

Properties of hydroxypropylated normal wheat, waxy wheat, and waxy maize starches and an improved ^1H NMR method to determine level of hydroxypropyl groups

by

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Abstract

Waxy wheat starch (WWS) containing little or no amylose has unique properties for food and industrial uses. Hydroxypropylation, a widely used means for modifying starches, could enhance functionalities of starch by substituting hydroxyl groups in starch. There are limited numbers of literatures regarding hydroxypropylation of WWS. WWS hydroxypropylated with 3.0-9.0% propylene oxide (PO) were prepared and compared to hydroxypropylated normal wheat starch (NWS) and hydroxypropylated waxy maize starch (WMS). The molar substitution (MS) of the resulting hydroxypropylated NWS, WWS and WMS was 0.055-0.151, 0.048-0.133, and 0.049-0.139, respectively. Gelatinization temperatures and enthalpy of hydroxypropylated starches were significantly lower than those of their unmodified counterparts and the extent of decrease was positively correlated to the MS. Differential scanning calorimetry (DSC) results showed that hydroxypropylation reduced the retrogradation of the starches during the storage after gelatinization but there were differences between the starches. For the same level of PO reaction (3 and 5% PO), hydroxypropylated WWS retrograded less than hydroxypropylated WMS, suggesting that for the same level of cold storage stability, less PO is needed for WWS. After reacted with 6% PO, MS was 0.092 and 0.094, respectively, for WWS and WMS, and no retrogradation was observed for those hydroxypropylated starches, indicating that at MS ~0.094, hydroxypropyl groups prevents amylopectin chains from re-associating and forming crystalline structures. In contrast, a higher level of PO (9%) was needed to react with NWS to achieve the hydroxypropylated starch with an MS of 0.151 that gave no retrogradation. Consistent with microscopic observation results, Micro-Visco-Amylograph (MVA) analysis showed hydroxypropylated starches developed viscosity at lower temperatures and had improved hot and cold viscosities.

The specific characteristics of hydroxypropylated starches are related to the degree of hydroxypropylation. Therefore, it is critical to determine the level of hydroxypropylation in modified starches. ^1H NMR is a simple and rapid means of determining hydroxypropyl (HP) group in modified starches. In this study, a method to prepare a HP starch for NMR analysis was improved. The optimum parameters proposed to hydrolyze HP starches were 10% starch solid content, 3.5% (wt.%) DCl in D_2O as the solvent, sodium acetate as an internal standard, heating at 90°C for 1 h. Optionally, 6% (v/v) trifluoroacetic acid- d as the water-peak shifting reagent. Six hydroxypropylated starch samples and two commercial cross-linked and hydroxypropylated starch samples were hydrolyzed with this modified method before ^1H NMR analysis, and the results of HP group content in the samples were in agreement with the results from an enzyme-catalyzed method, which indicate that the improved acid hydrolysis method is applicable for both hydroxypropylated starch and cross-linked and hydroxypropylated starch.

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Dedication

This dissertation is dedicated to my family.

Chapter 1 - Introduction

Starch is the predominant component in wheat grain, which has a critical influence on the texture, appearance, and quality of wheat flour-based food products. Waxy wheat, which firstly developed in early 1990s in Japan, provided a possibility to alter the unique structures and specific characteristics of starch used in food and other industry applications. In this thesis, properties of hydroxypropylated normal wheat, waxy wheat, and waxy maize starches were investigated, and an improved ^1H NMR method to determine level of hydroxypropyl groups was developed. Waxy wheat flour showed a weak dough forming property compared to normal wheat flour. Protein is another important component in flour, therefore, it is also significant to investigate the properties and structures of proteins besides starch.

Chapter 2 summarized literature search result regarding hydroxypropylation of starch and methods of determining hydroxypropyl groups in modified starch.

Chapter 3, the first research chapter, was focused on characterization of properties of hydroxypropylated normal wheat, waxy wheat, and waxy maize starches. Specific experiments were performed to: (1) prepare hydroxypropyl starches with four levels (3, 5, 6, 7, and 9%, dry starch base) of propylene oxide (PO) from normal wheat, waxy wheat, and waxy maize starches, (2) determine the pasting properties of the hydroxypropylated and unmodified starches at 6% solids in water by Micro Visco-Amylograph (MVA), (3) record changes in both unmodified and hydroxypropylated starch granules during heating with a light microscope, (4) measure the settling volume of unmodified and hydroxypropylated starches at 25°C and after being heated at 70°C for 20 min, (5) analyze gelatinization of hydroxypropylated and unmodified starches, and the extent of re-association (retrogradation) of the gelatinized starches after being stored at 4°C for 1 week by differential scanning calorimetry (DSC).

Chapter 4, the second research chapter, a published method of preparing starch samples for ^1H NMR analysis was improved. The effects of acid concentration and temperature on hydrolysis and solubilization of hydroxypropylated starch were studied. Additionally, the improved acid hydrolysis method was applied on 6 hydroxypropylated starch samples and two cross-linked and hydroxypropylated starch samples, and the results were compared with the results obtained by an enzyme-catalyzed method, which indicate that the improved acid hydrolysis method is applicable for both hydroxypropylated starch and cross-linked and hydroxypropylated starch.

Chapter 5 summarized the conclusions of the research chapters and discussed the future work.

Chapter 2 - Literature Search and Review

2.1. Literature search and review on hydroxypropylation of starch

Database: Scifinder

Date: 10/01/2018

Keywords: 'hydroxypropylation' and 'starch'

Journals/ patents/ reviews in English, 2196 articles were found, because there were 9001 articles if other languages were included. All the articles were screened by names and abstract, some to full text, and 40 papers were specifically regarding preparing and characterizing hydroxypropylated starch, which were listed below. Among the 40 articles, only 3 papers were related to hydroxypropylation of waxy wheat starch (Reddy & Seib, 2000; Hansen, Jackson, Wehling, & Graybosch, 2010; Graybosch & Hansen, 2016). However, none of them studied systematical comparison of the properties of WWS, WMS and NWS hydroxypropylated at different substitution levels. In addition, no report correlated the degree of substitution with the degree of retrogradation in hydroxypropylated WWS, WMS, and NWS although there are studies that have reported that retrogradation generally decreases after hydroxypropylation of starches (Perera & Hoover, 1999; Lan et al., 2010).

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2. Chun, E. H., Oh, S. M., Kim, H. Y., Kim, B. Y., & Baik, M. Y. (2016). Effect of high hydrostatic pressure treatment on conventional hydroxypropylation of maize starch. *Carbohydrate Polymers*, 146, 328-336.
3. Chun, S. Y., & Yoo, B. (2007). Effect of molar substitution on rheological properties of hydroxypropylated rice starch pastes. *Starch-Stärke*, 59(7), 334-341.
4. De Graaf, R. A., & Janssen, L. P. (2003). The hydroxypropylation of starch in a self-wiping twin screw extruder. *Advances in Polymer Technology: Journal of the Polymer Processing Institute*, 22(1), 56-68.

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21. Lan, C., Liu, H., Chen, P., Yu, L., Chen, L., Li, X., & Zhang, X. (2010). Gelatinization and retrogradation of hydroxypropylated cornstarch. *International Journal of Food Engineering*, 6(4).
22. Lawal, O. S. (2011). Hydroxypropylation of pigeon pea (*Cajanus cajan*) starch: Preparation, functional characterizations and enzymatic digestibility. *LWT-Food Science and Technology*, 44(3), 771-778.
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39. Yousefi, A. R., Razavi, S. M., & Norouzy, A. (2015). In vitro gastrointestinal digestibility of native, hydroxypropylated and cross-linked wheat starches. *Food & Function*, 6(9), 3126-3134.
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2.2. Literature search on methods of determining hydroxypropyl groups in modified starches

Database: Scifinder

Date: 10/05/2018

Keywords: 'determination' and 'hydroxypropyl group' and 'starch'

Journals/ patents/ reviews, 72 articles were found, and 12 of them were specifically regarding methods of determining hydroxypropyl groups in modified starches, which were listed below. Among the 12 articles, 6 of them discussed 1H-NMR method.

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2. Forrest, B. (1992). Identification and Quantitation of Hydroxypropylation of Starch by FTIR. *Starch - Stärke*, 44(5), 179-183.
3. Han, J.-A., & BeMiller, J. N. (2006). Influence of reaction conditions on MS values and physical properties of waxy maize starch derivatized by reaction with propylene oxide. *Carbohydrate Polymers*, 64(2), 158-162.
4. Johnson, D. P. (1969). Spectrophotometric determination of the hydroxypropyl group in starch ethers. *Analytical Chemistry*, 41(6), 859-860.
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Chapter 3 - Gelatinization, Pasting and Retrogradation Properties of Hydroxypropylated Normal Wheat, Waxy Wheat, and Wwaxy Maize Starches

Abstract

Normal wheat starch (NWS), waxy wheat starch (WWS) and waxy maize starch (WMS) were hydroxypropylated with 3-9% propylene oxide (PO). The objectives of this study were to correlate morphology changes to pasting properties of hydroxypropylated starches and establish the relationship between molar substitution (MS) and degree of retrogradation of the starches. Gelatinization temperatures and enthalpy of starches decreased after hydroxypropylation, and the extent of decrease was positively correlated to MS. Differential scanning calorimetry results showed that hydroxypropylation reduced retrogradation during cold storage after gelatinization, but the effects varied among starches. For the same level of PO (3 and 5%) reaction, hydroxypropylated WWS retrograded less than WMS. WWS and WMS had no retrogradation after reacted with 6% PO. However, a higher level of PO (9%) was needed to react with NWS to achieve no retrogradation. Consistent with microscopic observation, Micro Visco-Amylograph analysis showed hydroxypropylated starches developed viscosity at lower temperatures and improved hot and cold viscosities.

3.1. Introduction

Starch, the predominant component in wheat grain, has a critical influence on the texture, appearance, and quality of wheat flour-based food products. The ratio of amylose to amylopectin and their structures determine physicochemical properties of starch, and hence affect the processing properties of flour and the quality of end-use products (Schirmer, Höchstötter, Jekle, Arendt, & Becker, 2013). Therefore, the development of waxy wheat, which contains no amylose in its endosperm starch, has provided a possibility to alter the unique structures and specific characteristics of starch used in food and other industry applications (Graybosch, 1998; Chibbar & Chakraborty, 2005; Hung, Maeda, & Morita, 2006; Maningat, Seib, Bassi, Woo, & Lasater, 2009). As a relatively new waxy starch compared to waxy maize starch (WMS), waxy wheat starch (WWS) has attracted increasing attention, and is superior to normal wheat starch (NWS) in (1) water retention capacity (Jung, Kim, Baik, & Park, 2015), (2) swelling properties (Hung, Maeda, & Morita, 2007; Garimella Purna, Shi, Guan, Wilson, & Graybosch, 2015), and (3) resistance to retrogradation (Guan, 2008; Garimella Purna, Miller, Seib, Graybosch, & Shi, 2011; Wang, Guan, Seib, & Shi, 2018).

Although WWS has some advantages compared to NWS, native WWS still has some shortcomings such as undesirable stringy paste texture and retrogradation during storage, which limit its applications in foods. Cross-linking may be used to improve the texture of WWS paste (Wang et al., 2018). Hydroxypropylation, another widely used means for modifying starches (Tuschhoff, 1987), could enhance functionalities of WWS by substituting hydroxyl groups in starch. As a result of hydroxypropylation, the modified starch exhibits improved starch solubility (Majzoobi, Saberi, Farahnaky, & Mesbahi, 2014), paste viscosity (Chuenkamol, Puttanlek, Rungsardthong, & Uttapap, 2007), paste clarity (Woggum, Sirivongpaisal & Wittaya, 2015),

swelling power (Lee & Yoo, 2011) and freeze-thaw stability (Reddy & Seib, 2000), decreased gelatinization and pasting temperatures (Chun & Yoo, 2007; Woggum, et al., 2015) and syneresis (Reddy & Seib, 2000; Chun & Yoo, 2007). The improved properties are due to the steric effects introduced by hydroxypropyl groups, which can inhibit the starch chain alignment, enhance the hydrophilicity of the starch, and weaken the internal bonds holding the starch granule structure.

A majority of the research reported in the literature regarding hydroxypropylation is carried out using corn, potato, tapioca, and normal wheat starches, but a few studies have investigated hydroxypropylation of WWS (Graybosch & Hansen, 2016; Hansen, Jackson, Wehling, & Graybosch, 2010; Reddy & Seib, 2000). Reddy & Seib (2000) compared cross-linked and hydroxypropylated WWS with WMS after dual modification of hydroxypropylation and cross-linking and found that modified WWS has a greater freeze-thaw stability and gives non-stringy pastes of almost equal consistency compared to modified WMS. Graybosch & Hansen (2016) analyzed pasting properties of hydroxypropylated waxy, partial waxy starch and wild-type wheat starches using a rapid visco analyzer (RVA), and found that both native and modified starches, partial waxy pasting curves displayed viscosities intermediate between those of waxy and wild-type starches, which is in agreement with the findings reported earlier (Hansen et al., 2010). However, there is no report on systematical comparison of the properties of WWS, WMS and NWS hydroxypropylated at different substitution levels. In addition, no report correlated the degree of substitution with the degree of retrogradation in hydroxypropylated WWS, WMS, and NWS although there are studies that have reported that retrogradation generally decreases after the hydroxypropylation of starches (Perera & Hoover, 1999; Lan et al., 2010).

The objectives of this study were to correlate the morphology changes to the pasting properties of hydroxypropylated WWS, WMS, and NWS, and establish the relationship between

molar substitution (MS) and degree of retrogradation of the starches. Specific experiments were performed to: (1) prepare hydroxypropyl starches with five levels (3, 5, 6, 7, and 9%, dry starch base) of propylene oxide (PO) from normal wheat, waxy wheat, and waxy maize starches; (2) determine the pasting properties of the hydroxypropylated and unmodified starches at 6% solids in water by Micro Visco-Amylograph (MVA); (3) record changes in both unmodified and hydroxypropylated starch granules during heating with a light microscope; (4) measure the settling volume of unmodified and hydroxypropylated starches at 25°C and after being heated at 70°C for 20 min; and (5) analyze gelatinization of hydroxypropylated and unmodified starches, and the extent of re-association (retrogradation) of the gelatinized starches after being stored at 4°C for 1 week by differential scanning calorimetry (DSC).

3.2. Materials and methods

3.2.1. Materials

NWS was obtained from MGP Ingredients Inc (Atchison, KS). WMS was obtained from Ingredion Inc (Bridgewater, NJ). One advanced waxy hard wheat cultivar (Mattern) was provided by Dr. Robert Graybosch (USDA/ARS, University of Nebraska). Waxy wheat starch was isolated with the procedure described in our previous paper (Wang et al., 2018). Propylene oxide (99%) and sodium sulfate were purchased from Fisher Scientific (Fair Lawn, NJ).

3.2.2. Preparation of hydroxypropylated starches

Starch (100 g, dry basis), anhydrous sodium sulfate (5.0 g) and deionized water (150 mL) were mixed in a beaker with an overhead mixer, and the pH was adjusted to 11.5 with 3% (w/v) sodium hydroxide solution, and the slurry was transferred to a glass jar. After 3.6, 6.0, 7.2, 8.4, or 10.8 mL (3, 5, 6, 7, or 9 wt % based on dry weight of starch) of propylene oxide (PO) was added with a glass syringe, the jar was sealed immediately and shaken (100 rpm) in a water bath at 45°C

for 20 h. After reaction, the slurry was adjusted to pH 5.5 with 25% (v/v) sulfuric acid, and the slurry was agitated with an overhead mixer at 250 rpm for 30 min and filtered with a vacuum filter. The starch cake was re-suspended in distilled water (300 mL) and filtered again, and this washing process was repeated three times. The starch cake was crumbed into small pieces by hands and dried in an air-forced oven at 35°C for 24 h, gently ground with a motor and pestle, and stored in sealed plastic bags at room temperature.

3.2.3. Determination of hydroxypropylation molar substitution

The hydroxypropyl (HP) content in the modified starches was determined by ¹H NMR. The procedure by Stahl & McNaught (1970) was modified to prepare the starch samples for NMR analysis. Anhydrous sodium acetate (125 mg) and deuterium chloride solution (2.5 mL, 35% w/w) were added in a 25-mL volumetric flask, and the volume was adjusted to 25 mL with deuterium oxide (Solution A). Each starch (200 mg) was weighed, recorded and mixed with 2 mL of Solution A in a glass vial. The vial was placed in a water bath at 90°C for 60 min with constant magnetic stirring and cooled to the room temperature. The solution was filtered with a 0.45-µm syringe filter. Approximately 0.8 mL of solution was transferred to a standard 3-mm NMR tube with a disposable Pasteur pipette and analyzed by NMR with the conditions reported by Bai & Shi (2011). The bound HP content and molar substitution (MS) was calculated according to Equation 1 and Equation 2, respectively.

$$\%HP = \left[\frac{\text{weight of Sodium acetate}}{\text{weight of starch}} \right] \times \left(\frac{2}{10} \right) \left[\frac{59.08 \times \text{Intensity at 1.13 ppm}}{82.05 \times \text{Intensity at 2.08 ppm}} \right] \text{ (Equation 1)}$$

Here, 59.08= molecular weight of hydroxypropyl group; and 82.05= molecular weight of sodium acetate.

$$MS = \frac{\text{moles of substituent}}{\text{moles of anhydro-glucose unit}} = \frac{\%HP \times 162}{59.08 \times (100 - \%HP)} \text{ (Equation 2)}$$

Here, 59.08= molecular weight of hydroxypropyl group; and 162= molecular weight of anhydroglucose unit.

3.2.4. Thermal properties

The thermal properties of the unmodified and HP starches were measured with a differential scanning calorimeter (Q200, TA instrument, Schaumburg, IL). The ratio of starch (~8 mg) to water was 1:2 (w/w) and high-volume stainless-steel pans were used. Gelatinization was determined by heating a starch-water mixture in a DSC pan from 10°C to 140°C at a heating rate of 10 °C/min. The onset (T_o), peak (T_p), and conclusion (T_c) temperatures and the enthalpy (ΔH) of the transition were determined by DSC software (TA Instruments, Schaumburg, IL, USA). To determine retrogradation of a gelatinized starch, the gelatinized sample in a DSC pan was stored at 4°C for 7 d and rescanned under the same heating conditions as used in the gelatinization test. The analysis was conducted in duplicates.

3.2.5. Pasting properties by Micro-Visco-Amylograph

Pasting properties of starches were determined by Micro-Visco-Amylograph (C.W. Brabender Instruments Inc., South Hackensack, NJ). Each starch (6.90 g, db) was suspended in distilled water (108.10 mL) to form 6% starch slurry, heated from 30°C to 95°C at a heating rate of 6.0 °C/min, held at 95°C for 5 min, cooled to 50°C at a cooling rate of 6.0 °C/min, and held at 50°C for 2 min. The viscosity of the starch suspension was expressed in Brabender Units (BU). The measurements were done in duplicates.

3.2.6. Microscopic observation of starch granules

Starch (0.03 g) and distilled water (3.0 mL) were added into vials with caps and dispersed by using a vortex mixer, and stirred in a water bath with a magnetic stirrer at 25°C, 45°C, or 70°C for 20 min. After that, the dispersion was stained with 100 μ L of 0.1 N iodine solution at 25°C.

The iodine solution was prepared by dissolving 3.8 g of potassium iodide in 2.4 mL of purified water, and adding 1.28 g iodine crystals, stirring until it was completely soluble in solution. The solution was transferred to a 100-mL volumetric flask with the aid of purified water and diluted to the volume with water and mixed. The stained slurry (15 μ L) was placed on a microscope slide and covered with a cover-slip. The starch was viewed with an Olympus BX-51 microscope using the 40X objective lens (Olympus America Inc., Melville, NY).

3.2.7. Settling volume

The settling volume was determined by the method reported by Wang, Guan, Seib, & Shi (2018). Starch (1.2 g, db) was stirred continually in 118.8 mL distilled water (total weight 120.0 g) in a water bath at 25°C or 70°C for 20 min. The slurry was cooled to 25°C with constantly gentle stirring, and 100 mL of the slurry was transferred into a 100-mL graduated cylinder. The cylinder was covered with plastic wrap and held at 25°C for 24 h, and the volume of sediment was read visually.

3.2.8. Statistical analysis

All data were analyzed using Statistical Analysis Software (SAS) (version 9.2, SAS Institute, Cary, NC) by analysis of variance (ANOVA), and the values are expressed as means \pm standard deviations from two replicates.

3.3. Results and discussion

3.3.1. Molar substitution

The MS of hydroxypropylated NWS, WWS, and WMS reacted with different levels (3-9%, dry starch base) of PO are shown in Table 1. When reacted with 3-9% PO, the MS values of hydroxypropylated NWS, WWS and WMS were 0.055-0.151, 0.048-0.133, and 0.049-0.139, respectively. HP content and MS values increased when the addition level of propylene oxide

increased, which are in agreement with the trends found in several previous studies. Chuenkamol et al. (2007) reported that the MS of hydroxypropylated canna starch were 0.01-0.11 when 0.5-10% (v/w) of PO were used. Chun & Yoo (2007) also reported that MS of hydroxypropylated rice starch were 0.03-0.14 when 3-12% (w/w) PO were added. The results in Table 1 also indicated the reaction efficiencies of NWS, WWS, and WMS were 57.81-65.75%, 51.57-57.21%, and 52.23-58.36%, respectively, which shows a decreasing trend with the increase of PO addition level. Tuschoff (1987) stated that the reaction efficiency is generally ~60% when 5-10% PO is used. WWS showed similar reaction efficiencies compared to WMS but had lower reaction efficiencies than NWS reacted with the same level of PO. This finding is consistent with the result that amylose was derivatized to a greater extent than amylopectin when potato starch (Kavitha & BeMiller, 1998) and common corn starch (Shi & BeMiller, 2000) were reacted with PO. Amylose molecules are in amorphous regions, and hydroxypropyl groups are primarily introduced in the amorphous regions of starch granules (Hood & Mercier, 1978; Steeneken & Smith, 1991; Kaur, Singh & Singh, 2004).

3.3.2. Gelatinization and retrogradation properties

The gelatinization and retrogradation properties of unmodified and hydroxypropylated NWS, WWS and WMS in excess water (67% water in slurry) are summarized in Table 3.2. For gelatinization, the onset temperature (T_o) of the unmodified NWS was close to that of WWS, but the peak (T_p) and conclusion (T_c) temperatures and the enthalpy of gelatinization (ΔH) of WWS were significantly higher than those of NWS. WWS and WMS had similar ΔH , but the gelatinization temperatures (T_o , T_p and T_c) of WWS were markedly lower than those of WMS, which is in agreement with the work by Reddy & Seib (2000) and Guan (2008). After the gelatinized samples stored at 4°C for 1 week, NWS, WWS and WMS exhibit closed T_o , T_p and

T_c, but WMW showed larger ΔH (5.7 J/g) of retrogradation than NWS (3.9 J/g) and WWS (3.5 J/g), which is in agreement with result reported by Guan (2008).

The endotherms were shifted to lower temperatures (T_o, T_p and T_c), and ΔH of gelatinization gradually decreased with the increase of PO treatments for all starches (Table 3.2). Onset gelatinization temperatures of hydroxypropylated starches decreased by 2-10°C compared to the unmodified starches. This result is in agreement with previous publications (Liu, Ramsden & Coke, 1999; Chuenkamol et al., 2007; Lee & Yoo, 2011; Yang et al., 2016). The decrease in gelatinization temperatures of hydroxypropylated starches are attributed to the disruption of hydrogen bonds between starch chains in the amorphous regions, which directly improved the mobility of the starch chains and indirectly decreased the melting temperature of the starch crystallites (Woggum et al., 2015). Generally, substituted starches exhibit a lower ΔH than native ones (Chuenkamol et al., 2007; Woggum et al., 2015; Yang et al., 2016). ΔH is correlated to the crystallinity of the starch granules ((Dhital, Shrestha, Hasjim, & Gidley, 2011)), and a lower ΔH value of the hydroxypropylated starch indicated a reduced crystallinity. Retrogradation of the starches reduced as molar substitution (MS) of hydroxypropyl groups increased (Table 3.2 and Figure 3.1). Hydroxypropyl groups interfere the rearrangement of molecular chains to ordered structures during the storage after gelatinization. For the same level of PO reaction (3 and 5% PO), hydroxypropylated WWS retrograded less than hydroxypropylated WMS (Table 3.2 and Figure 3.1), suggesting that for the same level of cold storage stability, less PO is needed for WWS. The initial experimental design did not include 6% PO, and it was found that waxy starches reacted with 7% PO showed no retrogradation. To accurately find out the degree of substitution that achieve no retrogradation, 6% PO was added. After reacted with 6% PO, MS was 0.092 and 0.094, respectively, for WWS and WMS (Table 3.1), and no retrogradation was observed for those

hydroxypropylated starches (Table 3.2 and Figure 3.1), indicating that at MS ~0.094, hydroxypropyl groups prevents amylopectin chains from associating and forming crystalline structures. In contrast, a higher level of PO (9% PO) was needed to react with NWS to achieve the hydroxypropylated starch with an MS of 0.151 that gave no retrogradation (Table 3.2 and Figure 3.1). PO probably reacted with more with amylose than amylopectin in NWS as observed in potato (Kavitha & BeMiller, 1998) and normal maize starch (Shi & BeMiller, 2000), and because of that, higher overall MS was needed to prevent amylopectin in NWS from recrystallization.

As shown in Table 3.3, hydroxypropylation remarkably decreased temperatures and enthalpy required to melt amylose-lipid complex in NWS compared to the unmodified NWS, and the extent of the decrease increased with the level of PO treatment. Similar trends of endothermic transition (T_o , T_p , T_c and ΔH) were also found when the gelatinized starch pastes were re-heated after being stored at 4°C for 7 d. The phenomena could be attributed to the introduction of hydroxypropyl groups. During hydroxypropylation, the amylose-lipid complex may be partially disrupted by alkaline treatment (Matsunaga & Seib, 1997; Gunaratne & Corke, 2007). Substitution of hydroxypropyl groups in amylose also weakens the complex between amylose and lipids.

3.3.3. Pasting properties

Pasting properties of starch may be explained by considering a paste as suspension of swollen granules in a dilute solution of starch molecules (Eliasson & Gudmundsson, 2006). The pasting curves of the unmodified and hydroxypropylated NWS, WWS and WMS as determined by MVA are shown in Figure 3.2. The unmodified NWS started pasting at 87.1°C and showed stable viscosity with a slight breakdown during the holding period at 95°C (Figure 3.2). Amylose and amylose-lipid complex inhibit the swelling of NWS granules (Morrison et al., 1993). As a result, NWS was more resistant to breakdown compared with WWS and WMS (Figure 3.2). After

hydroxypropylation, the hydroxypropylated NWS showed a lower pasting temperature but a higher breakdown and setback viscosity (Table 3.4). Lower pasting temperatures were also observed on hydroxypropylated WWS and WMS (Figure 3.2 and Table 3.4). The modified granules swelled to a greater extent, resulting in a higher peak viscosity. The pasting and peaking temperature reduction for hydroxypropylated NWS (20-25°C, 11-20°C) was much larger than those of modified WWS (5-11°C, 1-7°C) and WMS (1-10°C, 2-9°C), indicating that amylose was substituted in NWS and less effective in inhibiting swelling of wheat starch granules. In other words, NWS granules were able to swell at lower temperatures and swell to greater extents after hydroxypropylation.

3.3.4. Morphology observation

Changes in morphology of unmodified and modified NWS, WWS and WMS granules were recorded after heating in water at 45°C and 70°C for 20 min, respectively (Figures 3.3-3.5). After being heated at 45°C for 20 min, the unmodified starch granules did not swell, while the hydroxypropylated starch granules swelled slightly. After being heated at 70°C for 20 min, the unmodified granule size increased drastically but there were still some intact granules. For hydroxypropylated starches, greater swelling was observed, as evidenced by dramatically enlarged size of the granules and even disappearance of intact granules with the increasing of PO treatment level. Hydroxypropylation of the starches in general appeared to induce the granules to swell at temperatures below the pasting temperatures of the unmodified starches. The higher the degree of hydroxypropylation, the higher extent of swelling of granules at 45-70°C as seen by their larger average size and by the lower intensity of the iodine-staining of granules (Figures 3.3-3.5).

There were some differences between the swelling of NWS, WWS and WMS. After heated at 70°C for 20 min, the unmodified WWS swelled more significantly than WMS followed by NWS

(Figures 3.3-3.5). NWS granules kept the intact round shape even after swelling at 70°C (3rd column of Figure 3.3), whereas WWS (3rd column of Figure 3.4) and WMS (3rd column of Figure 3.5) disintegrated into many small fragments after being heated at 70°C for 20 min. The result was in agreement with our previous study (Wang et al., 2018). Amylose and amylose-lipid complex in NWS inhibited swelling and maintained the integrity of swollen starch granules (Morrison, Tester, Snape, Law & Gidley, 1993).

3.3.5. Settling volume

The settling volumes of the unmodified and modified starches at 25°C were low in magnitude (2.5-3.1 mL), and the hydroxypropylation showed no measurable effect on settling volume (Table 3.5). However, after the starches were heated at 70°C for 20 min, the settling volume values differed dramatically between the modified starches and their unmodified counterparts as well as among the various starches. After being heated at 70°C for 20 min, modified starch granules were highly swollen (Figures 3.3-3.5), especially the WWS and WMS reacted with 7-9% PO (Figures 3.4-3.5). Those modified starches had no or little starch precipitate after being heated at 70°C for 20 min (Table 3.5). The photomicrographs of the unmodified WWS and WMS (top rows in Figures 3.4-3.5) show that the starch granules at 70°C were swollen and partially disintegrated when compared with NWS (the top row in Figure 3.3). Therefore, the settling volume of NWS was much smaller than those of WWS and WMS. The unmodified and modified NWS reacted with 3-5% PO gave a settling volume of 10-20 mL (Table 3.5) after being heated at 70°C because their granules were moderately swollen at that temperature (the top three rows of Figure 3.3). However, the settling volume increased to 74-81 mL after NWS was reacted with 7 and 9% PO (Table 3.5, reflecting that those modified starches were highly swollen at 70°C (Figure 3.3).

The increased settling volume correlated with the higher pasting viscosity (Table 3.4 and Figure 3.2).

3.4. Conclusions

The pasting properties and thermal properties of NWS, WWS and WMS were significantly altered by hydroxypropylation. The pasting temperature and peak temperature decreased with the increasing of MS, while the peak viscosity and breakdown increased. The gelatinization temperatures and enthalpy of the hydroxypropylated starches decreased with increasing of MS. For the same level of PO reaction (3 and 5% PO), hydroxypropylated WWS retrogradated less than hydroxypropylated WMS, suggesting that to achieve for the same level of cold storage stability, less PO is needed for WWS. No retrogradation was observed for the hydroxypropylated WWS and WMS after they were reacted with 6% PO and MS was ~0.094. In contrast, a higher level of PO (9% PO) was needed to react with NWS to achieve the hydroxypropylated starch with an MS of 0.151 that gave no retrogradation.

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Table 3.1 Weight percentage and molar substitution (MS) of hydroxypropyl groups in normal wheat starch (NWS), waxy wheat starch (WWS), and waxy maize starch (WMS) reacted with 3, 5, 6, 7, and 9% (dry starch basis) propylene oxide (PO).

Sample	Hydroxypropyl group (%)	Reaction efficiency (%)	Molar substitution (MS)
NWS-3%PO	1.93±0.16 g *	65.75±1.54 a	0.055±0.001 i
NWS-5%PO	3.01±0.17 ef	61.80±1.62 ab	0.087±0.002 fg
NWS-6%PO	3.55±0.10 cde	59.17±0.80 bcd	0.101±0.002 de
NWS-7%PO	4.00±0.19 bc	59.96±0.19 bc	0.120±0.001 c
NWS-9%PO	5.00±0.15 a	57.81±0.64 bcde	0.151±0.001 a
WWS-3%PO	1.72±0.07 g	57.21±1.24 bcdef	0.048±0.001 i
WWS-5%PO	2.79±0.23 f	55.79±1.68 cdefg	0.079±0.002 h
WWS-6%PO	3.23±0.18 def	53.83±0.20 efg	0.092±0.002 f
WWS-7%PO	3.71±0.22 cd	52.93±0.91 fg	0.106±0.002 d
WWS-9%PO	4.64±0.21 a	51.57±0.80 g	0.133±0.003 b
WMS-3%PO	1.75±0.06 g	58.36±1.92 bcde	0.049±0.002 i
WMS-5%PO	2.86±0.08 f	57.29±1.54 bcdef	0.081±0.002 gh
WMS-6%PO	3.31±0.08 def	55.00±1.12 defg	0.094±0.002 ef
WMS-7%PO	3.66±0.18 cd	52.23±1.35 g	0.104±0.003 d
WMS-9%PO	4.83±0.18 ab	53.64±0.70 efg	0.139±0.002 b

* Mean values in the same column not sharing a common letter are significantly different at $P \leq 0.05$.

Table 3.2 Gelatinization and retrogradation properties of normal wheat (NWS), waxy wheat (WWS), and waxy maize (WMS) starches hydroxypropylated with 3, 5, 6, 7, and 9% (dry starch basis) propylene oxide (PO) as determined by differential scanning calorimetry with ratio of starch: water=1:2.

Sample	Temperature * (°C)			Enthalpy* (J/g)
	To	Tp	Tc	ΔH
<i>Gelatinization</i>				
NWS	60.1±0.1 a	65.3±0.1 a	76.5±0.4 a	13.0±0.0 a
NWS-3%PO	55.8±0.2 b	61.8±0.3 b	74.7±0.1 b	9.3±0.3 b
NWS-5%PO	54.7±0.4 c	60.5±0.5 c	74.7±0.3 b	9.0±0.2 b
NWS-6%PO	53.4±0.4 c	59.1±0.5 cd	73.0±0.3 c	8.9±0.4 b
NWS-7%PO	52.9±0.1 d	58.4±0.3 d	71.5±0.3 d	8.5±0.6 b
NWS-9%PO	50.2±0.0 e	57.0±0.1 e	70.7±0.3 d	6.7±0.2 c
WWS	59.8±0.2 a	68.3±0.2 a	82.0±0.2 a	17.5±0.3 a
WWS-3%PO	57.8±0.1 b	65.8±0.2 b	80.1±0.3 b	14.8±0.5 b
WWS-5%PO	56.3±0.2 c	63.6±0.3 c	75.2±0.1 c	12.8±0.2 c
WWS-6%PO	54.3±0.2 d	62.0±0.5 d	74.7±0.3 c	12.3±0.2 c
WWS-7%PO	53.8±0.5 d	61.6±0.1 d	73.4±0.1 d	11.3±0.2 d
WWS-9%PO	53.1±0.2 d	61.3±0.3 d	72.7±0.1 d	11.1±0.3 d
WMS	66.4±0.2 a	73.9±0.4 a	86.0±0.2 a	17.1±0.2 a
WMS-3%PO	62.6±0.3 b	70.3±0.4 b	81.9±0.5 b	15.5±0.3 b
WMS-5%PO	60.7±0.1 c	69.2±0.3 b	81.1±0.3 b	14.4±0.4 c
WMS-6%PO	59.6±0.4 cd	68.0±0.5 bc	79.7±0.1 c	14.3±0.2 c
WMS-7%PO	59.0±0.3 d	67.5±0.3 c	78.5±0.5 cd	13.8±0.1 cd
WMS-9%PO	56.9±0.3 e	66.2±0.4 c	77.3±0.5 d	12.9±0.3 d
<i>Retrogradation</i>				
NWS	45.4±0.1 a	57.3±0.1 a	71.1±0.6 a	3.9±0.1 a
NWS-3%PO	48.1±0.1 b	58.1±0.2 a	69.9±0.2 a	2.0±0.0 b
NWS-5%PO	50.9±0.4 c	59.1±0.6 ab	69.4±0.2 a	1.3±0.1 c
NWS-6%PO	53.4±0.4 c	59.5±0.5 b	70.0±0.3 a	0.9±0.3 cd
NWS-7%PO	53.8±0.6 d	59.4±0.3 b	68.9±0.2 a	0.3±0.0 d
NWS-9%PO			**	
WWS	45.1±0.2 a	57.0±0.4 a	70.4±0.4 a	3.5±0.3 a
WWS-3%PO	48.4±0.5 b	59.5±0.1 b	70.7±0.5 a	0.7±0.0 b
WWS-5%PO	52.9±0.5 c	59.3±0.1 b	70.1±0.1 a	0.3±0.0 b
WWS-6%PO			**	
WWS-7%PO			**	
WWS-9%PO			**	
WMS	45.9±0.6 a	58.1±0.5 a	70.8±0.5 a	5.7±0.2 a
WMS-3%PO	50.2±0.4 b	59.7±0.2 a	70.9±0.7 a	2.0±0.2 b
WMS-5%PO	53.7±0.2 c	60.7±0.2 b	69.6±0.5 a	0.5±0.0 c
WMS-6%PO			**	
WMS-7%PO			**	
WMS-9%PO			**	

* Data are means \pm standard deviation of duplicates. Starch solid was 33.3%. Means in the same column not sharing a common letter are significantly different at $P \leq 0.05$.
** No melting peaks were detected.

Table 3.3 Thermal properties of amylose-lipid complex in normal wheat starch (NWS) reacted with 3, 5, 6, 7, and 9% propylene oxide (PO, dry starch basis) as determined by differential scanning calorimetry with ratio of starch: water=1:2.

Sample	Temperature (°C)			Enthalpy (J/g)
	T _o	T _p	T _c	ΔH
<i>Melting</i>				
NWS-0	95.0±0.7 a*	103.1±0.2 a	115.2±0.2 a	2.3±0.1 a
NWS-3%PO	84.0±0.7 b	94.0±0.8 b	107.3±0.4 b	1.1±0.0 b
NWS-5%PO	82.2±0.5 bc	92.4±0.6 b	103.9±0.3 c	1.0±0.0 b
NWS-6%PO	80.5±0.7 cd	90.1±0.8 bc	100.7±0.3 d	0.7±0.2 bc
NWS-7%PO	79.9±0.6 cd	88.6±0.3 c	99.0±0.0 e	0.5±0.1 c
NWS-9%PO	79.0±0.6 d	87.1±0.1 c	96.2±0.7 f	0.4±0.0 c
<i>Re-melting</i>				
NWS-0	98.9±0.3 a	105.9±0.3 a	115.4±0.2 a	2.0±0.0 a
NWS-3%PO	80.9±0.4 b	90.7±0.1 b	104.7±0.1 b	1.7±0.1 a
NWS-5%PO	79.6±0.3 b	89.4±0.3 b	102.3±0.5 c	1.2±0.1 b
NWS-6%PO	78.0±0.2 c	87.1±0.9 c	100.5±0.1 d	1.0±0.2 b
NWS-7%PO	77.5±0.6 c	86.6±0.7 c	98.3±0.2 e	1.0±0.0 b
NWS-9%PO			**	

* Means in the same column not sharing a common letter are significantly different at $P \leq 0.05$.

** No melting peaks.

Table 3.4 Pasting properties of normal wheat starch (NWS), waxy wheat starch (WWS), and waxy maize starch (WMS) hydroxypropylated with 3, 5, 7, and 9% (dry starch basis) propylene oxide (PO) determined at 6% solids by Micro Visco-Amylograph.

Sample	Pasting temp (°C)	Peak viscosity (BU)	Peak temp (°C)	Peak time (min)	Breakdown (BU)	Setback (BU)
NWS	87.1±0.4 a	236.0±0.0 c	95.5±0.1 a	12.0±0.1a	18.5±3.5 c	204.0±2.8 d
NWS-3%PO	66.9±0.1 b	328.5±0.7 b	84.1±1.5 b	9.2±0.2 b	144.0±4.2 b	316.5±2.1 a
NWS-5%PO	64.7±1.3 b	339.0±0.0 b	80.5±0.6 c	8.5±0.2 c	152.5±2.1 b	280.5±6.4 b
NWS-7%PO	63.8±0.7 b	384.0±15.6 a	77.1±0.2 d	7.9±0.0 d	183.0±7.1 a	267.0±8.5 b
NWS-9%PO	62.2±0.7 b	395.1±12.6 a	76.0±0.4 d	7.6±0.0 d	194.0±5.0 a	235.0±4.5 c
WWS	67.6±1.5 a	795.0±35.3 a	71.2±1.4 a	6.6±0.3 a	462.5±33.2 c	139.0±2.8 a
WWS-3%PO	62.6±1.2 b	923.0±11.3 b	69.9±0.2 a	6.1±0.1 a	555.5±9.1 b	176.5±6.3 b
WWS-5%PO	61.0±0.3 b	950.0±36.7 b	67.2±1.3 a	6.1±0.0 a	565.5±28.9 b	175.0±7.0 b
WWS-7%PO	59.6±0.1 b	965.5±26.1 b	68.0±0.8 a	5.9±0.0 ab	598.0±25.4 b	181.5±3.5 b
WWS-9%PO	56.3±0.1 c	1029.5±21.0 b	64.2±0.6 a	5.6±0.1 b	680.2±15.0 a	193.0±7.5 b
WMS	71.1±0.5 a	807.5±13.5 b	78.6±0.6 a	8.3±0.2 a	463.5±0.7 b	129.0±8.5 c
WMS-3%PO	70.0±0.1 ab	784.5±27.6 b	76.8±1.9 ab	7.9±0.4 ab	444.0±21.2 b	143.0±5.1 bc
WMS-5%PO	68.9±0.2 b	790.5±20.5 b	73.4±0.1 bc	7.3±0.1 bc	461.5±0.0 b	147.0±1.4 b
WMS-7%PO	64.7±0.3 c	855.5±20.5 ab	71.4±0.5 c	6.8±0.1 c	487.5±20.5 b	176.5±0.7 a
WMS-9%PO	60.6±0.5 c	915.5±20.5 a	69.1±0.7 c	6.7±0.1 c	560±20.0 a	174.9±3.5 a

* Data represent the mean ± standard deviation. Values in the same column with different letters are significantly different ($P < 0.05$).

Table 3.5 Settling volume at 25°C from a 1.0% aqueous mixture of wheat (NWS), waxy wheat (WWS), and waxy maize (WMS) starches and their hydroxypropylated starches after holding a mixture at 25°C or 70°C for 20 min

Starch	Settling volume ¹ (mL)	
	25°C	70°C
NWS	2.5±0.1 b	10.2±0.3 j
NWS-3%	2.6±0.1 b	14.5±0.7 j
NWS-5%	2.5±0.1 b	20.5±0.7 i
NWS-7%	2.5±0.2 b	74.0±1.4 f
NWS-9%	2.7±0.2 abc	81.8±1.8 e
WWS	2.9±0.1 abc	67.0±1.2 g
WWS-3%	3.0±0.1 ab	87.5±1.1 cd
WWS-5%	3.1±0.3 a	94.0±0.8 ab
WWS-7%	3.1±0.1 a	97.0±1.6 a
WWS-9%	3.1±0.2 a	No ²
WMS	2.8±0.2 abc	43.3±0.4 h
WMS-3%	2.8±0.1 abc	84.5±1.1 de
WMS-5%	2.7±0.1 abc	90.0±1.4 bc
WMS-7%	2.7±0.1 abc	96.0±0.4 a
WMS-9%	2.9±0.2 abc	No ²

1 Means in the same column for a given starch that do not share a common letter are significantly different at $P \leq 0.05$.

2 No settling of precipitate from the mixture.

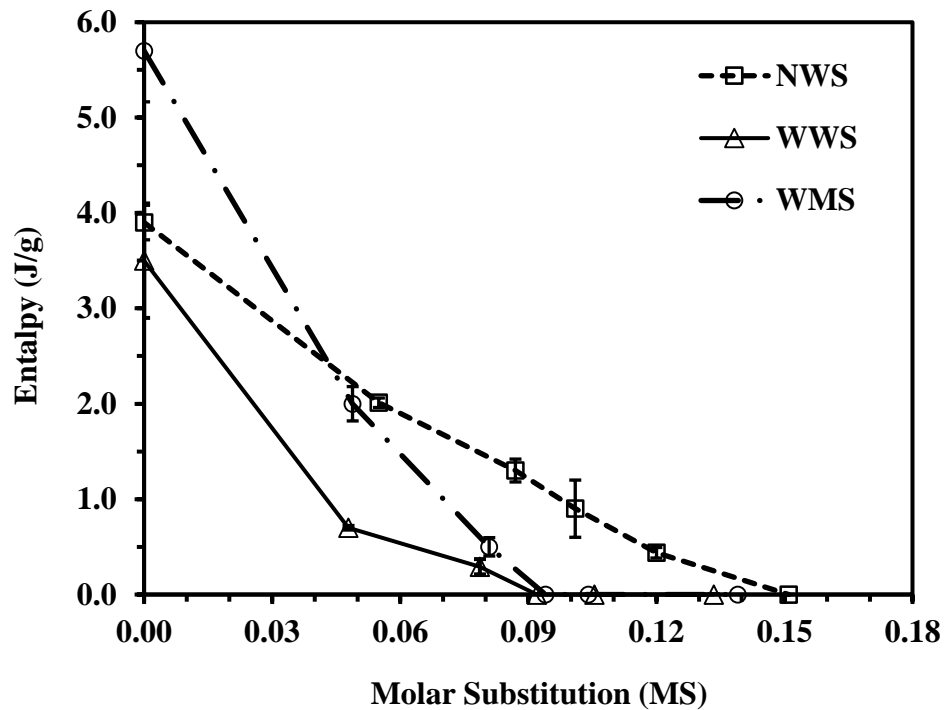


Figure 3.1 The relationship between molar substitution (MS) of hydroxypropyl normal wheat starch (NWS), waxy wheat starch (WWS), and waxy maize starch (WMS) and their retrogradation enthalpy measured by DSC.

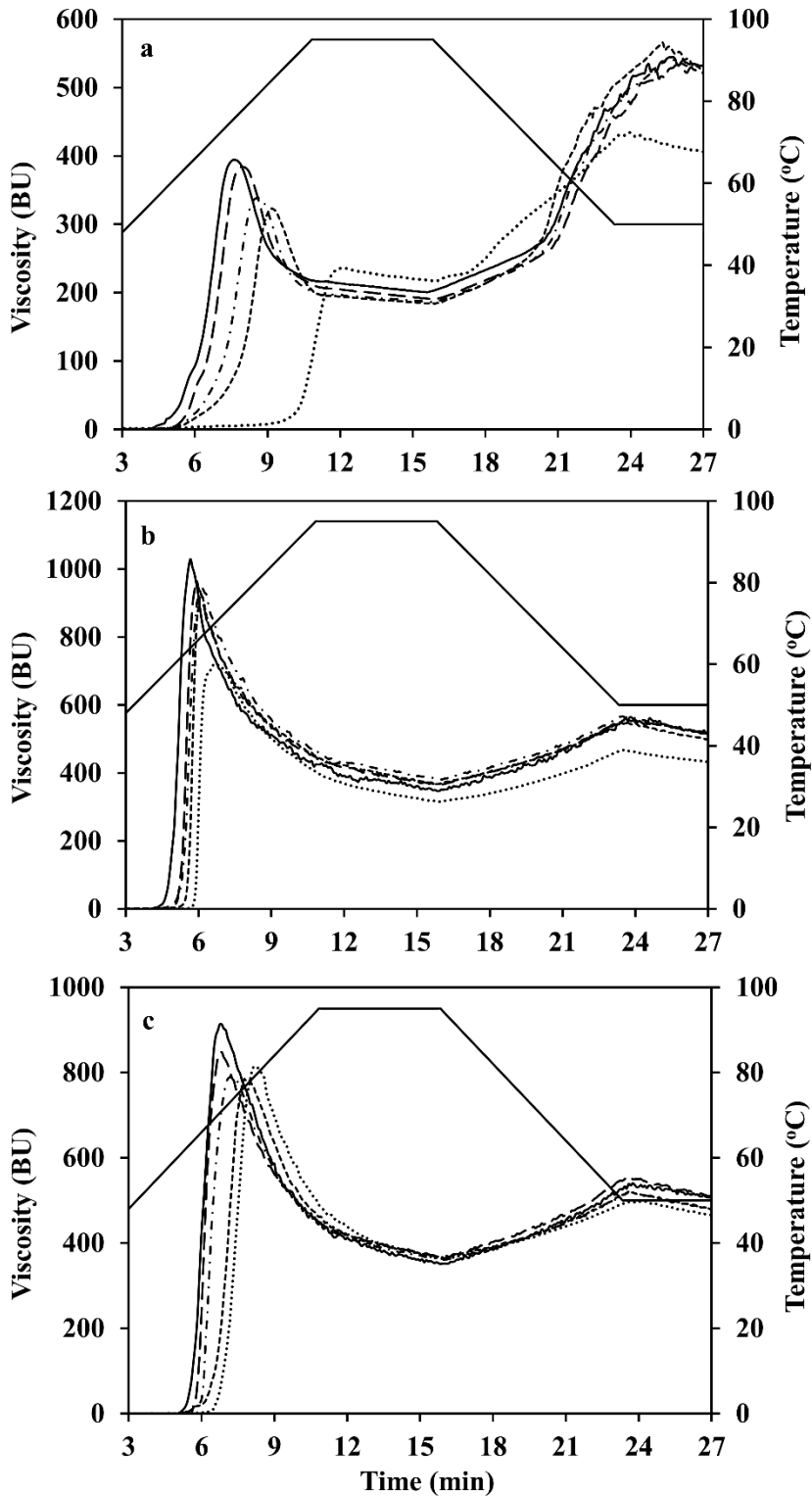


Figure 3.2 Pasting profiles of native and hydroxypropylated normal wheat starch (NWS) (a), waxy wheat starch (WWS) (b), and waxy maize starch (WMS) (c) reacted with 0% (·····), 3% (---), 5% (-·-·-), 7% (- - -) and 9%(—) propylene oxide (PO, w/w dry starch basis).

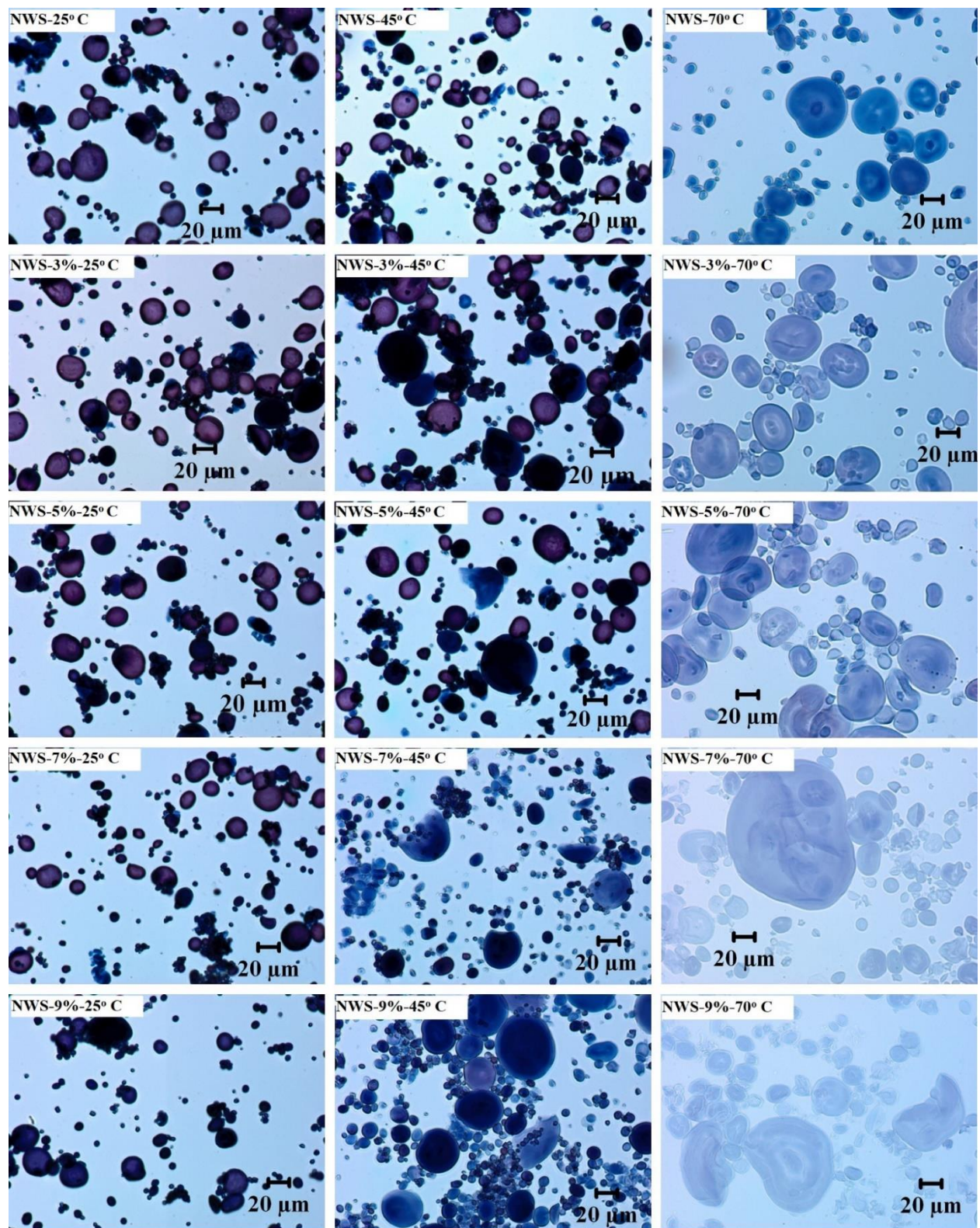


Figure 3.3 Photomicrographs of 1% normal wheat starch (NWS) and its hydroxypropylated starches heated at 25°C, 45°C, and 70°C for 20 min, and stained with I₂/KI. A scale of 20 µm is shown on each photomicrograph.

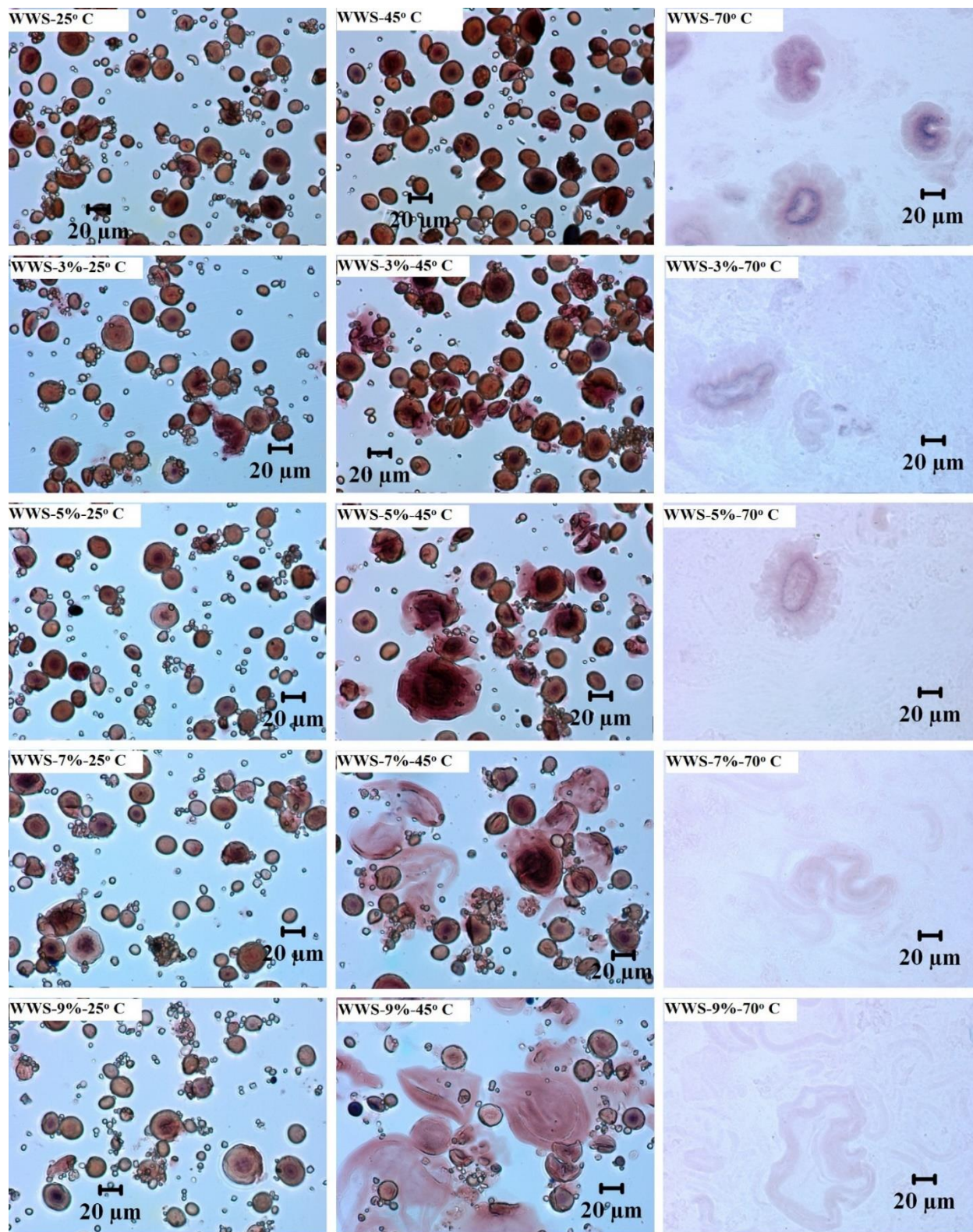


Figure 3.4 Photomicrographs of 1% waxy wheat starch (WWS) and its hydroxypropylated starches heated at 25°C, 45°C, and 70°C for 20 min, and stained with I₂/KI. A scale of 20 µm is shown on each photomicrograph.

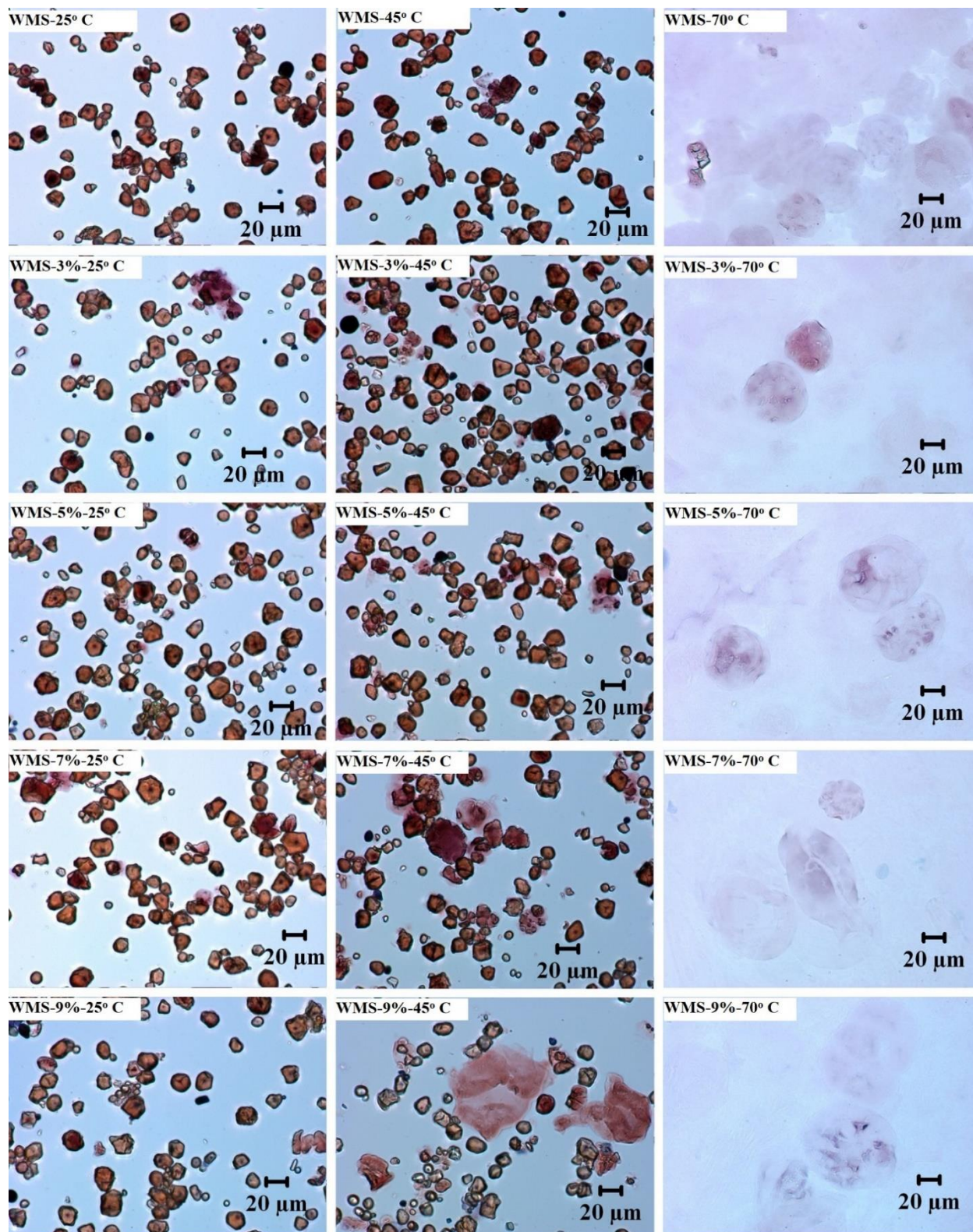


Figure 3.5 Photomicrographs of 1% waxy maize starch (WMS) and its hydroxypropylated starches heated at 25°C, 45°C, and 70°C for 20 min, and stained with I₂/KI. A scale of 20 µm is shown on each photomicrograph.

Chapter 4 - An Improved Method for to Determine the Level of Hydroxypropyl Group in Modified Starches by ^1H NMR

Abstract

^1H NMR is a rapid means of determining the hydroxypropyl (HP) group content in modified starches. However, the sample preparation method for NMR analysis by acid hydrolysis in the literature has some drawbacks including undissolved particles, brownish color, and low detected HP content. The objective of this study was to improve the sample preparation method. The effects of acid (DCl) concentration and temperature on hydrolysis and solubilization of hydroxypropylated starches were examined. The optimal conditions proposed to hydrolyze starches were 10% starch solid content, 3.5% (wt.%) DCl in D_2O as the solvent, sodium acetate as an internal standard, and heating at 90°C for 1 h. Optionally, 6% (v/v) TFA-d may be added as the water-peak shifting reagent. Six hydroxypropylated starches and two cross-linked and hydroxypropylated starches were hydrolyzed with this improved acid method, and the results of the HP content were in agreement with those from an enzyme-catalyzed method.

4.1. Introduction

Hydroxypropyl (HP) starches are used as thickeners and stabilizers in foods because the physicochemical properties of starches could be significantly altered by hydroxypropylation, such as improved freeze/thaw stability, cold storage stability and paste clarity (Wurzburg, 1986). The specific characteristics of the etherified starches are related to the degree of hydroxypropylation. Therefore, it is critical to determine the level of hydroxypropylation in modified starches. There have been numbers of analytical methods for determining the level of HP group in modified starches, including distillation (Lortz, 1956), colorimetric (Johnson, 1969), Fourier transform infrared (FTIR) spectroscopy (Forrest, 1992), and ¹H-NMR spectroscopy (Xu & Seib, 1997) methods.

In the distillation method (Lortz, 1956), hot, constant-boiling hydriodic acid cleaves ether linkages, and the hydroxyalkyl groups decompose quantitatively into ethyl iodide and ethylene. Ethyl iodide and ethylene could be determined volumetrically in standard solutions of silver nitrate and bromine, respectively. Gas chromatography may be used to quantify ethyl iodide. Gas chromatography can be used to quantify ethyl iodide (Van der Bij, 1967). However, the distillation method is slow and tedious. The colorimetric method (Johnson, 1969) involves hydrolysis of the hydroxypropyl group to propylene glycol which in turn is dehydrated to propionaldehyde and the enolic form of allyl alcohol. These products are measured spectrophotometrically after they are reacted with ninhydrin to form a purple color. Different amounts of propylene glycol are used as standards to build a standard curve. Tsai and Hsieh (1982) added sodium bisulfite in the dehydration step, which improved both color stability and sensitivity of the procedure. However, the colorimetric method and its modification give variable results between analysts, and the method is time-consuming (Xu & Seib, 1997). Forrest (1992) reported that the maximal difference

between the FTIR spectra of native starch and its hydroxypropylated counterpart is in the region of the peak at 2974 cm^{-1} . The content of hydroxypropyl groups can be calculated from the second derivative of difference spectra between that of the hydroxypropylated starch and the native starch. The area under the peak centered at 2974 cm^{-1} is calculated, and hydroxypropyl group content is determined by using a calibration curve. However, this FTIR method, being established and calibrated by using colorimetry method and NMR method, is suitable for a quick routine quality assay but not optimal for quantification (Forrest, 1992).

^1H NMR can effectively determine the content of hydroxypropyl groups in starch because chemical shifts of protons from methyl groups in the hydroxypropyl group and starch polysaccharides are different (Casu, Gallo, Reggiani, & Vigevani, 1968). Compared to the colorimetric and distillation methods, ^1H NMR method is much simpler, quicker, and more reproducible (Xu & Seib, 1997). For NMR analysis, HP starch samples need to be degraded and solubilized. Two approaches have been used to depolymerize and solubilize HP starch with a low viscosity for NMR analysis. One is acid hydrolysis (Stahl & McNaught, 1970; Graaf, Lammers, Janssen, & Beenackers, 1995), and the other is enzymolysis (Han & BeMiller, 2006; Kavitha & BeMiller, 1998; Xu & Seib, 1997).

In the acid hydrolysis method developed by Stahl & McNaught (1970), a HP starch was hydrolyzed with 10% hydrochloric acid at 100°C with acetic acid as an internal standard. The results for HP content were consistent with the colorimetric method (Johnson, 1969), but lower than the distillation method (Lortz, 1956). However, when we depolymerized HP starch samples with this method, the hydrolysate showed a brownish dark color and there were many undissolved particles in the slurry after being boiled for 30 min, which may affect the HP content obtained by NMR analysis. The objective of this study was to improve the acidic hydrolysis conditions, so that

HP starches were degraded and solubilized without generating off color. The effects of acid concentration and temperature on hydrolysis and solubilization were investigated. Optionally, trifluoroacetic acid-d (TFA-d) was added to shift water peak in the NMR spectrum. In addition, the hydroxypropyl group content (HP%) and molar substitution (MS) results obtained from the optimized acid hydrolysis conditions were compared with those results obtained from the enzyme-catalyzed method by Xu & Seib (1997). Furthermore, two commercial cross-linked and hydroxypropylated starches were hydrolyzed with the optimized acid hydrolysis method to evaluate the applicability of the method in dual modified starches.

4.2. Materials and Methods

4.2.1. Materials

Waxy maize starch (WMS) and a cross-linked and hydroxypropylated tapioca starch were obtained from Ingredion, Inc (Bridgewater, NJ). A hydroxypropyl wheat starch and a cross-linked and hydroxypropylated wheat starch were obtained from MGP Ingredients, Inc (Atchison, KS). A normal maize starch (NMS) was obtained from Tate & Lyle (Decatur, IL).

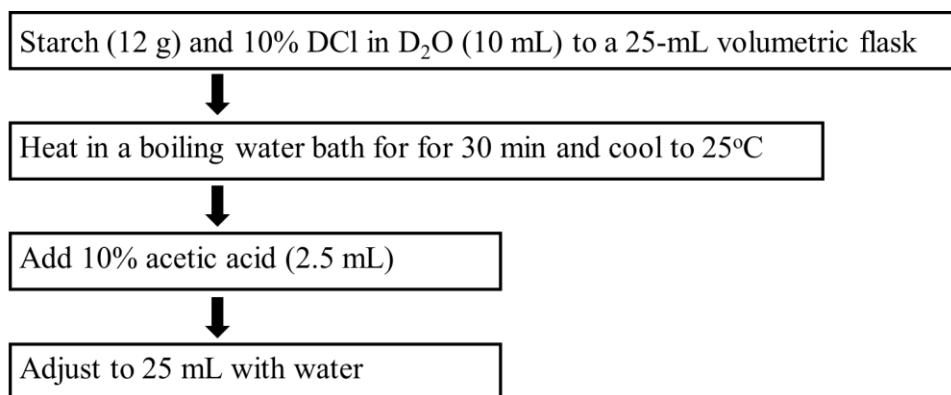
Six hydroxypropylated starches with varied HP contents were prepared in our lab and used to compare the optimized acid-catalyzed hydrolysis and enzyme-catalyzed hydrolysis methods. NMS or WMS (50 g, dry base), sodium sulfate (2.5 g) and distilled water (75 mL) were mixed in a beaker with an overhead mixer, and the pH value of the slurry was adjusted to 11.5 with 3% NaOH. The starch slurry was transferred into a glass jar and mixed with different levels of propylene oxide (PO), and the jar was sealed and shaken in a 45°C water bath for 20 h. After reaction, pH was adjusted to 5.5 with 25% (v/v) sulfuric acid, and the starch was filtered with a vacuum filter and washed with water for three times. The starch cake was crumbed into small

pieces by hands and dried in an air-forced oven at 35°C for 24 h, gently ground with a motor, and pestle and stored in sealed plastic bags at room temperature.

Deuterium oxide (D₂O, 99.9 atom % D contains 0.05 wt.% 3-(trimethylsilyl) propionic-2,2,3,3-d 4 acid, sodium salt), hydrochloric acid-d (DCl, ≥99 atom % D, 35 wt. % in D₂O), and trifluoroacetic acid-d (TFA-d, 99.5 atom % D), were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2. The acid method by Stahl and McNaught (1970) to prepare a HP starch for ¹H NMR analysis

The acid-catalyzed hydrolysis procedure reported by Stahl & McNaught (1970) is shown in Scheme 1. The clear filtrate (0.7 mL) was transferred to a NMR tube and analyzed.



Scheme 1. Acid hydrolysis procedure by Stahl & McNaught (1970) to prepare a hydroxypropylated starch for ¹H NMR analysis

The HP group content (HP%) and the molar substitution (MS) of the modified starch were calculated by Equation 1 and Equation 2, respectively.

$$\text{HP\%} = \frac{0.2623 \times \frac{59.08 \times \text{Intensity at 1.13 ppm}}{60.05 \times \text{Intensity at 2.08 ppm}}}{12.00 \text{ g starch}} \times 100 = 2.151 \times \frac{\text{Intensity at 1.13 ppm}}{\text{Intensity at 2.08 ppm}} \quad (\text{Equation 1})$$

Here, 0.2623= grams of acetic acid in samples; 59.08= molecular weight of hydroxypropyl group; and 60.05= molecular weight of acetic acid.

$$MS = \frac{\text{moles of substituent}}{\text{moles of anhydro-glucose unit}} = \frac{HP\% \times 162}{59.08 \times (100 - HP\%)} \quad (\text{Equation 2})$$

Here, 59.08= molecular weight of hydroxypropyl group; and 162= molecular weight of anhydroglucose unit.

4.2.3. An improved hydrolysis procedure to prepare a HP starch for ¹H NMR analysis

As shown in the Scheme 2, some modifications were made on the method by Stahl & McNaught (1970) to improve solubility and reduce off color of the hydrolysate. The modifications include:

- 1) Starch solid content was decreased from 48% to 10%.
- 2) The concentration of DCl in heating process was decreased from 10% to 3.5%.
- 3) Starch slurry was heated at 90°C for 60 min instead of 100°C for 30 min.
- 4) Sodium acetate was used as an internal standard in place of acetic acid.
- 5) All water was replaced with D₂O.
- 6) Optionally, TFA-d was added to shift the water peak in NMR spectrum.

Starch (200.0 mg), anhydrous sodium acetate (10.0 mg), deuterium chloride solution (0.2 mL, 35% w/w) and deuterium oxide (1.8 mL, contains 0.05 wt. % TMS-d₄) were added in a 15-mL glass vial.



The vial was placed in a water bath at 90°C for 60 min with constant magnetic stirring, and cooled to the room temperature.



The solution was filtered with a 0.45-μm syringe filter.



0.7 mL of supernatant was added to a standard 3-mm NMR tube and analyzed by ¹H NMR

Scheme 2. Improved acid hydrolysis procedure to prepare a hydroxypropylated starch for ¹H NMR analysis

HP% and MS of the modified starch were calculated by Equation 3 and Equation 2, respectively.

$$\text{HP\%} = \left[\frac{\text{weight of Sodium acetate}}{\text{weight of starch}} \right] \times \left(\frac{2}{10} \right) \left[\frac{59.08 \times \text{Intensity at 1.13 ppm}}{82.05 \times \text{Intensity at 2.08 ppm}} \right] \text{ (Equation 3)}$$

Here, 59.08= molecular weight of hydroxypropyl group; and 82.05= molecular weight of sodium acetate.

4.2.4. Optimization of the modified acid method

To improve the acid-catalyzed hydrolysis of starch, the key factor, including temperature, concentration of DCl, and addition level of TFA-d, were studied and optimized.

4.2.4.1. Temperature

In the preliminary experiment, it was found that heating at 100°C would lead to a yellow color of the starch solution, which could affect the NMR result. Therefore, lower temperatures, 80 and 90°C, were used and compared to the samples heated at 100°C.

4.2.4.2. Concentration of DCl

In the acid-catalyze hydrolysis method reported by Stahl & McNaught (1970), 10% DCl was used during heating process, and the final DCl content in the final solution was 4%. The dark brownish color of the hydrolysate may be due to the high acid concentration. As a result, lower DCl concentrations, 3.5, 2.8, 2.1, and 1.4% (wt.%), were used to hydrolyze starch samples and the results were compared.

4.2.4.3. Addition level of TFA-d

To avoid the influence of residual moisture on a ^1H NMR spectrum, TFA-d was added to shift the water peak. The chemical shift of TFA-d in D_2O is ~ 11.5 ppm, which could help shift water peak to downfield. The addition levels of TFA-d, 3, 6, and 9% (v/v), were compared.

4.2.5. Starch solubility

To determine and confirm the extent of starch solubilization, starch (1.0 g) and 3.5, 2.8, 2.1, or 1.4% hydrochloric acid solution (10.0 mL) were added in a 25-mL vial and stirred with a magnetic stirrer. After being heated at 90°C for 1 h and cooled to room temperature, the hydrolysate was transferred to a centrifuge tube and centrifuged at $10000\times g$ for 10 min. The supernatant was discarded, and the sediment was washed with water ($\sim 5\text{mL}$) and centrifuged ($10000\times g$ for 10 min). The precipitate was dried in an air forced oven at 60°C for 48 h. The weight of dried sediment was weighed. The starch solubility was calculated by using Equation 4.

$$\text{Starch solubility (\%)} = 100 - \frac{\text{weight of dried sediment}}{\text{weight of starch material}} \times 100 \text{ (Equation 4)}$$

4.2.6. An enzyme-catalyzed method

Starch samples were prepared by the enzymatic hydrolysis following the method reported by Xu & Seib (1997). The HP% and MS were calculated by Equation 5 and Equation 2, respectively.

$$\text{HP\%} = \frac{I_{\text{HP}} \times m_{\text{NaOAc}} \times 59.08 \times 100}{I_{\text{Ac}} \times m_{\text{starch}(db)} \times 82.05} \text{ (Equation 5)}$$

Here, 59.08= molecular weight of hydroxypropyl group; and 82.05= molecular weight of sodium acetate.

4.2.7. NMR Spectroscopy

^1H NMR spectrum was recorded on a Varian 500 NMR System (Varian Inc., Palo-Alto, CA) at 25°C with the procedure reported by Bai & Shi (2011). The NMR spectrometer was

equipped with a 3-mm diameter, triple-resonance, inverse-detection, pulse-field-gradient probe operating at 499.85 MHz. The spectra were collected in 128 individual scans with a sweep width of 12 ppm and a delay time of 1 s. Chemical shifts are reported in parts per million (ppm).

4.2.8. Statistical analysis

Data are presented as mean \pm SD (n=2). Difference between means was compared using Tukey's test ($P < 0.05$). Statistical analysis was performed using Minitab software (Version 17.0, Minitab, Inc., State College, PA).

4.3. Results and discussion

4.3.1. Comparison of the depolymerization method by Stahl & McNaught (1970) and the improved method

Waxy maize starch reacted with 7% PO was hydrolyzed with the Stahl & McNaught (1970) method and the improved method in this study. The appearance of the solutions prepared by the two methods was visibly different (Figure 4.1A). Starch hydrolyzed by the Stahl & McNaught (1970) method showed a dark brownish color and there were some undissolved particles (~6.3 wt. %) in the slurry after being boiled, whereas the starch prepared with the improved method was clear with slight particulates (~0.7 wt. %). Because starch depolymerized by the Stahl & McNaught (1970) method contained many undissolved particles in the slurry after boiling treatment, the hydrolysate was filtered through a 0.45- μ m syringe filter in this study. As shown in Figure 4.1B, after the undissolved particles were removed, the solution prepared by the Stahl & McNaught (1970) method was clear, but the color of the solution was much darker than those hydrolyzed with the improved method. The color of the samples prepared with the improved method became darker with the increase of heating temperature (Figure 4.1B).

The ^1H NMR spectra and HP content of samples prepared by the Stahl & McNaught (1970) method are shown in Figure 4.2A and Table 4.1. The HP content of the samples, hydroxypropylated waxy maize starch and wheat starch, prepared by the Stahl & McNaught (1970) method was significantly lower than those of samples prepared by the modified improved method in this study. In addition, the water peak in the Stahl & McNaught method (~ 5.5 ppm) was much larger than that of the improved method in this study (~ 5.3 ppm) (Figure 4.2 B).

4.3.2. Effect of heating temperature

As showed in Figure 4.1, 100°C may cause a yellow color. Therefore, lower temperatures, 80 and 90°C , were used, but the starch could not be dissolved well when 80°C was applied. As a result, samples heated at 90°C and 100°C were compared (Table 4.1 and Figure 4.3). When 90 and 100°C were used, HP content were respectively 4.00% and 3.94% for the WMS treated with 7% propylene oxide (PO). To avoid excessive side reactions, 90°C was used in other experiments.

4.3.3. Effect of DCl concentration

In the preliminary test, it was observed that dark color occurred when the concentration of hydrochloric acid (HCl) was above 5% . To avoid off colors in starch hydrolysate, lower acid concentrations, 3.5 , 2.8 , 2.1 and 1.4% (wt. %), were used and the solubility of the starch was compared. The solubility of WMS in HCl solutions are shown in Table 4.2. The lower HCl concentration was, the lower the solubility of the starch, but 3.5% HCl was able to achieve a solubility above 99% . Thus, 3.5% acid concentration was chosen to hydrolyze a HP starch for ^1H NMR analysis.

4.3.4. Effect of TFA-d

To prevent the interference of water peak, TFA-d was added ~ 3 minutes before NMR analysis. The effect of TFA-d on water peak was showed in Figure 4.4. Water peak was shifted to

downfield by adding TFA-d. With the addition level of TFA-d increasing from 3 to 9%, the water peak was gradually shifted from 5.27 ppm to 5.40-5.65 ppm. By adding 6% TFA-d, water peak could be shifted to 5.55 ppm, which is enough to separate water peak from OH-2 and OH-3 peaks (~5.24 ppm).

4.3.5. Comparison of the optimized acid method with Xu & Seib (1997) enzyme method

The HP% content of six hydroxypropylated starch samples hydrolyzed by both the modified acid and enzyme (Xu & Seib, 1997) methods is shown in Table 4.3. The two methods gave similar HP content. Therefore, the improved acid-catalyzed hydrolysis method developed in this study, which is relatively simpler and faster, is proposed to prepare a HP starch for hydroxypropyl determination by ^1H NMR analysis.

4.3.6. Application of the modified acidic method on two cross-linked and hydroxypropylated starches

The optimized acidic method developed in this study was used to prepare two commercial cross-linked and hydroxypropylated starches for ^1H NMR analysis and compared with the enzyme method reported by Xu & Seib (1997). The solubility of the two samples were higher than 99. The HP and MS of both samples obtained through the optimized acid hydrolysis method in this study and Xu & Seib (1997) method are similar (Table 4.4), suggesting that the modified acidic method is also applicable for HP content analysis of cross-linked and hydroxypropylated starches.

4.4. Conclusion

An improved acid-catalyzed hydrolysis method was developed to hydrolyze HP starches for ^1H NMR analysis. The optimal conditions are 10% starch solid content, 3.5% (wt.%) DCl in D_2O as the solvent, sodium acetate as an internal standard, heating at 90°C for 1 h. Optionally, 6%

(v/v) TFA-d may be added as the water-peak shifting reagent. The HP content and MS results of hydroxypropylated starches analyzed with the improved acid-catalyzed method were consistent with those obtained from the enzyme-catalyzed method (Xu & Seib, 1997). ¹H NMR may be used as a simple and rapid means of determining hydroxypropyl group content in both hydroxypropylated starches and cross-linked and hydroxypropylated starches.

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Table 4.1 Hydroxypropyl (HP) content results of samples prepared by Stahl & McNaught (1970) method and the modified method and measured by ¹H NMR.

Sample ^a	HP Content (%) ^b		
	Stahl and McNaught method 100°C, 10% DCl, 48% starch	Improved method I 100°C, 3.5% DCl, 10% starch	Improved method II 90°C, 3.5% DCl, 10% starch
WMS	0	NM ^c	0
WMS 3	0.98±0.01 ^b	NM	1.97±0.05 ^a
WMS 7	2.10±0.02 ^b	3.94±0.08 ^a	4.00±0.10 ^a
HP wheat starch	1.56±0.00 ^b	NM	2.86±0.12 ^a

^a WMS- waxy maize starch; WMS 3- waxy maize starch reacted with 3% (w/w) propylene oxide; WMS 7- waxy maize starch reacted with 7% (w/w) propylene oxide; HP wheat starch- a commercially hydroxypropylated wheat starch obtained from MGP Ingredients.

^b Means with different superscripts within the same row are significantly different at $P < 0.05$. Value is represented as the mean ± standard deviation (n = 2).

^c NM= not measured.

Table 4.2 Solubility of waxy maize starch in acid solutions with different HCl contents (wt./wt.) after being heat at 90°C for 1 h.

HCl concentration (%)	Solubility (%) ^a
3.50	99.30±0.07 a
2.80	98.92±0.21 ab
2.10	98.34±0.47 ab
1.40	97.86±0.25 b

^a Results not sharing a common letter are significantly different at $P < 0.05$. Value is represented as the mean \pm standard deviation (n=2).

Table 4.3 Comparison of NMR results of hydroxypropylated starch (HP) samples made in our lab hydrolyzed with the modified acidic method and the enzymatic method reported by Xu & Seib (1997)

Sample	HP (%) ^a		MS ^a	
	acid	enzyme	acid	enzyme
WMS-4% PO	2.09±0.04 ^a	2.16±0.04 ^a	0.059±0.001 ^a	0.061±0.001 ^a
WMS-5% PO	2.91±0.08 ^a	3.10±0.16 ^a	0.082±0.008 ^a	0.088±0.005 ^a
NMS-4% PO	2.74±0.07 ^a	2.65±0.04 ^a	0.077±0.002 ^a	0.075±0.001 ^a
NMS-7% PO	4.19±0.08 ^a	4.55±0.34 ^a	0.120±0.003 ^a	0.131±0.010 ^a
NMS-8% PO	5.21±0.18 ^a	5.42±0.10 ^a	0.151±0.006 ^a	0.157±0.003 ^a
NMS-10% PO	5.89±0.11 ^a	6.12±0.21 ^a	0.172±0.004 ^a	0.179±0.007 ^a

^a Values from acid and enzyme method for the same sample not sharing a common letter are significantly different at $P < 0.05$. Values are represented as the mean \pm standard deviation (n= 2).

Table 4.4 Comparison of hydroxypropyl (HP) content of the waxy maize starch (WMS) reacted with different levels of propylene oxide (PO) as measured by ¹H NMR using the improved acid hydrolysis method (acid) and the enzymatic hydrolysis method by Xu & Seib (enzyme).

Sample	HP (%) ^a		MS ^a	
	acid	enzyme	acid	enzyme
WMS-4% PO	2.09±0.04 ^a	2.16±0.04 ^a	0.059±0.001 ^a	0.061±0.001 ^a
WMS-5% PO	2.91±0.08 ^a	3.10±0.16 ^a	0.082±0.008 ^a	0.088±0.005 ^a
NMS-4% PO	2.74±0.07 ^a	2.65±0.04 ^a	0.077±0.002 ^a	0.075±0.001 ^a
NMS-7% PO	4.19±0.08 ^a	4.55±0.34 ^a	0.120±0.003 ^a	0.131±0.010 ^a
NMS-8% PO	5.21±0.18 ^a	5.42±0.10 ^a	0.151±0.006 ^a	0.157±0.003 ^a
NMS-10% PO	5.89±0.11 ^a	6.12±0.21 ^a	0.172±0.004 ^a	0.179±0.007 ^a

^a Values from acid and enzyme method for the same sample not sharing a common letter are significantly different at $P < 0.05$. Values are represented as the mean ± standard deviation (n= 2).

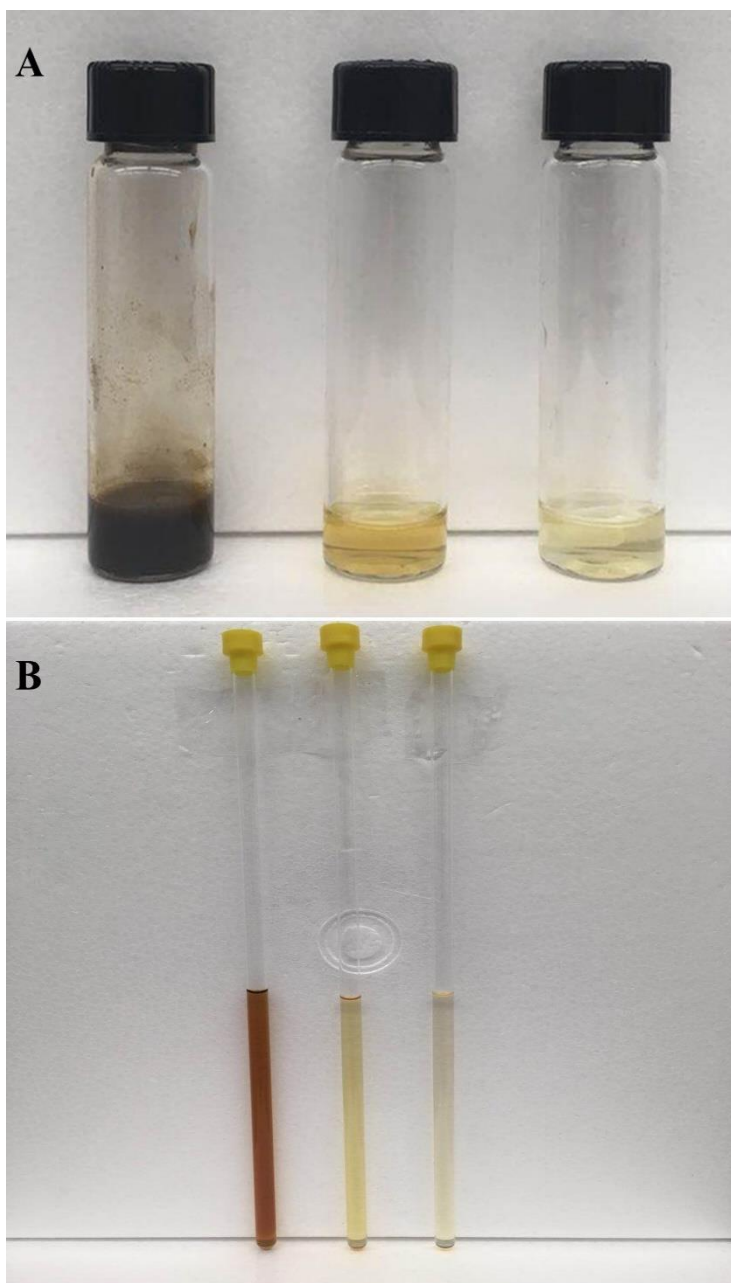


Figure 4.1 Appearance of the waxy maize starch reacted with 7% propylene oxide (WMS 7) prepared by different acid hydrolysis methods (A) before and (B) after being filtered through a 0.45- μm syringe filter; samples from left to right were prepared with the Stahl & McNaught method (100°C, 10% DCl, 48% starch), modified method I (100°C, 3.5% DCl, 10% starch), and modified method II (90°C, 3.5% DCl, 10% starch), respectively.

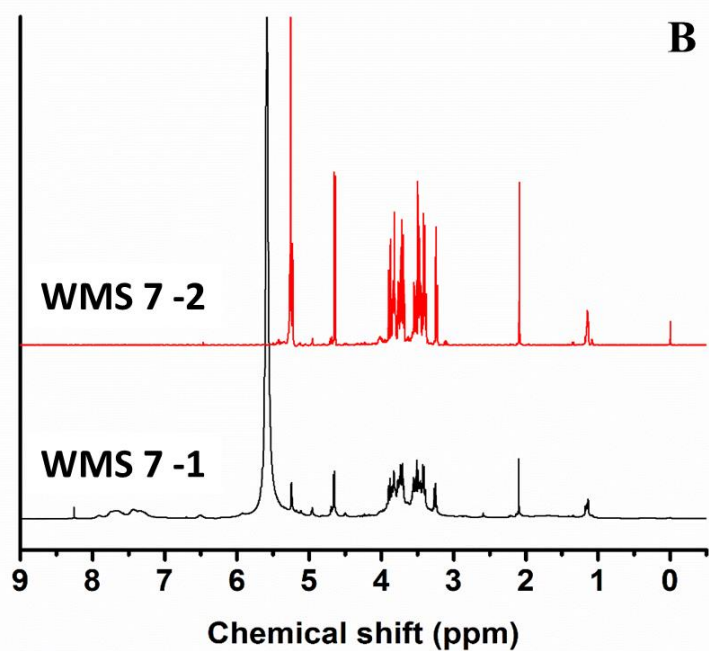
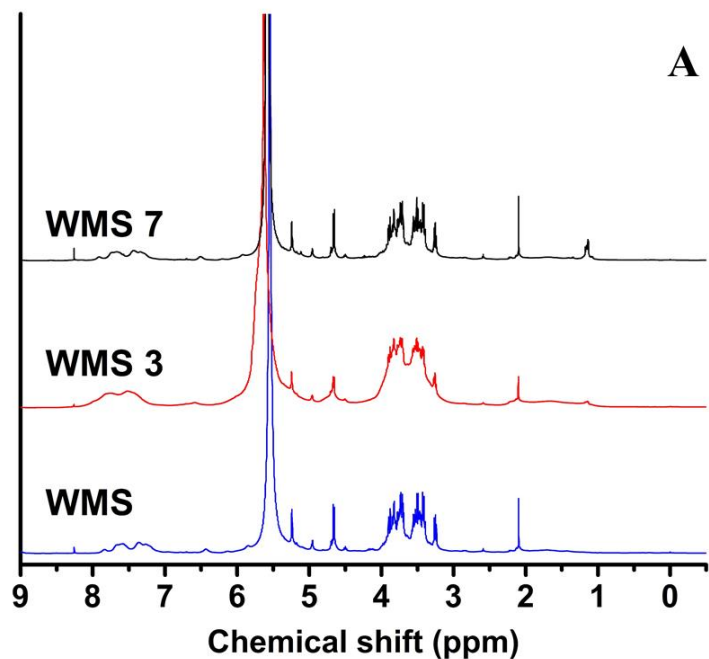


Figure 4.2 NMR spectra of (A) the unmodified and hydroxypropyl waxy maize starch (WMS) prepared by the Stahl & McNaught method (WMS 3- waxy maize starch reacted with 3% (w/w) propylene oxide (PO); WMS 7- waxy maize starch reacted with 7% (w/w) PO); (B) waxy maize starch etherified with 7% PO and depolymerized with Scheme 1, the Stahl & McNaught method (WMS 7-1) and Scheme 2, the modified method - 90°C, 3.5% DCI, 10% starch (WMS 7-2).

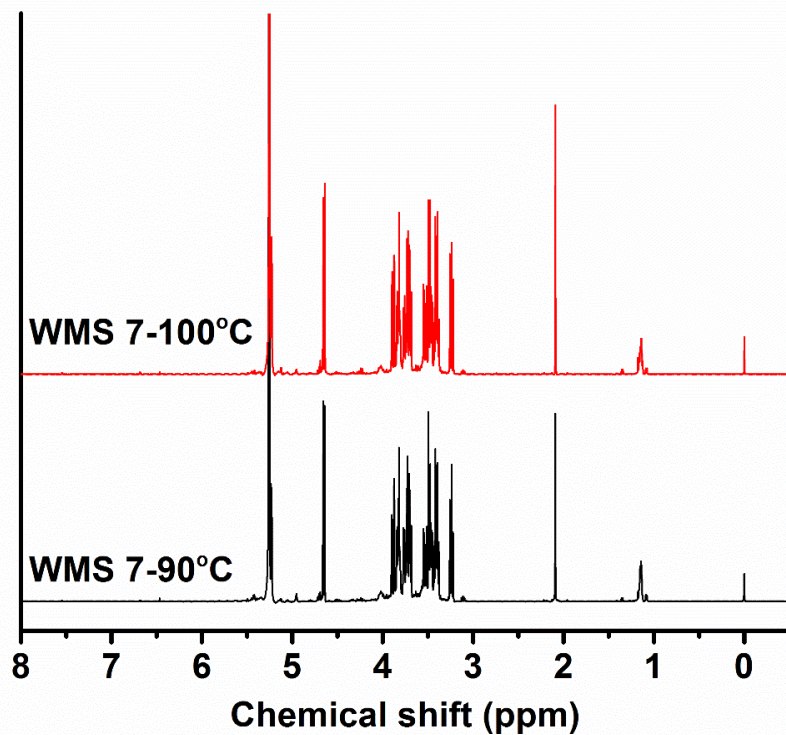


Figure 4.3 NMR spectra of the waxy maize starch reacted with 7% (w/w) propylene oxide (WMS 7) prepared with the modified acid hydrolysis method (Scheme 2) heated at different temperatures.

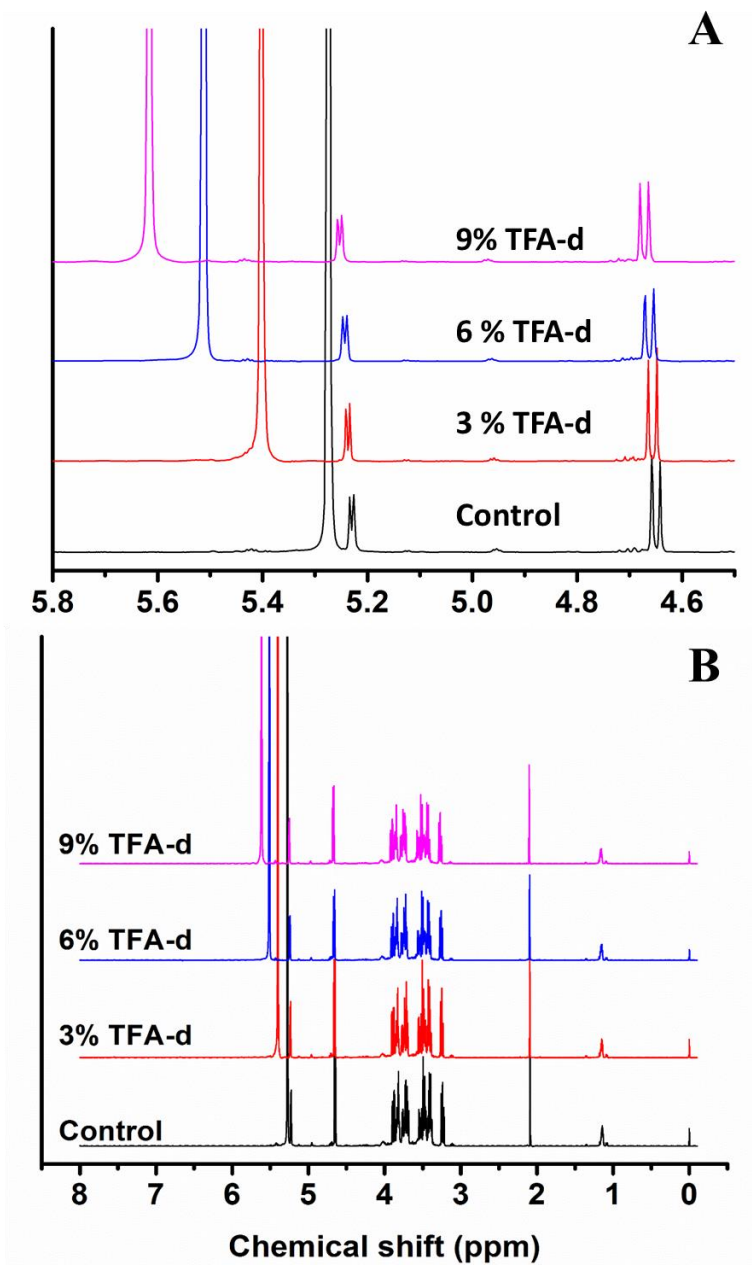


Figure 4.4 NMR spectra of the waxy maize starch reacted with 7% (w/w) propylene oxide prepared with the modified method by adding different levels of trifluoroacetic acid-d (v/v) (Scheme 2) (B), and partial spectra (A) between 5.8 and 4.5 ppm were expanded.

Chapter 5 - Conclusions and Perspectives

The pasting properties and thermal properties of NWS, WWS and WMS were significantly altered by hydroxypropylation. The pasting temperature and peak temperature decreased with the increasing of MS, while the peak viscosity and breakdown increased. The gelatinization temperatures and enthalpy (ΔH) of the hydroxypropylated starches decreased with increasing of MS. For the same level of PO reaction (3 and 5% PO), hydroxypropylated WWS retrogradated less than hydroxypropylated WMS, suggesting that to achieve for the same level of cold storage stability, less PO is needed for WWS. No retrogradation was observed for the hydroxypropylated WWS and WMS after they were reacted with 6% PO and MS was ~ 0.094 . In contrast, a higher level of PO, 9%, was needed to react with NWS to achieve the hydroxypropylated starch with an MS of 0.151 that gave no retrogradation.

The specific characteristics of the etherified starch are related to the degree of hydroxypropylation. Therefore, it is critical to determine the level of hydroxypropylation in modified starches. An improved acid-catalyzed hydrolysis method was developed to hydrolyze HP starches for ^1H NMR analysis. The optimal conditions are 10% starch solid content, 3.5% (wt.%) DCl in D_2O as the solvent, sodium acetate as an internal standard, heating at 90°C for 1 h. Optionally, 6% (v/v) TFA-d may be added as the water-peak shifting reagent. The HP content and MS results of hydroxypropylated starches analyzed with the improved acid-catalyzed method were consistent with those obtained from an enzyme-catalyzed method (Xu & Seib, 1997). ^1H NMR may be used as a simple and rapid means of determining hydroxypropyl group content in both hydroxypropylated starches and cross-linked and hydroxypropylated starches.

Our work on sample preparation method for NMR analysis focused on modified waxy and normal starches. Further research is needed to include hydroxypropylated high amylose starches and further improve the method.