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Effect of octenylsuccinic anhydride modification on β-amylolysis of starch

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ABSTRACT

The effects of octenylsuccinic anhydride (OSA) modification of waxy maize and sorghum starches on subsequent β-amylolysis are examined. Hydrolysis with β-amylase is a method by which OSA starches may be structurally modified for industrial purposes. The hydrolysis of both granular and gelatinised forms of both starches follows first-order kinetics regardless of the OSA used as a percent of starch mass (0–24%). The highest hydrolysis rate coefficients for granular starches are at modification with 6% OSA/starch. The largest molecular sizes of β-amylase hydrolysed OSA-modified gelatinised starches are found at modification with 24% OSA/starch. The results suggest that octenylsuccinyl groups have an action-blocking effect on β-amylolysis of gelatinised starch, but the effect of semi-crystalline granular structure is more pronounced than that of OSA modification. Hence β-amylolysis can be used under appropriate conditions to modify the structure of gelatinised OSA-modified starches.

1. Introduction

Starch is commonly modified with octenylsuccinic anhydride (OSA) to produce amphiphilic emulsion stabilisers. OSA-modified starches are used for many purposes, including as thickeners, beverage emulsifiers and encapsulating agents for non-polar compounds. The functions of OSA-modified starch depend to some extent on the structure of the starch substrate. However, it is difficult to determine which structural feature(s) are important for the functional qualities of OSA-modified starch, as starch structure is highly heterogeneous with multiple levels of scale. Previous studies indicate that average molecular weight plays an important role. Higher molecular weight can lead to more viscous solution and better stabilisation of emulsion (Dokic, Dokic, Dapcevic, & Krstonosic, 2008), although lower molecular weights obtained through enzyme treatments have also been shown to improve stabilising properties (Liu et al., 2008), possibly due to the ability of smaller molecules with higher diffusion rate to migrate quickly to an oil-droplet surface. However, under turbulent flow emulsion preparation (a widely used technique), convective transport seems to preferentially transport larger molecules to interfaces (Nilsson & Bergenstahl, 2006). Moreover, molecules with very low molecular size are more likely to have no OS groups, thus lacking amphiphilic properties. The degree of branching (DB) of starch is also important for emulsion stability: for example, phytoglycogen, which is highly and randomly branched, becomes an excellent stabiliser after OSA substitution (Scheffler, Huang, Bi, & Yao, 2010). Further structural modifications of the starch such as enzyme degradation have been performed prior to (Huang et al., 2010), during (Xu et al., 2012) and after (Liu et al., 2008) the addition of the octenylsuccinyl (OS) group to enhance desirable qualities such as esterification reaction efficiency (where structural modification precedes OSA reaction), emulsion capacity and gel properties. β-Amylase hydrolysis is one such structural modification technique that has not been widely investigated with OSA-modified starch.

β-Amylase is an exo-hydrolase that specifically cleaves α-(1→4) glycosidic linkages two D-anhydroglucose monomers inwards from the non-reducing ends of starch branches, producing a maltose for every successful hydrolysis. The hydrolysis process is stopped by the presence of branching points, such as α-(1→6) glycosidic linkages and possibly other moieties from the substitution reaction on the hydroxyl groups of α-D-glucopyranosyl monomers, thereby trimming the outer branches of starch molecules. From this process, DB is increased because of the relative loss of

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α-(1 → 4) glycosidic linkages. Previous studies using other amylolytic enzymes, such as α-amylase, have indicated an action-blocking effect of the OS (octenylsuccinyl) groups on the hydrolysis action of these enzymes, which is more pronounced at higher degree of substitution (DS) (Han & BeMiller, 2007; He, Liu, & Zhang, 2008; Zhang et al., 2011). Hence β-amylase hydrolysis, along with the alteration of DS, can provide a useful means to sculpt the structure of OSA-modified starch to a desirable form for certain applications, such as those requiring high DB for greater solubility.

The objective of the current study is to understand the impacts of OS substitution of starch on the subsequent β-amylase hydrolysis of both granular and gelatinised forms. While most OSA modification reactions are performed with the starch in granular form, structural modifications are performed either as granules or gelatinised starch (Sweedman, Tizzotti, Schäfer, & Gilbert, 2013). It has also been shown that OSA starch may be maintained in granular form all the way to end use in Pickering emulsions (Rayner, Sjöö, Timgren, & Dejmek, 2012). Although higher DS is preferred for certain applications, food-grade OSA-modified starch must meet the regulatory guidelines, limiting the amount of OSA used for the substitution reaction to 3% of starch weight (EFSA, 2010). Thus β-amylase hydrolysis can be used after OSA modification to increase the DS and the solubility of OSA-modified starch. Both DS and DB are increased as unmodified monomers are removed at α-(1 → 4) linkages, and the solubility increases as the molecules are made smaller and more highly branched. In order to investigate the effects of OSA modification of starch on subsequent enzyme action, this study uses much higher DS than would otherwise be used. The progress of the β-amylolysis of OSA-modified starch in the present study was monitored by following the development of reducing sugars. The molecular structures of the remaining starch in the insoluble hydrolysate were investigated by size exclusion chromatography (SEC, also known as gel permeation chromatography, GPC) and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Data on changes in structure during hydrolysis provides information on the effects of the OS groups on the degradation mechanism of β-amylase with starch.

Most studies on OSA-modified starch have been performed on waxy maize (*Zea mays* L.) starch as the substrate. Waxy starches are composed almost entirely of amylopectin (>98%) although some "waxy" starches, such as those from barley (*Hordeum vulgare* L.) mutants, may contain up to 8% amylose. Amylopectin is highly branched with molecular weight $\sim 10^{7-8}$. Amylose, the other type of molecule in non-waxy starches, is primarily linear with few long branches and lower molecular weight, $\sim 10^{5-6}$. The present study uses waxy maize starch for comparison with the results from literature of products made without β-amylase treatment, and waxy sorghum (*Sorghum bicolor* L.) starch to identify the effects from different botanical origins. Waxy sorghum starch is an unexplored substrate for the synthesis of OSA-modified starch. Since sorghum is an excellent dry-climate crop widely grown in arid and semiarid areas where most cereal crops are not suitable, it may be an important starch source in the near future because of climate change. The results from the two-grain sources are compared and may provide new food and other industrial applications for waxy sorghum starch.

2. Materials and methods

Waxy sorghum grains (A1*F_B004215) were gifts from the Queensland Department of Agriculture, Fisheries and Forestry (DAFF). Waxy maize starch was purchased from Penford Australia Ltd. (Lane Cove, NSW, Australia). 2-Octen-1-ylsuccinic anhydride (OSA, 97%), β-amylase (Type II-B, from barley), and D-(+)-maltose monohydrate (≥99%), dimethyl sulfoxide (DMSO)-*d*6, trifluoroacetic acid (TFA)-d_1, and LiBr (ReagentPlus) were purchased from Sigma–Aldrich Pty. Ltd. (Castle Hill, NSW, Australia). DMSO (GR for analysis ACS) was purchased from Merck & Co., Inc. (Kilsyth, VIC, Australia). Protease from *Streptomyces griseus* was purchased from Sigma (P5147) and isoamylase from *Pseudomonas sp*. was obtained from Megazyme International Ltd. (Bray, Co., Wicklow, Ireland). Other chemical reagents are analytical grades.

2.1. Isolation of starch from waxy sorghum grains

Starch was isolated from 160 g raw sterile waxy sorghum grains by a laboratory wet-milling process following the method of Syahariza, Li, and Hasjim (2010) with slight modifications. Grains were soaked overnight in excess sodium bisulfite solution (made from 0.45%, w/w, sodium metabisulfite in distilled water), requiring no acidification (Hoover & Sosulski, 1985) to weaken the disulfide bonds of the (kafirin) protein network (not as a preservative), followed by grinding the grains in the same solution using a household blender. This method without using acidification has been used for many years in wet-milling. The slurry was passed through a 0.55-μm sieve, and the filtrate was centrifuged at 4000 × *g* for 10 min to collect solid materials, which are mostly starch granules and protein. The solid materials were then washed with water to remove bisulfite and subsequently treated with protease 1.8 U mL^{-1} in tricine buffer (250 mM, pH 7.5), for 2 h. After being centrifuged at 4000 × *g* for 10 min, the solid materials were washed three times with ethanol and once with acetone, dried at 65 °C and sieved through 0.55-μm openings again to remove clumps of non-starch materials. The nitrogen content of the extracted waxy sorghum starch, analysed in duplicate using a LECO TruSpec CHN analyser (LECO Corp., St. Joseph, MI, USA) was 0.331 wt%, equivalent to crude protein content of approximately 1.87% with a conversion factor of 5.65 (Mosse, 1990). It is anticipated that the residual protein at this low content will not affect β-amylolysis of starch and further analyses.

2.2. OSA modification of starches

The isolated waxy sorghum starch and commercial waxy maize starch were chemically modified with OSA to various DS using the method of Song, He, Ruan, and Chen (2006). OSA was first dissolved in ethanol to a ratio of 1:2 (OSA:ethanol, by weight). The OSA–ethanol mixture was added drop-wise over 2 h to an aqueous suspension of starch (15 g in 60 mL water) with stirring at 35 °C to yield 3, 6, 12 and 24% (w/w) OSA on the basis of dry starch weight. Incubation was continued for another hour. The pH was adjusted to 8.5 with 0.2 M NaOH during the addition of OSA and further incubation. At the end of the reaction, the starch suspension was neutralised with 0.02 M HCl, the OSA-modified starch was then washed twice with ethanol and once with acetone, and dried overnight at 65 °C. A sample subjected to the same treatment apart from the addition of OSA is denoted by 0%. OSA modification was confirmed by measuring the DS using solution ^{1}H NMR.

2.3. β-Amylase digestion of OSA-modified starch

The OSA-modified starches were subjected to β-amylase hydrolysis in granular (as-is) and gelatinised forms following the method of Mendez-Montealvo, Wang, and Campbell (2011) with slight modifications. Since raw starch in semi-crystalline granular form is more resistant to enzyme degradation than amorphous gelatinised starch (Tester, Qi, & Karkalas, 2006), the β-amylolysis of granular starch was performed over a longer period of time than that of gelatinised starch. Mendez-Montealvo et al. (2011) used a similar procedure with granular starch for up to 7 days.

The β-amylolysis of OSA-modified granular starch (20 mg) was performed at 40 °C in 15-mL centrifuge tubes with β-amylase (0.1 g L^{-1}, ~5.5×10^{-2} U L^{-1}) in a sodium acetate buffer solution (1 mL, 0.05 M, pH 4.5) containing 0.01% (w/w) sodium azide as an antimicrobial agent. Hydrolysates were collected from separate tubes at 0, 5, 15, 30, 90, 240, and 480 min, then 1, 2 and 4 d, and centrifuged immediately at 4000 *g* for three min. The precipitates were washed twice with ethanol and once with acetone, and then dried overnight at 60 °C. The supernatants were frozen at −16 °C for at least 24 h for subsequent reducing sugar analysis.

For gelatinised starch samples, OSA-modified granular starch (50 mg) was dispersed in 9 mL water and heated at 90 °C for at least an hour. The gelatinised starch was cooled to 40 °C and β-amylase (0.1 g L^{-1}) in a sodium acetate buffer solution was added (1 mL, 0.05 M, pH 4.5) containing 0.01% (w/w) sodium azide, for a final β-amylase concentration of approximately 5.5×10^{-3} U L^{-1} in a 0.005 M buffer. Hydrolysates were collected at 0, 1, 2, 5, 10, 30, 60, 90, 120 and 180 min, and the reaction was stopped by heating the hydrolysates in a boiling water bath for at least 20 min. An aliquot (1 mL) of each hydrolysate was collected for reducing sugar analysis, and the remaining hydrolysate was quenched in liquid nitrogen and freeze-dried. The dried hydrolysate was dissolved in 5 mL DMSO solution containing 0.5% (w/w) LiBr at 80 °C, and the starch in the dissolved hydrolysate was precipitated with at least 20 mL absolute ethanol followed by centrifugation at 3000 × *g* for 5 min. The precipitate was further washed twice with ethanol. Residual ethanol was discarded by draining, and the starch hydrolysate was dried overnight at 60 °C in preparation for structural analysis.

2.4. Reducing sugar test

The degree of β-amylase hydrolysis was determined as the concentration of reducing sugars using the well-established methods by Somogyi (1945) and Nelson (1944), which require three separately prepared solutions. Reagent A and B solutions were mixed in a volume ratio of 25:1, respectively, immediately before each analysis. A calibration curve was generated using six maltose standard solutions in water ranging from 0 to 250 mg L^{-1}, which were prepared fresh for each assay. The samples from the early stage of β-amylase hydrolysis were analysed without further dilution. The samples from later stage of hydrolysis were diluted with water to between 1/5th and 1/100th of the original concentration, as appropriate to bring the concentrations of reducing sugars into the sensitive range of the assay. Each sample or maltose standard was combined with an equal volume of the mixture of Reagent A and B solutions and heated in a boiling water bath for 20 min. After cooling under running water, the result was mixed with a volume of Reagent C equal to the original solution and allowed to stand for 20 min. The mixture was then diluted to 1/10th concentration with distilled water for optimum colour detection, and the absorbance was collected at 520 nm using a UV-1700 PharmaSpec (Shimadzu, Kyoto, Japan).

The reducing sugar development was fitted with the first order model:

$$[m] = [m]_{MAX}\,(1 - e^{-kt}) \tag{1}$$

Here $[m]$ is the maltose concentration at time t, $[m]_{MAX}$ is the maltose developed at completion and k is the hydrolysis rate coefficient. Values for $[m]_{MAX}$ and k were non-linear least-squares fitted using "Solver" (Excel, Microsoft); the resulting values of $[m]_{MAX}$ where then fixed, and values of k recalculated by a linear least-squares fit of $\ln(1 - [m]/[m]_{MAX})$ against t.

2.5. Nuclear magnetic resonance

Approximately 2 mg of each β-amylase hydrolysed OSA-modified starch or unhydrolysed OSA-modified starch was dissolved in 1 mL DMSO-d_6 containing 0.5% (w/w) LiBr at 80 °C. TFA-d_1 (~20 mg) was added directly into the sample medium immediately before analysis. ^{1}H NMR spectra were obtained as previously described (Tizzotti, Sweedman, Tang, Schaefer, & Gilbert, 2011). The NMR spectra were recorded at 70 °C using an Avance NMR spectrometer (Bruker Biospin, Rheinstetten, Germany), operating at an observation frequency of 749.15 MHz for ^{1}H, equipped with a TXI5z probe (Bruker Biospin) (8.80 μs 90° pulse, a repetition time of 15.98 s composed of an acquisition time of 3.98 s and a relaxation delay of 12 s, 128 scans, DMSO-d_6).

Data were processed using Bruker TOPSPIN software (v2.1; Bruker Biospin). All spectra were manually phased and baseline-corrected. A Lorentzian fit was used for spectral deconvolution. DB was calculated from the results of ^{1}H NMR using the integral measurements from the following peaks: α-(1 → 6) glycosidic linkages ($I_{\alpha\text{-}(1\to6)}$) at approximately 4.75 ppm, α-(1 → 4) glycosidic linkages ($I_{\alpha\text{-}(1\to4)}$) around 5.11 ppm, as follows:

$$\mathrm{DB} = \frac{I_{\alpha\text{-}(1\to6)}}{I_{\alpha\text{-}(1\to6)} + I_{\alpha\text{-}(1\to4)}} \times 100\% \tag{2}$$

DS is the fraction of OS groups, identified using the proton signal at the C3 position of the octenyl tail, approx. 2.05 ppm (I_{OSA}), per glucose monomer as follows:

$$\mathrm{DS} = \frac{I_{OSA}}{2(I_{\alpha-(1\to6)} + I_{\alpha-(1\to4)})} \tag{3}$$

The amount of reducing ends (I_{re}) is negligible in large polysaccharide molecules, such as amylose and amylopectin, and can be ignored. The reaction efficiency (RE) is the ratio of actual DS of OSA-modified starch to the maximum (theoretical) DS that can be achieved from the amount of OSA used for the reaction, as follows:

$$\mathrm{RE} = \frac{\text{Actual DS}}{\text{Theoretical DS}} \times 100\% \tag{4}$$

2.6. Debranching of starch samples

Approximately 3 mg each of β-amylase hydrolysed OSA-modified starch or unhydrolysed OSA-modified starch was debranched using isoamylase following the method of Tran et al. (2011). The starch sample was dispersed in 0.9 mL of deionised water, and the dispersion was heated in a boiling water bath for 15 min and then cooled to room temperature. Sodium azide solution (5 μL, 0.04 g mL^{-1}) and 100 μL sodium acetate buffer (0.1 M, pH 3.5) were added to the starch suspension, followed by 2.5 μL isoamylase solution. The mixture was incubated in a water bath at 37 °C for 3 h, after which it was neutralised to pH 7 with drop-wise addition of 0.1 M NaOH, heated to 80 °C for 2 h, and centrifuged. The supernatant containing debranched starch was snap-frozen in liquid nitrogen to prevent retrogradation and freeze-dried overnight. Although the residues from the debranching procedure, such as denatured enzyme and salts, were not removed from the debranched starch samples, the analysis of these substances alone showed that they did not interfere with sample peaks.

2.7. Size exclusion chromatography

The molecular sizes of whole (fully branched) and debranched starches were analysed using SEC after they were dissolved in DMSO containing 0.5% (w/w) LiBr overnight at 80 °C to yield

final concentrations of 2 and 4 g L^{-1}, respectively. Controls were given treatment without β-amylase and were unmodified (0%) or modified with the highest level of OSA modification (24%). The dissolution technique has been proven to completely dissolve starch molecules with minimal degradation (Syahariza, Li, & Hasjim, 2010). SEC is known to induce shear scission with larger, highly branched molecules (Cave, Seabrook, Gidley, & Gilbert, 2009) and may undergo elongation effects which can give false results (Makan, Otte, & Pasch, 2012). However, amylopectin is a highly branched molecule with mainly very short branches, so elongation effects will be minimal. Further, the, enzyme treated molecules are smaller than whole amylopectin, reducing but not eliminating the amount of shear scission. Now, the purpose of the present work is comparison between samples of similar structure, and because the SEC characterisation is carried out under the same conditions for each sample, shear scission will be the same in each case and thus meaningful relative comparisons are valid (Cave et al., 2009). The SEC analyses of whole and debranched starches were performed using an Agilent 1100 series (Agilent Technologies, Wadlbronn, Germany) consisting of an isocratic pump, an autosampler that injected 100 μL per sample without temperature control, and a column oven set at 80 °C. A series of SEC columns consisting of GRAM Pre-Column, 30 and 3000 analytical columns (Polymer Standard Services GmbH (PSS), Mainz, Germany) were used for whole starch molecules and those consisting of GRAM Pre-Column, 10 and 1000 analytical columns (PSS) were used for debranched starch molecules. DMSO containing 0.5% (w/w) LiBr was used as eluent and the flow rates were set at 0.3 and 0.6 mL min^{-1} for whole and debranched starches, respectively. A refractive index (RI) detector (RID-10A, Shimadzu, Kyoto, Japan) operating at 635 nm and thermostated at 45 °C was used to detect the molecules eluted after SEC separation. The signals from the RI detector were collected and processed using WinGPC Unity (PSS). The relationship between elution volume and hydrodynamic volume V_h (or equivalent hydrodynamic radius R_h) was obtained using pullulan standards (PSS) with peak molecular weight (M_p) ranging from 342 to 2.35×10^6, which were converted to V_h using the Mark–Houwink equation (Liu, Halley, & Gilbert, 2010) using a dn/dc value of 0.0717 mL g^{-1}. This method of converting calibration with linear standards of given molecular weight to hydrodynamic size is well established (Vilaplana & Gilbert, 2010). As discussed in the cited review, there are no linear standards of sufficiently large size to cover the size range used here, and sizes outside this range are obtained by extrapolation. Our interest in the present paper is comparison between similar polymers, and so this comparison is independent of absolute uncertainties in the size arising from this extrapolation. For highly branched polymers, such as whole starch molecules, there is no unique relationship between hydrodynamic size (the separation parameter of SEC) and molecular weight, and the data were plotted as the SEC weight distributions $w(\log V_h)$ against R_h and normalised to yield the same height of the highest peak.

2.8. Statistical analysis

All samples for this study were analysed in duplicate. The *p*-values of digestion fitting were 0.180 for granular samples and 0.016 for gelatinised, calculated using Minitab 16 (State College, Pennsylvania).

3. Results and discussion

3.1. DS and DB of OSA-modified starches

The presence of OS groups on starch molecules after modification was confirmed by NMR spectra, as indicated by an increase in the signal intensity of the ^1H at the 5th carbon of the octenyl tail. The DS increases, and the RE decreases, with the higher concentration of OSA used for the reaction for both waxy starches (Table 1). The DS for both starches are similar at each level of OSA modification, showing that the reaction affects the two waxy starches to a similar extent. These RE values are by no means the optimum possible levels, but do fall within those of similar procedures reported in literature (Jeon, Viswanathan, & Gross, 1999). As the purpose of this study is to investigate the effects of OSA modification on the structural changes by the post-modification β-amylolysis of starch, it is important that the OSA modification scheme chosen does not itself cause much molecular degradation, which has been seen in higher RE values of OSA modification achieved using pyridine (Viswanathan, 1999) and in the reaction coupled with enzyme degradation (Xu et al., 2012).

3.2. Kinetics of β-amylase hydrolysis of OSA-modified waxy starches

The digestion profiles of samples at all levels of OSA modification in granular and gelatinised forms are shown in Fig. 1A and C for waxy maize and B and D for waxy sorghum, respectively. For granular OSA-modified starches, the degrees of hydrolysis reached a maximum of ~20% at 4 days of hydrolysis, whereas the gelatinised OSA-modified starch reached a maximum degree of hydrolysis of at least 30% within 180 min (Fig. 1). The hydrolysis profiles of granular and gelatinised OSA-modified starches at all levels of OSA modification display good visual fit and good R^2 values (Supplementary Data, Fig. S1), indicating the appropriateness of the first order model for β-amylolysis of OSA-modified starch. This also indicated the activity of the enzyme was limited by a single factor for this duration, the factor being the access to activity sites rather than the factors affecting the enzyme directly. The hydrolysis rate coefficients (k) for the β-amylolysis of OSA-modified waxy starches in granular and gelatinised forms are listed in Table 1. It is expected that higher DS would have greater hindrance for β-amylolysis, as β-amylase is an exo-enzyme, cleaving only the α-(1 → 4) glycosidic linkages from the non-reducing ends. Although in all cases the starches with the highest level of OSA modification show the lowest amount of reducing sugars released in the hydrolysis profiles (Fig. 1), the k for granular and gelatinised OSA-modified waxy starches show no consistent correlations with the concentration of OSA used in the reaction, indicating that the increase in the DS with OS groups on the starch molecules may affect the rate of the β-amylolysis in other ways beside molecular binding. Zhang et al. (2011) also reported that the susceptibility of OSA-modified high-amylose maize starch to digestion by α-amylase was affected in a non-linear manner with DS, correlating negatively from just above the lowest levels of modification. Furthermore, for granular OSA-modified starches of both waxy maize and sorghum, the k values appear to peak at DS ~0.022–0.025 (with 6% OSA used in the reaction), whereas the β-amylolysis of gelatinised starches shows no such trend. While the increase in DS may ultimately hinder the β-amylolysis of starch at the molecular level, at lower DS the starch substrate in granular form may be more accessible by β-amylase due to partial disruption of the crystalline structure, facilitating the swelling of starch granules in excess water. The granular structure has been destroyed in the gelatinised starch, and thus the effect of granule swelling is not observed with this substrate. Hence the effect of OS groups on the β-amylolysis of gelatinised waxy maize starch is mostly at a molecular level (or molecular binding) and there is a linear negative relationship between the concentration of OSA used for the substitution reaction and the hydrolysis rate coefficients k, although this trend is not apparent with gelatinised waxy sorghum starch. Further analysis is needed to understand the

Table 1
Experimental values of OSA-modified waxy starches before and after β-amylase hydrolysis.[a]

Starch	OSA (%)	Initial DS	Initial DB (%)	RE (%)	After β-amylase hydrolysis					
					Granular form[b]			Gelatinised form[c]		
					Final DB (%)	ΔDB (%)	k	Final DB (%)	ΔDB (%)	k
Waxy maize	0	0	3.63	n/a	4.26	0.62	4.2×10^{-4}	11.92	8.29	4.91×10^{-2}
	3	0.014	3.74	60	4.03	0.28	8.1×10^{-4}	10.61	6.87	3.38×10^{-2}
	6	0.023	3.78	49	4.10	0.31	8.8×10^{-4}	10.70	6.92	2.45×10^{-2}
	12	0.033	3.85	36	4.09	0.24	8.3×10^{-4}	9.80	5.95	2.39×10^{-2}
	24	0.054	3.81	29	3.86	0.06	4.0×10^{-4}	8.25	4.44	1.52×10^{-2}
Waxy sorghum	0	0	3.57	n/a	3.92	0.36	3.9×10^{-4}	9.95	6.38	3.90×10^{-2}
	3	0.013	3.64	56	3.95	0.31	4.5×10^{-4}	11.13	7.49	7.25×10^{-2}
	6	0.025	3.70	52	3.94	0.24	6.4×10^{-4}	10.14	6.44	5.33×10^{-2}
	12	0.036	3.63	38	3.95	0.32	5.9×10^{-4}	9.65	6.02	6.29×10^{-2}
	24	0.053	3.71	27	3.85	0.14	5.0×10^{-4}	9.51	5.80	3.06×10^{-2}

[a] DS = degree of substitution, DB = degree of branching, RE = reaction efficiency, k = rate coefficient for β-amylase activity.
[b] After 4 d of β-amylase hydrolysis.
[c] After 180 min of β-amylase hydrolysis.

relationship between DS and the hydrolysis rate coefficients k of gelatinised OSA-modified waxy sorghum starch.

3.3. Structural changes induced by β-amylase hydrolysis of OSA-modified waxy starches

3.3.1. Degree of branching

The DBs of OSA-modified starches for both granular and gelatinised forms increase after β-amylolysis (Table 1), supporting the suggestion that branching points stop β-amylolysis of starch, as well established for the hydrolysis pattern of β-amylase (Bernfeld, 1955). The final DBs of both granular and gelatinised forms decrease with increasing initial DSs of the OSA-modified waxy maize and sorghum starches, where the samples with the highest initial DS result in the least increase in the final DB after β-amylolysis. The results confirm that OSA modification has a protective effect on α-(1 → 4) glycosidic linkages towards β-amylase hydrolysis.

Comparison of the changes in the final DBs between granular and gelatinised samples of the OSA-modified waxy maize and sorghum starches shows that the final DBs and the differences between initial and final DBs of gelatinised OSA-modified waxy starches are higher than those of the granular OSA-modified counterparts (Table 1): more α-(1 → 4) glycosidic linkages in the gelatinised samples were hydrolysed by β-amylase in 180 min than in the granular samples in 4 days. The differences between the final DBs of granular and gelatinised samples indicate a protective effect of the semi-crystalline granular structure towards β-amylase hydrolysis, separate from the effect of OS groups. Furthermore, the presence of OS groups can create steric hindrance, which destabilises the granular structure and promote the granule swelling in excess water (Bhosale & Singhal, 2007). Hence the counter-effects of granular structure and OS groups result in a small difference in the final DBs of granular samples at different DS, especially at 0–12% OSA modification. However, since the granular structure does not exist in gelatinised samples, β-amylase has greater access to hydrolyse the amorphous starch molecules, showing a greater effect of OS groups in stopping the hydrolysis of starch molecules by β-amylase.

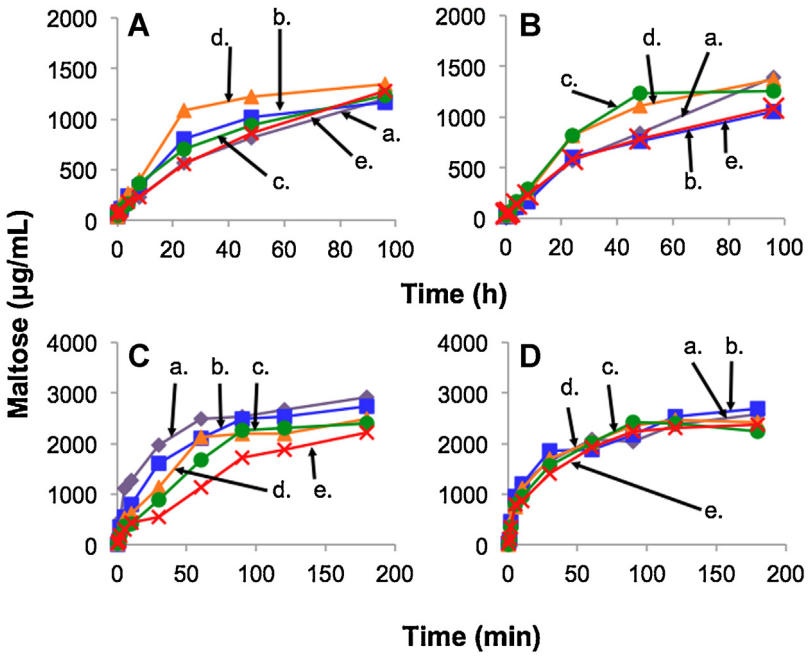

Fig. 1. Hydrolysis profiles of OSA-modified starches by β-amylase: granular starch from (A) waxy maize and (B) waxy sorghum, as well as gelatinised starch from (C) waxy maize and (D) waxy sorghum. OSA treatments: a, 0%; b, 3%; c, 6%; d, 12%; and e, 24%.

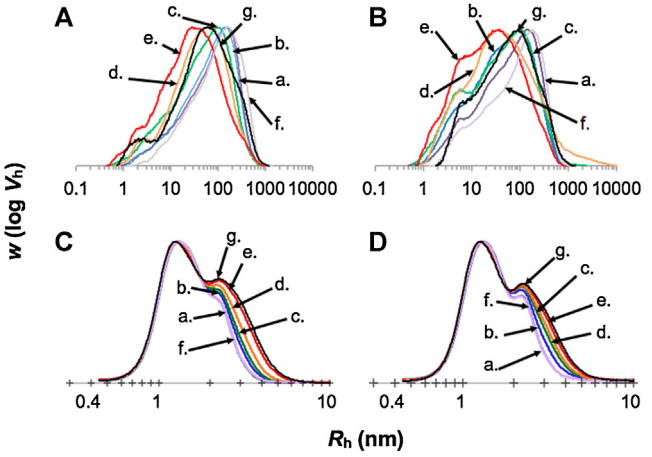

Fig. 2. Weight distributions of OSA-modified starches after 4 d of β-amylase hydrolysis in granular form: whole (fully branched) starches from (A) waxy maize and (B) waxy sorghum, as well as debranched starches from (C) waxy maize and (D) waxy sorghum. Controls are those without β-amylase enzyme. OSA treatments: a, 0%; b, 3%; c, 6%; d, 12%; e, 24%; f, 0% control, and g, 24% control.

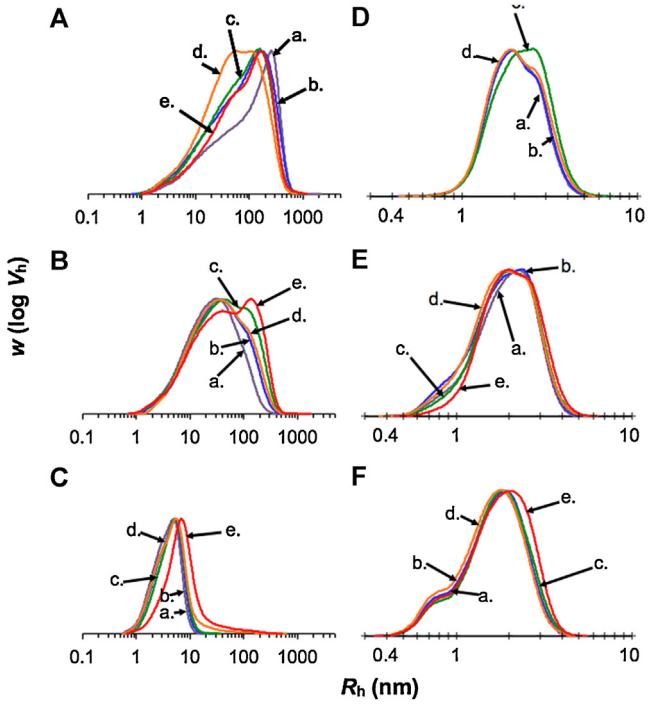

Fig. 3. Weight distributions of (A–C) whole molecules and (D–F) debranched OSA-modified waxy maize starch after β-amylase hydrolysis in gelatinised form for (A and D) 1, (B and E) 30, and (C and F) 180 min. OSA treatments: a, 0%; b, 3%; c, 6%; d, 12%; and e, 24%.

3.3.2. *Molecular structure of granular starches*

The SEC weight distributions of whole OSA-modified waxy maize and sorghum starches with and without β-amylolysis in granular form show only amylopectin molecules (R_h up to 1000 nm) with peak R_h ranging between 20 and 200 nm (Fig. 2A and B for waxy maize and sorghum starches, respectively). A shoulder is also observed at R_h < 10 nm, which is more pronounced in waxy sorghum starch. Both waxy maize and sorghum starches showed similar behaviour with OSA modification and β-amylolysis: the peak R_h is shifted to smaller molecular size. The peak height of the shoulder at R_h < 10 nm increases with increasing DS, suggesting that more molecules were hydrolysed by β-amylase at higher DS, probably due to greater granule swelling with a larger amount of bulky OS groups on starch molecules. The OSA modification also seems to cause mild degradation of starch molecules, as the peak R_h is shifted to smaller molecular size and the peak height of the shoulder at R_h < 10 nm increases with increasing DS, even when the OSA-modified waxy starches were not hydrolysed by β-amylase (controls). Because granular starch is more resistant towards enzyme hydrolysis than freshly gelatinised starch, the reduction of the molecular size after β-amylolysis is quite small (compared to that observed with gelatinised samples in Fig. 3, which will be discussed later). This is consistent with the small differences in the final DBs of granular OSA-modified waxy starches after the β-amylolysis compared with their initial DBs (Table 1). Mendez-Montealvo et al. (2011) also reported that the β-amylolysis of native waxy maize starch in granular form resulted in only a slight decrease in the weight-average molecular weight of whole starch.

The SEC weight distributions of debranched OSA-modified waxy maize and sorghum starches with and without β-amylolysis in granular form show two populations of amylopectin branches, which are short branches (R_h between 0.5 and 2 nm) and long branches (R_h between 2 and 10 nm) (Fig. 2C and D for waxy maize and sorghum starches, respectively). These are the familiar amylopectin branches grouped as A chains carrying no branches and B chains carrying at least one branch, and longer B chains can be further grouped into B1, B2, B3, ... for those spanning 1, 2, 3, ... amorphous/crystalline lamellae. The structural changes induced by OSA modification are more pronounced than those induced by β-amylolysis. The molecular size of long amylopectin branches increases with the DS, suggesting that the preference of OSA modification on the long amylopectin branches due to their abundance in the amorphous region. On the other hand, the distributions of the debranched OSA-modified waxy starches after β-amylolysis are virtually superimposable with those of the control counterparts without β-amylolysis, which is consistent with the small differences between the SEC weight distributions of whole OSA-modified waxy starches with and without β-amylolysis (Fig. 2A and B, respectively) and the small differences between the initial DBs before β-amylolysis and the final DBs after β-amylolysis (Table 1). The results indicate that the degree of β-amylolysis of granular starch is too low to induce changes in the starch branch-chain length. Mendez-Montealvo et al. (2011) also reported that the β-amylolysis of native waxy maize starch in granular form resulted in only a slight changes of the branch-chain length distributions; the increase or decrease of each branch population with DP > 5 was less than 2% from the total branch population.

3.3.3. *Molecular structure of gelatinised starches*

The SEC weight distributions of whole OSA-modified waxy maize and sorghum starches in gelatinised form show more noticeable reduction in molecular size after β-amylolysis (Fig. 3A–C and Fig. 4A–C for waxy maize and sorghum starches, respectively) than those of their granular counterparts (Fig. 2A and B for waxy maize and sorghum starches, respectively). Three time-points at 1, 30, and 180 min of β-amylolysis were chosen to demonstrate the time evolution of the starch molecules for waxy maize and sorghum starches at each concentration of OSA used for the substitution reaction. In general, the peak of the weight distributions of whole OSA-modified waxy maize and sorghum starches in gelatinised form are shifted from 100–120 to 11–13 to 5–8 nm for 1, 30, and 180 min of β-amylolysis, respectively. The greater reduction in molecular size after β-amylolysis of gelatinised OSA-modified starches compared

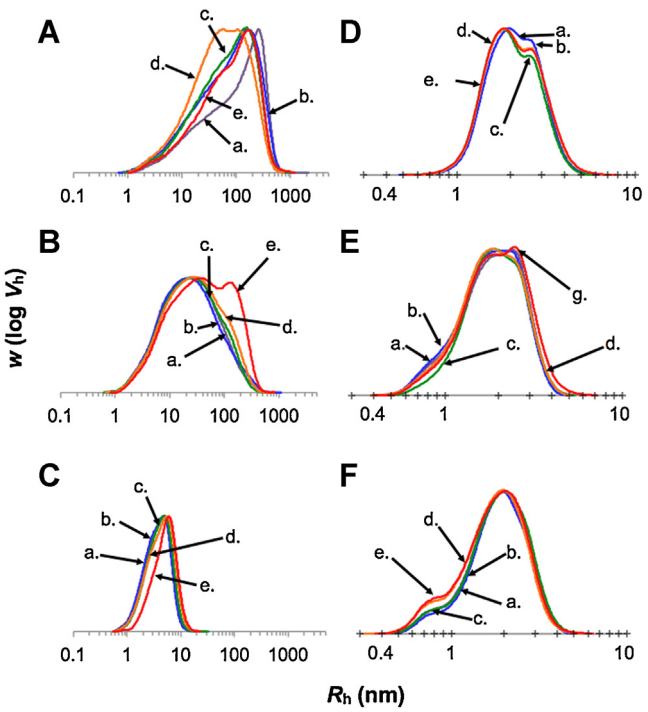

Fig. 4. Weight distributions of (A–C) whole molecules and (D–F) debranched OSA-modified waxy sorghum starches after β-amylase hydrolysis in gelatinised form for (A and D) 1, (B and E) 30, and (C and F) 180 min. OSA treatments: a, 0%; b, 3%; c, 6%; d, 12%; and e, 24%.

with granular counterparts is attributed to the loss of starch semi-crystalline structure after gelatinisation, converting the semi-crystalline starch molecules to an almost completely amorphous conformation and increasing the accessibility of β-amylase to hydrolyse all starch molecules. These results are also consistent with the higher final DBs after β-amylolysis of gelatinised samples compared with those of granular counterparts (Table 1).

At 30 min of β-amylolysis, the OSA-modified waxy maize starches in gelatinised form have larger amounts of large whole starch molecules (R_h > 100 nm) than the waxy maize starch in gelatinised form without OSA modification (0% OSA) (Fig. 3B). In addition, the peak at R_h = 110 nm, which is the major peak for most OSA-modified waxy maize starches in gelatinised form after 1 min of β-amylolysis (Fig. 3A), is still visible after 30 min of β-amylolysis in the weight distribution of waxy maize starch substituted with 24% OSA. Furthermore, at 180 min of β-amylolysis, waxy maize starch substituted with 24% OSA has the largest molecular size with a peak at R_h at 8 nm, whereas the weight distributions of whole starch from other OSA-modified waxy maize starches are superimposable on the waxy maize starch without OSA modification (0% OSA) (Fig. 3C). The results show the action-blocking effects of OS groups on the β-amylolysis of starch molecules, especially at the highest DS or highest concentration of OSA used for the substitution reaction, consistent with the decreasing hydrolysis rate coefficient *k* of β-amylolysis and final DB of waxy maize starch in gelatinised form with the increases in the DS or the concentration of OSA used for the substitution reaction (Table 1). The similar distributions of whole starch among different OSA-modified waxy maize starches after 180 min of β-amylolysis, except that substituted with 24% OSA, could mean that the enzyme hydrolysis has reached β-limit dextrins or that the molecules are smaller than the lower separation limit of the SEC columns (equivalent size of pullulan at 1080 Da).

On the other hand, all OSA-modified waxy sorghum starches in gelatinised form after 30 and 180 min of β-amylolysis (Fig. 4B and C, respectively), except that substituted with 24% OSA, have almost superimposable weight distributions of whole starch, including that without OSA modification (0% OSA). This is consistent with no correlation with OSA concentration of the hydrolysis rate coefficients *k* of β-amylolysis among the OSA-modified waxy sorghum starches in gelatinised form (Table 1). However, similar to the waxy maize starch, the waxy sorghum starch substituted with 24% OSA has the largest molecular size after 30 and 180 min of β-amylolysis and the lowest hydrolysis rate coefficient *k* among all OSA-modified waxy sorghum starches, indicating the action-blocking effect of high amount of OS groups on the β-amylolysis of waxy sorghum starch in gelatinised form.

Similar to the whole starch molecules, the SEC weight distributions of debranched OSA-modified waxy maize and sorghum starches in gelatinised form show more noticeable molecular size reduction after β-amylolysis (Fig. 3D–F and Fig. 4D–F for waxy maize and sorghum starches, respectively) than those of their counterparts in granular form (Fig. 2C and D for waxy maize and sorghum starches, respectively), consistent with previously studies (Syahariza, Sar, Hasjim, Tizzotti, & Gilbert, 2013; Witt, Gidley, & Gilbert, 2010). In general, at 1 min of β-amylolysis of OSA-modified waxy maize and sorghum starches in gelatinised form, two peaks are observed at R_h = 1.5 and ~2.5 nm. At 30 min of β-amylolysis, the two peaks merge and become a broader peak, and a shoulder at smaller R_h < 1 nm emerges. At 180 min of β-amylolysis, the shoulder of smaller R_h < 1 nm becomes apparent and the primary peak is shifted to R_h = 2 nm. The results suggest that short branches (R_h between 0.5 and 2 nm), which include outer A chains, were hydrolysed to much smaller molecular sizes (R_h < 1 nm), mostly maltose and maltotriose. On the other hand, long branches (R_h between ~2 and 8 nm), including B2, B3, . . . chains, retain some of their lengths (R_h between 1 and 5 nm) because of the presence of the branching points on the B branches. There is, however, no clear trend between the branch-chain length after β-amylolysis and the DS of either waxy starch.

The similarity in the branch-chain lengths of waxy maize at different DSs and sorghum starches at different DSs can be attributed to the similarity of their amylopectin molecules and the heterogeneous location of OS groups on starch molecules (Shogren, Viswanathan, Felker, & Gross, 2000).

Changes in chain length distribution were consistent with measurements of DB, a trivial result because DB is the reciprocal of the number-average of the chain-length distribution.

Only the samples modified with 24% OSA modification stand out from the other OSA-modified and unmodified starches (0% OSA) in the distributions of whole starch after 180 min of β-amylolysis. Two possible explanations are that the physical distribution of OS groups on starch molecules is either near the branching points or beyond the first branching points (in the direction of the reducing end) at 3–12% OSA modification. The most likely explanation is that the OSA substitution only occurred near the surface of each granule, where most of the molecules in the inner part of the granule were not modified, especially at 3–12% OSA modification, in agreement with previous studies (Huang et al., 2010; Wetzel, Shi, & Schmidt, 2010a; Wetzel, Shi, & Reffner, 2010b; Zhang et al., 2011). In addition, it is understood that the level of modification is heterogeneous among branch populations (Shogren et al., 2000), thus, the size distributions of both whole and debranched starches (the latter being the chain-length distribution, CLD) from gelatinised OSA-modified waxy starches show only slight or no differences after 180 min of β-amylolysis, as most of the molecules can still be hydrolysed to similar sizes. At 24% OSA modification, probably due to slight granule swelling with increasing repulsion force of the bulky OS groups, more molecules in the inner part of the granule were modified, resulting in different distributions compared with other OSA-modified starches at lower levels of modification.

4. Conclusions

Hydrolysis of OSA-modified starch by β-amylase provides a useful method of structural modification. The data obtained here suggest that this hydrolysis is partially hindered by the presence of OS groups on the starch chain, by blocking the enzyme from adhering to the starch chain. Controls of hydrolysis in the granular form show that structural changes to the starch granule primarily result from aspects of the treatment other than the β-amylase: perhaps the result of interactions with the sodium acetate buffer used in the case of granular β-amylolysis. The increase in ionic strength of the buffer solution probably assists the dissolution of hydrophilic starch regions, and the same effect is opposed by the presence of hydrophobic OS groups.

The information from gelatinised starch is most useful, though enzyme reaction kinetics for all samples were affected by the presence of OS groups, leading to lower hydrolysis rate coefficients (k) in most highly substituted starches, and overall less degradation, as indicated by changes to the starch structure. Low values for k were also observed in the unsubstituted starches, indicating that these starches were less accessible the action of the enzyme. This can be explained by the lack of steric hindrance in unmodified starch, allowing some possible localised interactions between neighbouring starch chains. From the very low DS values, the steric hindrance caused by OS groups allows greater enzyme access, but at very high levels blocks the progress of the enzyme.

Structural properties of the final product show clear but minor effects from the presence of OS groups. Highest DS samples consistently result in slightly larger products after enzyme hydrolysis. Why this effect is not more pronounced could be the result of the heterogeneity of the OSA esterification reaction. OS groups clustered around branch points result in less effective change than those further towards the non-reducing ends of starch chains. There is a much higher number of branch points than OS groups on a given starch molecule and as such the presence of branch points plays a more important role than does DS. This is reflected in both gelatinised and granular analysis, the latter still being affected by the high number of branch points, as both OS esterification and β-hydrolysis were restricted by the structure to the same areas of the granule.

That OS groups block the action of β-amylase is also reflected in the change in *DB* in relation to the initial *DS* of the samples. The presence of OS groups clearly protects from enzyme attack a significant number of α-(1 → 4) linkages, indicating loss of enzyme progress in digesting linear sections. Such modification logically results in a greater presence of hydrophobic groups at the periphery of OSA starch molecules, and will be of further interest for its effects on functional properties. While this is consistent with other studies using different enzyme systems, this is the first study conclusively demonstrating the hindrance of β-amylase by OS groups. Using this information about the actions of β-amylase on OSA starches allows much greater versatility in the structural modification of these important stabilisers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.04.041.

References

Bernfeld, P. (1955). Amylases, α and β. In S. P. Colowick, & N. O. Kaplan (Eds.), *Methods in enzymology* (vol. 1) (pp. 149–158). Academic Press.
Bhosale, R., & Singhal, R. (2007). Effect of octenylsuccinylation on physicochemical and functional properties of waxy maize and amaranth starches. *Carbohydrate Polymers*, *68*(3), 447–456.
Cave, R. A., Seabrook, S. A., Gidley, M. J., & Gilbert, R. G. (2009). Characterization of starch by size-exclusion chromatography: the limitations imposed by shear scission. *Biomacromolecules*, *10*, 2245–2253.
Dokic, P., Dokic, L., Dapcevic, T., & Krstonosic, V. (2008). Colloid characteristics and emulsifying properties of OSA starches. *Progress in Colloid and Polymer Science*, *135*, 48–56.
Panel on food additives, & Nutrient sources added to food. (2010). Scientific opinion on the use of gum acacia modified with octenyl succinic anhydride (OSA) as a food additive. *EFSA Journal*, *8*(3), 1539–1562.
Han, J.-A., & BeMiller, J. N. (2007). Preparation and physical characteristics of slowly digesting modified food starches. *Carbohydrate Polymers*, *67*(3), 366–374.
He, J., Liu, J., & Zhang, G. (2008). Slowly digestible waxy maize starch prepared by octenyl succinic anhydride esterification and heat-moisture treatment: Glycemic response and mechanism. *Biomacromolecules*, *9*(1), 175–184.
Hoover, R., & Sosulski, F. (1985). Studies on the functional characteristics and digestibility of starches from *Phaseolus vulgaris* biotypes. *Starch – Stärke*, *37*(6), 181–191.
Huang, Q., Fu, X., He, X. W., Luo, F. X., Yu, S. J., & Li, L. (2010). The effect of enzymatic pretreatments on subsequent octenyl succinic anhydride modifications of cornstarch. *Food Hydrocolloids*, *24*(1), 60–65.
Jeon, Y.-S., Viswanathan, A., & Gross, R. A. (1999). Studies of starch esterification: Reactions with alkenylsuccinates in aqueous slurry systems. *Starch – Stärke*, *51*(2,3), 90–93.
Liu, W.-C., Halley, P. J., & Gilbert, R. G. (2010). Mechanism of degradation of starch, a highly branched polymer, during extrusion. *Macromolecules*, *43*, 2855–2864.
Liu, Z., Li, Y., Cui, F., Ping, L., Song, J., Ravee, Y., et al. (2008). Production of octenyl succinic anhydride-modified waxy corn starch and its characterization. *Journal of Agricultural and Food Chemistry*, *56*(23), 11499–11506.
Makan, A. C., Otte, T., & Pasch, H. (2012). Analysis of high molar mass branched polybutadienes by SEC-MALLS and AF4-MALLS. *Macromolecules*, *45*(12), 5247–5259.
Mendez-Montealvo, G., Wang, Y.-J., & Campbell, M. (2011). Thermal and rheological properties of granular waxy maize mutant starches after β-amylase modification. *Carbohydrate Polymers*, *83*(3), 1106–1111.
Mosse, J. (1990). Nitrogen-to-protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content. *Journal of Agricultural and Food Chemistry*, *38*(1), 18–24.
Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry*, *153*(2), 375–380.
Nilsson, L., & Bergenstahl, B. (2006). Adsorption of hydrophobically modified starch at oil/water interfaces during emulsification. *Langmuir*, *22*(21), 8770–8776.
Rayner, M., Sjöö, M., Timgren, A., & Dejmek, P. (2012). Quinoa starch granules as stabilizing particles for production of Pickering emulsions. *Faraday Discussions*, (158), 139–155.
Scheffler, S. L., Huang, L., Bi, L., & Yao, Y. (2010). In vitro digestibility and emulsification properties of phytoglycogen octenyl succinate. *Journal of Agricultural and Food Chemistry*, *58*(8), 5140–5146.
Shogren, R. L., Viswanathan, A., Felker, F., & Gross, R. A. (2000). Distribution of octenyl succinate groups in octenyl succinic anhydride modified waxy maize starch. *Starch – Stärke*, *52*(6,7), 196–204.
Somogyi, M. (1945). A new reagent for the determination of sugars. *Journal of Biological Chemistry*, *160*(1), 61–68.
Song, X., He, G., Ruan, H., & Chen, Q. (2006). Preparation and properties of octenyl succinic anhydride modified early indica rice starch. *Starch – Stärke*, *58*(2), 109–117.
Sweedman, M. C., Tizzotti, M. J., Schäfer, C., & Gilbert, R. G. (2013). Structure and physicochemical properties of octenyl succinic anhydride modified starches: A review. *Carbohydrate Polymers*, *92*(1), 905–920.
Syahariza, Z. A., Li, E., & Hasjim, J. (2010). Extraction and dissolution of starch from rice and sorghum grains for accurate structural analysis. *Carbohydrate Polymers*, *82*(1), 14–20.
Syahariza, Z. A., Sar, S., Hasjim, J., Tizzotti, M. J., & Gilbert, R. G. (2013). The importance of amylose and amylopectin fine structures for starch digestibility in cooked rice grains. *Food Chemistry*, *136*(2), 742–749.
Tester, R. F., Qi, X., & Karkalas, J. (2006). Hydrolysis of native starches with amylases. *Animal Feed Science and Technology*, *130*(1,2), 39–54.
Tizzotti, M. J., Sweedman, M. C., Tang, D., Schaefer, C., & Gilbert, R. G. (2011). New ^{1}H NMR procedure for the characterization of native and modified food-grade starches. *Journal of Agricultural and Food Chemistry*, *59*(13), 6913–6919.

Tran, T. T. B., Shelat, K. J., Tang, D., Li, E., Gilbert, R. G., & Hasjim, J. (2011). Milling of rice grains. The degradation on three structural levels of starch in rice flour can be independently controlled during grinding. *Journal of Agricultural and Food Chemistry*, *59*(8), 3964–3973.

Vilaplana, F., & Gilbert, R. G. (2010). Characterization of branched polysaccharides using multiple-detection size separation techniques. *Journal of Separation Science*, *33*(22), 3537–3554.

Viswanathan, A. (1999). Effect of degree of substitution of octenyl succinate starch on the emulsification activity on different oil phases. *Journal of Environmental Polymer Degradation*, *7*(4), 191–196.

Wetzel, D. L., Shi, Y.-C., & Schmidt, U. (2010). Confocal Raman and AFM imaging of individual granules of octenyl succinate modified and natural waxy maize starch. *Vibrational Spectroscopy*, *53*(1), 173–177.

Wetzel, D. L., Shi, Y. C., & Reffner, J. A. (2010). Synchrotron infrared confocal microspectroscopical detection of heterogeneity within chemically modified single starch granules. *Applied Spectroscopy*, *64*(3), 282–285.

Witt, T., Gidley, M. J., & Gilbert, R. G. (2010). Starch digestion mechanistic information from the time evolution of molecular size distributions. *Journal of Agricultural and Food Chemistry*, *58*(14), 8444–8452.

Xu, J., Zhou, C.-w., Wang, R.-z., Yang, L., Du, S.-s., Wang, F.-p., et al. (2012). Lipase-coupling esterification of starch with octenyl succinic anhydride. *Carbohydrate Polymers*, *87*(3), 2137–2144.

Zhang, B., Huang, Q., Luo, F.-x., Fu, X., Jiang, H., & Jane, J.-I. (2011). Effects of octenyl-succinylation on the structure and properties of high-amylose maize starch. *Carbohydrate Polymers*, *84*(4), 1276–1281.