

Effects of phosphorylation and cross-linking on the pasting properties and molecular structure of sago starch

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Abstract

Native sago starch was phosphorylated with 5% sodium tripolyphosphate (STPP) or cross-linked with 4% phosphorous oxychloride (POCl_3) at pH 8 to 11. The modified starches were characterized for degree of substitution (DS), pasting properties, crystallinity and molecular structure. The results indicated that DS of phosphorylated sago starch prepared at pH 9 was 0.008, which was the highest value among those prepared at any other pH. For cross-linked sago starch, increase in the pH of the reaction mixture resulted in increase in DS. The swelling properties indicated unrestricted swelling of phosphorylated sago starch, while that of cross-linked sago starch showed restricted swelling. The restricted swelling might be related to the formation of inter-molecular bridges by phosphate group. The phosphorylated and cross-linked sago starches had degrees of crystallinity ranging from 22.96-24.76% and a C type of X ray diffraction pattern. The ^{31}P NMR spectrum of phosphorylated sago starch (DS 0.008) indicated that the phosphorylation resulted in monostarch phosphate showing the phosphate groups attached at C-6 and C-3, while that of cross-linked sago starch (DS 0.018) showed a signal for distarch phosphate with a small proportion of cyclic monostarch phosphate.

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Introduction

Native starches generally contain small amounts (< 0.1%) of phosphorus (Lim and Seib, 1993; Yoneya *et al.*, 2003), mainly linked directly to amylopectin molecules as phosphate group (Bertoft, 2004; Jane, 2009). Although phosphorus is present in small quantities, it can affect the properties and behaviour of the starch (Yoneya *et al.*, 2003; Carmona-Garcia *et al.*, 2009). Phosphorus content in potato starch appeared to be an important factor affecting the rheological, thermal, structural and nutritional properties of potato starches, compared to amylose content (Lu *et al.*, 2012).

In nature, native sago starch contains chemically bound phosphorus at levels <0.01% (Muhammad *et al.*, 2000; Srichuwong *et al.*, 2005). The phosphate groups affect the properties and behaviour of the sago starch (Muhammad *et al.*, 2000; Polnaya *et al.*, 2012). To obtain higher phosphorus content, starch may be chemically phosphorylated.

Starch phosphates may be grouped into two classes: monostarch phosphate and distarch phosphate (cross-linked starch) (Lim and Seib, 1993). Monostarch phosphate can be obtained by esterification of native starch using sodium

tripolyphosphate (STPP) (Lim and Seib, 1993); or mixtures of sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate twehydrate (Zhang and Wang, 2009). Distarch phosphate can be obtained using phosphorous oxychloride (POCl_3) (Carmona-Garcia *et al.*, 2009) and sodium trimetaphosphate (Ratnayake and Jackson, 2008).

Monostarch phosphate exhibit increased paste clarity, viscosity, water binding capacity (Lim and Seib, 1993; Muhammad *et al.*, 2000). On the other hand, the formation of distarch phosphates may help to maintain the granule integrity and to make starch paste more resistant to retrogradation, high temperature, and low pH than native starch (Choi and Kerr, 2004). Polnaya *et al.* (2012) showed that phosphorylation of sago starch with 5% STPP increased the paste clarity while viscosity decreased, but that with 4% POCl_3 decreased both properties. X-ray diffraction and amylography have been used to characterize the crystalline/amorphous structure and amylograph of starches, respectively. The X-ray diffraction pattern of native sago starch was a C-type (Ahmad *et al.*, 1999; Kaur *et al.*, 2011). The pasting properties of native sago starch might be separated into two categories, i.e., type 1 which was specified by a maximum consistency at 75 or 85°C immediately

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followed by a sharp decrease, or type 2 distinguished by a plateau when the maximum consistency was reached (Ahmad *et al.*, 1999).

The two different reagents react with starch through different mechanisms. At pH below 9.0 the terminal phosphate groups of STPP are protonated and produce monometaphosphates, which can react rapidly with starch hydroxyl groups to produce monostarch phosphates (Lim and Seib, 1993; Deetae *et al.*, 2008). At a reaction pH above 10, the ionized hydroxyls can attack the STPP central phosphate to form starch pyrophosphates, which can be further attacked by starch hydroxyl groups to give distarch phosphate. Initial reaction of POCl₃ with water in starch slurry produces mostly dichlorophosphate. The dichlorophosphate then diffuses into starch granules, and react with the polymer chains. Cross-linking of starch molecules to give a distarch phosphate was favored by alkalinity above pH 10 in the presence of a neutral sodium salt. Otherwise, monostarch phosphates are formed (Singh *et al.*, 2007).

The objectives of this study were to investigate the effect of reagent types (STPP and POCl₃) and reaction pH between 8 and 11 on the pasting properties and molecular structure in the phosphorylated sago starches.

Materials and Methods

Materials

Native sago starch was donated by PT. Tiga Pilar Sejahtera Food Tbk., Indonesia. Native sago starch was stored in a high-density polyethylene bag before analysis and phosphorylation with STPP or POCl₃. The water content of the sample ranged between 12-13%. POCl₃, sodium hydroxide (NaOH), hydrochloric acid (HCl), and sodium sulfate (Na₂SO₄) were purchased from Merck, while STPP was obtained from a local market and was food grade. Thermally stable α amylase (63 U/mL) and glucoamylase (151 U/mL) were donated by Sumber Manis factory (Pati, Center of Java). All chemicals for analyses were analytical grade.

Chemical starch modification

Phosphorylated sago starch was prepared using the method of Lim and Seib (1993). Native sago starch (300 g) was phosphorylated at various pH levels (from 8 to 11) with 15 g STPP in the presence of 15 g Na₂SO₄. The impregnation of STPP with aqueous starch slurry was carried out for 1 h at 27°C and dried to 10-15% moisture at 40°C in a forced-air oven. The dried starch cake was heated for 2 h at 130°C in a forced-convection oven. After being

cooled to 27°C, the reaction mixture was dispersed in distilled water (350 mL). The starch was recovered by centrifugation (2,054 × g, 10 min) (IEC UV Centrifuge, Damon/IEC Division, UK) and redispersed in 600 mL of distilled water. The dispersion was adjusted to pH 6.5. The starch was washed with water (4 ×, 600 mL) and dried at 40°C.

Cross-linked sago starch was prepared using the method of Felton and Schopmeyer (1943). Native sago starch (50 g, dry basis) was cross-linked at various pH levels (from 8 to 11) with 4% POCl₃ in the presence of 7.5 g Na₂SO₄. POCl₃ was added drop wise over a 20 min, while maintaining the pH with the 1.0 M NaOH. The starch dispersion was stirred at 25°C for 1 h, and then adjusted to pH 6.5 with 1.0M HCl. The modified starch was collected by centrifugation (2,054 × g, 10 min), washed with water (4 ×, 100 mL), and dried at 40°C.

Determination of degree of substitution of sago starches

The degree of substitution (DS) of mono (Passauer *et al.*, 2006) and distarch phosphate (Singh and Nath, 2011) were calculated according to

$$DS \text{ (monostarch phosphate)} = 162P / (3100 - 103P)$$

$$DS \text{ (distarch phosphate)} = 162P / (3100 - 102P)$$
 with P being the colorimetric determined percentage of phosphorus content, 162 the molecular weight of the anhydroglucose unit, 3100 the atomic weight of the phosphorus multiplied by 100, 103 the molar mass of the phosphate in monostarch phosphate, and 102 the molar mass of the phosphate in distarch phosphate. The phosphorus content of starch was determined spectrophotometrically following the method of Smith and Caruso (1964).

Pasting properties of sago starches

The pasting properties of the samples were determined with a Rapid Visco Analyser (Model RVA-4SA, Newport Scientific Pty Ltd. Warriewood, Australia). The viscosity was recorded in Relative Viscosity Unit (RVU). Starch sample (2.5 g of starch) was dispersed in distilled water (25 mL) and stirred in an RVA canister at 960 rpm for the initial 10 s, and then maintained at 160 rpm for the rest of the test. The paddle was placed centrally inside the canister and then inserted into the RVA machine. The measurement cycle was initiated and the profile was determined over 12 minute periode. The time-temperature regime used was: idle temperature 50°C for 1 min, heated from 50°C to 95°C in 3 min 45 s, held at 95°C for 2 min 30 s and cooled to 50°C over a 3 min 45 s followed by a period of 2 min where the temperature was controlled at 50°C. The total

test time was 13:00 min. The interval between the viscosity and temperature readings was 4 s (195 data points per RVA curve). The values measured from the pasting profile were pasting temperature (PT), peak viscosity (PV), trough viscosity (TV) (minimum viscosity at 95°C), final viscosity (FV) (viscosity at 50°C), breakdown viscosity (BV) (PV – TV), and setback viscosity (SV) (FV – TV).

X-ray diffraction

The degree of crystallinity of the samples was quantitatively estimated employing an X-ray diffractometer (Lab X 116 XRD-6000, Shimadzu Japan) using a copper anode X-ray tube (Cu-K α radiation). The starch powders were packed tightly into sample holders. Each sample was exposed to the X-ray beam at 30 kV and 30 mA. The scanning region of the diffraction angle ranged from $2\theta = 5^\circ$ to 50° at in increments 0.40 with a count time of 1.0 s; the rotary speed of the sample holder was 30 min⁻¹. A smooth curve, which connected peak baselines, was computer-plotted on the diffractograms (Figure 1). The area above the smooth curve was taken as the crystalline portion, and the lower area between smooth curve and the linear baseline which covered the 2θ range from 5° to 35° was taken as the amorphous section. The ratio of the upper area to total diffraction was used as the degree of crystallinity. The degrees of crystallinity were calculated according to

$$\text{Crystallinity (\%)} = A_c / (A_c + A_m) \times 100$$

with A_c the crystalline area on the X-ray diffractogram and A_m the amorphous area on the X ray diffractogram.

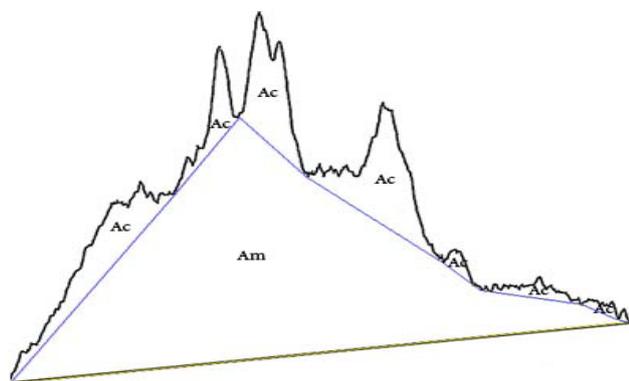


Figure 1. Measurement degree of crystallinity using image tool software. Ac = crystallinity region; Am = amorphous region

³¹P NMR analysis

Purified phosphorylated starch was digested by a modification of the starch-hydrolysing conditions described in Sang *et al.* (2007). Phosphorylated

starch (1.0 g, db) was added to water (50 mL) and the pH was adjusted to 5.5 by adding 1M HCl. An α -amylase solution (100 μ L) was added, and the beaker was covered with aluminium foil and heated in a boiling water bath to 95-100°C for 30 min with vigorous stirring. The digested starch was cooled to 30°C and the pH was adjusted to 5.5 with 1M HCl. Then α -amylase solution (100 μ L) was added and the digestion step was repeated. Upon cooling, the pH of the digested starch was adjusted to 4.5 with 1M HCl. Glucoamylase (200 μ L) was added, and the digested starch was heated to 60°C and allowed to digest for 1 h. The digested starch was cooled, centrifuged, and the supernatant freeze-dried. The recovered α, γ -dextrins amounted to >950 mg, which represented at least 95% of the weight of the phosphorylated starch.

The freeze-dried digest of phosphorylated starch (α, γ -dextrin, ~950 mg) was dissolved in D₂O (1.0 mL) containing 0.02% sodium azide as preservative, and the resulting solution was adjusted to pH 8.0. The proton-decoupled ³¹P NMR data were acquired on a 11.75 Tesla spectrometer, operating at 500 MHz for ¹H and 202.34 MHz for ³¹P, respectively, with a 3 mm NMR probe. The experiments were performed at 25°C using a delay of 6 s between pulses (pulse width 15.0 μ s), sweep width of 12370 Hz and 400 transients for each spectrum. Chemical shifts were reported in δ (ppm) from the reference signal of external 85% phosphoric acid.

An aliquot (850 μ L) of the concentrated digested starch (10 mL) prepared from the total standard reaction mixture was added to 100 μ L of a D₂O solution of NAD (30 mM, pH 8.0) in an NMR tube. The 40 μ L of EDTA (500 mM, pH 8.0, in D₂O) and 10 μ L of sodium azide (0.2%, pH 8.0, in D₂O) were added to the tube.

Statistical analysis

Data were statistically analyzed by analysis of variance test procedure and significant different were identified by Tukey's test ($P < 0.05$) using SAS 9.0 software (SAS, Inc.).

Result and Discussion

Degree of substitution

The sago starch phosphate contained significantly higher phosphorus contents and DS ($P < 0.05$) compared to native sago starch (Table 1). The cross-linked sago starch ($DS \geq 0.013$) showed a significantly higher phosphorus content and DS ($P < 0.05$) than that of phosphorylated sago starch. This might have been caused by differences in the reagents used and the pH of the phosphorylation conditions. The starch

Table 1. Phosphorus content (%) and degree of substitution of native sago starch, STPP and POCl_3 treated sago starches with different reagent and pH reaction

Reagent (% sb*)	pH reaction condition	Phosphorus (%)	DS
Native starch			
0	-	0.0085 ^e	0.0004 ^e
STPP			
5	8	0.0837 ^d	0.004 ^d
	9	0.1466 ^c	0.008 ^c
	10	0.1125 ^d	0.006 ^d
	11	0.1003 ^d	0.005 ^d
POCl_3			
4	8	0.0202 ^c	0.001 ^c
	9	0.2788 ^b	0.013 ^b
	10	0.2938 ^b	0.016 ^b
	11	0.3483 ^a	0.018 ^a

* sb = starch basis

Means in the same column without the same letter differ ($P < 0.05$) according to the least significant difference test.

phosphorylation was conducted at pH 8 to 11, though the reaction at pH 9 gave the highest DS (Table 1). This might have been due to the competition of phosphorus binding between the starch and NaOH used in the phosphorylation. At pH higher than 9 the phosphorus tended to react with NaOH. This was in accordance with the report of Muhammad *et al.* (2000) that the highest phosphorus content (0.21%) was obtained by phosphorylated sago starch at pH 9. In this study, phosphorylation using the same level of STPP as reported by Muhammad *et al.* (2000) gave lower phosphorus content. This difference most likely due to differences in properties of the native starch and the method of phosphorus analysis. The DS for cross-linked sago starch had the tendency to increase with the increase of pH. The results confirm that the DS was affected by pH of reaction mixture. This was expected because a higher pH catalyses starch phosphorylation (Nabeshima and Grossmann, 2001). The increase in DS might be due to higher reactivity of OH groups at C6 which was dependent on the alkalinity of the reaction mixture (Wootton and Haryadi, 1992).

Pasting properties

All of the phosphorylated sago starches had higher DS and easier to paste than native sago starch (Table 2), indicating the incorporation of the hydrophylic phosphate group enhance the gelatinization. Phosphorylated sago starches had higher swelling indicating the formation monostarch phosphate, due to the phosphate groups diminished the hydrogen bonding among adjacent molecules, which allowed greater water penetration and swelling.

Cross-linked sago starches showed in general restricted swelling compared to the phosphorylated sago starch and the native counterpart, indicating the formation of cross-linking (Ratnayake and Jackson, 2008) which reinforced the intermolecular linkages in the starch granule resulting more difficult to paste

Table 2. RVA pasting properties of the native sago starch, STPP and POCl_3 treated sago starches with different reagent and degrees of substitution

Reagent (% sb)	DS	P_{temp} (°C)	Viscosity (RVU)				
			PV	TV	BV	FV	SV
Native starch							
0	0.0004	78.8	300	94.5	205.5	148	53.5
STPP							
5	0.004	71.5	379.5	161	218.5	256	95
	0.005	76.3	393.5	144.5	249	237.5	93
	0.006	73.9	405.5	138	266.5	233.5	94.5
	0.008	67.0	435.5	137	298.5	223.5	86.5
POCl_3							
4	0.001	79.1	789.5	360.5	429	493.5	133
	0.013	81.2	231.5	208	23.5	291.5	83.5
	0.016	nr	64	37	27	60	23
	0.018	nr	44	17	27	21.5	5.5

DS = degree of substitution; PV = peak viscosity; TV = trough viscosity; breakdown viscosity (BV) = PV - TV; FV = final viscosity; setback viscosity (SV) = FV - TV; nr = not recorded.

as reported by Choi and Kerr (2004). Cross-linked sago starch prepared at pH 8 resulted with DS 0.001 having higher parameters of pasting properties than native sago starch. This phenomenon was probably owing to monostarch phosphate predominantly formed, since Manoi and Rizvi (2010) reported that POCl_3 treatment of starch resulted in a mixture containing distarch phosphate, monostarch phosphate and the others as indicated by ^{31}P NMR spectrum.

The pasting properties of cross-linked sago starch varied widely depending on the DS. Cross-linked sago starch with DS 0.001 had highest viscosity among the samples, but sample with DS 0.013 to 0.018 had lower viscosity (Table 2). Earlier workers reported that the level of cross-linking made starch difficult to swell and gelatinize, so the pasting viscosity was very low (Tran *et al.*, 2007) or confirmed by the absence of the pasting peak in the viscograph (Chung *et al.*, 2004).

Native sago starch began to gelatinize at 78.8°C (Table 2). While Tie *et al.* (2008) reported that native sago starch gelatinized at a range of 78.0 to 78.8°C. The increase in the DS of cross-linked sago starch gave increase in the gelatinization. Suryani and Haryadi (1998) reported that the consequence of higher DS was the higher gelatinization temperature. The higher PV and BV values of all STPP treated sago starches (Table 2) suggested that the phosphorylation enhanced the swelling of starch granules. Highly swollen granules were more susceptible to rupture, thus exhibiting a greater PV and BV. The PV and BV for cross-linked sago starch had the tendency to decrease with the increase of DS, indicates that cross-linking had taken place. The granular structure became more compact while swelling and solubility reduced as the DS of cross-linked sago starch increased (Polnaya *et al.*, 2012). A decrease in the PV of cross-linked sago starch prepared at pH 10 has been reported (Muhammad *et al.*, 2000). The TV for phosphorylated sago starch had the tendency to decrease with the increase of DS, indicate that

phosphorylation of starch reduced the intermolecular bonding strength of starch. Cross-linked sago starch with $DS \geq 0.016$ exhibited a lower TV than other treatments because the pasting viscosity was very low. Native sago starch showed a FV significantly lower than that of sago starch phosphate, except for cross-linked sago starch with $DS \geq 0.016$. A higher value of SV indicated a greater degree of retrogradation and vice versa (Singh *et al.*, 2007). Cross-linked sago starch with DS 0.001 had highest SV among the samples, but sample with DS 0.013 to 0.018 had lower SV (Table 2). The SV for cross-linked sago starch had the tendency to decrease with the increase of DS, indicate that starch phosphate decreases in the retrogradation rate.

Crystallinity of phosphorylated starch

Native sago starch (Figure 2a) was characterized by weak diffraction patterns at $2\theta = 5.67^\circ$ and broad peaks at $2\theta = 15.30^\circ$, 17.12° , 18.08° , and 23.46° , indicated the characteristics of C-type crystalline pattern. This observation was also supported by the report of Leong *et al.* (2007), who showed that native sago starch has weak diffraction pattern at $2\theta = 5.6^\circ$ and broad peaks at $2\theta = 17.2^\circ$, 17.9° , and 23.4° . This type are known to be present in starches with relatively high amylose content (Ahmad *et al.*, 1999). Polnaya *et al.* (2012) reported that the amylose content of native sago starch was 33%. Phosphorylated and cross-linked sago starches showed a slightly different pattern than that of native sago starch (Figure 2b, c). The peak around $2\theta \approx 18^\circ$ of native sago starch disappeared, while phosphorylated and cross-linked sago starches showed new peaks at $2\theta = 17.88^\circ$ and 17.84° , respectively.

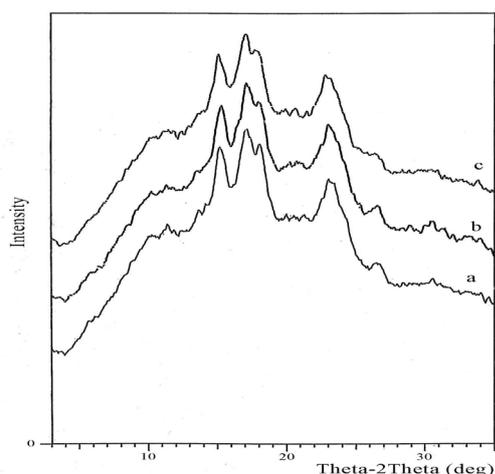


Figure 2. X-ray diffraction patterns of the: a) native sago starch; b) STPP treated sago starch, DS 0.008; and c) $POCl_3$ treated sago starch, DS 0.018.

It could be seen that native and sago starch phosphate showed similar degree of crystallinity

Table 3. X-ray diffraction intensities of peak and crystallinity of native sago starch, STPP treated sago starch at pH 9 and $POCl_3$ treated sago starch at pH 11

Starch	2 θ with intensities					Crystallinity type	Crystallinity (%)
	5 $^\circ$	15 $^\circ$	17 $^\circ$	18 $^\circ$	23 $^\circ$		
NSS	5.67(20)	15.30(210)	17.12(261)	18.08(216)	23.46(187)	C	23.09 ^a
STPP-9	5.75(18)	15.23(198)	17.06(253) 17.88(195)	-	23.38(194)	C	22.96 ^a
$POCl_3$ -11	5.60(10)	15.29(189)	17.20(233) 17.84(187)	-	23.34(170)	C	24.76 ^a

NSS = native sago starch; STPP-9 = STPP treated sago starch at pH 9, DS 0.008; $POCl_3$ -11 = $POCl_3$ treated sago starch at pH 11, DS 0.018. Means with the same superscripts are not significantly different.

($P > 0.05$) (Table 3). This result suggested that phosphorylation with STPP or cross-linking with $POCl_3$ did not dramatically alter the crystalline pattern of native sago starch. Zhang and Wang (2009) reported that, if compared to native starch, the crystallinity of monostarch phosphate remains nearly the same, suggesting that the layer structure was not destroyed in the phosphorylation.

^{31}P NMR spectra of phosphate compound in phosphorylated starches

The ^{31}P NMR spectrum (Figure 3a) of the α,γ -dextrins of phosphorylated sago starch showed two signals for monostarch phosphate at δ 3.41 and 4.94 ppm. The two signals at monostarch phosphate indicating the phosphorylation of hydroxyl groups at C-6 and C-3, respectively. Sang *et al.* (2007) showed a strong set of three signals for monostarch phosphate at δ 3.8-5.2 ppm, indicated the phosphorylation of hydroxyl groups at C-2, C-3, and C-6. Passauer *et al.* (2006) showed that the signal at 3.6 ppm was caused by a primary phosphate group at the C-6 position of the anhydroglucose unit and the signal at 4.7 ppm could obviously be assigned to phosphate groups at the C-2 and C-3 position.

On the other hand, cross-linked sago starch with DS 0.018 produced predominantly distarch phosphate in the modified starch and much less cyclic monostarch phosphate (Figure 3b). During the reaction with $POCl_3$ under alkaline condition, the hydrophilic phosphorus group reacts immediately with the OH of the starch, producing a distarch phosphate (Carmona-Garcia *et al.*, 2009). The ^{31}P NMR spectrum (Figure 3a) of cross-linked sago starch showed a strong signals for distarch phosphate at the range δ -0.51 to 0.20 ppm and a weak signal for cyclic monostarch phosphate ester at δ 15.05 ppm. The signals at δ 0.0, 3.6-4.6, and 15.5 ppm were attributed to the phosphodiester, phosphomonoester, and cyclic phosphate glucan,

respectively (Manoi and Rizvi, 2010).

The multiplicity of the two sets of peaks observed in the δ -0.51 to 0.20 ppm region of the ^{31}P NMR spectra indicated that the phosphate groups on cross-linked sago starch occur on at least two positions on the anhydroglucose unit along the starch chain. Most likely the 6-OH and 3-OH were the positions of substitution in the distarch phosphate in accordance with the findings of Manoi and Rizvi (2010). The weak signal at δ 2.03 ppm was not assigned monostarch phosphate, because it was absent at the same region as that of monostarch phosphate (Sang *et al.*, 2007) and it might be correspond to Pi (inorganic phosphate) (Manoi and Rizvi, 2010).

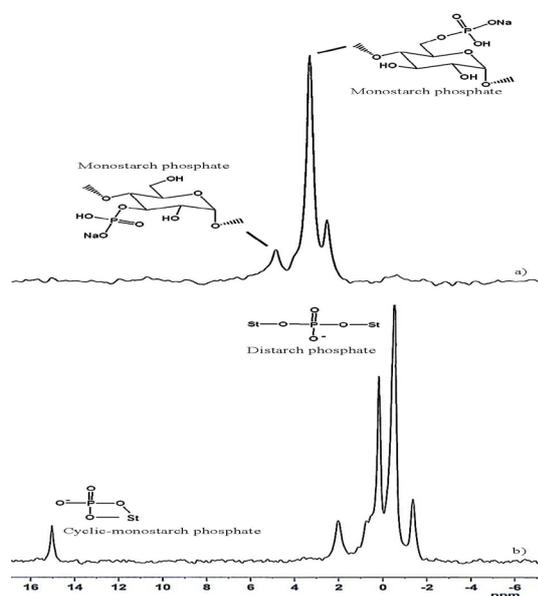


Figure 3. ^{31}P NMR spectra of the: a) STPP treated sago starch, DS 0.008; and b) POCl_3 treated sago starch, DS 0.018.

Conclusions

Phosphorylation and cross-linked procedures applied to the native sago starch, using different reagents at various pH of the reaction had a considerable effect on the pasting properties and molecular structure of the processed starch. The highest DS values resulted from the STPP treatment of the starch was achieved at pH 9, while that from the POCl_3 treatment at pH 11. It was found that the viscosity of the phosphorylated sago starch increased in comparison with the native sago starch. The cross-linked sago starch showed a restricted swelling than native sago starch and was also characterized by lowered viscosity. These sago starches exhibited a C-type diffraction pattern and X-ray diffraction confirmed that the crystalline structure of native sago starch was not affected. The phosphorylation of

sago starch at pH 9 produced monostarch phosphate, while the cross-linking with POCl_3 at pH 11 produced mainly distarch phosphate.

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