

Full Length Research Paper

Preparation and physicochemical characteristics of cross-linked resistant starch under heat-moisture treatment

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Sweet potato starch was subjected to cross-linking to achieve high content of resistant starch (RS) under heat-moisture treatment and the physicochemical properties of cross-linked sweet potato starches were investigated. The cross-linking agent was sodium trimetaphosphate and sodium tripolyphosphate (STMP/STPP). A maximum RS content (72.45%) was obtained after 10% STMP/STPP (99/1% w/w) at 120°C and pH 11.5 with a 20% moisture level. Through the analysis of ³¹P (phosphorus) nuclear magnetic resonance (³¹P NMR) spectroscopy, the RS-72.45% contained distarch monophosphate (DSMP) and monostarch monophosphate (MSMP) was not detected. Fourier-transform infrared spectroscopy (FTIR) presented the cross-linking reaction, confirmed by the comparison of native curves. After cross-linking, in the starches with high moisture treatment (≥25%) a loss of birefringence and surface erosion were observed. X-ray diffraction patterns showed that the crystal type was unchanged but with slight alteration in the relative crystallinity of cross-linked starches. Differential scanning calorimetry (DSC) showed gelatinization temperature increased as the moisture content increased, while gelatinization enthalpy apparently decreased.

Key words: Sweet potato starch, Heat-moisture treatment, ³¹P Nuclear magnetic resonance spectroscopy, optimization, X-ray diffraction (XRD), scanning electron microscopy (SEM), polarized light microscopy.

INTRODUCTION

Generally, RS is divided into physically inaccessible starch (RS₁), native granular starch with the B-type crystal pattern (RS₂), retrograded starch (RS₃), and chemically modified starch (RS₄) typically through esterification and cross-linking. It can be added to baked and extruded food to improve its processing technology and product quality,

such as improving the brittleness and expansibility of the product. As a type of high RS, cross-linked starch has increasingly drawn broad interest for functional properties and health benefits (Hung and Morita, 2004; Yeo and Seib, 2009; Miller et al., 2011). Many researches have been conducted on cross-linking reagents of POCl₃,

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sodium tripolyphosphate (STPP), epichlorohydrin (EPI), sodium trimetaphosphate (STMP), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (Lim and Seib, 1993; Hirsch and Kokini, 2002; Woo and Seib, 2002; Zhang and Wang, 2009), being capable of forming either ether or ester intermolecular linkages between hydroxyl groups on starch molecules (Rutenberg and Solarek, 1984). Normally, STMP and STPP are regarded as appropriate cross-linking agents to prepare food-grade RS (Ashwar et al., 2017). This starch is low in toxicity with no reports on its adverse effects on humans (Woo and Seib, 1997); just by comparison, both POCl_3 and EPI are toxic and flammable (MSDS, 1996; Woo and Seib, 1997), while $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ show low effect for cross-linking. Regarding the cross-linking reaction condition, there are many reports such as slurry reaction condition, semi-moist reaction condition (Woo and Seib, 2002), ultra high pressure (Hwang et al., 2009; Kim et al., 2012), supercritical fluid extrusion (Landerito and Wang, 2005; Manoi and Rizvi, 2010).

Apart from heat-moisture treatment been defined as a physical modification that involves incubation of starch granules at low moisture level (< 35% water (w/w)) during a certain period of time, at a temperature above the glass transition temperature but below the gelatinization temperature (Jacobs and Delcour, 1998), no information exists regarding it as a condition for cross-linking reaction except Sang and Seib (2006) who reported that simultaneous heat-moisture treatment and phosphorylation of high-amylose corn starch achieved high RS with good boiling-stability for food use. The paper mainly studied the effect of RS content under the heat-moisture condition.

Accordingly, in this research, the cross-linked sweet potato starch with high RS_4 content was prepared under the condition of heat-moisture treatment. The modification conditions were optimized using single factor experiment. The objective of this study is to establish optimum conditions for the production of starches with the highest RS_4 content. Moreover, the structure of the cross-linked starch was characterized by FT-IR and ^{31}P NMR and the physicochemical properties were investigated.

MATERIALS AND METHODS

Sweet potato starch was obtained from Quxian national grain storage, Sichuan, China. Resistant starch assay kit was purchased from Megazyme International Ireland Limited, including pancreatic α -amylase, amyloglucosidase and glucose-oxidase-peroxidase-aminopyrine (GOPD) reagent enzymes. STPP, STMP, sodium hydroxide, hydrochloric acid and other chemicals were analytical grade reagents.

Preparation of cross-linked resistant starch (RS_4)

Sweet potato starches were cross-linked by the method of heat-moisture treatment. Sweet potato starch (50 g, dry basis) was stirred for 60 min at ambient temperature in water (75 mL)

containing sodium sulfate (5 g), and the mixed reagents STMP/STPP (99/1) was different levels (1, 2, 4, 8, 10, and 12% (w/w), based on dry basis). The mixture was adjusted by adding 1 M NaOH solution to pH (10, 10.5, 11, 11.5 and 12). Then dried to suitable moisture content (15, 20, 25 and 30%), at high temperature (100, 110, 120, 130 and 140°C) reaction for 4 h. After cooling to room temperature, the reaction mixture was dispersed in 100 mL distilled water, the suspensions were neutralized to pH 6.0 with 0.1 M HCl solution, washed with distilled water ($300\text{mL} \times 7$), dried at 40°C for 24 h in an oven, and sifted through a 100 mesh sieve.

Determination of phosphorus content

Phosphorus content was colorimetrically determined by the reaction with ammonium molybdate according to the method of Smith and Caruso (1964).

Determination of resistant starch content

Resistant starch content was analysed using resistant starch assay kit based on AOAC (2002.02) and McCleary et al. (2018). Starch sample (100 mg, db) was equilibrated horizontally in a shaking water bath with 4.0 mL enzyme mixture (pancreatic α -amylase, 10 mg/mL; amyloglucosidase, 3 U/mL) for 16 h (37°C, 200 strokes/min). Afterwards, 4.0 mL ethanol (99% v/v) was used to terminate the reaction and the residue obtained was washed with ethanol (50% v/v) twice, and treated with KOH solution in an ice bath for 20 min (4 M, 2 mL) to solubilize the RS. The RS solution obtained was added 8 mL of 1.2 M sodium acetate buffer (pH 3.8). After incubation with amyloglucosidase (0.1 mL, 3300 U/mL) at 50 for 30 min, the samples were centrifuged at $1500 \times g$ for 10 min. 3 mL GOPD was added to aliquots (0.1 mL) of the supernatant, and the mixture was incubated at 50°C for 20 min. Absorbance was measured using a spectrophotometer at 510 nm.

^{31}P NMR spectroscopy

Purified phosphorylated starch was digested by the procedure of Sang et al., (2007). Cross-linked starch (1.0 g, db) was suspended in calcium chloride solution (2.0 mM, pH 8.2). Heat-stable α -amylase solution (100 μL , 3000 Ceralpha U/mL) was added, and the suspension was heated in a boiling water bath for 30 min with vigorous stirring. The digest was cooled and its pH was readjusted from 7.5 to 8.2. Then α -amylase solution (100 μL) was added and the digestion step was repeated. After cooling, the digest was adjusted to pH 4.5 by adding 1 M hydrochloric acid. Glucoamylase (200 μL) was added, and the digest warmed to 60°C and allowed to digest for 1 h. The digest was cooled, centrifuged, and the supernatant freeze-dried.

The freeze-dried digest of a phosphorylated starch (α , β -dextrin) was dissolved in deuterium oxide (1.0 mL) containing 0.02% sodium azide as preservative, and the resulting solution was adjusted to pH 8.0 ± 0.1 . ^{31}P NMR spectra were acquired on NMR spectrometer (Varian INOVA-400, Bruker, Germany), operating at 400 MHz for ^1H and 202.34 MHz for ^{31}P respectively, with a 3 mm NMR probe. The ^{31}P NMR experiments were performed at 25°C using a delay of 6 s between pulses (pulse width 15.0 μs), sweep width of 12730 Hz and 400 transients for each spectrum. ^{31}P chemical shifts were referenced with respect to 85% H_3PO_4 .

Scanning electron microscopy (SEM)

Starch granules were mounted directly onto aluminum stubs using

double-sided adhesive tape, and then coated with 20 nm gold under vacuum. Images of starch granules were obtained with a field emission SEM (EVO 18, Zeiss, Germany) at an acceleration potential of 10 kV and magnification of $\times 3000$.

Polarized light microscopy

Birefringence of starch granules were observed under an optical microscope (Model BH-2, Olympus, Japan). All samples were dispersed in solution (glycerine/deionised water; 1:1 v/v) and the images were recorded at 500 \times magnification under polarized light.

X-ray diffraction (XRD)

X-ray patterns were performed using X-ray diffractometer (D8 Advance, Bruker, Germany). Starch samples were equilibrated in a saturated relative humidity chamber for 24 h at ambient temperature. The operating conditions were 40 kV and 40 mA with Cu α -radiation, the samples were scanned in the range of 4-35 $^{\circ}$ (2 θ). Relative crystallinity was calculated as the ratio of the areas of crystalline and amorphous regions of X-ray diffractograms (Nara and Komiya, 1983).

Swelling factor (SF)

SF of native and cross-linked starches (2%, w/w) was measured according to the method of Tester and Morrison (1990). The mixture was heated at 80 $^{\circ}$ C for 30 min, centrifuged (3500 \times g, 15 min) and the supernatant was removed to aluminum box and weighted at 105 $^{\circ}$ C. The SF is reported as the ratio of the volume of swollen granules to the volume of dry starch.

Differential scanning calorimetry

Thermal characteristics were measured with a Perkin Elmer differential scanning calorimetry (DSC) 8000 (Norwalk, CT, USA) instrument. Starch (4.5 mg, db) was weighed in a gold DSC pan adding 10.5 μ L distilled water. To ensure complete equilibration of water and sample, pans were kept at ambient temperature for about 12 h. The pan was heated from 30 to 150 $^{\circ}$ C at 5 $^{\circ}$ C/min; an empty pan was used as a reference. The DSC software was applied to analyze onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c), and gelatinization enthalpy (ΔH).

FT-IR spectroscopy

During the cross-linked, amplitudes of hydroxyl groups peaks of starch molecules differed as could be confirmed by FTIR. All FT-IR patterns were recorded on a Vector 33 spectrometer (Bruker, Germany) using an attenuated total reflection method. FT-IR samples were prepared by mixing the starch with KBr and compressed to the sheet. Each spectrum was recorded over the wavenumber range from 4000 to 400 cm^{-1} with a spectral resolution of 4 cm^{-1} by 64 scans at room temperature. For each spectrum, the FT-IR spectra were adjusted by subtraction of the KBr spectra (background).

Statistical analysis

The data reported are averages of triplicate observations. Mean values and standard deviations were analyzed and reported using the Origin Program 8.0 (Origin Lab Company, USA). Analysis of

variance with a significance level of 5% was done and Duncan's test applied to determine differences between means using the commercial statistical package (SPSS 17.0).

RESULTS AND DISCUSSION

Optimization of reaction conditions

In the case of STMP/STPP, the reaction was located by the phosphorus (P) level boundary of less than 0.4% and the resistant starch (RS) boundary at its highest level. In this study, reaction conditions including cross-linking agents, pH, moisture content and reaction temperature in the process of preparation resistant starch was analyzed, and it was found that RS content could be greatly increased by cross-linking reaction under heat-moisture treatment.

A comparison among samples showed that increased RS content (from 18.25 to 76.60%) was directly correlated with increased amount of cross-linking agent (from 1 to 12%) under pH 11.5, 25% moisture, 120 $^{\circ}$ C condition (Figure 1). Consequently, 12% cross-linking agent was eliminated on account of phosphorus content beyond the edible denatured starch prescribed maximum of 0.4%. The 10% cross-linking agent was appropriate.

The reaction pH is another important factor in the production of RS. Effect of pH on the RS content under 10% cross-linked agent, 25% moisture, 120 $^{\circ}$ C condition is shown in the Figure 2. Appropriate pH would facilitate molecular hydroxyl activation, which could increase the reactivity so that STMP/STPP nucleophilic attacks to starch molecular. The RS yield increased gradually as the reaction pH increased from 10 to 11.5, reaching a maximum of 68.10% at pH 11.5. However, a marked reduction (68.10 to 50.21%) was observed when the pH increased from 11.5 to 12, indicating that excessive alkaline which probably destroyed the starch granule structure was adverse to formation of resistant starch.

For determining optimum moisture content at pH 11.5, 25% moisture and 120 $^{\circ}$ C, some different moisture contents were used. As shown in Figure 3, the result presented that the 20% moisture content generated the greatest increase in RS content (72.45%). Under the condition of higher moisture (more than 20%), RS content tended to decrease for the two possible reasons: one is hydroxyl groups of water competed for the phosphorylating agent with the hydroxyls of starch, which resulted in decrease of RS. Another is over moisture lead to swelling of starch which hindered the preparation of RS.

Regarding the reaction temperature, 120 $^{\circ}$ C was favorable for production of RS under 10% cross-linked agent, pH 11.5 and 20% moisture (Figure 4). When the temperature was higher than 120 $^{\circ}$ C, RS content straightly decreased. The reason to that was probably because temperature at over 120 $^{\circ}$ C provides enough energy to break the intra-molecular structure, resulting

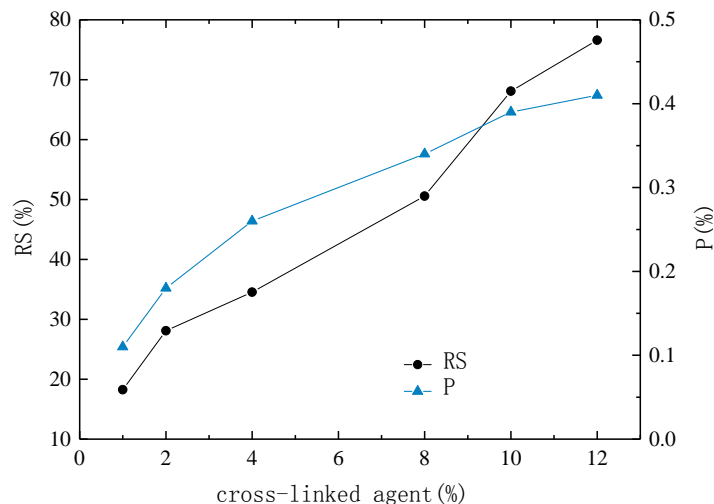


Figure 1. Effect of cross-linked agent amount on the RS content under the pH 11.5, 25% moisture and 120°C condition.

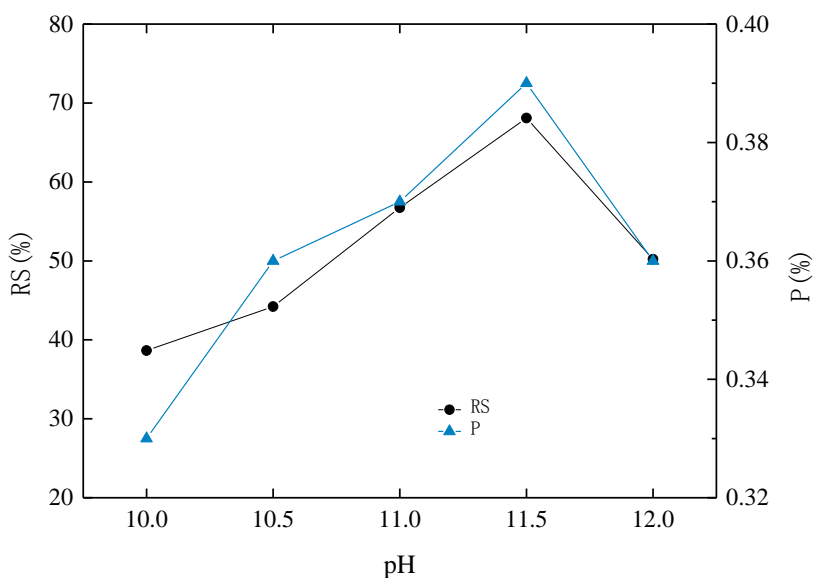


Figure 2. Effect of pH on the RS content under 10% cross-linked agent, 25% moisture and 120°C condition.

in partly gelatinization of starch granules.

Based on the results with all factors (amount of cross-linking agent, pH, moisture content and reaction temperature), sweet potato starch was cross-linked with 10% STMP/STPP (99:1) in 20% moisture, pH 11.5 and 120°C, produced the highest contents of type 4 resistant starch (RS₄).

³¹P NMR spectrum of RS-72.45%

The key feature of ³¹P NMR spectrum is the ability to

identify product of crosslinking reaction. According to the results of Sang et al., (2007); wheat starch cross-linked with STMP in alkaline slurry was found to contain DSMP ester, whereas with STPP reacted to produce two kinds of products, including MSMP and DSMP. In this study, it was observed that in case of heat-moisture treatment phosphorylated sweet potato starch (RS-72.45%) with 0.39% P prepared by roasting the starch with STMP/STPP (99/1) at an initial pH 11.5 was shown to favor the formation of DSMP ester. The ³¹P NMR spectrum (Figure 5) of the α,γ -dextrin of RS-72.45% showed strong signals for DSMP at δ 0 to + 1 ppm.

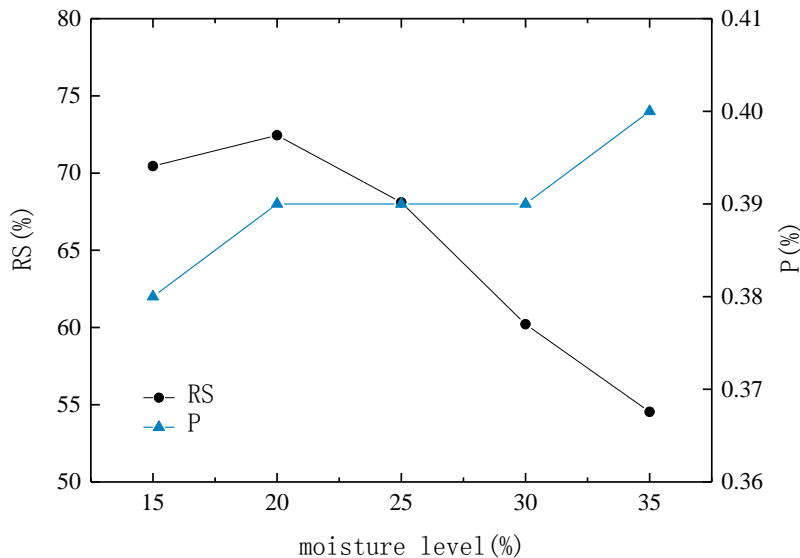


Figure 3. Effect of moisture level on the RS content under 10% cross-linked agent, pH 11.5 and 120°C, condition.

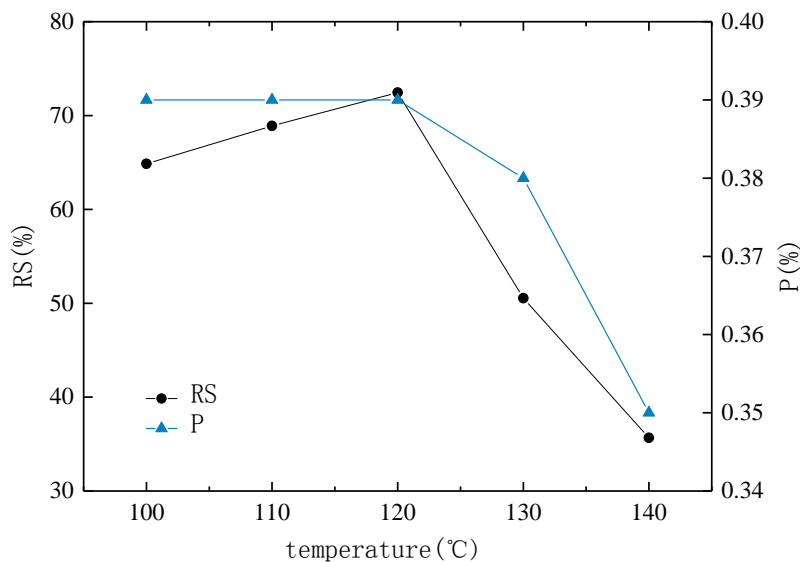


Figure 4. Effect of temperature on the RS content under 10% cross-linked agent, pH 11.5 and 20% moisture.

Surprisingly, MSMP linkages (δ 3.5-5.0 ppm) were not observed in the digest of the total reaction mixture. A similar outcome that MSMP structures were lost in the non-soluble portion of the digest was reported by Sang et al., (2007) who explained the monophosphate groups associated with STMP perhaps gave strong localized chelation of calcium ion and denatured the α -amylase. The high content RS of CLM-20 (the cross-linked reaction under the 20% moisture) could ascribe the formation of DSMP which played a key role in making the

phosphorylated starch resistant to α -amylase and amyloglucosidase digestion (Sang et al., 2010).

SEM and microscopy

While scanning electron microscope is superior to detect the granule shape and surface characteristics of starch, polarized light microscopy gives comprehensive view to image the average radial orientation of helical structures.

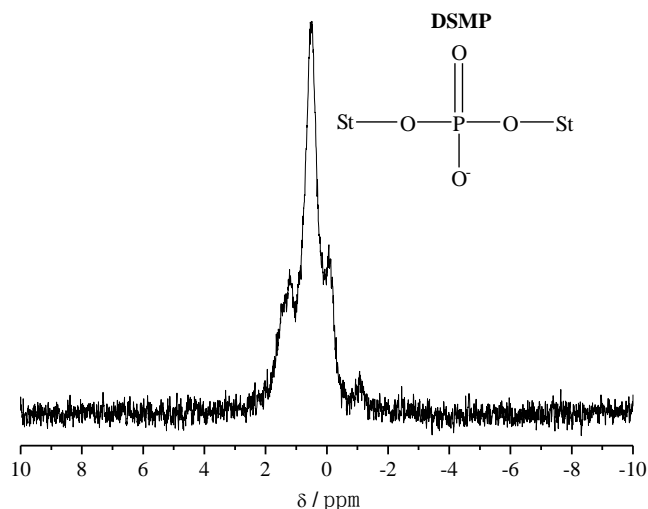


Figure 5. ^{31}P NMR spectra of α , γ -dextrins prepared from RS-72.45% starch. No signal was observed beyond the regions of 10 and -10 ppm.

The SEM images and birefringence patterns of native and cross-linked sweet potato starches are clearly shown in Figure 6. Native (Figure 6A) and CLM-20 starch granules (Figure 6B) had similar morphology; they were spherical or polygonal in shape with smooth surfaces, whereas CLM-30 and CLM-35 starch granules exhibited surface erosion with slight fragments and concaves. These characteristics in the surface of cross-linked starch granules indicate different heat-moisture treatments consequently led to different structural changes.

Similarly, the intensity of birefringence of CLM-20 (Figure 6F) starch almost remained unchanged compared with native starch (Figure 6E). However, birefringence loss occurred in the CLM-30 (Figure 6G) and CLM-35 (Figure 6H) starches (started at the hilum or botanical center of the granules and spreaded gradually to the periphery), the extent of this loss increased with increase in the moisture content treatment. This conclusion was in agreement with the investigation of Chung et al., (2009), who demonstrated that HMT with high moisture content could facilitate double helices move, which lead to a loss of radial orientation. Moreover, the weaker birefringence intensity and the lower RS content suggested that resistance to enzyme of cross-linked starch correlated with starch granule structure integrity. As shown in Figure 6, all the gelatinization starts at the hilum or botanical center of the granules and spreads rapidly to the periphery (de Castro et al., 2018; Ye et al., 2018).

XRD

The X-ray diffraction pattern and relative crystallinity of native and cross-linked starches are shown in Figure 7.

There was no apparent difference between the native and modified starches in the X-ray diffraction pattern. All the phosphorylated starches showed similar diffraction patterns with peaks at 2θ of around 5.6 , 15 , 17 , 18 and 23° compared to native starch, which are typical characteristics of C-type starch (Zobel, 1988). Similar results were observed for corn starch whereby unchanged diffraction pattern was speculated, and the cross-linking reaction mainly took place in the amorphous regions of starch granule and did not change the crystalline patterns of starches (Hoover and Sosulski, 1986; Koo et al., 2010).

Relative crystallinities of all the cross-linked starches except CLM-35 slightly increased (crystallinity variation 0.9-6.3%), which indicated that covalent bonds introduced in the process of cross-linking were much stronger than hydrogen bonds which fastened interaction of starch molecular. However, the decrease in relative crystallinity of CLM-35 compared with native starch was perhaps due to the damage of crystal structure in high moisture reaction, which could be proved from the result of birefringence (Figure 6). In other words, cross-linked starches with higher moisture levels reaction showed larger reductions in relative crystallinity.

DSC

Gelatinization parameters of native and cross-linked sweet potato starches (Table 1) are presented. The result showed that the gelatinization temperature was increased, while the enthalpy of all the cross-linked starches compared with native starch was decreased. The differences in gelatinization temperatures among the starches can be attributed to the two aspects. On the one

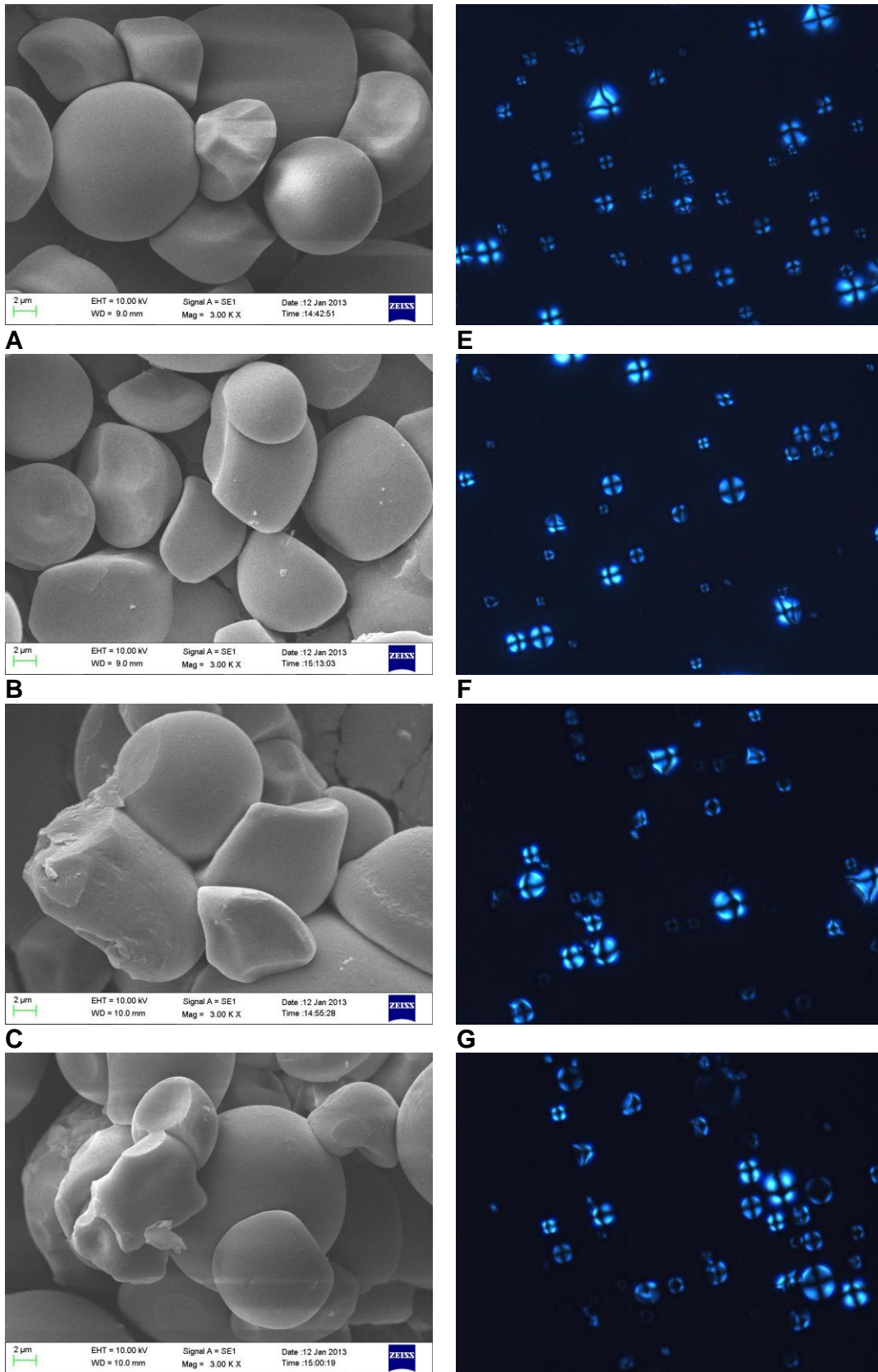


Figure 6. Native, CLM-20, CLM-30, CLM-35 of sweet potato starch visualized by SEM (A, B, C, and D respectively), at 3000× magnification; and native, CLM-20, CLM-30, CLM-35 of sweet potato starch viewed by polarized light microscopy (E, F, G and H respectively), at 500× magnification.

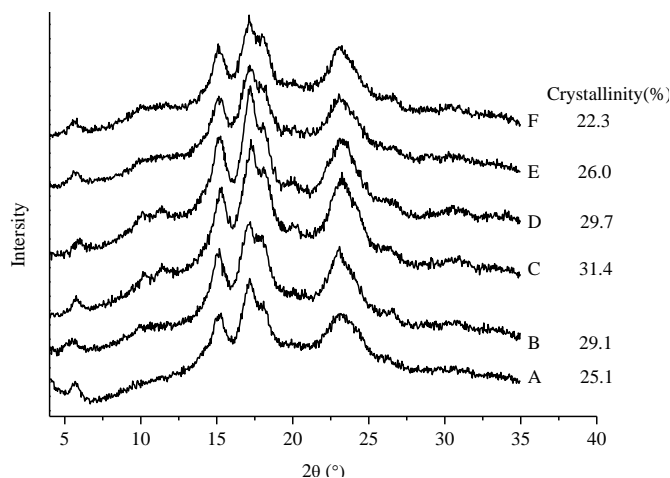


Figure 7. X-ray diffraction patterns (XRD) and relative crystallinity of native and cross-linked sweet potato starches prepared under 10% cross-linked agent, pH 11.5 and 120°C condition and different moisture contents. (A) Native starch, (B) CLM-15, (C) CLM-20, (D) CLM-25, (E) CLM-30, (F) CLM-35.

Table 1. Thermal characteristics of native and cross-linked starches.

Sample	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g ⁻¹)	SF (g/g)
Native starch	60.05 ± 0.41 ^a	76.38 ± 0.56 ^a	85.08 ± 0.43 ^a	14.89 ± 0.22 ^e	17.31 ± 0.92 ^d
CLM-15	68.01 ± 1.12 ^b	81.30 ± 0.21 ^b	90.97 ± 0.14 ^c	12.43 ± 0.53 ^d	5.21 ± 0.37 ^b
CLM-20	74.65 ± 0.33 ^d	84.42 ± 1.45 ^d	87.74 ± 0.18 ^b	8.11 ± 0.27 ^c	3.38 ± 0.42 ^a
CLM-25	73.36 ± 0.36 ^c	83.12 ± 0.39 ^c	91.31 ± 0.49 ^{cd}	7.27 ± 0.51 ^b	4.21 ± 0.33 ^{ab}
CLM-30	75.01 ± 0.53 ^{de}	84.21 ± 0.20 ^{cd}	91.59 ± 0.43 ^{cd}	6.01 ± 0.47 ^a	4.82 ± 0.24 ^b
CLM-35	76.00 ± 0.36 ^e	84.48 ± 0.17 ^d	92.02 ± 0.36 ^d	5.41 ± 0.32 ^a	6.33 ± 0.71 ^c

Values are given as mean ± standard deviation. Different letters indicate significantly different ($p < 0.05$) when analyzed by Duncan's New Multiple Range Test.

hand, introduction of cross-linking covalent groups into the starch by STMP could reinforce the interchain association tensioning the starch structure, which facilitated less water penetration into the starch granules with a consequent decrease in swelling (Table 1), leading to an increase in the gelatinization temperature. On the other hand, increase in T_o , T_p , and T_c was due to interaction between amylose-amylose and/or amylose-amylopectin chains, and to the formation of additional complexes between amylose and starch lipids (Hoover and Vasanthan, 1994).

In general, the great reduction ΔH in all the cross-linked starches is most probably due to the melting of imperfect amylopectin-based crystals, with potential contributions from both crystal packing and helix melting enthalpies (Lopez-Rubio et al., 2008). That decline in enthalpy of gelatinization probably resulted from partial unwinding of double-helices during the modification reaction at 120°C, sufficient moisture and Ph initial 11.5 (Sang and Seib, 2006). Starch cross-linked with high

moisture markedly weakened its bonding forces and structurally weaker complex may require less energy to complete the thermal transition. Cross-linked starches treated with higher moisture levels showed larger reductions in gelatinization enthalpy than those treated at low moisture levels. The decreased ΔH values following the cross-linked sweet potato starches reflect the loss of double helices and some crystallites. The conclusion was confirmed by the NMR spectrum.

FT-IR spectroscopy

Figure 8 showed the FT-IR spectra of native starch and RS-72.45%. The characteristic peak position in the infrared spectrum of sweet potato starch is mainly: 3440 cm^{-1} is O-H stretching vibration, 2931 cm^{-1} is C-H stretching vibration, 1642 cm^{-1} is the bending vibration of H_2O , and 1017 cm^{-1} is the stretching vibration of C-O connected with the hydroxyl group of C_6 primary alcohol.

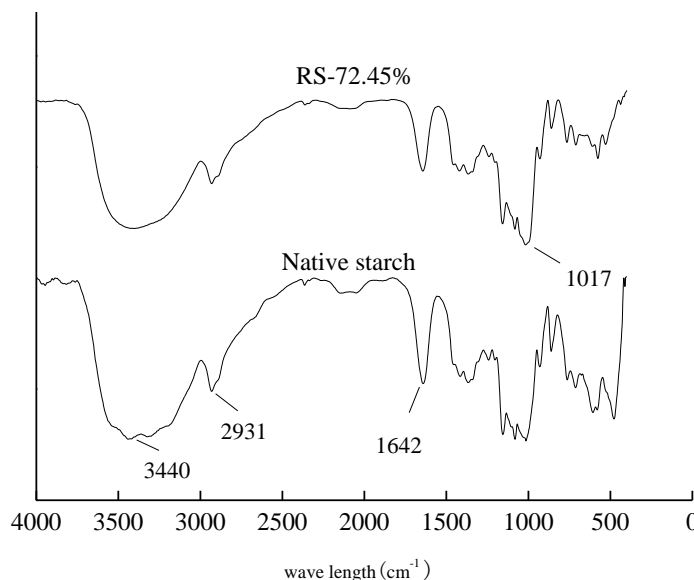


Figure 8. FT-IR spectra of native starch and RS-72.45%.

The peak of RS-72.45% was obviously changed, compared with the native starch. The absorption peak of RS-72.45% was enhanced at 1017 cm^{-1} . The reason may be that the wave length of $995\text{ to }1050\text{ cm}^{-1}$ was stretching vibration of P-O-C, corresponding to the C₆-O-H stretching vibration band in starch, which indicated that C₆-O-P was formed in the reaction process.

However, the peaks of RS-72.45% at 3440 cm^{-1} , 2931 cm^{-1} and 1642 cm^{-1} were weaker than the native starch, which indicated that the stretching vibration of O-H, C-H and bending vibration of H₂O was weak dealing with the cross-linking and heat-moisture. The essence of the crosslinking reaction of starch molecules with multidimensional space mesh structure was formed by alcohol hydroxyl and crosslinking covalent bond, with hydrogen bond was replaced by the cross-linking of covalent bond. The cross-linking reaction was confirmed by the comparison of infrared spectrum curves.

Conclusion

RS₄ was prepared with mix reagents STMP/STPP (99/1%, w/w) under heat-moisture treatment. The highest content of RS (72.45%) was obtained under the following condition: 10% STMP/STPP, pH 11.5, 20% moisture level, and temperature of 120°C . ³¹P nuclear magnetic resonance spectroscopy showed that RS-72.45% was DSMP. According to the single factor experiment and ³¹P nuclear magnetic resonance analysis, the resistance to digest of cross-linked starch was related to the starch granule structure integrity and the space steric hindrance of DSMP. The reason may be that the interaction of amylase of glycosidic bond was limited. It suggested that the birefringence and fragments of cross-linked starch

were weakened under the excessive moisture reaction (especially CLM-35). The X-ray diffraction result also showed that the relative crystallinity of CLM-35 decreased compared to native starch. The DSC demonstrated that cross-linked starches under heat-moisture treatment had better heat stability than native starch. The FT-IR confirmed that the cross-linking reaction occurred by the comparison of infrared spectrum curves.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests..

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