching the working pressure was 120 s. The temperature inside the pressure chamber averaged 20 ± 2 °C. Pressurized starch pastes and gels were dried at 50 °C to constant weight. The dry samples were pulverized with an agate mortar.

The non-phosphorylated samples, used as references, and phosphorylated ones, were treated in a similar way.

2.2.3. Thermal treatment

Starch samples of about 30 mg were placed in EPR quartz tubes (inner diameter = 3 mm), heated in an oven for 30 min at 150 °C and afterwards for 30 min at 210 °C or 230 °C. During heating the tubes were open, i.e., the samples were in contact with air. After treatment the tubes were closed with a paraffin membrane.

2.2.4. X-ray diffraction and determination of degree of crystallinity

The X-ray diffraction patterns of native, chemically treated as well as chemically treated and afterwards pressurized starches were obtained by using the Philips diffractometer type X’Pert Pro in the Bragg–Brentano geometry, with a copper anode (Cu-K_α radiation, $\lambda = 1.54173$ Å), graphite monochromator and scintillation counter. The scanning voltage of the X-ray tube was 40 kV, the current 30 mA, the exposure time 1 s/step and the measured angle, 2θ, was from 5 ° to 35 °. The scanning step was 0.02 °.

The overall degree of crystallinity (CD) of native and modified starches was calculated as the ratio of the area of the crystalline reflections to the overall area of the X-ray diffraction patterns obtained for the analyzed starch samples (Matveev, van Soest, Nieman, Wasserman, Protserov, Ezernitskaja, & Yuryev, 2001). The calculations were made using Micro Image 4.0 for Windows (Olympus Optical Co., Europe).

2.2.5. Differential scanning calorimetry (DSC)

The DSC analyses were performed in a differential scanning calorimeter equipped with an Intra-cooling system (Diamond DSC AS, Perkin–Elmer). The amount of sample used for measurement was 8 mg with three-fold higher water addition. The samples were sealed in stainless steel pans, equilibrated for 2 h, and scanned. The heating rate was 10 °C/min over the temperature range 30–110 °C. An empty pan was used as a reference.

The values of the gelatinization degree (GD) were calculated using the following equation:

$$GD = \{(\Delta H_{ns} - \Delta H_{ts})\Delta H_{ns}{}^{-1}\} \times 100\%,$$

where ΔH_{ns} and ΔH_{ts} are the melting enthalpies of native and treated starches, respectively.

2.2.6. Light microscopy (LM) studies

The starch powders were suspended in a drop of water on a microscopic glass, covered by cover slip, and they were observed under optical microscope (OLYMPUS BX60) using polarized light.

2.2.7. EPR technique

EPR measurements were performed at room temperature with a Bruker ELEXSYS 500 spectrometer (Karlsruhe, Germany) operating in X-band (9.2 GHz) at modulation frequency 100 kHz, modulation amplitude 0.3 mT and microwave power 3 mW. 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 centers.

$VOSO_4 \cdot 5H_2O$ diluted with diamagnetic K_2SO_4 (5×10^{19} spins/g) was used as a primary standard. All necessary precautions, discussed in papers (Dyrek, Madej, Mazur, & Rokosz, 1990; Dyrek, Rokosz, & Madej, 1994) were followed in order to assure good precision of the quantitative EPR measurements. Generation of radicals was investigated on native, reference and phosphorylated samples before and after pressurization and heat treatment. EPR parameters of the radicals were determined by a simulation procedure using the program EPR SIM 32 (Spałek, Pietrzyk, & Sojka, 2005). The accuracy of determination of g values was ±0.001 and that of A values was ±0.5 G.

3. Results and discussion

3.1. Influence of phosphorylation and pressure treatment on crystalline structure, thermodynamic parameters and microstructure of starch granules

The results of phosphorylation of the starch, performed according to procedure elaborated by Lim and Seib (1992) and Sang and Seib (2006) in acidic or alkaline medium are presented in Table 1. Phosphorylation is accompanied by hydrolysis of the starch (Tomasik & Schilling, 2004; Błaszczak et al., in press) which leads to different products, depending on the pH values of the starch suspension (see Section 2). Therefore, the results will be discussed in relation to the reference samples obtained by the same procedure as applied for starch transformed into monostarch or distarch phosphates but without adding the phosphorylating agents. In this way the effect of phosphorus itself could be extracted from the experimental data.

Table 1
Determination by chemical analysis of the amount of phosphorus incorporated into the starch.

Sample	P (weight%)	
	Monostarch phosphate	Distarch phosphate
Waxy maize	0.4 ± 0.1	0.4 ± 0.05
Hylon VII	0.7 ± 0.03	0.4 ± 0.2

The diffraction patterns of waxy maize and Hylon VII as well as their derivatives (Fig. 1) reveal the presence of amorphous and crystalline phases. The reference sample of monostarch phosphate, which underwent hydrolysis in acidic medium, does not show noticeable lowering of crystallinity in respect to the native waxy maize (Fig. 1A). Also phosphorylation in acidic medium does not change visibly the crystalline structure of the monostarch waxy maize phosphate. These results suggest, that the both reactions: hydrolysis and phosphorylation leading to monostarch phosphate formation occur mostly in the amorphous regions of the waxy maize structure. On the other hand, phosphorylation in alkaline medium, resulting in appearance of distarch phosphate, is accompanied by certain drop in the crystallinity (Fig. 1B), which means

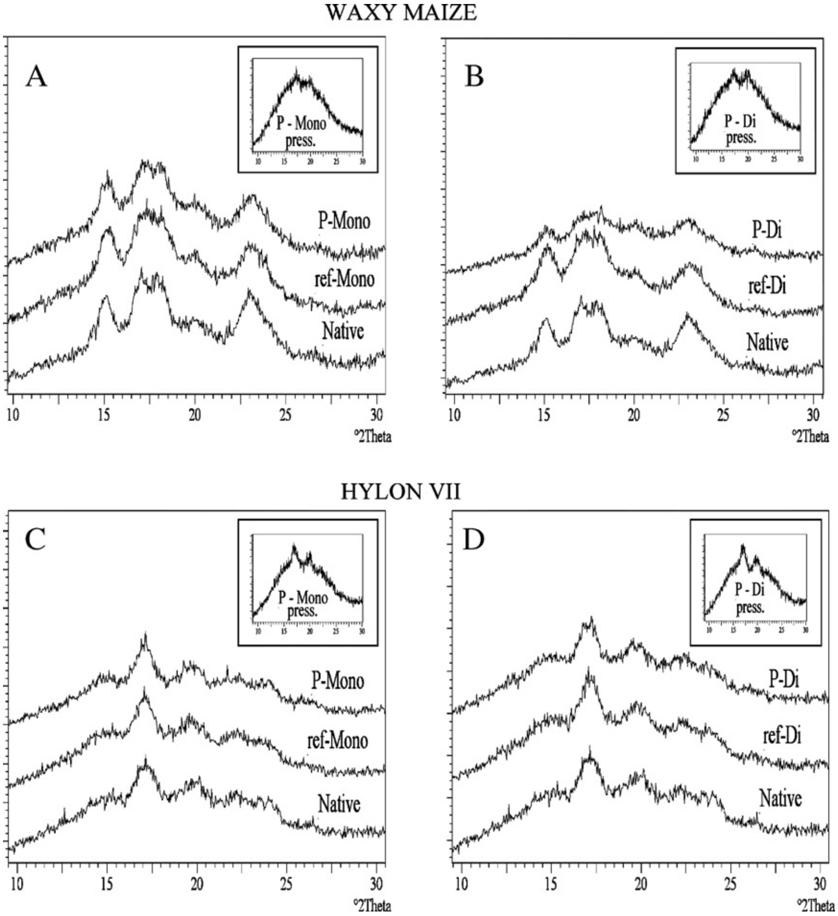

Fig. 1. Diffraction patterns of native and phosphorylated starches: waxy maize (A and B) and Hylon VII (C and D). The inserts contain data concerning pressurized samples.

Table 2
The values of melting parameters (temperature T_m, enthalpy ΔH, gelatinization degree GD) and degree of crystallinity (CD).

Sample	T_m (°C)	ΔH (J/g)	GD (%)	CD (%)
Waxy maize native	72.43	28.84		44
Reference Mono	67.41	23.55	18	38
Monostarch phosphate	66.81	18.76	35	35
Reference Di	67.92	20.47	29	39
Distarch phosphate	73.72	9.48	67	30
Hylon VII native	74.41	19.06		18
Reference Mono	74.16	10.56	45	18
Monostarch phosphate	65.79	9.28	51	18
Reference Di	76.04	7.11	63	18
Distarch phosphate	67.73	9.72	49	15
Hylon VII pressurized				
Reference Mono pressurized	86.04	3.88	80	16
Monostarch phosphate pressurized	77.99	0.85	95	7
Reference Di pressurized	84.2	3.25	83	14
Distarch phosphate pressurized	74.47	1.05	94	9

that this process occurs – at least partially – in the crystalline areas of the waxy maize granule. These observations are reflected by values of the degree of crystallinity (DC) presented in Table 2. The degrees of crystallinity of native Hylon VII and its derivatives almost do not change upon hydrolysis or phosphorylation as indicated by the diffraction patterns (Fig. 1C and D) and DC values in Table 2. It means, that both processes occur predominantly in the amorphous regions of the structure.

The high pressure treatment of waxy maize phosphates and corresponding reference samples (inserts in Fig. 1A and B) resulted in significant loss of granule crystallinity. The effect of pressurization resulting in decrease of crystallinity is present also in the case of phosphorylated Hylon VII (inserts in Fig. 1C and D), but it is less pronounced than that observed for waxy maize starches.

More information about the influence of phosphorylation on the structural and thermodynamic properties of waxy maize and Hylon VII starch were obtained from DSC studies. The analysis of the calorimetric data showed that modification of waxy maize and Hylon VII starch resulted in changes in the thermodynamic data of all the investigated starches (Table 2). The values of melting temperature (T_m) and melting enthalpy (ΔH) obtained for native waxy maize starch characterize melting of crystalline lamellae formed by A and B type of amylopectin structures. The value of melting enthalpy can be assigned to presence of hydrogen-bonded supramolecular complexes of polysaccharides with water molecules (Yuryev, Wasserman, Andreev, & Tolstoguzov, 2002).

Analysis of the experimental data reveals a decrease in both, the T_m and ΔH values for the reference samples of waxy maize starch compared to its native form. A decrease in thermodynamic parameters was found independently on pH medium (acidic or alkaline) used for treatment of the reference material. It may be thus concluded that this effect results rather from the destabilizing influence of temperature on the crystalline structure than from pH conditions used upon treatment. The waxy maize monostarch phosphate demonstrates lower T_m and ΔH values compared to native starch. The phosphorous substitution resulted probably in disruption of the inter- and intra-molecular hydrogen bonds. On the contrary, an increase in T_m value along with a significant decrease in ΔH one was found for distarch phosphate. An opposite relation between melting parameters found for distarch phosphate may suggest, that apart from substitution, some cross-linking reactions probably occur. An increase in gelatinization temperature was already reported in the literature for cross-linked starches (Sang & Seib, 2006; Singh, Kaur, & Mc Carthy, 2007), the effect being strongly dependent on the concentration and type of cross-linking reagent as well as on reaction conditions used in modification process. Since the same reagents (STMP/STPP, 99/1 w/w) were used to obtain mono- and distarch phosphates, it may be supposed that the cross-linking reaction was prone to undergo in alkaline medium (pH = 11) than in acidic one.

In opposite to waxy maize starch, the thermodynamic parameters obtained for Hylon VII describe the melting of B-type (B and B*) as well as V-type crystallites (Błaszczak, Fornal, Kisieleva, Yuryev, Sergeev, & Sadowska, 2007). The DSC and X-ray studies of native waxy maize and Hylon VII starches distinctly demonstrated that amylose content determined significantly the extent of crystallinity and melting enthalpy values (Table 2). A higher amylose content a lower chain packing density in the crystalline lattice of the starch was found (Matveev et al., 2001; Yuryev et al., 2002).

The reference samples of Hylon VII starch showed only slight changes in melting temperature compared to native starch, whereas a significant drop in their melting enthalpy values was noticed. The pH values used during treatment of the reference material had a more decisive effect on changes of the melting enthalpy than on those of melting temperature. The gelatinization degree calculated for the references of Hylon VII starch indicates that majority of granules are influenced by the treatment applied, the effect being more pronounced for alkaline medium. However, both reference starches show no changes in degree of crystallinity (Table 2 and Fig. 1C and D). Such phenomenon might be assigned to a fact that only amorphous regions in starch structure were affected by the treatment. It is known that upon heating of starch granules in excess of water the hydration of the amorphous growth rings precedes the melting of the crystalline structures (Rubens & Heremans, 2000). However, the treatment of reference starches was performed in a limited amount of water, i.e., after its evaporation up to 15–20%. Svensson and Eliasson (1995) found a minor reduction in the crystallinity of starch granules heated in a limited amount of water. Thus, it might be stated that treatment of reference starches at temperature 45–50 °C was enough to evoke amylose hydration, whereas, further increase of temperature up to 130 °C at already limited amount of water resulted in diffusion of hydrated amylose into granule's centre.

Incorporation of phosphorous into Hylon VII starch granules resulted in significant decrease in both T_m and ΔH values. An interesting phenomenon was observed for distarch phosphate, which manifested T_m and ΔH values similar to those of monostarch phosphate. A lack of differences in thermodynamic parameters between mono- and distarch phosphates might indicate that phosphorylation of Hylon VII starch occurs in the same way, independently on pH value.

After high pressure treatment the waxy maize starch preparations (reference and phosphorylated) became completely amorphous and no thermodynamic parameters were obtained in their DSC studies. Evidently, high hydrostatic pressure destroys effectively the ordered structures that were predominantly formed by the amylopectin chains in the double helical form (crystalline lamellae). The corresponding samples of Hylon VII are also significantly affected by the high pressure treatment (Table 2).

The microscopic observation (LM) of reference and phosphorylated samples obtained from waxy maize and Hylon VII starch is in close relation to the data obtained from X-ray and DSC studies.

The reference samples and monostarch phosphate obtained from waxy maize starch are birefringent under polarized light (Fig. 2A, C, E) showing a Maltese cross. This fact indicates a radial orientation of the crystallities formed by the amylopectin chains in the double helical form (Yuryev et al., 2002) and confirms that the treatment (hydrolysis and phosphorylation in acidic medium) does not affect significantly crystallinity of reference samples and monostarch phosphate, since it occurs in the amorphous region of the granule structure, mostly. On the contrary, some changes

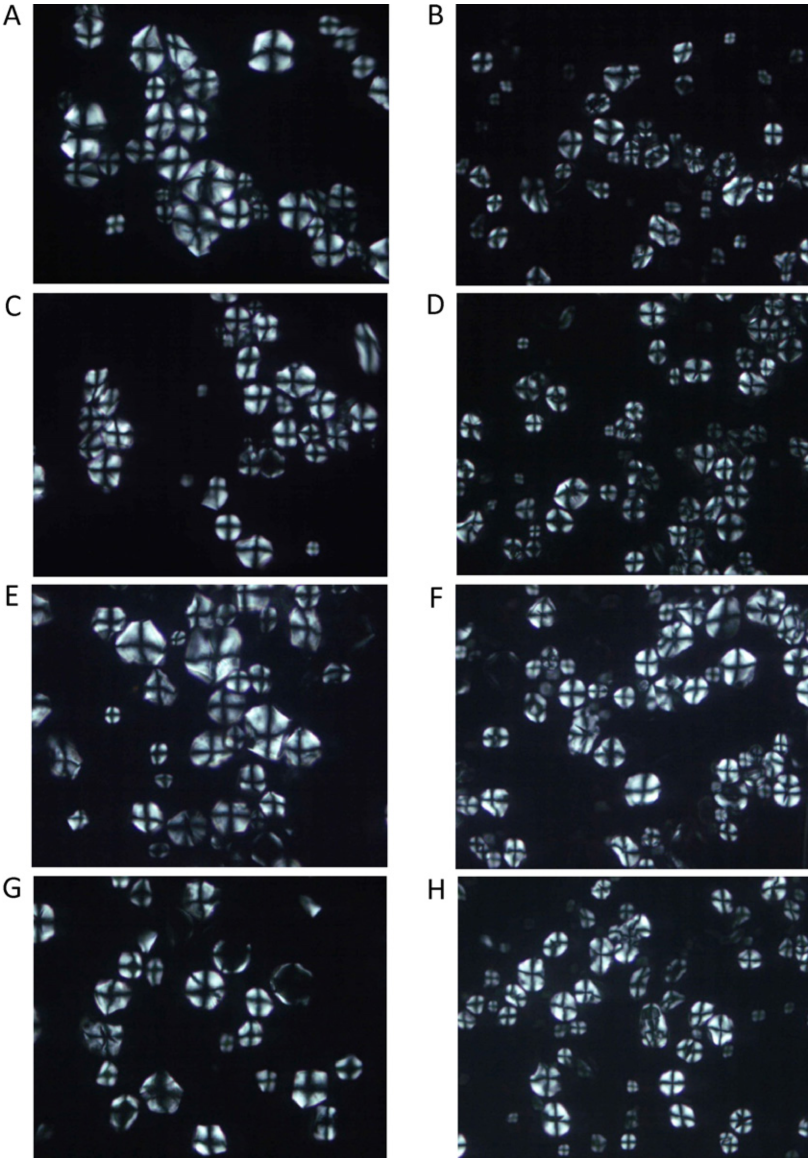

Fig. 2. LM micrographs of phosphorylated waxy maize and Hylon VII starches and corresponding reference samples: reference Mono of waxy maize (A); reference Mono of Hylon VII (B); reference Di of waxy maize (C); reference Di of Hylon VII (D); monostarch phosphate of waxy maize (E); monostarch phosphate of Hylon VII (F); distarch phosphate of waxy maize (G); distarch phosphate of Hylon VII (H).

in birefringence of distarch phosphate observed in Fig. 2G and in diffraction patterns (Fig. 1B) resulted from the fact, that the phosphorylation in alkaline medium, affected probably both: the crystalline and amorphous regions of the waxy maize granule (Table 2 and Fig. 1B).

All the preparations obtained from the Hylon VII starch were birefringent under polarized light (Fig. 2B, D, F, H). However, a hole-like appearance observed in central part of granules was assigned to the amorphous region (hillum) in starch structure that was affected by the treatment applied. On the contrary, the outer part of granules retained crystallinity demonstrated by Maltese cross indicating that no disruption of the granules occurred.

After pressurization the waxy maize starch preparations formed – independently on previous chemical treatment – completely amorphous structure (Fig. 3A, C, E, G), while the high pressure – treated reference samples of Hylon VII starch preserved Maltese cross (Fig. 3B and D). Nevertheless, the birefringence of phosphorylated samples of Hylon VII was visibly affected by the high pressure treatment (Fig. 3F and H). These results indicate that pressure treatment of waxy maize starch and its derivatives resulted in effective destruction of the crystalline lamellae, whereas the phosphorylated Hylon VII showed after pressurization rather advance disruption than complete melting of the crystalline lamellae.

The above discussed results lead to the conclusions concerning differences between changes in structures of amylose and amylopectin occurring upon phosphorylation and high pressure treatment. The major differences found in the degree of crystallinity values between phosphorylated waxy maize and Hylon VII starches allow to conclude, that phosphorous incorporation into high amylose Hylon VII starch occurs mainly in amorphous region within its structure, whereas the phosphorylation of waxy maize starch involves also crystalline lamellae. Another difference was revealed by the treatment with high hydrostatic pressure, which is more effective in destruction of the ordered branched chains of amylopectin, than non-branched, simple chains typical for amylose.

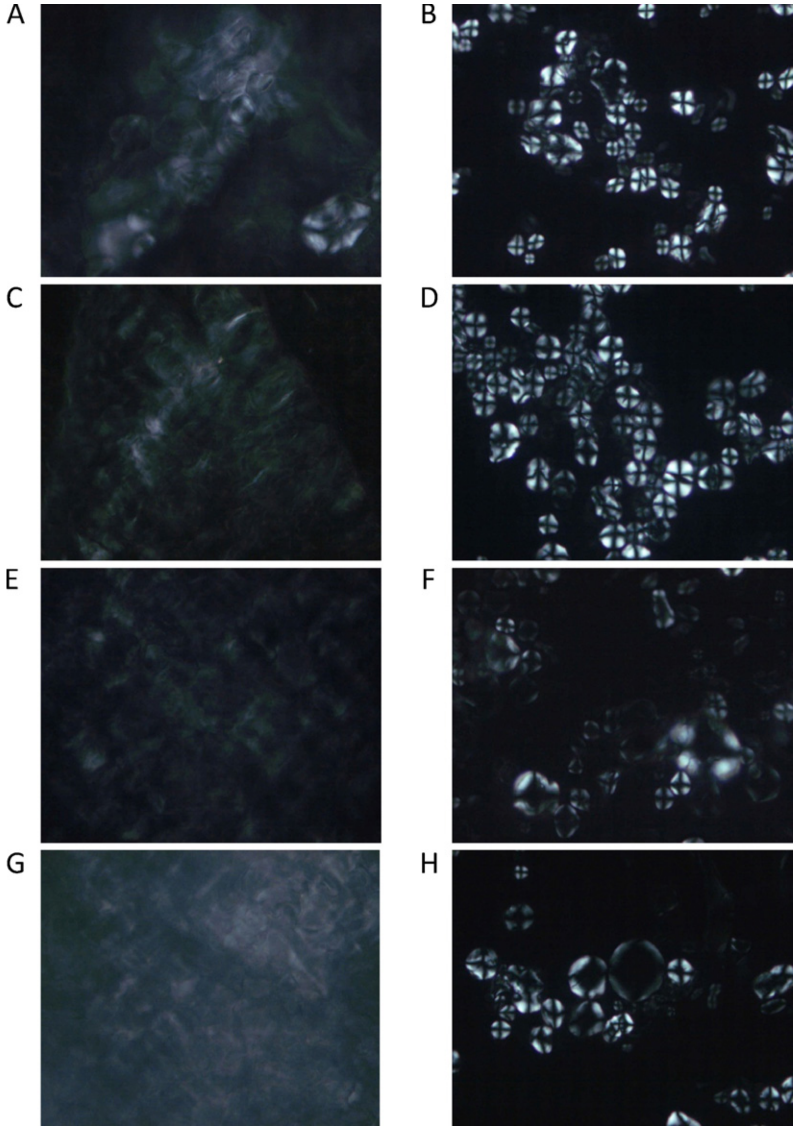

Fig. 3. LM micrographs of phosphorylated and high pressure treated waxy maize and Hylon VII starches and corresponding reference samples: pressurized reference Mono of waxy maize (A); pressurized reference Mono of Hylon VII (B); pressurized reference Di of waxy maize (C); pressurized reference Di of Hylon VII (D); pressurized monostarch phosphate of waxy maize (E); pressurized monostarch phosphate of Hylon VII (F); pressurized distarch phosphate of waxy maize (G); pressurized distarch phosphate of Hylon VII (H).

3.2. EPR spectra

The samples of native and phosphorylated waxy maize starch, Hylon VII and the reference samples did not show any EPR signal before heat treatment. After heating in the temperature range 150–230 °C the EPR signals appear, which are similar to typical EPR spectra of thermally treated native potato and corn starches (Ciesielski & Tomasik, 1996; Dyrek, Bidzińska, Łabanowska, Fortuna, Przetaczek, & Pietrzyk, 2007).

3.2.1. Influence of phosphorylation on the number of radicals

The effect of thermal treatment of the native and phosphorylated waxy maize and Hylon VII, as well as of the corresponding reference samples on the number of radicals generated at 210 °C and 230 °C is presented in Fig. 4. The number of spins is the smallest in the native samples of waxy maize and Hylon VII and increases in their reference samples, which before the thermal treatment underwent hydrolysis in acidic or basic medium, respectively (see Section 2). The most significant increase in the number of radicals is observed in the phosphorylated samples, especially in monostarch phosphates of both: waxy maize and Hylon VII. Evidently, hydrolysis of the starch as well as incorporation of phosphorus result in easier thermal degradation of its structure and formation of radicals. The increase in the number of spins generated at 210 °C, caused by incorporation of 0.4% of P into the waxy maize in the form of monostarch phosphate (Table 1), calculated in respect to the corresponding reference sample, is equal to 0.4×10^{16} spins/gram, whereas for distarch phosphate the increase amounts 0.2×10^{16} spins/gram. The corresponding numbers for Hylon VII are: 0.3×10^{16} spins/gram for monostarch and 0.2×10^{16} spins/gram for distarch phosphate, respectively (in the case of monostarch phosphate of Hylon VII the results were recalculated per 0.4% of phosphorus). Smaller effect observed for the same amount of phosphorus incorporated into the starch structure in the form of distarch phosphates, compared to monostarch ones, may be caused by some cross-linking of the distarch species which was indicated by calorimetric data (Section 3.1). Such process stabilizes the phosphate structure and makes the

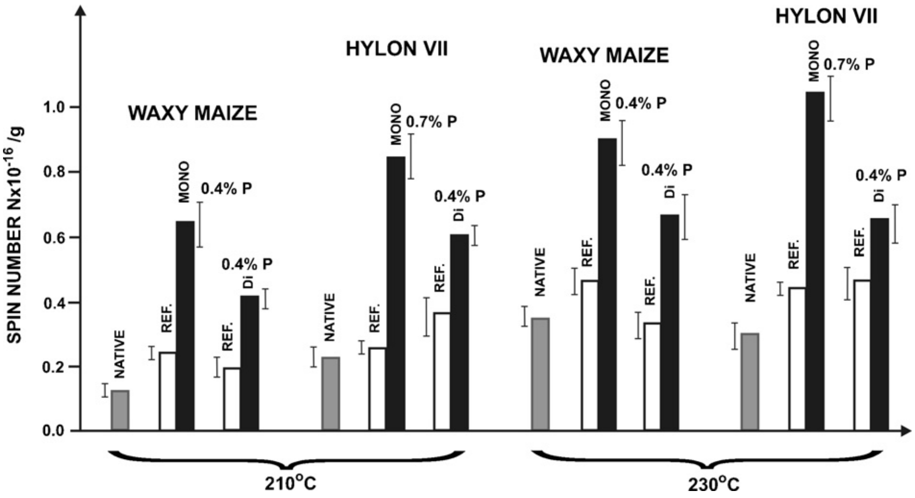

Fig. 4. Effect of phosphorylation of waxy maize starch and of Hylon VII on the number of thermally generated radicals.

radical formation more difficult. Effect of phosphorus after heating the samples at 230 °C is similar but less pronounced (Fig. 4) because at increasing temperature the thermal processes became dominating over other parameters influencing the number of radicals.

The obtained results are in agreement with recently reported data concerning phosphorylated potato starch where localization of phosphorus at terminal positions generated more significant number of radicals upon thermal treatment than in the case of its localization in distarch phosphate (Błaszczak et al., 2010, in press). It may be thus concluded, that the increase in number of thermally generated radicals in phosphorylated starches is more distinctly determined by the way of phosphorus incorporation (mono- or distarch phosphate) than by the type of the starch (waxy maize, Hylon VII or potato starch).

3.2.2. Influence of high hydrostatic pressure on the number of radicals

The already reported increase in the number of thermally generated radicals upon phosphorylation of the starch is in common opinion not desirable, for the reasons mentioned in the introduction. Therefore, in the present work we tried to reduce this effect by pretreatment of the phosphorylated starch with high hydrostatic pressure. It was namely shown that the number of radicals formed by heating of the native waxy maize and Hylon VII, which were pressurized before thermal treatment, is smaller than that in the non-pressurized samples (Błaszczak et al., 2008).

The influence of initial pressurization of the phosphates of waxy maize and Hylon VII on their thermal stability is presented in Fig. 5. After heating at 210 °C the number of thermally generated radicals in monostarch phosphates of both waxy maize and Hylon VII is reduced by about 0.3×10^{16} spins/gram in comparison to non-pressurized samples, whereas in distarch phosphates no visible decrease is observed. Similar effect was found after thermal treatment at 230 °C. Evidently, the increase in the number of radicals caused by incorporation of phosphorus into the starch structure as monostarch phosphates may be reduced by pretreatment of the starch with high hydrostatic pressure, while this reduction does not occur in the case of distarch phosphates. The decrease in the number of radicals may be due to the water molecules squeezed into the starch granules upon treatment of the monostarch phosphates–water suspension with high hydrostatic pressure. It is known that the presence of water accelerates disappearance of radicals generated in starch by heating at elevated temperatures (Ciesielski & Tomasik, 1996; Błaszczak et al., 2008). The penetration of water in distarch phosphates is evidently not so effective as in monostarch structures, most probably due to some cross-linking, which makes the influence of pressure negligible.

In the present work the quantitative measurements of the number of radicals by using EPR spectroscopy appeared an effective method of differentiation between monostarch and distarch phosphates of Hylon VII when compared with XRD and calorimetric results. Both types of Hylon VII phosphates presented almost identical diffraction patterns and similar thermodynamic parameters, independently on the reaction medium during synthesis (see Section 3.1). However, distinguishing of these phosphates was possible on the basis of EPR data, because only monostarch phosphate of Hylon VII revealed after pressurization the decrease in the number of thermally generated radicals. Such decrease was also observed for phosphorylated potato starch (Błaszczak et al., 2010), where, similarly as in the present work, the effect was pronounced only for monostarch and not for distarch phosphates. In all the investigated cases the effect of high hydrostatic pressure on the number of ther-

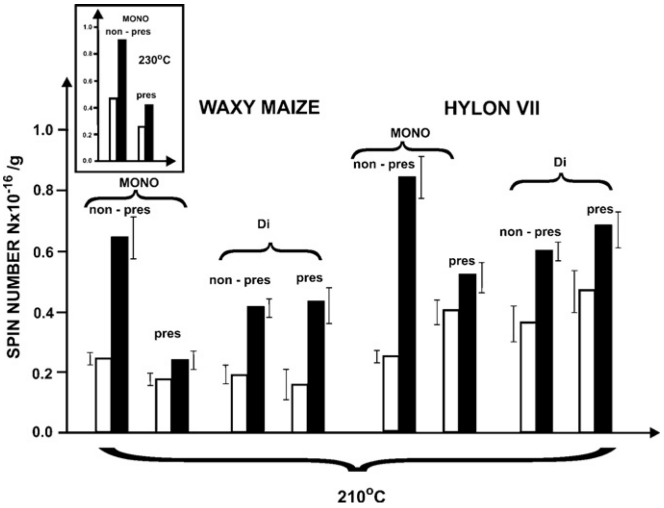

Fig. 5. Effect of pressurization on the number of radicals generated thermally in phosphorylated waxy maize starch and Hylon VII and in the corresponding reference samples.

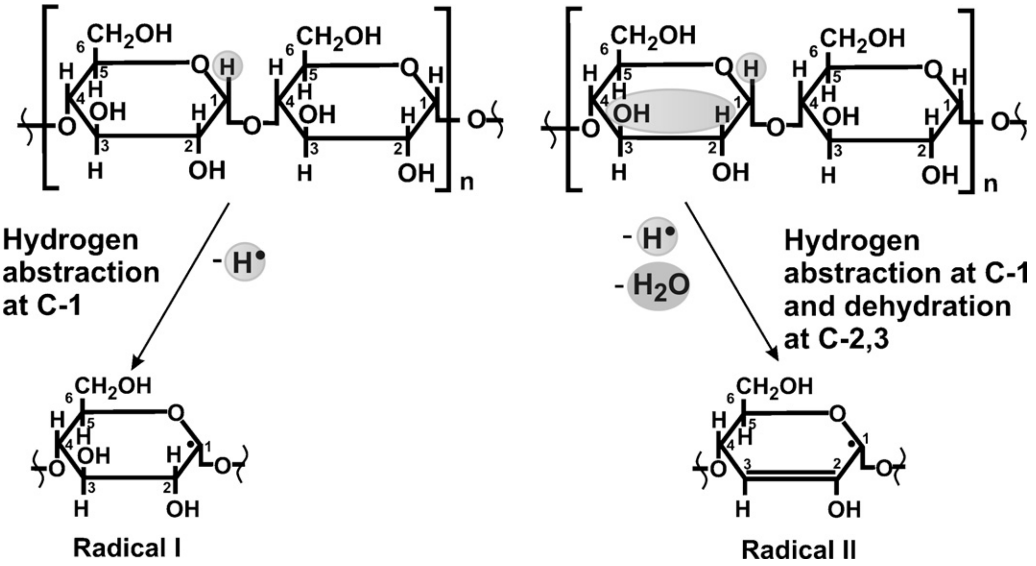

Fig. 6. Scheme of the formation of thermally generated radicals in starch.

mally generated radicals depended more on the way of phosphorus incorporation into the starch than on the kind of the starch.

3.2.3. *Influence of phosphorylation and pretreatment with high hydrostatic pressure on EPR parameters of thermally generated radicals*

It was shown by us previously (Dyrek et al., 2007) that EPR signals of radicals generated thermally in native potato and corn starches, in the range of temperatures commonly used for preparing food, are an envelope of two component signals related with radical I and radical II (Fig. 6). Radical I is generated by abstraction of hydrogen atom from C-1 atom of the glucose unit. The EPR signal of radical I exhibits hyperfine structure (HFS), due to the interaction of unpaired electron localized at C-1 with nuclear spin of vicinal hydrogen atom, localized at C-2. The EPR signal of radical II, without HFS, represents radical species with unpaired electron localized also at C-1, which, however, is not interacting with any neighboring hydrogen. The generation of this radical

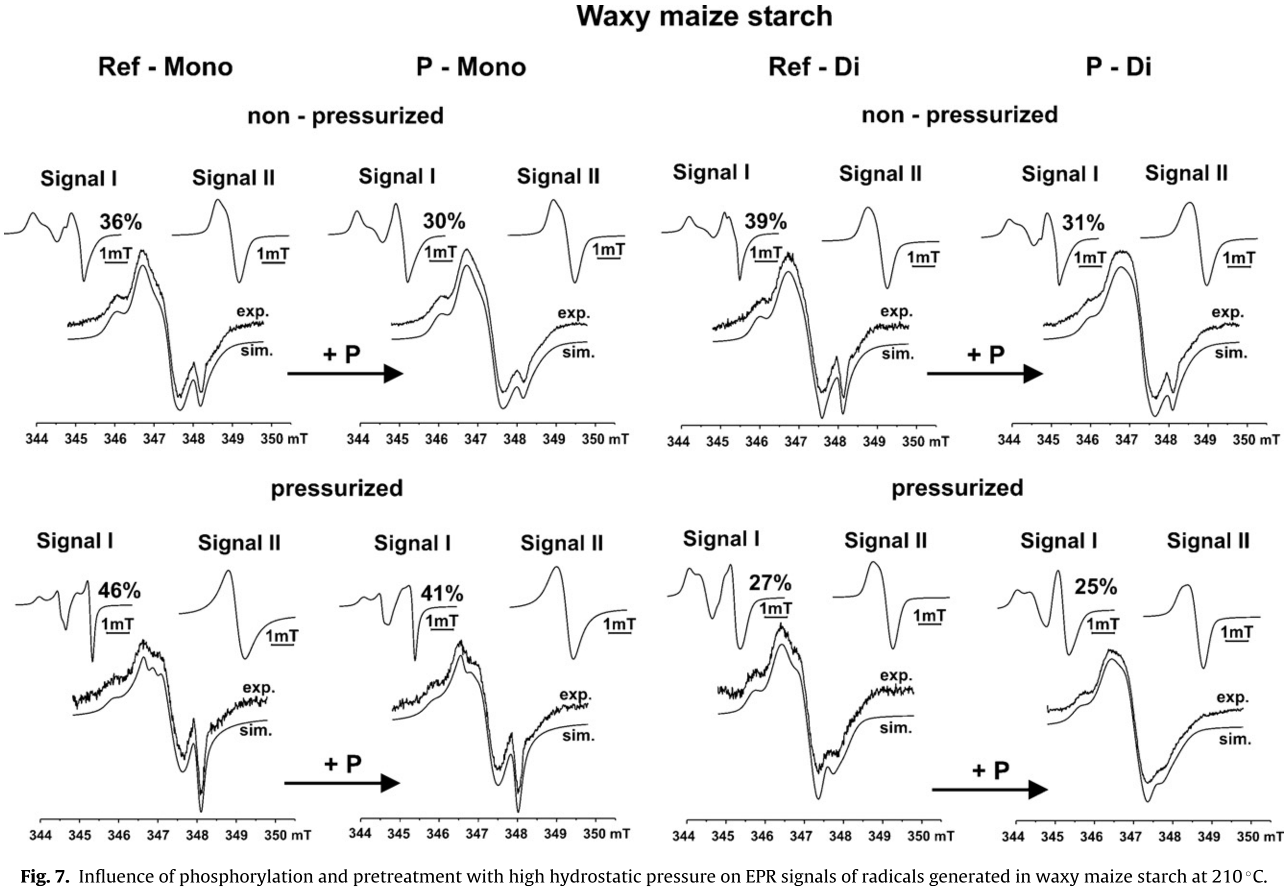

Fig. 7. Influence of phosphorylation and pretreatment with high hydrostatic pressure on EPR signals of radicals generated in waxy maize starch at 210 °C.

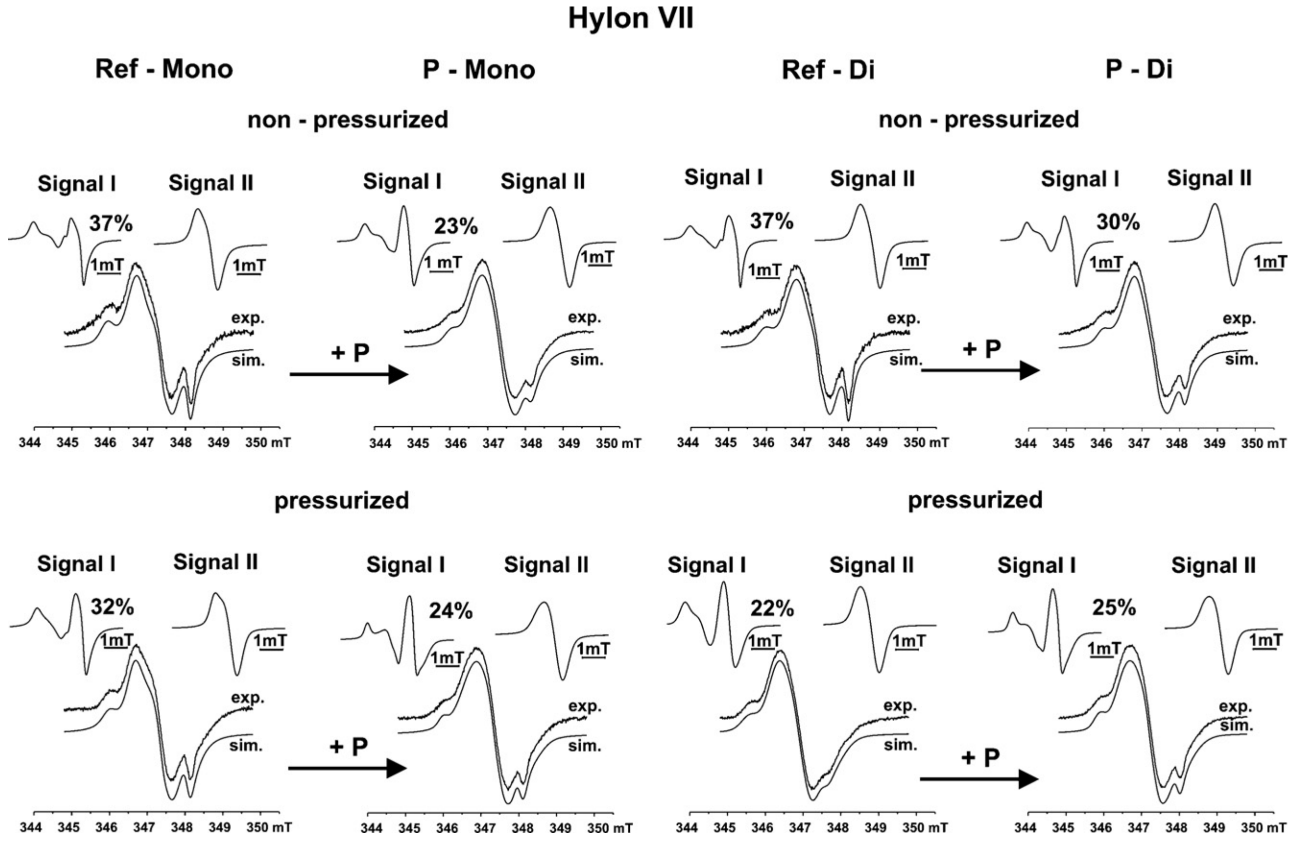

Fig. 8. Influence of phosphorylation and pretreatment with high hydrostatic pressure on EPR signals of radicals generated in Hylon VII at 210 °C.

Table 3
EPR parameters of radicals generated by heating of the phosphorylated waxy maize starch at 210 °C.

Sample	Treatment	Radical I				Radical II	
		Content (%)	A (G)	g_{av}	ΔB_{av} (G)	g_{av}	ΔB_{av} (G)
Native waxy maize	Non-pressurized	43	12.7	2.006	2.5	2.006	5.5
Reference of monostarch phosphate	Non-pressurized	36	11.4	2.006	4.1	2.006	4.7
	Pressurized	46	13.3	2.009	1.9	2.008	4.7
Monostarch phosphate	Non-pressurized	30	11.1	2.005	4.1	2.006	4.7
	Pressurized	41	13.2	2.008	2.2	2.008	5.7
Reference of distarch phosphate	Non-pressurized	39	10.4	2.006	3.9	2.007	4.6
	Pressurized	27	11.4	2.009	3.3	2.009	4.7
Distarch phosphate	Non-pressurized	31	11.3	2.006	3.9	2.006	5.0
	Pressurized	25	11.8	2.009	4.0	2.009	4.1

requires simultaneous abstraction of hydrogen atom from C-1 and water molecule (formed by hydrogen atom abstracted from C-2 and OH group abstracted from C-3) (Fig. 6).

Similar two-component signals exhibit radicals created at 210 °C and 230 °C in native and phosphorylated waxy maize and Hylon VII and in the corresponding reference samples (Figs. 7 and 8; Tables 3 and 4) indicating that the same mechanism of radical generation operates in all these cases. However, there are some differences between waxy maize and Hylon VII and their derivatives in the relative content and EPR parameters of radicals after hydrol-

Table 4
EPR parameters of radicals generated by heating of the phosphorylated Hylon VII at 210 °C.

Sample	Treatment	Radical I				Radical II	
		Content (%)	A (G)	g_{av}	ΔB_{av} (G)	g_{av}	ΔB_{av} (G)
Native Hylon VII	Non-pressurized	48	12.7	2.005	2.2	2.005	6.4
Reference of monostarch phosphate	Non-pressurized	37	12.0	2.006	3.7	2.006	4.6
	Pressurized	32	12.6	2.007	3.8	2.007	4.7
Monostarch phosphate	Non-pressurized	26	11.8	2.005	4.2	2.006	5.2
	Pressurized	24	12.6	2.005	3.1	2.006	5.8
Reference of distarch phosphate	Non-pressurized	37	12.6	2.006	3.0	2.006	5.1
	Pressurized	22	12.6	2.006	4.2	2.007	5.4
Distarch phosphate	Non-pressurized	30	11.6	2.007	3.6	2.007	5.7
	Pressurized	25	12.8	2.006	3.7	2.007	5.5

ysis, phoshorylation and pretreatment with the high hydrostatic pressure.

The relative content of the radical I (with HFS) in the EPR spectra of the investigated preparations decreased in reference samples and still more in the phosphorylated starches (Tables 3 and 4). The effect of phosphorus incorporation on percentage amount of radical I was extracted by comparing phosphorylated with reference samples. The decrease in distarch phosphates of waxy maize and Hylon VII was almost the same, hence it was not specific for the branched or non-branched chains. On the other hand, the decrease in the content of radical I for monostarch phosphate of Hylon VII was more significant than that for monostarch phosphate of waxy maize. These results may indicate that dehydration with simultaneous abstraction of hydrogen vicinal to localization site of unpaired electron (Fig. 6) is easier in amylose than in amylopectin after phosphorylation in the form of monostarch phosphate. Another explanation of the observed effect may be shorter lifetime of radical I generated in the monostarch phosphate of Hylon VII than in corresponding waxy maize sample.

The pressurization of the phosphorylated starches before thermal treatment caused sharpening of the EPR signals of radical I, particularly visible in the case of monostarch phosphate of waxy maize starch (Fig. 7 and Table 3), which points to the less restricted motion of radical species. This result is consistent with previously published data (Błaszczak et al., 2008; Kruczala, Varghese, Bokria, & Schlick, 2003), proving that upon high pressure treatment crystallinity zones in starch diminish, which makes the paramagnetic species more free in rotation leading to decrease of their anisotropy.

The hyperfine splitting constant A of radical I decreased in all investigated samples upon phosphorylation (Tables 3 and 4) evidencing weakening of the HFS interactions between unpaired electron and vicinal hydrogen. After pressurization the values of A do not change visibly.

The above mentioned changes in the content and shape of radical I, caused by phosphorylation and high pressure treatment, respectively, reveal that the more sensitive to external factors are monostarch esters containing phosphorus localized at terminal position of the glucose unit. The corresponding distarch samples are more resistant to modifications, most probably due to cross-linking occurring between the polymer chains. The starch main component (amylose or amylopectin) has also some influence on the observed effects. The changes in the width of the EPR signals of thermally generated radicals occurring upon pressurization are more visible in monostarch phosphate of amylopectin whereas the changes in content of radical I caused by phosphorylation are more significant in monostarch phosphate of amylose.

4. Conclusions

Phosphorylation of the waxy maize occurs mostly in amorphous but partly in the crystalline phase whereas in Hylon VII mainly in amorphous phase. Pressurization leads to the total deterioration of the waxy maize structure – independently if phosphorus is present or not – and advanced weakening of the phosphorylated Hylon VII structure. Hence, high hydrostatic pressure treatment is more effective in destruction of the far distance ordering of branched chains, present in amylopectin, than that, typical for amylose which consist on arrangement of simple, non-branched chains.

Calorimetric data indicate that phosphorus is incorporated into waxy maize in two different ways, as mono- or distarch phosphates, depending on initial pH value of the starch solution, the distarch phosphates revealing formation of cross-linked structure. In the case of Hylon VII substitution of phosphorus leads to products of similar thermodynamic parameters, independently on the reaction medium.

From EPR measurements it results that incorporation of phosphorus into the starch structure causes in both waxy maize and Hylon VII significant increase in number of thermally generated radicals, the effect being more pronounced for monostarch than for distarch phosphates. Pretreatment with high pressure has an opposite influence, i.e., decrease of the number of radicals but only in monostarch phosphates. The distarch phosphates are less receptive to changes due to cross-linking stabilizing their structure. Because of this feature the phosphates of Hylon VII, indistinguishable on the basis of thermodynamic parameters, can be differentiated by EPR measurements of the number of radicals generated thermally in pressurized samples.

The changes in the shape of radical I (with HFS) caused by high pressure treatment of the starch, reveal that the more sensitive to external factors is monostarch ester while the corresponding distarch sample is more resistant to modifications. The starch main component has also some influence on the observed phenomena. The changes occurring upon pressurization in the width of the EPR signals of thermally generated radicals are more visible in phosphates of amylopectin than in those of amylose.

Acknowledgement

This work was partially financed from the grant Nr N N312 103438 awarded by the Polish Ministry of Science and Higher Education (MNiSW).

References

Ashok, B. T., & Ali, R. (1999). The aging paradox: Free radical theory of aging. *Experimental Gerantology*, *34*, 293–303.

Błaszczak, W., Bidzińska, E., Dyrek, K., Fornal, J., & Wenda, E. (2008). Effect of high hydrostatic pressure on the formation of radicals in maize starches with different amylose content. *Carbohydrate Polymers*, *74*, 914–921.

Błaszczak, W., Bidzińska, E., Dyrek, K., Fornal, J., & Wenda, E. (2010). EPR study of the influence of high hydrostatic pressure on the formation of radicals in phosphorylated potato starch. *Carbohydrate Polymers*, *82*, 1256–1263.

Błaszczak, W., Bidzińska, E., Dyrek, K., Fornal, J., Michalec, M., & Wenda, E. Effect of phosphorylation on thermally induced processes in potato starch. In P. Tomasik, G. Gilbert, L. Wasserman, G. Zaikov (Eds.), *Latest Progress in Starch Science*. NY: Nova Science Publishers (Chapter 5), in press.

Błaszczak, W., Fornal, J., Kisieleva, V. I., Yuryev, V. P., Sergeev, I., & Sadowska, J. (2007). Effect of high pressure on thermal, structural and osmotic properties of waxy maize and Hylon VII starch blends. *Carbohydrate Polymers*, *68*, 387–396.

Błaszczak, W., Valverde, S., & Fornal, J. (2005). Effect of high pressure on the structure of potato starch. *Carbohydrate Polymers*, *59*, 377–383.

Ciesielski, W., & Tomasik, P. (1996). Starch radicals. Part I. Thermolysis of plain starch. *Carbohydrate Polymers*, *31*, 205–210.

Dyrek, K., Bidzińska, E., Łabanowska, M., Fortuna, T., Przetaczek, I., & Pietrzyk, S. (2007). EPR studies of radicals generated in starch by microwaves or by conventional heating. *Stärke*, *59*, 318–325.

Dyrek, K., Madej, A., Mazur, E., & Rokosz, A. (1990). Standards for EPR measurements of spin concentration. *Colloids Surfaces*, *45*, 135–144.

Dyrek, K., Rokosz, A., & Madej, A. (1994). Spin dosimetry in catalysis research. *Applied Magnetic Resonance*, *6*, 309–332.

Hemar, Y., Hardacre, A., Hedderley, D. I., Clark, S., Illingworth, D., Harper, J. W., & Boland, M. (2007). Relationship between the pasting behaviour and the phosphorus content of different potato starches. *Stärke*, *59*, 149–155.

Kruczala, K., Varghese, B., Bokria, J. G., & Schlick, S. (2003). Thermal aging of heterophasis propylene–ethylene copolymers: Morphological aspects based on ESR, FTIR and DSC. *Macromolecules*, *36*, 1899–1908.

Lim, S., & Seib, P. A. (1992). Preparation and pasting properties of wheat and corn starch phosphates. *Cereal Chemistry*, *70*, 137–144.

Matveev, Y. I., van Soest, J. J. G., Nieman, C., Wasserman, L. A., Protserov, V. A., Ezernitskaja, M., & Yuryev, V. P. (2001). The relationship between thermodynamic and structural properties of low and high amylose starches. *Carbohydrate Polymers*, *44*, 151–160.

Ohr, L. M. (2004). Nutraceuticals and functional foods. *Food Technology*, *58*(2), 71–75.

Rubens, P., & Heremans, K. (2000). Pressure-temperature gelatinization phase diagram of starch: An in situ Fourier transform infrared study. *Biopolymers*, *54*, 524–530.

Sang, Y., & Seib, P. A. (2006). Resistant starches from amylose mutants of corn by simultaneous heat-moisture treatment and phosphorylation. *Carbohydrate Polymers*, *63*, 167–175.

Singh, J., Kaur, L., & Mc Carthy, O. J. (2007). Factors influencing the physico-chemical, morphological, thermal and rheological properties of some chemically modified starches for food application. *Food Hydrocolloids*, *21*, 1–22.

Spałek, T., Pietrzyk, P., & Sojka, Z. (2005). Application of genetic algorithm joint with Powell method to non-linear least-squares fitting of powder EPR spectra. *Journal of Chemical Information and Modelling*, *45*, 18–29.

Steinberg, D. (1995). Clinical trials of antioxidants in artherosclerosis: Are we doing the right thing? *Lancet*, *346*, 36–38.

Svensson, E., & Eliasson, A. C. (1995). Crystalline changes in native wheat and potato starches at intermediate water level during gelatinization. *Carbohydrate Polymers*, *26*, 171–176.

Tomasik, P., & Schilling, C. H. (2004). Chemical modification of starch. *Advances in Carbohydrate Chemistry and Biochemistry*, *59*, 176–322.

Wongsagonsup, R., Shopsngob, S., Oonkhanond, B., & Varavinit, S. (2005). Zeta potential and pasting properties of phosphorylated or crosslinked rice starches. *Stärke*, *57*, 32–37.

Yuryev, V. P., Wasserman, L. A., Andreev, N. R., & Tolstoguzov, V. B. (2002). Structural and thermodynamic features of low- and high amylose starches. A review. In V. P. Yuryev, A. Cesaro, & W. J. Bergthaller (Eds.), *Starch and starch containing origins* (pp. 23–55). NY: Nova Science Publisher.