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Level and position of substituents in cross-linked and hydroxypropylated sweet potato starches using nuclear magnetic resonance spectroscopy

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ABSTRACT

Sweet potato starch was cross-linked using sodium trimetaphosphate and hydroxypropylated using propylene oxide. The level and position of phosphorus and hydroxypropyl groups within cross-linked and hydroxypropylated sweet potato starch was investigated by phosphorus and proton nuclear magnetic resonance spectroscopy (^{31}P, ^{1}H NMR). The cross-linking reaction produced monostarch monophosphate and distarch monophosphate in a molar ratio of 1:1.03, indicating that only half of the introduced phosphorus resulted in a possible cross-link. One cross-link per approximately 2900 glucose residues was found. Phosphorylation leading to monostarch monophosphate mainly occurred at O-3 and O-6 (ratio 1:1). It was inferred that the majority of the cross-links formed in distarch monophosphate were between two glucose residues positioned in different starch chains, while a minor part of the cross-links may be formed between two glucose residues within the same starch chain. Hydroxypropylation under alkaline conditions resulted in the formation of intra-molecular phosphorus cross-links, subsequent hydroxypropylation following cross-linking lowered both the level of intra- and inter-molecular cross-linking.

Using ^{1}H NMR the molar substitution of hydroxypropylation was determined to be 0.155–0.165. The hydroxypropylation predominantly occurred at O-2 (61%), and the level of substitution at O-6 (21%) was slightly higher than that at O-3 (17%). In dual modified starch, the preceding cross-linking procedure resulted in a slightly lower level of hydroxypropylation, where the substitution at O-6 decreased more compared to the substitution at O-2 and O-3.

1. Introduction

Starches are often modified to improve their functional properties for specific food applications. Cross-linking and hydroxypropylation are two important chemical modifications commonly used in the starch industry (Goff, 2004). For cross-linking, starch can be treated with a variety of multiple functional reagents, such as phosphorus oxychloride ($POCl_3$), sodium trimetaphosphate (STMP), sodium tripolyphosphate (STPP), or epichlorohydrin (EPI), forming either ester or ether cross-links between starch through their hydroxyl groups (Singh, Kaur, & McCarthy, 2007). Cross-links between starch chains reinforce the starch granule resulting in a good stability against heating, shear force and acid conditions (Wurzburg, 1986). Hydroxypropylation is one of the methods to make starch more stable towards retrogradation and represents a base-catalysed nucleophilic substitution reaction of propylene oxide and starch. Hydroxypropylation is used to increase paste consistency and clarity, and to improve freeze-thaw and cool-storage stabilities (Tuschhoff, 1986). Cross-linking of starch is often combined with hydroxypropylation (Wurzburg, 1986).

Even a very few cross-links can significantly change the paste and gel properties of starch (Singh et al., 2007). For controlling the modification reactions and hence, to control and optimise the production and use of modified starches, it is necessary to be able to correlate the extent and location of cross-links along the starch backbone with the functionality obtained. Depending on the type

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of starch, phosphorus may be present in the native starch at a low level (0.002–0.089%, w/w, dry basis) and may be divided over different ester forms (Dona et al., 2007; Jane, Kasemsuwan, Chen, & Juliano, 1996). Depending on starch source, phosphorus can even be present at low levels as phosphate or phospholipids. Therefore, the accurate determination of the level and location of phosphorylation within the starch is rather complicated. During the crosslinking reaction using STMP, distarch phosphate cross-links are formed, while also monoesters are formed as monostarch monophosphate (MSMP) without connecting starch molecules (Singh et al., 2007). For this reason, the spectrophotometric method to determine the degree of cross-linking according to the change in phosphorus content before and after cross-linking reaction might not be correct (Kasemsuwan & Jane, 1996).

^{31}P nuclear magnetic resonance (NMR) spectroscopy has been used to analyse the location of phosphorylation in native and modified starches. Phosphorus present in different forms results in different chemical shifts in the NMR spectrum. The results of phosphorus quantified by ^{31}P NMR show that phosphate monoesters were primarily present in potato, mung bean, and tapioca starch (0.0062–0.086%), while phospholipids were mainly present in wheat, maize, and high amylose maize starch (0.0097–0.058%) (Kasemsuwan & Jane, 1996). The phosphorylation of wheat starch prepared with sodium tripolyphosphate under semidry conditions was mainly at the O-6 position of glucose units with concomitant lower extents of phosphorylation at the O-3 and O-2 positions (Lim & Seib, 1993). In phosphorylated cross-linked wheat starch prepared with sodium trimetaphosphate and sodium tripolyphosphate, the molar ratio of distarch monophosphate and monostarch monophosphate formed was around 1:1 (Sang, Prakash, & Seib, 2007).

^{1}H NMR spectroscopy has also been used to determine the substitution of hydroxypropyl groups in hydroxypropylated starch. It has been shown that hydroxypropylation of the glucose unit occurred primarily (67–79%) at the O-2 position for potato, wheat and maize starch (Richardson, Nilsson, Bergquist, Gorton, & Mischnick, 2000; Xu & Seib, 1997). However, previous studies focussed only on the hydroxypropylation of single modified starch and the effect of two subsequent modifications on the achieved level of cross-linking and hydroxypropylation is still unknown.

The aim of this study was to investigate the level of modification and the position of substituents on glucose residues in cross-linked and/or hydroxypropylated sweet potato starches. The starches were hydrolysed with enzymes and the hydrolysates were analysed with ^{31}P and ^{1}H NMR.

2. Materials and methods

2.1. Starch samples

Native sweet potato starch (NT-SPS, SuShu2 species) was isolated as described previously (Chen, Schols, & Voragen, 2003). The modified starches were prepared at the AVEBE facilities and all modifications were conducted in aqueous suspensions of the granular starches (Chen et al., 2003). The cross-linked sweet potato starch (CL-SPS) was obtained by adding 0.002 mol sodium trimetaphosphate per mole glucose residue within the starch. The hydroxypropylated sweet potato starch (HP-SPS) was prepared by addition of 0.2 mol propylene oxide per mole glucose residue. The dual modified starch (cross-linked and hydroxypropylated sweet potato starch, CLHP-SPS) was prepared first with sodium trimetaphosphate, followed by the propylene oxide treatment (Zhao et al., 2012). The glucose/starch content was measured as described previously (Zhao et al., 2012).

Crystallised *Bacillus* sp. α-amylase (Type II-A, A6380, ≥1500 units/mg protein), and Rhizopus sp. amyloglucosidase (A7255, 11,600 units/g) were purchased from Sigma-Aldrich (St. Louis, MO, USA). One unit of α-amylase will liberate 1.0 mg of maltose from starch in 3 min at pH 6.9, 20 °C. One unit of amyloglucosidase will liberate 1.0 mg of glucose from soluble starch in 3 min at pH 4.5, 55 °C. Nicotinamide adenine dinucleotide (NAD) was obtained from Boehringer Mannheim (Germany). Sodium azide, Ethylene diamine tetraacetic acid disodium salt dehydrate were purchased from Merck (Darmstadt, Germany). Deuterium oxide (99.9 atom% D) was from Sigma–Aldrich.

2.2. Sample preparation for ^{31}P NMR analysis

Each starch sample was converted to α, γ-limit dextrins by enzymatic digestion before ^{31}P NMR spectroscopy according to published methods (Sang et al., 2007) with minor modification. Starch (1 g, dry basis) was dispersed in 45 mL of distilled water containing 2.0 mM calcium chloride ($CaCl_2$). Alpha-amylase (200 units) was added to the mixture, followed by gradual heating in a boiling shaking water bath for 15 min to enable gelatinisation with vigorous stirring in 3 min intervals. The suspension was cooled down and the pH was adjusted to 6.5. Then, additionally, 200 units of α-amylase were added and the mixture was incubated at 70 °C for 1 h. The hydrolysate was adjusted to pH 4.5 with 0.5 M hydrochloric acid (HCl) and amyloglucosidase (200 units) was added and the mixture was sequentially incubated at 60 °C for 2 h in a shaking water bath. Next, the mixture was boiled for 15 min to inactivate the enzymes. After cooling to ambient temperature, the mixture was adjusted to pH 7; centrifuged (3000 × *g* for 10 min) and the supernatant obtained was freeze-dried. The recovered soluble hydrolysates accounted at least for 97% weight (dry basis) of original samples and allowed to make high concentrations of starch with relative low viscosity in order to accelerate the NMR measurement and to obtain sharp peak signals.

2.3. Sample preparation for ^{1}H NMR analysis

Each starch (0.1 g, dry basis) was converted to α-limit dextrins by α-amylase treatment before the ^{1}H NMR measurement, using the similar treatment as described above leaving out the amyloglucosidase digestion. The recovered soluble hydrolysates accounted for at least 95% weight (dry basis) of the original samples.

2.4. ^{31}P NMR

The freeze-dried starch hydrolysates were mixed with 1.5 mL distilled water, 0.2 mL nicotinamide adenine dinucleotide solution (5 mg/mL), 0.2 mL ethylene diamine tetraacetic acid, disodium salt dihydrate (EDTA, 0.2 M), 0.05 mL sodium azide (NaN_3, 1 mg/mL), and 0.2 mL deuterium oxide. The solution was adjusted to pH 8 ± 0.1 by adding 0.5 M sodium hydroxide, and then transferred to a 10 mm NMR tube (Wilmad-LabGlass, Vineland, NJ, USA).

The proton decoupled ^{31}P NMR spectra were acquired on a Bruker AVANCE 300WB NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 10 mm probe operating at a frequency of 121.5 MHz. For each spectrum, 2500–6000 transients were collected with 10 s relaxation delay between pulses at a temperature of 25 °C. The spectra were processed with Bruker TopSpin 1.3 software. Chemical shifts are reported in δ (ppm) based on the external reference signal of 85% phosphoric acid at 0 ppm. The NAD signal (−10.9 ppm) was used as internal reference for the calculation of phosphorus content using integrals of the signals. This calculation of phosphorus content was based on the ratio of peak area of phosphorus in the sample as compared to the peak area of the internal reference phosphorus NAD with known concentration. A small amount of inorganic phosphorus was found in the

amylolytic enzymes, which was subtracted from the phosphorus content of the hydrolysed starches.

2.5. ^{1}H NMR

Each freeze dried starch α-limit dextrin (10 mg) was solubilised in 0.5 mL deuterium oxide in a 5 mm NMR tube, and spectra were recorded on a Bruker AVANCE 500 NMR spectrometer (Bruker). Spectra were measured at 25 °C, 1.6 s acquisition time, 10 s delay, 8 scans per spectrum, using a standard pulse sequence including presaturation of the water signal.

2.6. Spectrometric determination of phosphorus and hydroxypropyl groups

The total phosphorus content in starch was determined using an ammonium molybdate and vanadate spectrophotometric method described previously (Zhao et al., 2012). The hydroxypropyl content in the starches was measured using a ninhydrin spectrophotometric method as described (Lim, Kasemsuwan, & Jane, 1994).

3. Results and discussion

3.1. Level and location of cross-links

The phosphorus contents of the various starches were measured by ^{31}P NMR and the total phosphorus content was calculated (Table 1). The total phosphorus contents were also measured using a spectrophotometric method (Zhao et al., 2012), which is sensitive for low phosphorus content in starch. The total phosphorus content ranged from 0.0171% to 0.0239% (w/w, db) by ^{31}P NMR and is slightly lower than the data measured by the spectrophotometric method (0.0197% to 0.0265% (w/w, db)). In addition to a systematic difference between the two methods, this difference might be partly due to the removal of a small quantity of insoluble residue after enzymatic starch hydrolysis. Since starch degrading enzymes are inhibited by the phosphorus and hydroxypropyl groups, the remaining small fragments resulting from an extended digestion by amylolytic enzymes can readily be used for NMR analysis (Lim & Seib, 1993; Lim et al., 1994; Sang et al., 2007).

3.1.1. Phosphorus in native starch

The ^{31}P NMR spectrum of native sweet potato starch hydrolysates is shown in Fig. 1A. The quartet peaks centred at −10.9 ppm originates from the phosphorus internal standard, nicotinamide adenine dinucleotide. The quartet peak is a doublet of doublets with two chemical shift values for the ^{31}P. The integral of the quartet peak was used to quantify the two ^{31}P atoms of NAD. The intense peak at 2.5 ppm was assigned to inorganic phosphate (Pi) (Kasemsuwan & Jane, 1994). The single peak at 3.5 ppm was assigned to monostarch monophosphate substituted to O-6 of a glucose residue (Lim et al., 1994). The peak centred at 4.4 ppm was assigned to MSMP substituted at the O-3 position of glucose (Sang, Seib, Herrera, Prakash, & Shi, 2010). The split of signal is probably due to the presence of different chain lengths of phosphorylated dextrins. Because the amylolytic enzymes cannot hydrolyse the glucosidic bonds near a phosphate group, the final hydrolysates containing phosphorus have various chain lengths. The two signals in the NMR spectrum might represent different chain length populations of the enzyme resistant dextrins. The small peak at 4.7 ppm was assigned to MSMP substituted at O-2 of a glucose residue.

The level and form of phosphorus present in the sweet potato starches are shown in Table 1. In native sweet potato starch, nearly half of the phosphorus is of inorganic nature, despite the washing procedures during starch isolation. The remaining part of phosphorus is present as MSMP. For the MSMP, the proportions of phosphorus at O-6, O-3 and O-2 in MSMP were 67%, 32% and 1%, respectively. This indicates that MSMP in native sweet potato starch is predominately located at O-6 and O-3 of glucose residue and that O-6 substitution is occurring more frequently than O-3 substitution. These results of phosphorus level and substitution in native sweet potato starch are rather similar to previous findings published for native potato starch (Hizukuri, Tabata, & Nikuni, 1970; Tabata & Hizukuri, 1971). There was no distarch monophosphate present in native sweet potato starch, indicating the absence of cross-links.

3.1.2. Phosphorus in cross-linked starch

The total phosphorus content of CL-SPS was 51% higher than that of native starch as measured by ^{31}P NMR indicating that the phosphorus of the STMP reagent reacted with the starch (Table 1). Inorganic phosphorus was still present in the NMR spectrum of CL-SPS (Fig. 1B, 2.5 ppm), but its content decreased from 0.0071% to 0.0027% (w/w, dry starch). Obviously, part of the inorganic phosphate has been removed during the cross-linking procedure which included a washing step. Signals for MSMP can also clearly be recognised in the NMR spectrum of CL-SPS (Fig. 1B). It can be seen that most of the MSMP esters are positioned at O-6 and O-3 of the glucose moieties with some minor substitution at O-2. This finding implies that the hydroxyl groups at O-6 and O-3 are significantly more reactive than at O-2 in the cross-linking process. In CL-SPS, 4 additional phosphorus signals are present in the ^{31}P NMR spectrum (Fig. 1B, 1.0 to −1.0 ppm), which were not present in native starch. These signals of phosphorus correspond to DSMP cross-linking of phosphate between two glucose moieties (Lack, Dulong, Picton, Le Cerf, & Condamine, 2007). The peaks were not sharp, probably because the cross-links are present within limiting dextrins of various lengths not being degraded further during the amylolytic digestion. For cross-linked starch, the two small peaks at 1.0 and 0.4 ppm were denoted as DSMP-1, and the other two peaks at −0.1 and −1.0 ppm were denoted as DSMP-2. Based on the abundance of the DSMP-2 signals and the description of these signals by Sang et al. (2007), the DSMP-2 signals at 0.1, −1.0 ppm were annotated to originate from phosphorus cross-linking two different starch molecules. Since the hydroxyl groups O-6 and O-3 are more reactive towards the STMP reagent than the hydroxyl at O-2 (Sang et al., 2007), the cross-links may occur solely within hydroxyl groups at O-6 and O-3. However, the resolution was insufficient to determine this. It is suggested that the DSMP-1 signals originate from phosphorus involved in the intra-molecular cross-linking of starch: i.e. between hydroxyl groups of glucose moieties present in the same chain. The ratio of intra-molecular cross-linking (DSMP-1) to inter-molecular cross-linking (DSMP-2) is 1:3.

3.1.3. Cross-linking efficiency

During the cross-linking reaction, starch reacted with STMP to form starch-grafted sodium tripolyphosphate, which reacts with starch sodium salt to produce a starch diester *or* reacts with water to form a starch monoester (Lack et al., 2007). The phosphorus increases during cross-linking were ≈0.0030%, 0.0034% and 0.0067% (w/w, dry basis) for MSMP (O-6), MSMP (O-3) and DSMP, respectively. The levels of DSMP indicate that only half of the phosphorus formed the cross-links, and that the other half of phosphorus only resulted in MSMP. This is consistent with observations for wheat starch treated with STMP in alkali (Sang et al., 2010). The pH in the cross-linking reaction as described by Lack et al. (2007) determines the ratio of methyl glucopyranoside phosphate diester to phosphate monoester.

According to the content of phosphorus in DSMP, the amount of cross-links can be calculated as 1 cross-link (involving 2 glucoses)

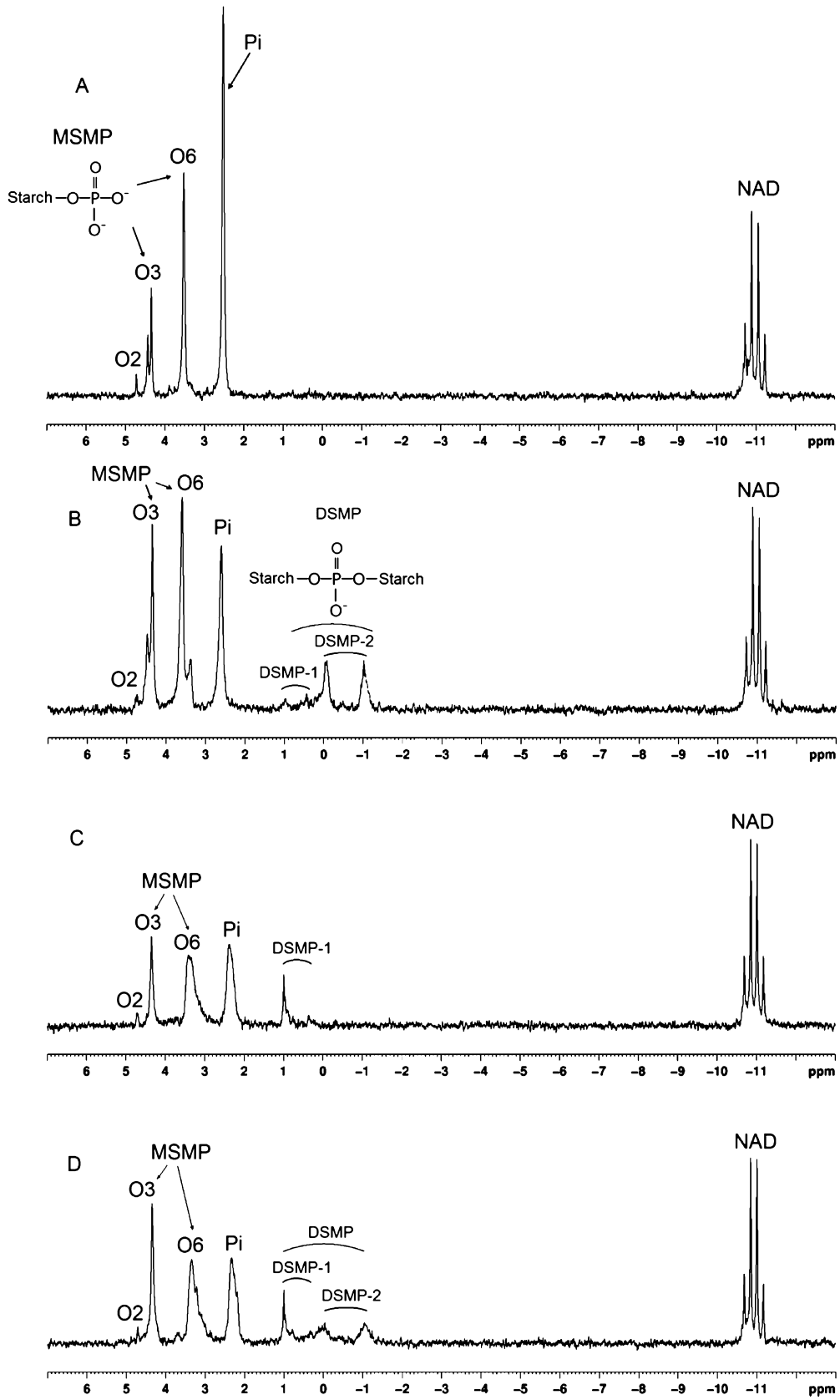

Fig. 1. Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectra of native (A) cross-linked (B), hydroxypropylated (C) and dual modified (D) sweet potato starches. MSMP: mono- starch mono-phosphate, DSMP: di-starch mono-phosphate, O-2, O-3, O-6: phosphate esterified to the hydroxyl at the 2nd, 3rd and 6th carbon of the glucose residue, respectively; glucose, Pi: inorganic phosphate, NAD: nicotinamide adenine dinucleotide.

Table 1
Phosphorus contents of different molecular forms in native and modified sweet potato starches (w/w %, db).

	NMR							Spectrophotometric assay[a]
Starches	MSMP (O-2)	MSMP (O-3)	MSMP (O-6)	Pi	DSMP-1	DSMP-2	Total phosphorus	Total phosphorus
Chemicalshift (ppm)	4.7	4.4	3.5	2.5	1.0, 0.4	−0.1, −1.0		
NT-SPS	0.0001 ± 0.0000	0.0032 ± 0.0001	0.0067 ± 0.0003	0.0071 ± 0.0004	0 ± 0.0000	0 ± 0.0000	0.0171 ± 0.0005	0.0197 ± 0.0008
CL-SPS	0.0002 ± 0.0000	0.0066 ± 0.0003	0.0097 ± 0.0004	0.0027 ± 0.0001	0.0018 ± 0.0001	0.0049 ± 0.0002	0.0259 ± 0.0008	0.0296 ± 0.0001
HP-SPS	0.0001 ± 0.0000	0.0030 ± 0.0001	0.0074 ± 0.0003	0.0019 ± 0.0001	0.0014 ± 0.0001	0 ± 0.0000	0.0137 ± 0.0004	0.0154 ± 0.0001
CLHP-SPS	0.0001 ± 0.0000	0.0055 ± 0.0002	0.0088 ± 0.0004	0.0029 ± 0.0001	0.0024 ± 0.0001	0.0042 ± 0.0002	0.0239 ± 0.0009	0.0265 ± 0.0011

NT: native; CL: cross-linked; HP: hydroxypropylated; CLHP: combined cross-linked and hydroxypropylated; SPS: sweet potato starch; MSMP: monostarch monophosphate; O-2, O-3, O-6: substituted on the position 2, 3, 6 of the glucose residues, Pi: inorganic phosphorus; DSMP-1; distarch monophosphate substituted between two glucose residue on same starch chain; DSMP-2: distarch monophosphate substituted between two glucose residue on different two starch chains;

[a] Data adapted from results in Zhao et al. (2015).

per 2900 glucose residues. Even though the number of cross-links was low, the viscosity of starch paste was affected significantly as has been described previously (Zhao et al., 2015).

3.1.4. Phosphorus level and state within hydroxypropylated starch

When using the NMR spectrum to calculate the phosphorus content (Fig. 1C; Table 1, HP-SPS), the total phosphorus content of hydroxypropylated starch was 20% lower than that of native starch, indicating that part of phosphorus was released from the starch during the hydroxypropylation procedure. This was consistent with our previous results using the spectrophotometric method (Zhao et al., 2012). The MSMP values did not change significantly when compared to those of the native starch. The signal of MSMP of HP-SPS was broader than that of native starch, probably due to wide distribution of the chain lengths of starch hydrolysates containing phosphorylation dextrin. The inorganic phosphorus content decreased with 73%, whereas the DSMP values increased to 0.0014% (w/w, db). It is surprising that cross-linking happened during the hydroxypropylation reaction, since the ^{31}P NMR spectrum of hydroxypropylated starch (Fig. 1C) indicates that not the commonly present DSMP signals at −0.1 and −1.0 ppm (DSMP-2) appeared, but signals at 1.0 and 0.4 ppm (DSMP-1), although in low quantities. This implies that during the hydroxypropylation reaction part of inorganic phosphorus (20%) reacted to yield DSMP-1, and another part (53%) of inorganic phosphorus has been lost during the reaction and washing procedure. No signals for the formation of inter-molecular cross-links (DSMP-2) were observed.

3.1.5. Phosphate level and state in dual modified starch

The dual modified sweet potato starch was cross-linked followed by hydroxypropylation. The spectrum of dual modified starch (Fig. 1D) contains the spectral features of both cross-linked and hydroxypropylated starch. The total phosphorus content in dual modified starch was slightly lower (8%) compared to that of cross-linked starch. This indicates that part of the phosphorus in cross-linked starch was lost during the sequential hydroxypropylation. The inorganic phosphorus content of CLHP-SPS was similar to that of the CL-SPS. The MSMP O-3 and O-6 values of CLHP-SPS were lower than those of CL-SPS and higher than those of HP-SPS (Table 1). This is considered to be the consequence of the fact that cross-linking increased the MSMP phosphorus level, whereas hydroxypropylation decreased the phosphorus during reaction as mentioned above. Comparing the phosphorus of DSMP in cross-linked starch (CL-SPS) and dual modified starch (CLHP-SPS), it was found that phosphorus content in DSMP-1 of dual modified starch was higher than that of cross-linked starch, while for DSMP-2, it was the opposite (Table 1). This finding indicates that DSMP-1, phosphorus cross-linking glucose residues within one starch chain, occur slightly more frequently after a hydroxylation reaction than after a cross-linking reaction. DSMP-2, phosphorus connecting two glucose residues of different starch chains, occurs only after adding the cross-linking agent. Furthermore, in dual modified starch, the amount of DSMP-2 produced during the cross-linking reaction decreased slightly during the subsequent hydroxypropylation, probably due to some hydrolysis of the ester linkages.

3.2. Level and location of hydroxypropyl groups in modified sweet potato starch

3.2.1. ^{1}H NMR spectra of native and cross-linked starches

The ^{1}H NMR spectra of hydrolysates of native and modified sweet potato starches are shown in Fig. 2 . The anomeric proton signal of native starch was present between 5.8 and 4.6 ppm of the ^{1}H NMR spectrum (Fig. 2A). The assignment of anomeric protons in starch is indicated in Fig. 2 as interpreted from literature

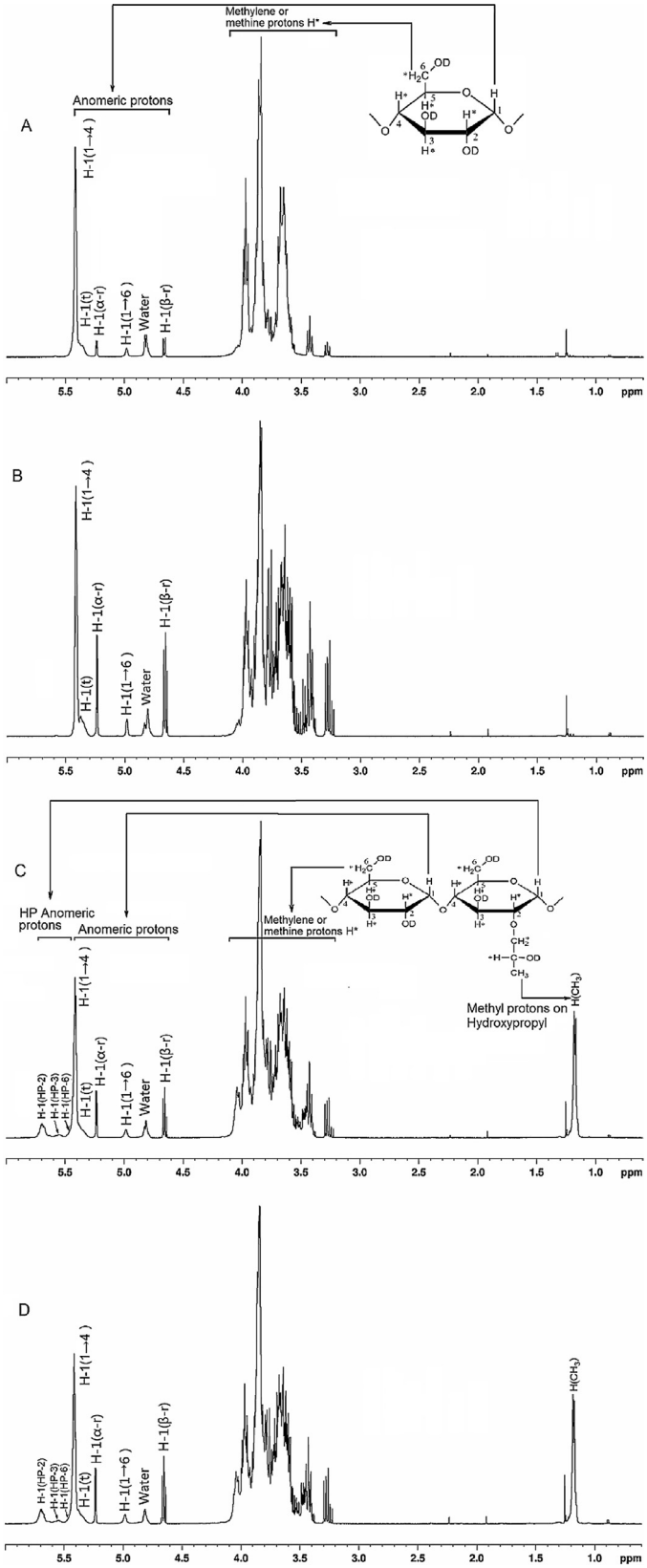

Fig. 2. Proton nuclear magnetic resonance (^{1}H NMR) spectra of native (A), cross-linked (B), hydroxypropylated (C) and dual modified (D) sweet potato starches. Protons marked with asterisk (*) in glucose residues or hydroxypropyl are methylene or methine protons. H-1(1→4): anomeric proton on α-(1→4) linked inner glucose unit, H-1(t): anomeric proton on terminal non reducing end of starch chain, H-1 (α-r): α-anomeric proton on reducing end of starch chain, H-1(1→6): anomeric proton on α-(1→6) linked terminal glucose unit, H-1 (β-r): β-anomeric proton on reducing end of starch chain, H-1(HP-2): anomeric proton on hydroxypropylated glucose unit substituted at carbon-2, H-1(HP-3): anomeric proton on hydroxypropylated glucose unit substituted at carbon-3, H-1(HP-6): anomeric proton on hydroxypropylated glucose unit substituted at carbon-6, H(CH_3): methyl proton in the hydroxypropyl group.

(Nilsson, Gorton, Bergquist, & Nilsson, 1996) and annotated according to Fig. 3. A set of peaks between 3.2 and 4.1 ppm are the methylene and methine non-anomeric protons of then glucose residues. The cross-linked starch showed a similar ^{1}H NMR spectrum (Fig. 2 B) compared to native starch, indicating that cross-linking did not affect the ^{1}H NMR spectrum in the anomeric proton region. In the case of hydroxypropylated starch, the anomeric protons of the substituted glucose moieties and the methyl protons on hydroxypropyl group give additional signals at 1.18, 5.52, 5.67 ppm of the ^{1}H NMR spectra (Fig. 2C and D). The doublet peak at 1.18 ppm is assigned to the methyl proton of hydroxypropyl groups (H(CH_3)). The peaks at 5.67 and 5.52 ppm were assigned to H-1 of glucose residues substituted at O-2 and O-3 by a hydroxypropyl group (denoted as H-1(HP-2), H-1(HP-3)), respectively (Fig. 2 and literature (Xu & Seib, 1997)). It is known that the substitution of propylene oxide occurs primarily at the O-2 hydroxyl in glucose residues, because this hydroxyl exhibits a relatively high acidity due to its proximity to the anomeric centre (Tuschhoff, 1986). Hence, the relatively bigger peak at 5.67 ppm was assigned to O-2 substitution and the smaller peak at 5.52 ppm was assigned to O-3 substitution. The shoulder on the downfield side of 5.40 ppm could be assigned to the anomeric proton on hydroxypropylated glucose unit substituted at O-6 (H-1(HP-6)) (Xu & Seib, 1997).

3.2.2. Molar substitution of hydroxypropylation

The total molar substitution (MS) was calculated as the ratio of one third of the integral of methyl proton of hydroxypropyl groups (H(CH_3)) at 1.18 ppm to the integral of anomeric protons (H-1) between 5.8 and 4.6 ppm (Eq. (1)) (Richardson et al., 2000). H-1(α-r) and H-1(β-r) denoted the proton (H-1) at the reducing end of the glucose in the α- or β- form, respectively.

$$\mathrm{MS} = \frac{\mathrm{Integral}\,[\mathrm{H}(\mathrm{CH_3})]/3}{\begin{array}{l}\mathrm{Integral}[\mathrm{H}-1(\mathrm{HP}-2)+\mathrm{H}\text{-}1(\mathrm{HP}\text{-}3)+\mathrm{H}\text{-}1(\mathrm{HP}-6)\\ +\mathrm{H}\text{-}1(1\rightarrow 4)+\mathrm{H}\text{-}1(t)+\mathrm{H}\text{-}1(\alpha-r)\\ +\mathrm{H}\text{-}1(1\rightarrow 6)+\mathrm{H}\text{-}1(\theta-r)]\end{array}} \quad (1)$$

The results are shown in Table 2. The total MS as determined by NMR is consistent with the results measured by the spectrophotometric method as indicated in the same table. The MS of dual modified starch was 14% lower than that of hydroxypropylated starch, indicating that, despite the same reaction conditions, cross-linking of the starch has hindered the hydroxypropylation. The possible reasons are that formation of cross-links hinders the penetration of propylene oxide reagent within the granules.

3.2.3. The ratio of hydroxypropyl substitution at different positions of the glucose units

The molar proportion of substitution at different positions on the glucose residues can be calculated as the ratio of integral of each anomeric proton on substituted glucose units to the sum of substituted peaks on the three positions (H-1(HP)). The peaks of H-1(HP-2) at 5.67 ppm and H-1(HP-3) at 5.52 ppm were resolved, so the integral can be obtained directly. However, the signal of H-1(HP-6) is a shoulder downfield of a peak at 5.40 ppm and could not be integrated precisely. Hence, the integral of anomeric proton of glucose residues substituted on O-6 (H-1(HP-6)) was calculated from the integral of methyl proton of hydroxypropyl and the anomeric protons of glucose residues substituted to O-2 and O-3. When the molar substitution (MS) is below 0.43, only mono substitution is reported to occur (Banks, Greenwoo, & Muir, 1973).

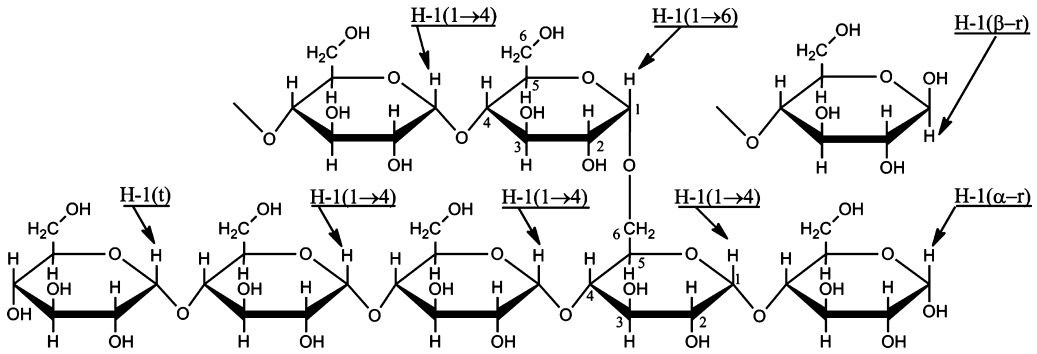

Fig. 3. Annotation of anomeric protons on glucose residues within starch.

Table 2
Molar substitution and the proportion of substitution at the various positions of the glucose residues in hydroxypropylated sweet potato starch.

		NMR				Spectrophotometric assay
Starches		HP-2 (5.67 ppm)	HP-3 (5.52 ppm)	HP-6 (5.40 ppm)	Total	Total
HP-SPS	Molar proportion, %	61.3	17.4	21.3	100	
	MS	0.101 ± 0.004	0.029 ± 0.001	0.035 ± 0.001	0.165 ± 0.007	0.176 ± 0.011
CLHP-SPS	Molar proportion, %	63.1	17.9	19.0	100	
	MS	0.098 ± 0.003	0.028 ± 0.001	0.029 ± 0.001	0.155 ± 0.006	0.151 ± 0.003

HP-1, HP-2, HP-3: hydroxypropylation on the position O-2, O-3, O-6 of glucose residues within the starch molecules. MS: molar substitution; CLHP-SPS: cross-linked hydroxypropylated sweet potato starch.

In that case the degree of substitution (DS) equals to the molar substitution (MS). Hence, the sum of the peak integrals of the protons at the anomeric carbon of glucose moieties substituted by hydroxypropyl equals to one third of the integral of the methyl protons in substituted hydroxypropyl groups H(CH_3). The integral of H-1(HP-6) can be calculated as one third of integral of H(CH_3) minus integral of H-1(HP-2) and H-1(HP-3). Therefore, the ratio of substitution on a different position can be calculated with the following equation:

$$\text{Molar proportion of substitution at position } y(\%) = \frac{\text{Integral[H-1(HP} - y)] \times 100}{\text{Integral[H(CH}_3)]/3} \quad (2)$$

The results shown in Table 2 indicate that position O-2 of the glucose residues of sweet potato starch was the predominant position (61.3%) for hydroxypropylation. The molar proportions of hydroxypropylation on O-3 and O-6 of sweet potato starch were 17.4% and 21.3%, respectively. This is consistent with data found for hydroxypropylated maize and wheat starch (Xu & Seib, 1997). Hence, this indicates that starch packing and another botanical origin does not affect at all the reactivity of specific substitution sites.

From the point of cross-linking, it was found that the chemical phosphorylation as MSMP mainly occurred on O-3 and O-6 (Table 1, CL-SPS). When comparing the MS of hydroxypropylation of CLHP-SPS and HP-SPS (Table 2), it can be seen that the absolute level of substitution on HP-6 of CLHP-SPS was significantly lower than that of HP-SPS. The MS on HP-2 of CLHP-SPS was slightly lower than that of HP-SPS, and MS on HP-3 was rather constant. Cross-linking the starch prior to hydroxypropylation obviously has an effect on the preferred position of hydroxypropylation.

4. Conclusions

Nuclear magnetic resonance is an efficacious method to analyse level and substitution position of cross-linking and hydroxypropylation of sweet potato starch, even at the low level of substitution used for food applications. Native sweet potato starch only contains endogenous monostarch monophosphate, while cross-linking using sodium trimetaphosphate induced the formation of additional monostarch monophosphate and distarch monophosphate in the ratio of 1:1. This information clearly indicates that the determination of the level of cross-linking by using the increase in phosphorus may lead to wrong values. In our NMR study, we noticed different forms of cross-linking, which suggests that low levels of intra-molecular cross-linking are present, both after crosslinking as well as after the hydroxypropylation. Hydroxypropylation occurred predominantly at the O-2 position of glucose for both single and dual modified starches; the reaction is slightly hindered by the presence of cross-links. Our findings on substitution of dual modified starches may help to explain functional properties of such starches.

Conflict of interest statement

The authors declare no competing financial interest.

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