**Analysis of octenylsuccinate rice and tapioca starches: Distribution of octenylsuccinic
anhydride groups in starch granules**

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Abstract

Characterization of the fine structure of octenylsuccinic anhydride (OSA) starch would lead to a better understanding of functional properties. OSA rice and tapioca starches were analyzed using microscopy, liquid chromatography and nuclear magnetic resonance. Chain length distribution of amylopectin changed significantly ($P < 0.05$) after OSA esterification. Weight averaged degree of polymerization (DP_w) decreased significantly ($P < 0.05$) from 16.47 to 13.29 and from 14.87 to 12.47 in native and OSA rice and tapioca starches, respectively. The chain length distribution of pure amylopectin fractions suggested that OSA groups were not present in the amylopectin portion of the starch. ^1H -NMR analysis of pure amylose and amylopectin fractions indicated that OSA substitution was present only in amylose fractions of rice and tapioca starches. Esterification with 3% OSA results in starch that has OSA substituted mainly on amylose chains or possibly on amylopectin chains that have been hydrolyzed from the amylopectin molecules during esterification.

Key words: octenylsuccinic starch (OSA), OSA group distribution, amylose, amylopectin

1. Introduction

Among carbohydrates, starch has a wide variety of uses as an ingredient in different food products. To improve starch functionality for specific applications, it is modified by physical, chemical and enzymatic processes (Hui, Qi-He, Ming-liang, Qiong, & Guo-qing, 2009). Octenylsuccinic anhydride (OSA) esterified starch is one type of chemically modified starch obtained by esterification of OSA groups on repeating anhydroglucose units along the starch chains (Bai, Kaufman, Wilson, & Shi, 2014). Its preparation involves the reaction in aqueous medium under mild alkaline conditions with the starch in its granular form. The alkalinity helps to reduce hydrogen bonding between starch chains which consequently favors granular swelling and the diffusion of OSA molecules within the swollen starch granule (Sweedman, Tizzotti, Schäfer, & Gilbert, 2013). Such modification imparts a partial hydrophobic character to the starch and weakens the internal bonding keeping the granules intact (Bhosale & Singhal, 2007). The partial hydrophobicity resulting from OSA esterification of the hydrophilic native starch results in an amphiphilic molecule with unique properties (Sweedman, Tizzotti, Schäfer, & Gilbert, 2013). These characteristics make OSA starch useful in stabilizing oil/water emulsions and impart resistance to re-agglomeration (Shogren, Viswanathan, Felker, & Gross, 2000). However, previous studies have found that the location and distribution of the OSA groups in the starch granules effect the physical properties of OSA modified starches (Wang, He, Fu, Huang, & Zhang, 2016)

The effect of OSA esterification on the starch structure has been investigated in regards to the overall extent of substitution, the substitution at the granular level and at the molecular level. Concerning the overall extent of substitution and the substitution at the granular level, it had been found that OSA substitution occurred mostly in the amorphous region without change

to the starch crystallinity with 3% OSA (Bai, Kaufman, Wilson, & Shi, 2014). Shogren, Viswanathan, Felker, and Gross (2000) reported that the distribution of OSA groups in a cross-section of waxy maize starch granules was homogenous. However, another study by Bai, Kaufman, Wilson, and Shi (2014) determined that the distribution of OSA groups was uneven in waxy maize starch granules at a specific DS (0.05). The reaction of OSA with the starch and the distribution of OSA in the granule can also be effected by the reaction conditions. Wang, He, Fu, Luo, and Huang (2015), found that applying high-speed shear during the modification process increased the degree of substitution. When the OSA distribution in different starch sources (maize and potato) was analyzed, it was found that OSA maize starch granules showed more OSA groups in the inner region of the granule when compared to the OSA groups present in OSA potato starch, indicating that maize starch displayed more homogeneous OSA reaction pattern than potato starch. Even so, it was reported that greater proportions of OSA groups were found in the periphery than in the core of the starch granule in both botanical sources. On the other hand, because pinholes and channels in the starch granules; it was suggested that most of the starch granules are accessible to react with OSA (Wang, He, Huang, Fu, Luo, & Li, 2013).

At the molecular level, a few studies have been conducted to determine the position of the OSA groups on the anhydroglucose of starch after the esterification process. After partial debranching with pullulanase of waxy maize starch, it was reported that most of the chains were neutral, suggesting heterogeneity in OSA substitution at the branch level (Shogren, Viswanathan, Felker, & Gross, 2000). Also, Bai and Shi (2011) found that OSA substitution occurred at the O-2, O-3 and O-6 positions and even at the reducing end. Bai, Kaufman, Wilson, and Shi (2014) reported that for waxy maize starch at low DS, most OSA groups were located near the branching points of the amylopectin, while in higher DS modifying groups were located near the

branching points and or close to non-reducing ends. A study conducted by Xu, Huang, Fu, and Jane (2015), found that hydrolysis of OSA modified waxy corn starch with β -amylase resulted in greater occurrence of hydrophobic groups near the periphery of the starch molecules. The majority of studies about the distribution of OSA in starches and their fine structure have been conducted on waxy type starches that do not contain amylose (Bai, Kaufman, Wilson, & Shi, 2014; He, Liu, & Zhang, 2007; Miao, Li, Jiang, Cui, Zhang, & Jin, 2014; Shogren, Viswanathan, Felker, & Gross, 2000; Wang, He, Fu, Huang, & Zhang, 2016; Xu, Huang, Fu, & Jane, 2015). However, the distribution of the OSA groups along the starch chains of amylose and amylopectin in non-waxy starches of different botanical origins is still unclear. So, investigation of the distribution of OSA groups on amylose and amylopectin fractions and the structural characterization of OSA starches; with a special focus on the starch chain branching structure, a complex parameter that influences the molecular size, composition and flexibility of the starch molecule is still required.

2. Material and methods

2.1 Materials

Non-waxy rice starch was purchased from Sigma-Aldrich and tapioca starch was provided by Ingredion (Westchester, IL). Octenylsuccinic anhydride (OSA) was purchased from Dixie Chemical Company (Pasadena, TX). OSA rice and tapioca starch were esterified with OSA at 3% of starch weight, according to the methodology described by Han and BeMiller (2007). The degree of substitution (DS) of OSA rice and tapioca starch was 0.026 and .018, respectively. The amylose content of the OSA rice starch was 25.4% and the OSA tapioca starch had 24.5% amylose (Simsek, Ovando-Martinez, Marefati, Sjö ö , & Rayner, 2015).

2.2 Confocal laser scanning microscopy (CLSM)

Native and esterified starch samples were dye-stained with methylene blue before visualization with confocal laser scanning microscopy (CLSM) (Zhang, Huang, Luo, Fu, Jiang, & Jane, 2011) with slight modifications. Starches (0.5g) were dispersed in distilled water (30 ml) and then the pH was adjusted to 8.0. To stain the starches, methylene blue solution (1%, 100 μ L) was added into each suspension. The samples were held at room temperature (25°C) for 30 minutes in a shaking water bath (150 rpm). After dyeing the starch samples, the excess dye was removed by washing with methanol three times. An AxioObserver Z1 microscope was used for detection of the fluorescence signal from the dye-stained starch granules. The microscope was equipped with LSM 700 laser scanning module using fixed settings of 625 nm laser and 20 x 0.8 objective. The mean intensity levels for the entire picture were measured with Zeiss Zen 2010 software in order to measure the level of dye incorporated into the starch granules.

2.3 Backscattered electron (BSE) imaging

The native and OSA starch samples were embedded and sliced prior to staining and backscattered electron imaging. LR White acrylic resin (Ted Pella, Inc., Redding, California, USA) polymerized in a 60 °C oven for 48 h was used to embed the starch granules. RMC MT XL ultramicrotome (Boeckeler Instruments, Tucson, Arizona, USA) with a dry diamond knife was used to slice the embedded starch granules to produce a smooth surface for staining and imaging. The staining was conducted by placing the sectioned embedded starch granules into a sealed container and then several drops of OsO₄ solution was added. The samples were held with the OsO₄ for four hours. Then the blocks were coated with carbon in a high vacuum carbon evaporative coater (Cressington 208c, Ted Pella, Inc., Redding, California, USA). Backscattered electron (BSE) images were obtained with a JSM-7600F scanning electron microscope (JEOL

USA, Inc., Peabody, Massachusetts) in compositional mode with an accelerating voltage of 5 kV and working distance of 4.5 mm.

2.4 Anion-exchange chromatography

Potassium phosphate buffer (15ml, 1mM, pH 6) was used to dissolve the native and OSA starch samples (13mg). The sample solutions heated (135 °C) for 20 minutes with constant stirring to completely dissolve the starch. Then, the samples were centrifuged (3000 g for 10 min) before loading four milliliters of the supernatant into the low pressure chromatography column (25x150 mm). The column was packed with DEAE-Sephadex gel (A25120, Sigma-Aldrich, St Louis, MO). The mobile phase was 1 mM phosphate potassium buffer with a linear gradient of 1 mM phosphate buffer with 2 N NaCl (Shogren, Viswanathan, Felker, & Gross, 2000), and the flow rate was determined to be approximately 0.4 mL/min. Fractions were collected every 2 minutes. Total carbohydrate was determined on every third fraction using the phenol-sulfuric assay following the method described by Ovando-Martínez, Bello-Pérez, Whitney, Osorio-Díaz, and Simsek (2011).

2.5 Gel permeation chromatography

Gel permeation chromatography (GPC) was used to fractionate the amylopectin and amylose of the native and OSA starches. Native and esterified starch (40 mg) was dissolved in sodium hydroxide (4 mL, 2M) and heated at 80 °C with constant stirring for 2 h. The dissolved starches were filtered through 1.5 µm nylon syringe filters prior to loading onto the GPC column (1.6x100 cm, Pharmacia Inc., Piscataway, NJ). The GPC column was packed with Sephadex CL-2B gel (Sigma-Aldrich, St Louis, MO) and the mobile phase was 10mM NaOH with a flow rate of approximately 0.4 mL/min. Fractions were collected every nine minutes for approximately 16 hours. Total carbohydrate content by phenol-sulfuric assay and the identification of amylose and

amylopectin by the blue value assay (Ovando-Martínez, Bello-Pérez, Whitney, Osorio-Díaz, & Simsek, 2011) were conducted on every third fraction.

The fractions containing amylopectin or amylose were combined before conducting dialysis to remove sodium hydroxide. The amylopectin and amylose fractions were placed in distilled water and dialyzed for 3 days at 4 °C. The dialyzed samples were freeze-dried for the analysis of chain length distribution of amylopectin and ¹H-NMR of both amylose/amylopectin fractions to analyze the OSA distribution in the starch molecules.

2.6 ¹H nuclear magnetic resonance spectroscopy

To remove as much water as possible, the amylopectin and amylose samples were purged with deuterium oxide (D₂O) three times (lyophilizing between each purge) prior to conducting the nuclear magnetic resonance (NMR) spectroscopy experiments. The amylopectin and amylose fractions were placed in NMR tubes (8 inch, 5mm, thin wall) after being dissolved in D₂O (0.6 ml, 85 °C x 2h) a final time. A Bruker 400 MHz NMR (Billerica, USA) was used to collect the ¹H spectra, and the analysis was conducted at 25°C for 64 scans with a delay time of 1 s. The degree of substitution (DS) of amylopectin and amylose fractions from GPC fractions was calculated according to the method reported by Shih and Daigle (2003): $DS = A_{0.8-0.9} / (3 \times A_{5.10-5.26})$, where A is the integral value of the resonance assigned. The equatorial proton of the anhydroglucose unit (AGU) of starch (5.2-5.4 ppm) was used as the internal standard for the calculations. Integration of the methyl protons of the OSA (0.8-0.9 ppm) was used to determine the level of OSA substitution for the amylopectin and amylose fractions.

2.7 Amylopectin branch chain length distribution

High performance anion exchange chromatography (HPAEC) was used to analyze the chain length distribution of native and OSA starches and amylopectin fractions (Lee, Oh, & Yoo,

2009). Prior to analysis by HPAEC the native and esterified starches were dissolved in dimethylsulfoxide (DMSO) (10 mg, 1 mL 90% DMSO, 80 °C, 15 min). The samples were then precipitated with 6 mL of absolute ethanol and centrifuged (3000 g x 15 min). Finally, the precipitate was dispersed with 1 mL of deionized water. The amylopectin samples (10 mg) were prepared for analysis by dissolution in 1 mL of deionized water by heating at 80 °C for 15 min with constant stirring in a heating block. The starch and amylopectin samples were diluted with sodium acetate buffer (4 ml, 10 mM, pH 3.5). Then the α -(1-6)-D-glucosidic branch linkages were hydrolyzed by incubation of the samples for 48 hours with 20 μ L of isoamylase (Sigma–Aldrich Chemical Co., MO, USA) in a shaking water bath (40 °C). After debranching the samples were freeze-dried, dissolved in water (1 mL) and boiled for 10 min with stirring. After dilution to 0.2 % and filtering through 0.45 μ m (cellulose acetate), the sample (25 μ L) was injected into the HPAEC system (Dionex, CA, USA) onto a Carbopac PA-100 column. The eluent gradient was as follows: A) 150 mM NaOH, B) 600 mM sodium acetate in 150 mM NaOH, with a gradient elution from 100 % eluent A to 100% eluent B for 100 min, increasing 1 % eluent B per min. The samples were monitored with an ED-40 detector in pulsed amperometric mode (Kittisuban, Lee, Suphantharika, & Hamaker, 2014). The amylopectin chain length distribution, weight-average degree of polymerization (DP_w) and number-average degree of polymerization (DP_n) were calculated using the Dionex software.

2.8 Statistical analysis

Chain length distribution of amylopectin of starches and pure amylopectin was performed in duplicate. The mean and SD (standard deviation) were reported for each sample. Comparison of the means among samples was performed by one-way analysis of variance. Tukey's multiple

comparison test was used to determine significant differences among means with a significant level computed at $P < 0.05$. Data was analyzed using JMP v 8.0.2 (SAS Institute, Cary, NC).

3. Results and discussion

3.1 Distribution of OSA groups on starch granules

Confocal laser scanning microscopy (CLSM) was used to visualize the distribution of OSA esterification of starch granules that had been stained with methylene blue (MB^+). **Figure 1** shows the intensity of fluorescence of native and esterified rice and tapioca starches that have been stained with MB^+ . Since native starches used in this study absorbed some of the stain and showed some level of fluorescence, the fluorescence intensity values for each sample were reported (Figure 1). The fluorescence of the native starches may be due to surface proteins that are sometimes found on commercially produced starches. Native rice starch granules showed very little fluorescence intensity (9.67) after being stained with MB^+ , while the intensity of fluorescence of the native tapioca starch (57.27) was almost six times higher than native rice starch. However, the fluorescence intensities of the OSA esterified starches were both considerably higher than their native starch counterparts. The OSA rice starch had an intensity of 48.75 and the intensity of the OSA tapioca starch was 74.72. MB^+ is a cationic fluorescent dye that has the ability to interact with anionic components such as the $-COO^-$ of OSA groups (Jayaraj, Umadevi, & Ramakrishnan, 2001; Zhang, Huang, Luo, Fu, Jiang, & Jane, 2011). It is clear that OSA rice showed more OSA groups in its granules since the difference in intensity between the native and OSA esterified starch was greater in the rice starch samples. This is consistent with the higher DS of rice starch compared to tapioca starch (data shown in materials section). It was also observed that OSA distribution on starch granules is more concentrated on the surface of both rice and tapioca granules than in the core. This indicated that amylose or long

chains of amylopectin may react with OSA droplets as well as with dissolved OSA during the modification process.

To corroborate the OSA distribution on starch granules, images were also taken of native and OSA starches stained with OsO_4 using backscattering electron microscopy (BSE) (**Figure 2**). The osmium solution (OsO_4) oxidizes the double bonds of OSA resulting in the deposition of solid OsO_2 in the starch granule. The OsO_2 deposits are shown as bright areas in the starch granule images (Shogren, Viswanathan, Felker, & Gross, 2000). Regarding this, native rice and tapioca starch did not show bright areas in the starch granules. However, OSA rice and tapioca showed bright areas mostly on the surface of the granule. These results are similar to those found with CLSM, both confirming the high DS value of rice and the OSA distribution groups into the starch granule. OSA groups may be located at the surface of the granule and also in the amorphous region of the starch granule depending on the botanical source.

3.2 DEAE-Sephadex chromatograms

The distribution of the OSA esterification at the molecular level of the starch was studied with the separation of the starch molecules on the basis of their charge utilizing DEAE-Sephadex (**Figure 3**). DEAE-Sephadex is an anion exchange resin that binds negatively charged ions, so the samples that have more charged molecules are more tightly bound to the resin, travel slowly through the column and are then eluted later. The native starch chromatograms depicted in **Figure 3** indicate that native starch having a neutral charge was bound less strongly to the DEAE-Sephadex and eluted off sooner from the column. OSA rice and tapioca starch showed the presence of a second peak. The esterified starch had a negative charge, which corresponds to the OSA groups introduced into the starch structure. On the other hand, OSA rice presented a more pronounced decrease in the first peak in comparison with OSA tapioca starch. The decrease

in the peak representing uncharged starch molecules is likely due to the higher DS in the OSA rice starch. The results observed in this study were similar to those reported by Shogren, Viswanathan, Felker, and Gross (2000), whom suggested that most of the molecules in OSA waxy maize starch have some covalently attached OSA groups. The results obtained were also in agreement with those found in microscopy analysis of the starch granules (Shogren, Viswanathan, Felker, & Gross, 2000).

3.3 Separation of amylose and amylopectin fractions

The majority of studies that have investigated the fine structure of OSA starch and the distribution of OSA substitution have used waxy maize starch (Bai, Kaufman, Wilson, & Shi, 2014; He, Liu, & Zhang, 2007; Miao, Li, Jiang, Cui, Zhang, & Jin, 2014; Shogren, Viswanathan, Felker, & Gross, 2000). However, when considering starches containing amylose, the same inferences about the distribution of OSA and fine structure cannot be made. After investigation of OSA distribution in the starch granules, further study was needed to investigate the distribution of OSA substitution at the molecular level. To begin the study of OSA substitution at the molecular level the amylopectin and amylose fractions were separated from each starch sample using GPC. The amylopectin and amylose fractions were identified with the phenol-sulfuric assay (**Figure 4a&b**) and the blue value assay (**Figure 4c&d**). In the chromatograms, the first peak represents the amylopectin, while the second peak is the amylose fraction. Jane, Xu, Radosavljevic, and Seib (1992) found greater blue values in the amylopectin peak in GPC profiles of cross-linked starches than for their native forms, concluding that such an increase resulted from amylose cross-linked to amylopectin. However, substitution and cross-linking modifications have different effects on starch structure and we cannot presume that the OSA starch structure would be similar to cross-linked starch. The introduction of OSA groups into the

starch structure, whether amylopectin or amylose, may have changed the hydrodynamic volume of the molecule and so increase the fraction of starch that eluted first during GPC separation (Simsek, Ovando-Martinez, Marefati, Sjö ö , & Rayner, 2015). The changes observed in the first peak (amylopectin), did not directly indicate that OSA groups are located in this molecule; but suggest that there is a change in the fine structure of the amylose and amylopectin. Overall, the OSA esterification process resulted in some minor changes to distribution of amylose and amylopectin fractions. However, it is not conclusive if these changes are due to OSA substitution or changed caused by the alkaline conditions of the esterification process.

3.4 Distribution of amylopectin chain length

Previous research has found that the distribution of OSA groups along starch chains in OSA waxy maize starch with low DS were found near branching points of the amylopectin molecules (Bai, Kaufman, Wilson, & Shi, 2014). Also, it has been reported that the fine structure of amylopectin in OSA starch plays an important role in the flexibility of the molecule, affecting its physicochemical properties (Bai, Kaufman, Wilson, & Shi, 2014). The chain length distribution and degree of polymerization of starch and are presented in **Table 1**. The amylopectin fine structure was classified into different ranges of degree of polymerization from short to long chains (DP<6, 6-12, 13-24, 25-36, and DP >37) according to the study by Hanashiro, Abe, and Hizukuri (1996). Native and OSA starches presented low amounts of small (0.95-1.78%) and B3 (3.06-4.44%) chains and high amounts of A (31.06-33.89%) and B1 (49.35-52.16%) chains (**Table 1**). In general, there were some significant ($P<0.05$) differences in the percent distribution of the chains in native and OSA starch from both botanical sources. When comparing the OSA starch to the native starch counter parts there were more significant ($P<0.05$) differences between OSA rice starch and native rice starch than for the tapioca starches.

OSA rice starch had significantly ($P<0.05$) higher percentage of A chains and significantly ($P<0.05$) lower percentage of B2 chains than the native rice starch. After OSA esterification of both rice and tapioca starches, numerically, there was a slight increase in the B1 chains. However, there were no statistically significant ($P<0.05$) in the chain length distributions between native and OSA starch. OSA esterified starches had slightly lower weight averaged degree of polymerization (DP_w) than their native starch counterparts. OSA rice starch (16.60) had significantly lower DP_w than the native rice starch (17.51). However, there was no significant ($P<0.05$) differences in number averaged degree of polymerization (DP_n) among the four starch samples.

Significant ($P<0.05$) differences were observed in chain length distribution and degree of polymerization between amylopectin fractions from native and OSA starches (**Table 1**). When the pure amylopectin fractions of native and OSA starch were analyzed, the small and A chains increased in amylopectin from OSA starches, although the difference was only significant ($P<0.05$) for the small chains. Also, all the B chains, especially B1 decreased in amylopectin from OSA starches, showing significant differences ($P<0.05$) for the B1 and B3 chains. The percent of B1 chains in the amylopectin from native and OSA rice starches was 48.27 and 39.23%, respectively. The percent B1 chains in amylopectin from native and OSA tapioca starches was 39.23 and 23.06%, respectively. The results may indicate that during OSA esterification the long chains (B2 and B3) of starch may have been hydrolyzed and which may have led to a reorganization of the starch structure (amylopectin fine structure of native and esterified starches). It may have been possible that the OSA reagent could not penetrate the crystalline domains of the starch. Then, during the esterification process, the linear long chains found in the periphery of the starch granule were hydrolyzed. This can be supported by the

results observed with the CLSM and backscattered images (**Figure 1** and **2**), where the bright areas were observed on the surface of the starch granules. Shogren, Viswanathan, Felker, and Gross (2000) suggested that OSA esterification occurs in the interior and amorphous domains of amylopectin as well on the outside of the granule in waxy maize starch. The esterification possibly occurred at the O-2 and O-3 positions of the glucose units of starch chains located at the granular surface, as was previously reported (Bai & Shi, 2011). These results indicate that OSA esterification is mostly confined to the outside of the granule because of the observed decrease in the amylopectin long chains (**Table 1**).

The DP_w and DP_n of amylopectin fractions from OSA starches were lower than their native starch counterparts. The DP_w of native and OSA rice amylopectins were 16.47 and 13.29, respectively, while the DP_w for the native and OSA tapioca amylopectins were 14.87 and 12.74, respectively. The DP_w was significantly ($P < 0.05$) lower in amylopectin from OSA esterified starches compared to amylopectin from native starch. There was also a significant ($P < 0.05$) decrease in the DP_n of the amylopectin from OSA rice starch compared to the amylopectin from native rice starch. This confirms that the OSA esterification process resulted in hydrolysis of the long chains of amylopectin. The long chains of amylopectin are located among the amorphous region of the starch structure and are more accessible to the alkaline solution during OSA esterification. However, it is known that isoamylase hydrolyzes unmodified branches from native and OSA starches. Consequently, the isoamylase will be prevented from hydrolyzing amylopectin branch point if there is an OSA group at the branching point. To further clarify the distribution of OSA on the starch molecules, additional analysis of the amylopectin and amylose fractions was needed.

3.5 Nuclear magnetic resonance spectroscopy of pure amylose and amylopectin fractions

Amorphous regions of starch are mainly formed by internal long chains of amylose and amylopectin found among the cluster of branches (Vamadevan, Bertoft, & Seetharaman, 2013). Also, amylose was found to be more concentrated at the periphery than in the core of the granule (Pan & Jane, 2000). Knowing OSA esterification occurs mostly in the amorphous regions and at the granular surface of the starch over certain positions of its glucose units (Bai, Kaufman, Wilson, & Shi, 2014; Shogren, Viswanathan, Felker, & Gross, 2000); the ^1H -NMR spectroscopy was used to determine the presence of OSA groups on the amylose and amylopectin fractions of OSA starches. This information could be useful to identify which starch fraction (amylose or amylopectin) present the OSA groups within their structure. The ^1H -NMR spectra of pure amylose and amylopectin fractions of native and OSA starches (separated by GPC) are depicted in **Figure 5**. The resonance observed for amylose and amylopectin fractions from native starch (**Figure 5ab, spectra 1 and 2**) were assigned according to previous results (Simsek, Ovando-Martinez, Marefati, Sjö ö , & Rayner, 2015), where the resonances at 4.10-3.18, 4.64, 4.96, and 5.38 ppm represent the hydrogen at the position 2, 3, 4 of the glucose units, respectively. According to Ao, Simsek, Zhang, Venkatachalam, Reuhs, and Hamaker (2007), the small resonance close to 5 ppm represents the α -(1-6) linkages of starch molecules, which are mainly found in the amylopectin molecules (**Figure 5ab, spectra 1 and 3**).

In our previous report (Simsek, Ovando-Martinez, Marefati, Sjö ö , & Rayner, 2015), OSA starch presented resonances between 0.80 and 2.7 ppm; which were associated with the OSA groups introduced to the starch molecule. Resonances at 0.8 and 1.2-2.7 ppm corresponded to the methyl proton of the OSA group and the methylene groups of the succinic anhydride, respectively. So, according to literature, at low degree of esterification (3% OSA) most of the OSA groups were located near to the branching points of the amylopectin in waxy maize starch

(Bai, Kaufman, Wilson, & Shi, 2014). However, because of the presence of amylose in non-waxy starches the same conclusions cannot be drawn about the location of OSA esterification on the starch molecules. Interestingly, the ^1H NMR spectra in this study showed that there were no OSA groups present in the pure amylopectin of OSA rice or tapioca starches (**Figure 5ab, spectra 3**). However, pure amylose fractions of OSA rice and tapioca starches (**Figure 5ab, spectra 4**) showed the characteristics resonances for the OSA molecules. The DS values were 0.0451 and 0.0692 for pure amylose from OSA rice and tapioca, respectively. Although these results are contrary to previous studies by Zhang, Huang, Luo, Fu, Jiang, and Jane (2011) and Bai, Kaufman, Wilson, and Shi (2014), those previous studies were conducted on waxy and high-amylose mutant starches that either lack amylose or have a different arrangement of amylopectin and amylose in the starch granule. This suggest that amylose is found in the granular surface and react mostly with OSA reagent during the esterification process, and depending of the botanical source (presence of pores) and OSA level. Also, the OSA reagent may have diffused through the interior of the granule and modified the amylopectin structure. It is also possible that OSA esterification may have occurred on some of the amylopectin molecules that were subsequently hydrolyzed during the modification process. The hydrolyzed amylopectin chains containing OSA groups may have eluted with the amylose during GPC fractionation. The ^1H -NMR spectra of pure amylose and amylopectin fractions of OSA starch in this work demonstrated that OSA groups are present in the linear (or smaller) molecules (amylose) of OSA rice and tapioca starches at 3% level.

4. Conclusion

The study of the structural properties of OSA starches was conducted. The CLSM and backscattered images of OSA starches showed that OSA groups are located mainly at the surface

of the granules. The amylopectin chain length distribution analysis suggested that amylopectin molecules underwent significant changes in structure due to the OSA esterification process. ¹H NMR spectra of amylose and amylopectin fractions from OSA esterified starches indicated that OSA groups were mainly located on the amylose chains instead of amylopectin. Such result confirmed that there was no inhibition of the isoamylase enzyme by OSA groups during the debranching of amylopectin fraction in rice and tapioca starch. This information led us to conclude that OSA groups are introduced into the long chains of the amylose, probably at its branching points at the 3% OSA level. Perhaps, when starch is esterified at higher OSA levels, the OSA group substitution may occur on the branching points of the amylopectin depending on the granular surface and the OSA migration through the amorphous and crystalline region. This research is contrary to other reports that OSA substitution occurs close to the branch points and amorphous region of the amylopectin at low DS. However, many of the previous studies were conducted on waxy starches that lack amylose. The differences in the granular surface of the starch (presence of pores or channels), which may influence the OSA migration and distribution of OSA groups into the starch granule must also be taken into account. It will be interesting to analyze the fine structure of the amylose of OSA starch and also, increase the esterification level and observe changes at the structural level with the amylose and amylopectin fractions of the starches studied.

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484

485 **Figure Captions**

486 **Figure 1.** Distribution of OSA groups through confocal laser scanning microscopy section of
487 native and OSA starch from rice and tapioca.

488 **Figure 2.** Backscattered electron microscopy images of osmium-stained native and OSA starch
489 granules from rice and tapioca.

490 **Figure 3.** DEAE-Sephadex column chromatographs of native and OSA starch from rice and
491 tapioca.

492 **Figure 4.** Gel permeation chromatography fractions of native and OSA starch; a & b) total
493 carbohydrates determined by phenol-sulfuric assay; c & d) amylose and amylopectin peaks
494 determined by blue value

495 **Figure 5.** ^1H -NMR (400 MHz) spectra of amylopectin and amylose fractions of native and OSA
496 starches. **a)** Rice and **b)** Tapioca. Where 1= amylopectin fraction of native starch, 2= amylose
497 fraction of native starch, 3= amylopectin fraction of OSA starch, 4= amylose fraction of OSA
498 starch. Inset spectra are enlargements of the signal from 0.0-2.8 ppm.

Table 1. Chain length distribution and degree of polymerization of native and OSA starch and amylopectin from rice and tapioca.

Starch Samples	Degree of polymerization (DP)					DP _w	DP _n
	Small (<6)	A (6-12)	B1 (13-24)	B2 (25-36)	B3 (>37)		
Native Rice starch	1.00±0.33a	31.63±0.62b	51.13±0.63a,b	11.80±0.78b	4.44±0.78a	17.51±0.43a	13.28±0.43a
OSA Rice starch	0.95±0.27a	33.89±0.84a	52.16±0.77a	9.94±0.74c	3.06±0.81a	16.60±0.37b	12.94±0.24a
Native Tapioca starch	1.78±1.59a	31.06±0.42b	49.35±0.64c	13.57±0.79a	4.24±0.63a	17.66±0.51a	12.87±1.14a
OSA Tapioca starch	1.49±0.59a	32.46±0.80a,b	50.01±0.19b,c	12.58±0.35a,b	3.45±0.37a	17.18±0.23a,b	12.74±0.48a
Amylopectin Samples	Degree of polymerization (DP)					DP _w	DP _n
	Small (<6)	A (6-12)	B1 (13-24)	B2 (25-36)	B3 (>37)		
Native Rice amylopectin	6.52±1.47c	30.52±1.12a	48.27±0.86a	10.63±0.62a	4.05±0.12a	16.47±0.16a	9.58±0.57a
OSA Rice amylopectin	17.85±0.37a	36.67±1.82a	34.85±0.78e	8.53±0.79a	2.10±0.13b,c	13.29±0.16c	7.48±0.06b
Native Tapioca amylopectin	12.23±1.87b	34.85±1.32a	39.23±0.35b	10.68±0.22a	3.01±0.41b	14.87±0.28b	8.68±0.69a,b
OSA Tapioca amylopectin	21.26±1.23a	36.37±2.11a	32.06±0.66c	8.51±0.40a	1.81±0.19c	12.74±0.02c	6.81±0.38b

Values represent the mean ± SD, n=2. Data with different letters in the same column for the same sample type are significantly different (P<0.05) by Tukey test. **AP**: amylopectin, **DP_w**: weight-average degree of polymerization, **DP_n**: number-average degree of polymerization.

Figure 1. Distribution of OSA groups through confocal laser scanning microscopy section of native and OSA starch from rice and tapioca.

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