Carbohydrate Polymers 97 (2013) 502–511

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Hydration properties and phosphorous speciation in native, gelatinized and enzymatically modified potato starch analyzed by solid-state MAS NMR

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ARTICLE INFO

Article history:
Received 17 January 2013
Accepted 8 May 2013
Available online 15 May 2013

Keywords:
Starch
Branching enzyme
β-Amylase
Hydration
Solid-state NMR
HPSEC

ABSTRACT

Hydration of granular, gelatinized and molecularly modified states of potato starch in terms of molecular mobility were analyzed by ^{13}C and ^{31}P solid-state MAS NMR. Gelatinization (GEL) tremendously reduced the immobile fraction compared to native (NA) starch granules. This effect was enhanced by enzyme-assisted catalytic branching with branching enzyme (BE) or combined BE and β-amylase (BB) catalyzed exo-hydrolysis. Carbons of the glycosidic α-1,6 linkages required high hydration rates before adopting uniform chemical shifts indicating solid-state disorder and poor water accessibility. Comparative analysis of wheat and waxy maize starches demonstrated that starches were similar upon gelatinization independent of botanical origin and that the torsion angles of the glycosidic linkages were averages of the crystalline A and B type structures. In starch suspension phosphorous in immobile regions was only observed in NA starch. Moreover phosphorous was observed in a minor pH-insensitive form and as major phosphate in hydrated GEL and BE starches.

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1. Introduction

Starch is a one of the most abundant polysaccharides and serves as the main energy storage molecule in higher plants. From a structural point of view starch consists of two main components: amylose and amylopectin. Both are glucose polymers, and amylose is a linear molecule of α-1,4-linked glucose units whereas the amylopectin is branched and consist of shorter α-1,4-linked glucose unit chains further linked by α-1,6 branch points. The amylose/amylopectin ratio as well as the chain length and clustering of the α-1,4-linked glucose units are very important parameters guiding granular structure, functional and digestive properties of the starch (Ao et al., 2007; Jobling, 2004). Native starches are present as semi-crystalline granules and the crystal structure depends on the botanical origin of the starch. Ever since the first suggestion of double helical structures in the crystalline domains of starch by Kainuma and French (Kainuma & French, 1972) based on X-ray crystallography a number of very detailed structural refinements have been performed (Imberty, Buléon, Tran, & Pérez, 1991; Imberty, Chanzy, Pérez, Buléon, & Tran, 1988; Imberty & Pérez, 1988, 1989; Wu & Sarko, 1978a, 1978b). By these studies it was shown that, the crystalline part of A-type starch adopts a B_2 structure, whereas B-type crystals adopt a $P6_1$ structure. Amylopectin and amylose exist as A- and B-polymorphs, but for amyloses a V-polymorph predominates. One important characteristic for V-amylose is that it crystallizes in single helices and has a smaller pitch than A- and B-amyloses. This allows for interaction with smaller molecules (Horii, Yamamoto, Hirai, & Kitamaru, 1987; Wu & Sarko, 1978a, 1978b).

Solid-state ^{13}C NMR spectroscopy is another very important analytical technique for structural elucidation of starch. In particular ^{13}C cross-polarization (CP) magic-angle-spinning (MAS) NMR has proven useful for identification of amorphous and crystalline starch polymorphs in dry and hydrated powders (Gidley, 1989; Gidley & Bociek, 1985, 1988; Gidley & Bulpin, 1989; Horii, Hirai, & Kitamaru, 1986; Horii et al., 1987; Marchessault, Taylor, Fyfe, & Veregin, 1985; Paris, Bizot, Emery, Buzaré, & Buléon, 1999; Paris, Bizot, Emery, Buzaré, & Buléon, 2001; Tan, Flanagan, Halley, Whittaker, & Gidley, 2007; Tang & Hills, 2003; Veregin, Fyfe, & Marchessault, 1987; Veregin, Fyfe, Marchessault, & Taylor,

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http://dx.doi.org/10.1016/j.carbpol.2013.05.014

1986; Veregin, Fyfe, Marchessault, & Taylor, 1987). These results facilitated determination of relative amounts of amorphous and crystalline starch (Gidley & Bociek, 1985; Paris et al., 1999, 2001; Tan et al., 2007) as well as correlations between C1 chemical shifts and dihedral angles (ψ,ϕ) of the α-1,4 glycosidic bond (Gidley & Bociek, 1988; Veregin, Fyfe, & Marchessault, 1987; Veregin, Fyfe, Marchessault, & Taylor, 1987). Recently, 2D ^{13}C INADEQUATE MAS NMR spectra of ^{13}C labeled B-amylose were recorded and assigned providing accurate chemical shifts of C2–C5 that was previously impossible due to spectral overlap (Roundeau-Mouro, Veronese, & Buleon, 2006).

By ^{13}C CP/MAS NMR experiments, resonances from carbons located in immobile regions of a sample will be enhanced resulting in spectra almost exclusively describing the immobile part of the sample, whereas all carbon sites will be detected quantitatively by ^{13}C single-pulse (SP) MAS NMR experiments. This means that carbons in mixed-phase/heterogeneous samples will be observed quantitatively in the SP/MAS spectra, whereas almost only the immobile/crystalline part of the sample is observed in the CP/MAS spectra. Combining information from SP/MAS and CP/MAS experiments has previously been successfully applied for assessment of hydration properties in rhamnogalacturonan-I as well as hydroxyethyl- and hydroxypropyl-cellulose (Larsen et al., 2011; Larsen, Schöbitz, & Schaller, 2012). Hence, ^{13}C MAS NMR provides an effective tool to investigate molecular mobility as a function of hydration. Due to the high content of phosphorous in potato starch (Hoover, 2001; Kasemsuwan & Jane, 1996; Lim, Kasemsuwan, & Jane, 1994) a similar strategy can be employed to assess the structural impact of hydration on the phosphorous sites using ^{31}P CP/MAS and SP/MAS NMR. Enzyme-assisted modification of starch provides a clean technique to functionalize starch (Pandey et al., 2000; Smith, Zeeman, & Smith, 2005) and is expected to fundamentally affect the hydration properties of the starch.

In the present study, potato starch was analyzed in its raw (native) and gelatinized states and the gelatinized starch was furthermore analyzed after enzymatic modification with either branching enzyme (BE) to generate a highly branched product or BE and β-amylase (BAM) in combination to generate an even more branched product by trimming of the outer glucan chains of the product. Wheat and waxy maize starches subjected to an identical procedure were included for comparison. Changes in the molecular structure of the starches and the corresponding products generated after enzyme catalysis were characterized by ^{13}C solid-state MAS NMR and by high performance size exclusion chromatography (HPSEC) with a right angle laser light scattering (RALLS), low angle laser light scattering (LALLS), refractive index (RI) and intrinsic viscosity (IV) detector to assess effects on the molecules in solution. Combined information from MAS NMR and HPSEC permit characterization of all parts of the sample and at different scales in relation to physical state and molecular structure.

2. Materials and methods

2.1. Sample preparation

Native wheat- and potato starch (KMC, Denmark) and waxy maize starch (Cargill Nordic A/S, Denmark) were processed in the following ways: 11.1–12.5% (w/v) slurry was prepared by mixing native starch with Milli Q-water in a glass reactor. To prepare a gelatinized sample the slurry was adjusted to pH = 6.1 at room temperature, heated to 75 °C, and kept at this temperature for 20 h, pH was then adjusted to 3.5, and the slurry incubated at 93 °C for 30 min and finally adjusted pH to 5.5 at 93 °C. The samples treated by branching enzyme (BE) were prepared from the starch slurries by adjusting pH to 6.1, adding 1000 U/g DM *Rhodothermus obamensis* amylo-(1,4 → 1,6)-transglycosylase (BE, EC 2.4.1.18) (Shinohara et al., 2001), raising the temperature to 75 °C and incubating the reaction mixture for 20 h or 120 h. Enzyme catalysis was terminated by lowering pH to 3.5 with HCl increasing the temperature to 93 °C and maintaining it for 30 min. After adjustment of pH to 5.5, the reaction mixture was filtered (mesh: 0.2 μm). Part of the BE treated sample (120 h) was further incubated with β-amylase (BAM, EC 3.2.1.2) (Pandey et al., 2000) for 24 h after BE inhibition. The reaction was terminated by adjusting pH to 5.5 with HCl and incubating for 15 min at 93 °C. Low molecular weight sugars formed by the enzymatic hydrolysis were subsequently removed by ultrafiltration (Amicon, cut off 3000 Da). Part of the gelatinized potato starch was pH adjusted for the ^{31}P NMR experiments by mixing of 25 μL of 1 M sodium citrate buffer (pH = 5.5) and 100 mg of gelatinized potato starch.

All samples were freeze dried prior to analysis.

The products generated are denoted NA for native granular starch, GEL for gelatinized starch, BE for BE treated starch (incubated for 20 h) and BB for combined BE (incubated for 120 h) and BAM treated starches. Previously the GEL, BE and BB samples have been analyzed regarding their digestion properties (Kasprzak et al., 2012).

2.2. NMR spectroscopy

Hydrated samples were prepared by weighing out starch powders in the rotor and subsequently adding D_2O using a Hamilton Microliter®#810 syringe. The needle was inserted into the rotor along the inner wall of the rotor and the D_2O was slowly released while the needle was removed from the rotor. Prior to acquisition the hydrated samples had been spinning at 9 kHz for at least 1 h to ensure proper mixing of powder and D_2O. Due to the delicate spinning module in the NMR probe stable spinning was only achieved when the sample was homogenously distributed within the rotor. This also ensured a proper mixture of powder and D_2O. In this study D_2O volumes of 52 μL and 65 μL, respectively, were used. The degree of hydration for each sample is supplied in weight% ($= 100\% \times m_{D_2O}/(m_{D_2O} + m_{powder})$).

The solid-state NMR experiments were performed using a Bruker Avance 400 spectrometer (9.4 T) operating at 400.13, 161.99, and 100.62 MHz for ^{1}H, ^{31}P, and ^{13}C respectively, employing a double tuned solid-state probe equipped with 4 mm (o.d.) spinners. The single-pulse (SP) MAS and cross-polarization (CP) MAS spectra were recorded using a spin-rate of 9 kHz, 600 scans, ramped CP (Metz, Wu, & Smith, 1994) with a contact time of 1 ms for CP/MAS spectra and ^{1}H TPPM decoupling (Bennett, Rienstra, Auger, Lakshmi, & Griffin, 1995) during acquisition (40.9 ms for ^{13}C and 41.3 ms for ^{31}P). Recycle delays of 16 s and 128 s were employed for the CP/MAS and SP/MAS experiments, respectively. These recycle delays were optimized for the dry samples and used for the hydrated samples as well. For ^{13}C MAS spectra rf-field strengths of 80 kHz were employed for ^{1}H and ^{13}C whereas ^{1}H and ^{31}P rf-field strengths of 41.7 kHz were utilized for the ^{31}P MAS spectra. All ^{13}C MAS spectra were referenced (externally) to the carbonyl resonance in α-glycine at 176.5 ppm, whereas the ^{31}P MAS spectra were referenced to (external) 85% H_3PO_4 (aq) at 0.0 ppm. All spectra were apodized by a Lorentzian linebroadening of 10 Hz

2.3. HPSEC

20 mg of material were added to a 50 mL plastic tube and 20 mL 0.05 M aqueous NaOH solution was added (Suortti, 1993). The solution was mixed by vortexing and transferred to a boiling water bath for 20 min. The samples were cooled to room temperature and filtered through a 0.2 μm minisart filter (Sartorius Stedim Biotech GmbH, Germany). Hereafter the filtrate (200 μL)

was injected into the high-performance size-exclusion chromatography (HPSEC) system equipped with a Viscotek automatic injector and a pump (VE 2001 GPC Solvent/Sample Module). A series of size exclusion columns: TSK gel GMPWxl (Tosoh Bioscience Gmbh, Stuttgart, Germany), OH-Pak SB-806 HQ and SB-806 M HQ (Shodex, Kanagawa, Japan) were connected and maintained at 40 °C. The mobile phase was Milli Q-water with 0.02% (w/v) NaN_3. The detection system consisted of 90° and 7° angle light scattering detectors with a constant optical power output laser diode working at a wavelength of 670 nm (RALLS, LALLS), RI and a four-capillary bridge viscometer (Viscotek, Houston, TX, US). The calibration was performed using a pullulan standard with a molecular weight of 112 kDa. The differential index of reflection (dn/dc) used for starch in water solutions (0.146 mL/g) was adapted from literature (You, Fiedorowicz, & Lim, 1999).

3. Results and discussion

3.1. Effects of starch molecular structure and assembly on hydration

The effects of hydration and/or enzymatic modification on starch molecular organization are visualized in the ^{13}C SP/MAS and CP/MAS spectra in Fig. 1A and the assignments detailed in Table 1. Assignments are also included on the two upper spectra. The SP/MAS and CP/MAS spectra were similar for the starch powders, whereas the two types of spectra were very different for the starch suspensions. A similar effect has previously been reported for amylose gels (Gidley, 1989). The spectra of the powders were characterized by broader resonances, reflecting either several distinct overlapping chemical shifts and/or molecular disorder in these samples, whereas narrower resonances were observed for the suspensions. Of particular importance is the lack of C1-resonances at ~95 ppm and C4′-resonances at ~83 ppm in the spectra of suspensions. This suggests that the chemical environment of these carbon sites is significantly modified upon hydration and that they are easily accessible for water. For the spectra of suspensions, only the native starch resulted in improved spectral resolution in CP/MAS as compared to SP/MAS. The two C1-resonances at ~100 ppm in the CP/MAS spectrum characteristic for a B-type starch (Horii et al., 1986, 1987; Veregin et al., 1986) along with the intense C3/C4 resonance at ~75 ppm (Roundeau-Mouro et al., 2006) verifies the high crystallinity of the immobile regions. For the GEL, BE and BB samples narrower resonances in the SP/MAS spectra were observed and the S/N ratio in the CP/MAS spectra was reduced as an effect of enzymatic treatment. The latter confirms that the amount of immobile (e.g. crystalline) regions is reduced as an effect of enzyme catalyzed branching and chain trimming. Due to the effects of BE and/or BAM the resonance of the terminal C4-carbon (C4t ~ 70.3 ppm) became observable in the SP/MAS spectrum of the BB suspension.

The line width of the resonances in the SP/MAS spectra of suspensions became narrower in the order NA > GEL > BE > BB whereas for the powders they were broadened in the same order. This is particularly obvious in for the spectral regions of C1 (90–110 ppm) and C6 (55–70 ppm). The spectra of powders displayed distributions of C1-resonances that were sensitive towards both hydration and enzymatic treatment. This effect was most obvious between spectra of NA and GEL where the chemical shift maximum changed from ~100 ppm to ~103 ppm due to gelatinization. The CP/MAS spectrum of the NA suspension displayed two distinct resonances originating from C1 as anticipated for a type B starch. These resonances were not observed in the spectra of the gelatinized samples demonstrating disentanglement of the double-helical regions of crystalline amylopectin due to gelatinization as expected.

Deconvolution of the C1-resonances can be performed using several different procedures (Gidley & Bociek, 1985; Paris et al., 1999, 2001), but it is not possible to obtain a unique deconvolution due to lack of constraints. As an alternative the methylene C6-resonance was targeted to get structural information related to enzymatic modification and hydration. Previously C6 has been reported to be more disordered than C1 or C4 in V-type crystalline amyloses – in particular for low crystalline samples (Veregin, Fyfe, & Marchessault, 1987; Veregin, Fyfe, Marchessault, & Taylor, 1987). The C6-resonance is easier to analyze than C1 since it can be roughly modeled as a single site. Hereby only the position and line width needs to be determined. The line width (full width at half maximum = FWHM) of the C6-resonance in either CP/MAS or SP/MAS spectra as a function of treatment (Fig. 1B) revealed several differences between powdered and suspended samples. First, the line widths in the spectra of powders were between 135 Hz (NA) and 406 Hz (BB) higher than in the spectra of the corresponding suspensions. This difference increased following: NA < GEL < BE < BB. Secondly, only small differences (10–20 Hz) in line widths between SP/MAS and CP/MAS spectra were observed for powders, whereas differences up to 122 Hz (GEL) were observed for the suspensions. For the NA sample the line width was narrower in the CP/MAS spectrum for both powder and suspension, whereas the line widths in the CP/MAS spectra of other samples were wider than in the SP/MAS spectra for both powders and suspensions. In terms of molecular order, these data indicate that the crystalline regions in the NA samples are slightly more ordered than the overall sample. On the contrary, the powder samples of GEL, BE and BB were more disordered/amorphous but upon hydration the partly modified starches adopt similar configurations and no significant amounts of immobilized/crystalline regions remains. The higher degree of disorder in the BE and BB samples may be a combined effect of the lower molecular weight and higher degree of branching of these samples, potentially increasing molecular mobility and gelatinization. Comparing the line width trends of the C6 resonance in Fig. 1B with the size exclusion data in Table 2, the increased disorder around C6 in the order NA < GEL < BE < BB for the powdered samples is correlated with a higher polydispersity index (PDI), a lower hydrodynamic radius (R_h) and to a smaller extent a lower mean molecular weight (M_w). This suggests that the smaller polymers in the powdered BE and BB samples were less uniform and more disordered than the larger polymers in the GEL sample. An immediate effect of this is that hydration of the BE and BB samples is more efficient due to higher mobility of the smaller molecules and lack of long-range order.

3.2. Hydration of α-1,6 glycosidic linkages

Both C1 and C4 carbons involved in the glycosidic α-1,4-linkages were readily detected in the spectra (Fig. 1A) and even the terminal C4t carbons were detected. In contrast, no distinct resonances from C1 and C6 carbons involved in the α-1,6-linkages were observed. Previous liquid-state NMR studies have shown that the chemical shifts of these carbons should be around 99 ppm and 68.5 ppm for C1 and C6, respectively (Hansen et al., 2008) positioning at least the C6 resonance in a non-overlapped spectral region. One of the reasons for the lack of the C1 and C6 resonances could be line broadening induced by dynamic exchange as modeling studies (Best, Jackson, & Naidoo, 2002) have shown that the α-1,6 branch point may adopt two conformations of equal energy and therefore in mutual exchange. Increasing the hydration level will favor a "liquid-state like" conformation of the α-1,6-linkages. To test whether this conformation could be promoted, ^{13}C CP/MAS and SP/MAS spectra of NA, GEL and BB potato samples as powders as well as suspensions with hydration levels of ~57% and ~70% were recorded (Fig. 2). The increased hydration level reduced the line width in the SP/MAS spectra and the S/N-ratio of the CP/MAS spectra for the GEL and BB suspensions. In contrast the spectra of the NA samples did not

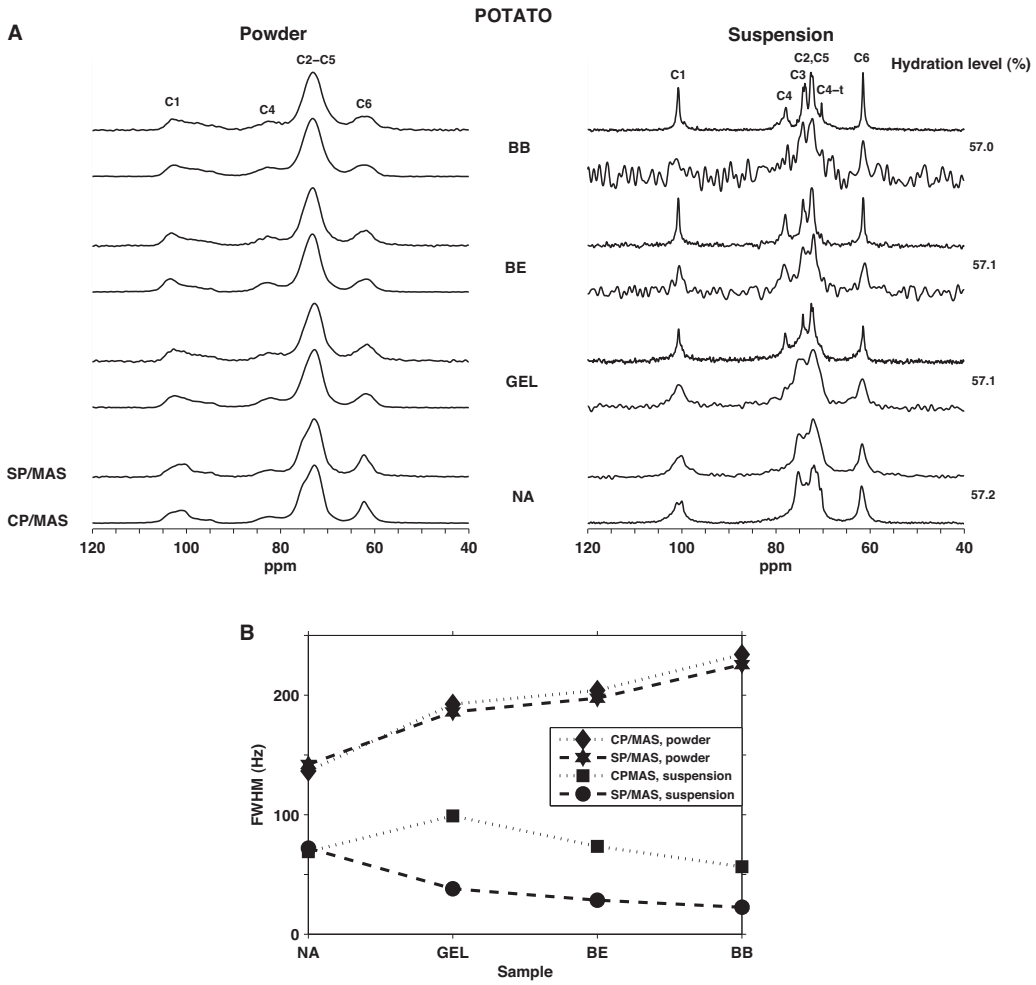

Fig. 1. (A) ^{13}C CP/MAS (lower spectra) and ^{13}C SP/MAS (upper spectra) NMR spectra of potato starch as native, gelatinized, treated with 1000 U BE for 20 h and treated with 1000 U BE for 120 h and BAM for 24 h. Left column: powders, right column: suspensions. Levels of hydration are listed for the suspensions. (B) Line widths (FWHM in Hz) of the C6 resonance in the spectra.

Table 1
Assignment of ^{13}C and ^{31}P NMR chemical shifts (ppm) in spectra of potato starch samples. Sn denotes site number *n*. When multiple ^{31}P sites are present their relative abundance (in %) is denoted in parenthesis.

Type	State	Exp.	Nucleus	S1	S2	S3	S4	S5	S6
NA	Powder	CP/MAS	^{13}C	103.3	70–77	70–77	70–77	70–77	62.3
	Powder	CP/MAS	^{13}C	101.0			82.3 (C4′)		
	Powder	CP/MAS	^{13}C	94.9					
	Powder	CP/MAS	^{31}P	0.7					
NA	Suspension	CP/MAS	^{13}C	101.0	71.4–72.3	75.2	73.5	70.4	61.8
	Suspension	CP/MAS	^{13}C	100.0					
	Suspension	CP/MAS	^{31}P	0.5					
	Suspension	SP/MAS	^{31}P	1.0					
B-amylose[a]	Powder	2D	^{13}C	100.2	72.1	75.1	74.3	70.2	61.3
	Powder	2D	^{13}C	99.3	71.3		72.6	69.8	
GEL (pH=7.0)	Powder	SP/MAS	^{31}P	5.0					
	Suspension	SP/MAS	^{31}P	4.2 (89%)	3.1 (11%)				
GEL (pH=5.5)	Powder	SP/MAS	^{31}P	2.2					
	Suspension	SP/MAS	^{31}P	0.8 (83%)	3.0 (17%)				
BE	Powder	SP/MAS	^{31}P	0.7					
	Suspension	SP/MAS	^{31}P	0.7 (84%)	1.5 (7%)	2.5 (9%)			
BB	Suspension	SP/MAS	^{13}C	100.7	72.4	74.2	77.8	72.1	61.4
	Suspension	SP/MAS	^{13}C	99.3			79.6		68.5
	Suspension	SP/MAS	^{13}C			70.2 (C4t)			
	Powder	SP/MAS	^{31}P	0.7					
	Suspension	SP/MAS	^{31}P	0.6 (78%)	1.1 (22%)				

[a] 2D ^{13}C–^{13}C INADEQUATE (Roundeau-Mouro et al., 2006).

Table 2
Degree of branching (DB = $\alpha_{1,6}/(\alpha_{1,4} + \alpha_{1,6})$), weight average molecular weight (M_w), polydispersity index (PDI), intrinsic viscosity (IV) and hydrodynamic radius of the molecule (R_h) by HPSEC RALLS/LALLS-RI-IV for GEL, BE and BB versions of waxy maize, wheat and potato starch.

Treatment	Starch source	DB (%)[a]	M_w (MDa)	PDI	IV (dL/g)	R_h (nm)
GEL	Waxy maize	3.6	36.64	1.1	0.6	67.9
	Wheat	2.6	11.55	2.0	0.5	40.6
	Potato	2.1	11.45	1.3	0.3	33.4
BE	Waxy maize	6.2	0.12	3.8	0.1	5.3
	Wheat	7.6	0.14	6.9	0.1	5.1
	Potato	5.1	0.14	5.9	0.1	5.6
BB	Waxy maize	13.4	0.07	5.6	0.1	3.9
	Wheat	13.2	0.08	13.1	0.0	3.7
	Potato	12.5	0.10	13.2	0.1	4.2

[a]Kasprzak et al. (2012).

change by increased hydration apart from slightly lower S/N-ratio due to less carbon in the sample. The most noticeable effect of increased hydration is the appearance of C1 (99.3 ppm) and C6 (68.5 ppm) resonances (marked by *) in the SP/MAS spectrum of the BB suspension with a hydration level of 70.6%. Furthermore a plausible C4 resonance for the 1,4,6-linked glucose residue at 79.6 ppm was observed.

For the BB suspension at hydration level of 70.6%, the C6* resonance was clearly visible in the SP/MAS spectrum but not in the CP/MAS spectrum. This indicates that the presence of immobile regions is highly correlated with the presence of disordered/non-hydrated α-1,6 glycosidic bonds. Previous data has demonstrated that, a large change in chemical shift occurs upon solvation of the oxygen site on C6 in α-glucose demonstrating that this site is very sensitive to hydration (Sefzik et al., 2007). The reduction of immobile regions is also correlated to disassembly of the crystalline double helical regions of amylopectin in the NA by gelatinization and further by the 115-fold reduction of the M_w (Table 2) when comparing the GEL to the BB sample. This illustrates that the immobile regions not only include crystalline regions (as in NA) but also hydrophobic regions created by large molecules (as in GEL).

The observed modification of hydrophobicity of the α-1,6-branch points can be relevant for enzymes with known side activity for α-1,6 linkages such as glucoamylase, maltase and α-glucosidase (Svensson, 1994) to more specifically direct catalysis towards the α-1,4 backbone at low hydration levels possibly reducing reversion reactions known to reduce the glucose yield in industrial starch processing.

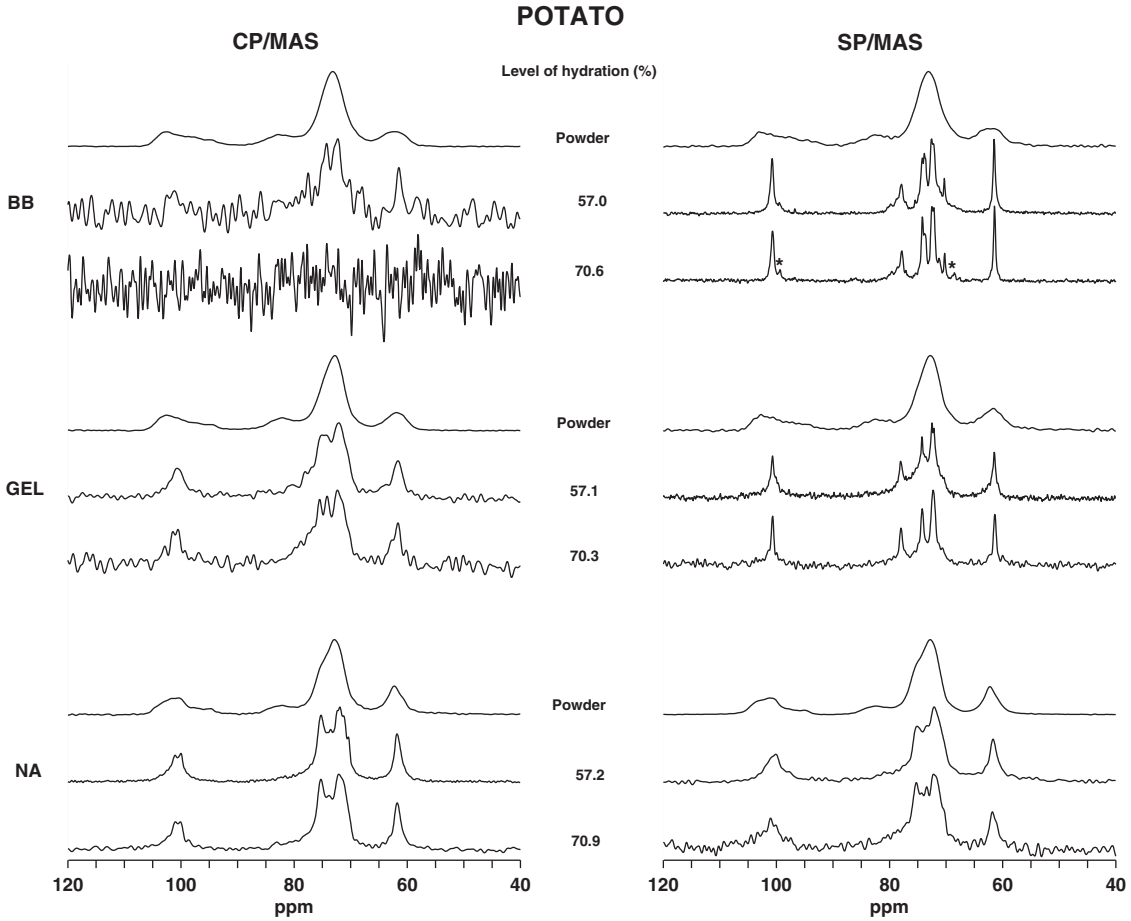

Fig. 2. ^{13}C CP/MAS (left column) and SP/MAS (right column) spectra of NA, GEL and BB potato starches as powders (upper spectra) and as suspensions having hydration levels of 57.0% (middle spectra) and 70.6% (lower spectra), respectively. Asterisks indicate C1 (99.3 ppm) and C6 (68.5 ppm) involved in the α-1,6 linkage.

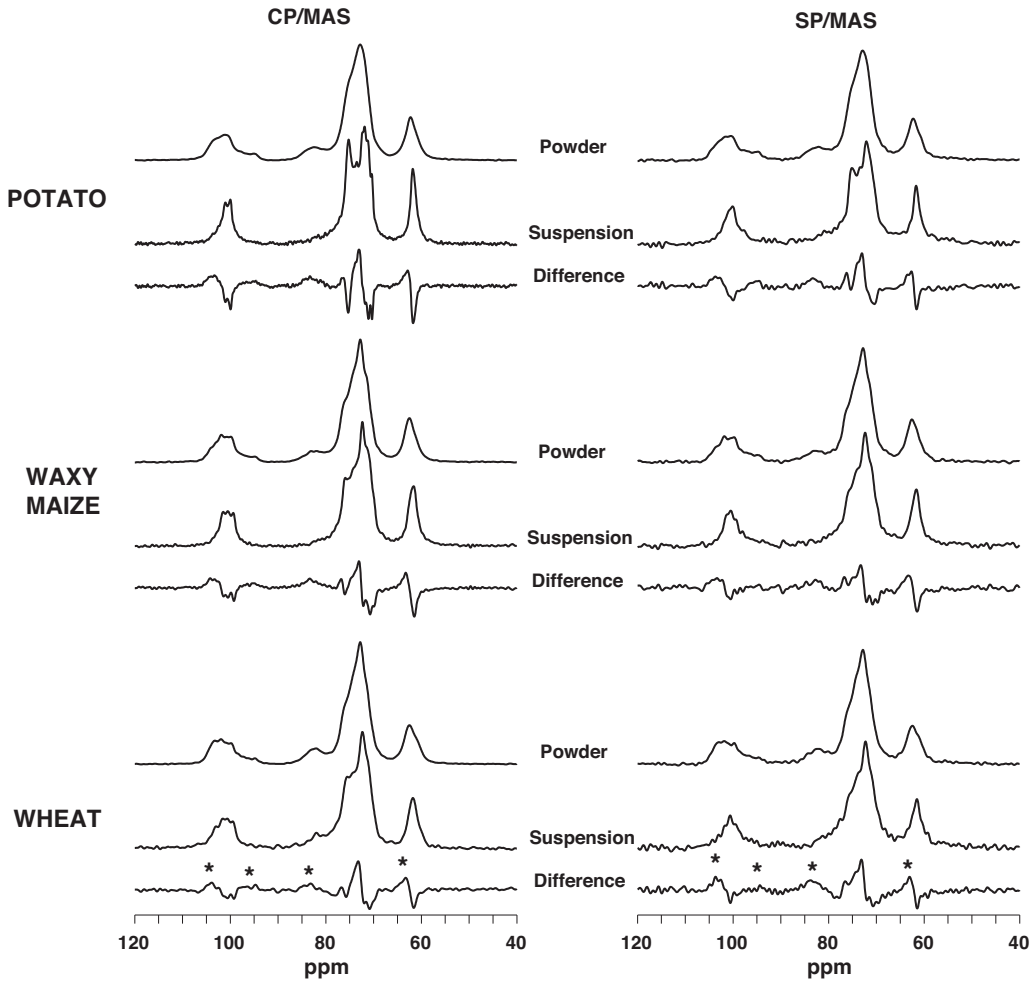

Fig. 3. ^{13}C CP/MAS (left column) and ^{13}C SP/MAS (right column) NMR spectra of NA samples of potato (top), waxy maize (middle) and wheat (bottom) starches as powders (top), suspensions (middle) and the difference between these (bottom). All CP/MAS and SP/MAS spectra were adjusted to a total integral of 1. For suspensions the hydration level was 57.2% for potato starch and 57.1% for waxy maize and wheat starches, respectively.

3.3. *Effects of crystalline polymorph*

To assess if the observed hydration effects are specific for B-type crystalline polymorphic starches two A-type starches, wheat starch and waxy maize starch, were subjected to a similar hydration procedure. NA samples were analyzed as powders and suspensions by ^{13}C CP/MAS and SP/MAS NMR and also the differences between spectra of powders and suspensions were examined (Fig. 3). The CP/MAS spectra of both wheat starch and waxy maize suspensions reveal three C1 resonances characteristic for A-type starches. In addition the spectrum of wheat starch suspension contains a C1 resonance at ~103 ppm and a C4 resonance at ~82 ppm suggesting the presence of V-amylose (Gidley & Bociek, 1988; Veregin, Fyfe, & Marchessault, 1987; Veregin, Fyfe, Marchessault, & Taylor, 1987). The resonances with negative intensity in the difference spectra mainly reflect the carbon sites in the immobile/crystalline regions of the samples, whereas the resonances with positive intensity are indicative of mobile/disordered regions. Resonances of positive intensity are located at ~63, 83, 95.9 and 103.7 ppm. The fact that the mobile/disordered regions were also present in wheat starch, suggests that these substructures are similar but not identical to V-amylose in agreement with previous findings (Gidley & Bociek, 1985; Veregin, Fyfe, & Marchessault, 1987; Veregin, Fyfe, Marchessault, & Taylor, 1987). Since V-amylose adopts a single helix conformation, possibly in complex with lipids (Gidley & Bociek, 1988; Veregin, Fyfe, & Marchessault, 1987; Veregin, Fyfe, Marchessault, & Taylor, 1987) it is highly likely that the single helix structure is predominant in the amorphous regions of starch.

In order to compare the structural impact of gelatinization on the three starch types, we focused on the C1 spectral region (90–110 ppm) of NA and GEL samples (Fig. 4). The CP/MAS and SP/MAS spectra of the powders were similar for each sample, whereas different distributions of C1 chemical shifts were observed for the samples of native starches according to the botanical origin. Similar chemical shift distributions were obtained for all the GEL samples independent of botanical origin. In the CP/MAS spectra of all hydrated starches the characteristic two resonances for B-type (potato) starch and three resonances for A-type (waxy maize) starches were observed. In addition, the hydrated wheat starch contained an additional resonance at ~103.0 ppm-assigned to V-amylose. This resonance was not present in the corresponding SP/MAS spectrum and enhanced by cross polarization indicating that this originates from immobile regions. This also reflects the poor hydration properties of V-amylose (Snape, Morrison, Maroto-Valer, Karkalas, & Pethrick, 1998). The presence of V-amylose in gelatinized wheat starch may also be the reason for higher PDI and IV values for wheat starch as compared to potato starch (Table 2). For powdered starch, the intensity of the V-amylose resonance was also more intense for wheat than for potato and waxy maize. An additional low intensity resonance in the CP/MAS spectrum of wheat starch powder at 32.0 ppm (data not shown) suggests the presence of a lipid-V-amylose complex (Snape et al., 1998). For the

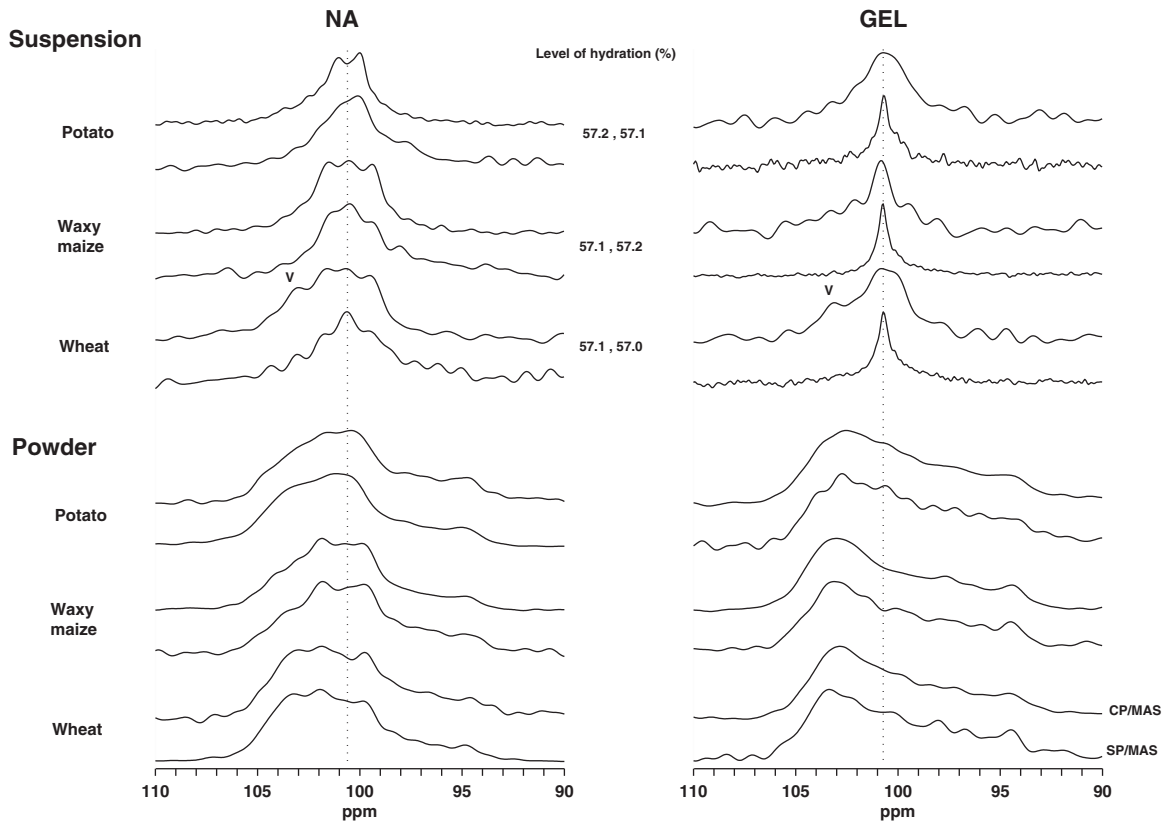

Fig. 4. ^{13}C SP/MAS (bottom) and ^{13}C CP/MAS (top) NMR spectra (spectral region 90–110 ppm: resonance of C1) of wheat, waxy maize and potato starch in the NA (left column) and the GEL (right column) state. Spectra of powders (lower part of figure) as well as suspensions (upper part of figure) are displayed. The "V" indicates V-amylose. Broken vertical lines at 100.7 ppm are inserted as a guide to the eye.

hydrated GEL samples, all of the SP/MAS spectra were characterized by a single resonance at ~100.7 ppm, whereas broader resonances were observed in the CP/MAS spectra – particularly for potato and wheat starch. This shift corresponds to the average chemical shift of the two B-type and the three A-type specific resonances of the hydrated NA starches as can be seen in Table 3. Previously the torsion angles (ϕ,ψ) for the α-1,4 glycosidic linkage have been shown to be proportional to ($|\phi| + |\psi|$) as well as $|\psi|$ (Gidley & Bociek, 1988; Veregin, Fyfe, & Marchessault, 1987; Veregin, Fyfe, Marchessault, & Taylor, 1987). In Table 3 the torsion angles from crystallographic analysis of crystalline A- and B-type starch as well as torsion angles of the second glucose unit in maltotriose (aq) are shown along with the calculated sum of these angles. It is noted that the sum of ϕ and ψ is close to −60 for each of the crystallographic sites in the starch whereas the sum is −32 and −88 for the two lowest energy conformations in maltotriose (aq). Assuming that these two conformations are in fast exchange only the average structure will be observed by NMR and the torsion angles describing the average structure are almost identical to the average of the average torsion angles for crystalline A- and B-type starches. On the basis of this, it is highly likely that the torsion angles (ϕ,ψ) for the hydrated gelatinized starches will be an average of the torsion angles of the crystalline A- and B-type starches. In view of this, the similarity of the powder spectra of GEL samples suggests that A- and B-type

Table 3
Torsion angles and ^{13}C chemical shifts for crystalline type A- and type B-starch as well as maltotriose (aq).

Compound	ϕ (deg)[a]	ψ (deg)[b]	Sum (deg)	Chemical shift (solid) (ppm)[c]	Chemical shift (GEL/liquid) (ppm)
A-starch[d]	91.8	−153.2	−61.4	101.4	
	85.7	−145.3	−59.6	100.5	
	91.8	−151.3	−59.5	99.4	
Average (*A*)	89.8	−149.9	−60.2	100.4	100.7
B-starch[e]	83.8	−144.6	−60.8	101.0	
	84.3	−144.1	−59.8	100.0	
Average (*B*)	84.1	−144.4	−60.3	100.5	100.7
Maltotriose[f]	67	−155	−88		
	106	−138	−32		
Average (Maltotriose)	86.5	−146.5	−60		99.7
Average (*A* + *B*)	86.9	−147.1	−60.2		

[a] Defined as O5–C1–O1–C4′.
[b] Defined as C1–O1–C4′–C5′.
[c] Tentative assignment.
[d] Imberty et al. (1988).
[e] Imberty and Pérez (1988).
[f] Hansen et al. (2008), second glucose unit.

crystalline structures result in the same structure or collection of structures. The latter term is more appropriate since the C1 chemical shift rage covers 12 ppm (94–106 ppm). Due to the correlation between C1 and torsion angles this also means that the distribution of torsion angles for the α-1,4 glucosidic linkages are the same for all gels independent of the botanical origin.

3.4. Hydration effects on phosphorous

Potato starch differs from wheat and waxy maize starches by being highly phosphorylated (Hoover, 2001; Kasemsuwan & Jane, 1996; Lim et al., 1994). In principle indirect information about phosphorylation could be obtained by ^{13}C NMR, but since only about 0.3% of the glucose residues are phosphorylated with about 1/3 of the P on C3 and about 2/3 of the P on C6 (Blennow, Bay-Smidt, Olsen, & Møller, 2000) this will require very good S/N-ratios. Moreover the anticipated chemical shifts for phosphorylated C3 and C6 sites ~77 ppm and ~64 ppm, respectively (Hansen et al., 2009), and only the C6-P resonance will be present in a non-overlapped spectral region. In addition, ^{31}P has a natural abundance of 100% (natural abundance for ^{13}C is 1.1%), making ^{31}P NMR an ideal tool for analyzing phosphorous sites in starch. There is ample data on liquid-state ^{31}P NMR for gelatinized or pre-treated starch in solution (Bay-Smidt, Wischmann, Olsen, & Nielsen, 1994; Kasemsuwan & Jane, 1996; Lim et al., 1994) and our work provides for the first time solid state NMR data for the phosphorous sites in starch. ^{31}P SP/MAS and CP/MAS data (Fig. 5) demonstrates that powders are very similar whereas significant differences were found for the suspensions due to major structural changes in the phosphorous environments upon hydration. An important observation is that phosphorous in immobile regions (^{31}P CP/MAS spectra) was only observed for NA suspensions demonstrating that all P is mobilized upon disruption of the starch granule by gelatinization even after intermittent drying. This effect is in agreement with previous observations (Larsen, Blennow, & Engelsen, 2008). Another very important observation is the broader chemical shift range for GEL powder compared to NA powder. This reflects more disordered phosphorous environments in the gelatinized and subsequently dried samples. Upon hydration two distinct ^{31}P resonances were observed for the GEL sample. Changing pH from 5.5 to 7.0, the chemical shift of the most intense resonance increased by 3.4 ppm as anticipated for P on C3 or C6 (Blennow, Bay-Smidt, Olsen, & Møller, 1998) whereas the chemical shift of the low intensity site only increased by 0.1 ppm (Table 1). This demonstrates that this site is situated in a pH-insensitive environment. By the action of branching enzyme the number of branch points is increased (Table 2), the molecular weight is significantly reduced and the abundance of pH-insensitive P is reduced. These effects are enhanced by the action of β-amylase completely removing the pH-insensitive P. The exact nature of this phosphorous is still to be disclosed.

To further detail the assignment of phosphorous sites (Table 1) it was observed that the phosphorous sites in the suspended GEL, BE and BB were characterized by a chemical shifts in the range 0.6–0.8 ppm – similar to the P-site in suspended NA at 1.0 ppm and very close to the resonance with maximum intensity in the spectra of NA, BE and BB powders at 0.7 ppm. Comparison with assignments of P on C6 (P6) and P on C3 (P3) in liquid-state NMR (Bay-Smidt et al., 1994) this suggest that the P-sites in the region 0.5–1.0 ppm originate from P6 sites, whereas resonances characterized by chemical shifts in the region 1.0–2.0 ppm originate from

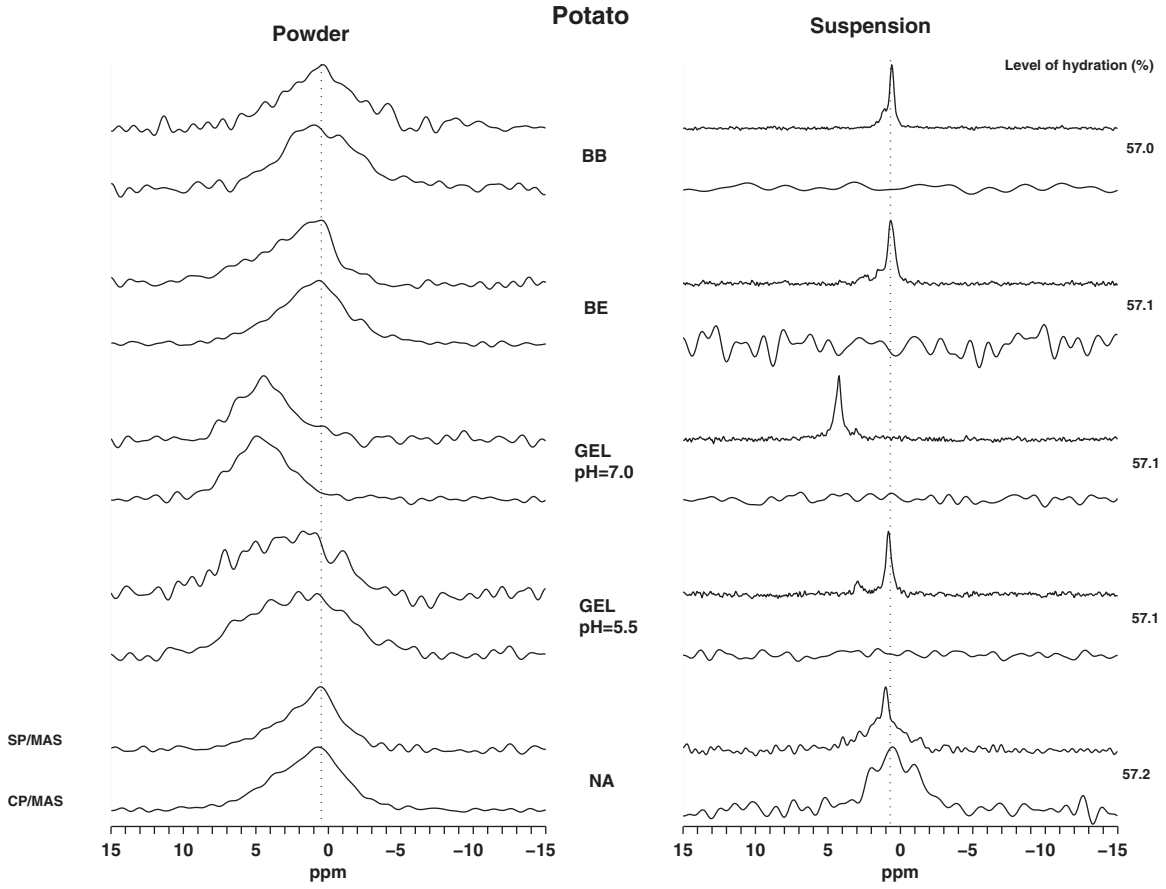

Fig. 5. ^{31}P CP/MAS (bottom) and ^{31}P SP/MAS (top) NMR spectra of NA, GEL, BE and BB potato samples. Samples were analyzed as powders (left column) and as suspensions (right column). Broken vertical lines at 0.7 ppm are inserted as a guide to the eye. GEL samples dried from suspensions with pH-values of both 5.5 and 7.0 are included.

P3 sites. In the spectrum of suspended NA, a narrow resonance on top of a broad resonance – both characterized by a chemical shift of 1.0 ppm – were observed. Due to the non-optimal S/N-ratio the corresponding CP/MAS spectrum can be interpreted as three resonances having chemical shifts of −1.0, 0.5 and 2.0 ppm or as one broad resonance with a chemical shift ∼0.5 ppm. The broad resonances observed in both spectra indicate either disorder or different chemical environments around the P sites. In the spectrum of suspended BB deconvolution of the two overlapping resonances resulted in a P3:P6-ratio of 22:78 in agreement with previous reports (Blennow et al., 2000).

All together this confirms that the main part of the phosphorous is present as P6 sites, but only in the spectrum of the BB sample it was possible to distinguish between P3 and P6 sites.

4. Conclusions

In this work, native (NA), gelatinized (GEL) and enzymatically modified (BE and BB) potato starches were analyzed as powders and aqueous suspensions using solid-state ^{13}C and ^{31}P CP/MAS and SP/MAS NMR. Gelatinization and enzyme-assisted branching and outer chain trimming enhanced the spectral resolution. Powder spectra of these samples were characterized by different distinct distributions of C1 chemical shifts and an increased C6 chemical shift range indicating increased disorder. The opposite trend was observed for spectra of suspensions indicating a higher degree of molecular order. The immobile regions in GEL exhibited a broader distribution of C6 chemicals shifts compared to NA and is an exception to this trend.

Hydration and ordering of the carbons in the α-1,6 glycosidic branch-linkages required a hydration level of around 70% indicating hydrophobicity of this linkage important for its susceptibility for e.g. industrial enzymes.

Difference spectra of powders/suspensions of NA starches suggest that the amorphous regions consist of structural units similar to V-amylose and therefore to be present as disordered single helices.

All hydrated GEL samples were characterized by an average C1 chemical shift of 100.7 ppm corresponding to the average chemical shift of the A and B crystalline parts of hydrated NA starches. Since the C1 chemical shift depends on the ψ- and ϕ-angles of the glycosidic linkage, this indicates that the molecular structure in the gel is an average structure of the crystalline structure.

Finally, only in NA samples phosphorous was present in the immobile regions. Following gelatinization phosphorous was mobilized. Interestingly, pH-insensitive phosphorous sites were observed in hydrated GEL and BE starches demonstrating phosphorous that is present in other forms besides phosphate, yet to be identified.

Acknowledgment

Financial support from the Danish Strategic Research Council through the project “Biofunctional Carbohydrates” (project no. 2101-08-0068) is gratefully acknowledged.

References

Ao, Z. H., Simsek, S., Zhang, G., Venkatachalam, M., Reuhs, B. L., & Hamaker, B. R. (2007). Starch with a slow digestion property produced by altering its chain length, branch density and crystalline structure. *Journal of Agricultural and Food Chemistry*, *55*, 4540–4547.

Bay-Smidt, A. M., Wischmann, B., Olsen, C. E., & Nielsen, T. H. (1994). Starch bound phosphate in potato as studied by a simple method for determination of organic phosphate and ^{31}P NMR. *Starch/Stärke*, *46*, 167–172.

Bennett, A. E., Rienstra, C. M., Auger, M., Lakshmi, K. V., & Griffin, R. G. (1995). Heteronuclear decoupling in rotating solids. *Journal of Chemical Physics*, *103*, 6951–6958.

Best, R. B., Jackson, G. E., & Naidoo, K. J. (2002). Modeling of the α(1 → 6) branch point of amylopectin in solution. *Journal of Physical Chemistry B*, *106*, 5091–5098.

Blennow, A., Bay-Smidt, A. M., Olsen, C. E., & Møller, B. L. (1998). Analysis of starch-bound glucose 3-phosphate and glucose 6-phosphate using controlled acid treatment combined with high-performance anion-exchange chromatography. *Journal of Chromatography A*, *829*, 385–391.

Blennow, A., Bay-Smidt, A. M., Olsen, C. E., & Møller, B. L. (2000). The distribution of covalently bound phosphate in the starch granule in relation to starch crystallinity. *International Journal of Biological Macromolecules*, *27*, 211–218.

Gidley, M. J. (1989). Molecular mechanisms underlying amylose aggregation and gelation. *Macromolecules*, *22*, 351–358.

Gidley, M. J., & Bociek, S. M. (1985). Molecular organization in starches: A ^{13}C CP/MAS NMR study. *Journal of the American Chemical Society*, *107*, 7040–7044.

Gidley, M. J., & Bociek, S. M. (1988). ^{13}C CP/MAS NMR studies of amylose inclusion complexes, cyclodextrins, and the amorphous phase of starch granules: Relationships between glycosidic linkage conformation and solid-state ^{13}C chemical shifts. *Journal of the American Chemical Society*, *110*, 3820–3829.

Gidley, M. J., & Bulpin, P. V. (1989). Aggregation of amylose in aqueous systems: The effect of chain length on phase behavior and aggregation kinetics. *Macromolecules*, *22*, 341–346.

Hansen, P. I., Larsen, F. H., Motawia, S. M., Blennow, A., Spraul, M., Dvortsak, P., et al. (2008). Structure and hydration of the amylopectin trisaccharide building blocks-synthesis, NMR and molecular dynamics. *Biopolymers*, *89*, 1179–1193.

Hansen, P. I., Spraul, M., Dvortsak, P., Larsen, F. H., Blennow, A., Motawia, S. M., et al. (2009). Starch phosphorylation–maltosidic restrains upon 3′- and 6′-phosphoryylation investigated by chemical synthesis, molecular dynamics and NMR spectroscopy. *Biopolymers*, *91*, 179–193.

Hoover, R. (2001). Composition, molecular structure and physicochemical properties of tuber and root starches: A review. *Carbohydrate Polymers*, *45*, 253–267.

Horii, F., Hirai, A., & Kitamaru, R. (1986). CP/MAS ^{13}C NMR spectroscopy of hydrated amyloses using a magic-angle spinning rotor with an o-ring seal. *Macromolecules*, *19*, 930–932.

Horii, F., Yamamoto, H., Hirai, A., & Kitamaru, R. (1987). Structural study of amylose polymorphs by cross-polarization-magic-angle spinning, ^{13}C NMR spectroscopy. *Carbohydrate Research*, *160*, 29–40.

Imberty, A., Buléon, A., Tran, V., & Pérez, S. (1991). Recent advances in knowledge of starch structure. *Starch/Stärke*, *43*, 375–384.

Imberty, A., Chanzy, H., Pérez, S., Buléon, A., & Tran, V. (1988). The double-helical nature of the crystalline part of A-starch. *Journal of Molecular Biology*, *201*, 365–378.

Imberty, A., & Pérez, S. (1988). A revisit to the three-dimensional structure of B-type starch. *Biopolymers*, *27*, 1205–1221.

Imberty, A., & Pérez, S. (1989). Conformational analysis and molecular modeling of the branching point of amylopectin. *International Journal of Biological Macromolecules*, *11*, 177–185.

Jobling, S. (2004). Improving starch for food and industrial applications. *Current Opinion in Plant Biology*, *7*, 210–218.

Kainuma, K., & French, D. (1972). Naegeli amylodextrin and its relationship to starch granule structure. II. Role of water in crystallization of B-starch. *Biopolymers*, *11*, 2241–2250.

Kasemsuwan, T., & Jane, J.-L. (1996). Quantitative method for the survey of starch phosphate derivatives and starch phospholipids by ^{31}P nuclear magnetic resonance spectroscopy. *Cereal Chemistry*, *73*, 702–707.

Kasprzak, M. M., Lærke, H. N., Larsen, F. H., Bach Knudsen, K. E., Pedersen, S., & Jørgensen, A. S. (2012). Effect of enzymatic treatment of different starch sources on the *in vitro* rate and extent of starch digestion. *International Journal of Molecular Sciences*, *13*, 929–942.

Larsen, F. H., Blennow, A., & Engelsen, S. B. (2008). Starch granule hydration – A MAS NMR investigation. *Food Biophysics*, *3*, 25–32.

Larsen, F. H., Byg, I., Damager, I., Diaz, J., Engelsen, S. B., & Ulvskov, P. (2011). Residue specific hydration of primary cell wall potato pectin identified by solid-state ^{13}C single-pulse MAS and CP/MAS NMR spectroscopy. *Biomacromolecules*, *12*, 1844–1850.

Larsen, F. H., Schöbitz, M., & Schaller, J. (2012). Hydration properties of regioselectively etherified celluloses monitored by ^{2}H and ^{13}C solid-state MAS NMR spectroscopy. *Carbohydrate Polymers*, *89*, 640–647.

Lim, S.-T., Kasemsuwan, T., & Jane, J.-L. (1994). Characterization of phosphorous in starch by ^{31}P-nuclear magnetic resonance spectroscopy. *Cereal Chemistry*, *71*, 488–493.

Marchessault, R. H., Taylor, M. G., Fyfe, C. A., & Veregin, R. P. (1985). Solid-state ^{13}C CP-MAS NMR of starches. *Carbohydrate Research*, *144*, C1–C5.

Metz, G., Wu, X., & Smith, S. O. (1994). Ramped-amplitude cross polarization magic-angle-spinning NMR. *Journal of Magnetic Resonance Series A*, *110*, 219–227.

Pandey, A., Nigam, P., Soccol, C. R., Soccol, V. T., Singh, D., & Mohan, R. (2000). Advances in microbial amylases. *Biotechnology and Applied Biochemistry*, *31*, 135–152.

Paris, M., Bizot, H., Emery, J., Buzaré, J. Y., & Buléon, A. (1999). Crystallinity and structuring role of water in native and recrystallized starched by ^{13}C CP-MAS NMR spectroscopy. 1. Spectral decomposition. *Carbohydrate Polymers*, *39*, 327–339.

Paris, M., Bizot, H., Emery, J., Buzaré, J. Y., & Buléon, A. (2001). NMR local range investigations in amorphous starchy substrates. I. Structural heterogeneity probed by ^{13}C CP-MAS NMR. *International Journal of Biological Macromolecules*, *29*, 127–136.

Roundeau-Mouro, C., Veronese, G., & Buleon, A. (2006). High-resolution solid-state NMR of B-type amylose. *Biomacromolecules*, *7*, 2455–2460.

Sefzik, T. H., Houseknecht, J. B., Clark, T. M., Prasad, S., Lowary, T. L., Gan, Z., et al. (2007). Solid-state ^{17}O NMR in carbohydrates. *Chemical Physics Letters*, *434*, 312–315.

Shinohara, M. L., Ihara, M., Abo, M., Hashida, M., Takagi, S., & Beck, T. C. (2001). A novel thermostable branching enzyme from an extremely thermophilic bacterial species. *Applied Microbiology and Biotechnology*, *57*, 653–659.

Smith, A. M., Zeeman, S. C., & Smith, S. M. (2005). Starch degradation. *Annual Reviews of Plant Biology*, *56*, 73–98.

Snape, C. E., Morrison, W. R., Maroto-Valer, M. M., Karkalas, J., & Pethrick, R. A. (1998). Solid state ^{13}C NMR investigation of lipid ligands in V-amylose inclusion complexes. *Carbohydrate Polymers*, *36*, 225–237.

Suortti, T. (1993). Size-exclusion chromatographic determination of β-glucan with postcolumn reaction detection. *Journal of Chromatography A*, *632*, 105–110.

Svensson, B. (1994). Protein engineering in the α-amylase family: Catalytic mechanism, substrate specificity, and stability. *Plant Molecular Biology*, *25*, 141–157.

Tan, I., Flanagan, B. M., Halley, P. J., Whittaker, A. K., & Gidley, M. J. (2007). A method for estimating the nature and relative proportions of amorphous, single and double-helical components in starch granules by ^{13}C CP/MAS NMR. *Biomacromolecules*, *8*, 885–891.

Tang, H., & Hills, B. P. (2003). Use of ^{13}C MAS NMR to study domain structures and dynamics of polysaccharides in the native starch granules. *Biomacromolecules*, *4*, 1269–1276.

Veregin, R. P., Fyfe, C. A., & Marchessault, R. H. (1987). Investigation of the crystalline V-amylose complexes by high-resolution ^{13}C CP/MAS NMR spectroscopy. *Macromolecules*, *20*, 3007–3012.

Veregin, R. P., Fyfe, C. A., Marchessault, R. H., & Taylor, M. G. (1986). Characterization of the crystalline A and B starch polymorphs and investigation of starch crystallization by high-resolution ^{13}C CP/MAS NMR. *Macromolecules*, *19*, 1030–1034.

Veregin, R. P., Fyfe, C. A., Marchessault, R. H., & Taylor, M. G. (1987). Correlation of ^{13}C chemical shifts with torsion angles from high-resolution, ^{13}C CP-MAS NMR studies of crystalline cyclomalto-oligosaccharide complexes, and their relation to the structures of the starch polymorphs. *Carbohydrate Research*, *160*, 41–56.

Wu, H.-C. H., & Sarko, A. (1978a). The double-helical molecular structure of crystalline B-amylose. *Carbohydrate Research*, *61*, 7–25.

Wu, H.-C. H., & Sarko, A. (1978b). The double-helical molecular structure of crystalline A-amylose. *Carbohydrate Research*, *61*, 27–40.

You, S. G., Fiedorowicz, M., & Lim, S. T. (1999). Molecular characterization of wheat amylopectins by multiangle laser light scattering analysis. *Cereal Chemistry*, *76*, 116–121.