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Aggregate and emulsion properties of enzymatically-modified octenylsuccinylated waxy starches

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ABSTRACT

Sorghum and maize waxy starches were hydrophobically modified with octenylsuccinic anhydride (OSA) and treated with enzymes before being used to emulsify β-carotene (beta,beta-carotene) and oil in water. Enzyme treatment with β-amylase resulted in emulsions that were broken (separated) earlier and suffered increased degradation of β-carotene, whereas treatment with pullulanase had little effect on emulsions. Combinations of surfactants with high and low hydrodynamic volume (V_h) indicated that there is a relationship between V_h and emulsion stability. Degree of branching (DB) had little direct influence on emulsions, though surfactants with the highest DB were poor emulsifiers due to their reduced molecular size. Results indicate that V_h and branch length (including linear components) are the primary influences on octenylsuccinylated starches forming stable emulsions, due to the increased steric hindrance from short amphiphilic branches, consistent with current understanding of electrosteric stabilization. The success of OSA-modified sorghum starch points to possible new products of interest in arid climates.

1. Introduction

Starches modified with octenylsuccinic anhydride (OSA) have been produced for many years for their useful surfactant properties. The starch most often used as the basis of octenylsuccinylated (OS) starch is waxy maize starch. Starches from other botanical sources may give products with different properties. Sorghum has been recognized as an important resource in drier climates such as Australia (Jordan, Hunt, Cruickshank, Borrell, & Henzell, 2012), and hence more tolerant to climate change. Several varieties have been bred to produce waxy starches with similar properties to waxy maize starch, but as yet there are no value-added applications of these as modified starches.

This paper examines the modification of waxy sorghum and waxy maize starch with OSA, which is used to create an amphiphilic molecule with surfactant properties. There is some uniformity in research done on the optimal OSA modification reaction conditions for the many starches (Sweedman, Hasjim, Tizzotti, Schäfer, & Gilbert, 2013; Sweedman, Tizzotti, Schäfer, & Gilbert, 2013), since the original conception of the process by Caldwell and Wurzburg (1953). This paper focuses on the structural similarities between the widely used waxy maize starch and the waxy sorghum starch of interest, and how and why these structural characteristics affect the resultant molecules' surface activity.

Elsewhere, we have highlighted the importance of branch structure in the colloidal stability of OS starch stabilized emulsions and chemical stability of substances within the oil phase, as well as the importance of molecular size, measured by hydrodynamic radius (R_h) or hydrodynamic volume (V_h), for emulsion stability (Sweedman, Hasjim, Schaefer, & Gilbert, 2014). The structural characteristics of waxy sorghum and waxy maize starches have previously been found to be quite similar (Taylor & Emmambux, 2010), mainly due to the former's non-starch components. In terms of functional analysis, one might expect the two starch species to behave similarly, and any difference is likely to be the result of more subtle architecture of the starch molecules.

OS starch has been previously compared to other stabilizers in the emulsion and chemical stability of β-carotene emulsions; however, these studies only used one commercial type of OS starch, which ignores the huge range of structures that may influence

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activity (Mao et al., 2009; Mao, Yang, Xu, Yuan, & Gao, 2010). In one set of studies (Mao et al., 2009), the OS starch compared unfavorably against Tween-20 (T20), decaglycerol monolaurate (DML), and whey protein isolate (WPI). After 12 days at 55 °C, they measured levels of just over 20% of initial β-carotene content compared to around 80%, 40% and 40% for WPI, DML and T20, respectively. Strangely, the same samples subjected to similar tests (reported a year later (Mao et al., 2010)) produced different results, with OS starch being comparable with T20 at ~55%, WPI strongly protective at ~70% and DML highly unfavorable at ~15%. The differences in these studies. which include some of the same authors, may perhaps highlight the great variability that occurs in emulsion studies due to the inherent instability of emulsions, but it also emphasizes that changes in the results can come from any number of variables. It should be noted that all these studies involved accelerated breakdown of the β-carotene (due to oxidation because of ambient air) in order to provide practicable analysis, whereas lower temperatures and the inclusion of anti-oxidants would facilitate storage of commercial products containing these ingredients.

These OSA-modified starches are examples of electrosteric stabilizers, wherein colloidal stability (and hence emulsification properties) are (with some simplification) from two effects: the enthalpic repulsion caused by the charged groups, and the entropic repulsion caused by the difficulty in compressing the water-soluble moieties when colloidal entities come too close. These precepts are useful in understanding the observations in this study.

This paper is the first to examine specifically effects of OS starch's molecular structure on emulsion stability and chemical stability of the oil phase, isolated from other constitutive properties like amylose content. Maintaining the oxidative stability of the oils suspended in emulsion systems is important for human consumables, both for quality assurance and because the breakdown products of lipid oxidation like aldehydes and ketones (Mordi, 1993) may be harmful to humans in higher doses (Siems et al., 2005). Oxidation of lipid-soluble compounds other than β-carotene have been investigated previously (Scheffler, Huang, Bi, & Yao, 2010; Scheffler, Wang, Huang, San-Martin Gonzalez, & Yao, 2010). The last-named study found the addition of ε-polylysine improved the stability of lipids in emulsions stabilized by highly branched OS phytoglycogen; another attested to the superior surfactant ability of OS phytoglycogen over OS amylopectin (Scheffler, Huang, et al., 2010; Scheffler, Wang, et al., 2010). Nevertheless, phytoglycogen is unlikely to supersede amylopectin as the most popular substrate for industrial OS polysaccharides, due to the substantial difference in their availabilities. What these studies do emphasize is that more highly branched parent polysaccharides are generally superior to less branched ones, which is consistent with research on amylose content (Song, Zhao, Li, Fu, & Dong, 2013) and industry (empirical) preferences in this field. The fundamental reason for this is that the more highly branched water-soluble moieties in a surfactant are, the less compressible they are as a result, which by the generally accepted model of steric stabilization (Napper, 1983) increases their stabilizing power.

As pointed out by a reviewer, the heterogeneous nature of the modification process means that most of the modified starches are at the surface of the granule, while those in the interior have no or little modification. Thus the emulsification properties examined here are those arising from starch molecules located toward the surface of the granule. Now, starch molecular structure does not vary strongly with location in the granule (Angellier-Coussy et al., 2009), and thus structure-property correlations deduced in this paper should be generally applicable, irrespective of the location of that molecule in the granule.

The degree of branching, DB, is inversely related to average chain length, but this latter, being a single measure, does not say anything about the underlying chain-length *distribution*: quite different distributions can have the same DB while subtleties of structure-property relations may result in significantly different properties. The goal of the current work is to extricate those structural aspects of OS starches from each other, so as to determine what properties of highly branched parent starches play the greatest role in their surfactant function. This is achieved by taking samples of specific, known and consistent architecture, and subjecting them to controlled enzymatic transformations of the desired qualities. These data also enable mechanistic explanations for the results to be deduced.

2. Methods and materials

2.1. Materials

Waxy sorghum grains (A1*F_B004215) were gifts from the Queensland Department of Agriculture, Fisheries and Forestry (DAFF). Mazaca waxy maize was purchased from Penford (Tamworth, NSW, Australia) and used as received. Hydrochloric acid (37%, analytical reagent) was from Lab Scan Analytical Sciences (Patumwan, Bangkok, Thailand). Pyrene (Sublimed, 99%), β-carotene (Type I, synthetic > 93%, C9750), sodium hydroxide (reagent grade, ≥98%, pellets, anhydrous), OSA (97% mixture of *cis* and *trans*, 416487, Batch #: 06515DA), β-amylase (Type II-B, from barley), protease from *Streptomyces griseus* (P5147), LiBr (Reagent Plus, ≥99%), DMSO-d_6 (99.5% atom D) and Trifluoroacetic acid, TFA-d_1 (99% atom D) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia) and used as received. All water was Milli-Q™ ultra-pure deionized with a resistivity of 18.2 MΩ cm. Dimethyl sulfoxide, DMSO (GR for analysis ACS), methanol, ethanol and isopropanol were purchased from Merck & Co., Inc. (Kilsyth, VIC, Australia). Isoamylase was from *Pseudomonas* sp. (210 U mg^{-1} (40 °C, pH 3.5, oyster glycogen), Megazyme, Wicklow, Ireland). Other chemical reagents are analytical grades.

2.2. Preparation of samples

2.2.1. Isolation of waxy sorghum starch

Waxy sorghum grain was wet-milled as described previously (Sweedman, Hasjim, et al., 2013; Sweedman, Tizzotti, et al., 2013). To loosen protein structures, grain (1 kg) was washed thoroughly with water and soaked overnight in sodium bisulfite solution (9 g L^{-1}). This was blended and passed through a 150 μm sieve. Permeate slurry was washed three times with water and subsequent centrifugation (3000 × *g*, 3 min), then treated with protease for 1.5 h, cleaned and dried as described in (Sweedman, Hasjim, et al., 2013; Sweedman, Tizzotti, et al., 2013). A crude starch/protein material (csp, as described later) was taken from the upper layer in the final stage of pelleted starch. This was dried at 65 °C in the same manner as the starch and used in samples "SP" as described later. The final starch and csp products were analyzed by combustion using a LECO (Baulkham Hills, NSW) TruSpec CHN analyser and found to have nitrogen contents of 0.31 and 3.14 wt%, which given a conversion factor of 5.65 (Mosse, 1990) equates to crude protein contents of 1.75 and 17.74 wt%, respectively.

2.2.2. Acid hydrolysis in alcohol

Acid hydrolysis was based on methods given by Tizzotti, Sweedman, Schäfer, and Gilbert (2013), which were slightly modified using information in Ma and Robyt (1987). The solvent system was chosen to provide molecular size distributions of degraded starch that are larger than those obtained using other alcohols and with less dispersity than systems containing significant amounts of water (Supplementary material, Fig. S1). The solvent used was 39.5%, 59.5% and 1.0% methanol, isopropanol and HCl (saturated,

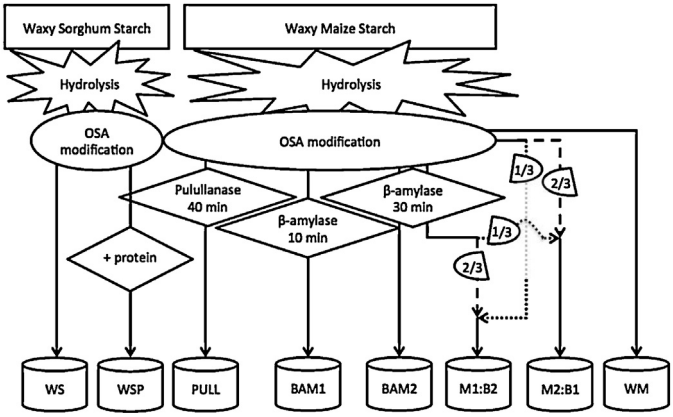

Fig. 1. Schema of experimental design outlining the 8 surfactant formulations.

37%), respectively. Granular starch was suspended in an equivalent weight of solvent and allowed to sit at room temperature with gentle stirring for 5 d. Upon completion, starch was recovered using a centrifuge (3000 × *g*, 1 min) and the sample washed three times with tricine buffer (pH 7.5, 250 mM), then washed twice by suspending it in ethanol, followed by centrifugation, before being allowed to dry overnight at 65 °C.

2.2.3. OSA modification of starches

OSA modification was performed based on methods previously published (Song, He, Ruan, & Chen, 2006). OSA (4.5 g) was dissolved in ethanol (22.5 g), as this ratio has been found optimal for other starches (Shi & He, 2012). Starch (150 g) was suspended in water (450 mL) at 35 °C. The pH was continually maintained at 8.5 with 0.2 M NaOH over a 3 h period as the OSA mixture was added dropwise during the first 2 h. Samples were neutralized with 0.02 M HCl, washed twice with ethanol and centrifugation (3000 × *g*, 3 min), and dried overnight at 65 °C.

2.2.4. Stabilizer preparation

Emulsions were prepared according to the schema in Fig. 1; in eight formulations using OS starch, six using a derivative of waxy maize starch (WM, Bam1, Bam2, M2:B1, M1:B2 and PULL), and two using a derivative of waxy sorghum starch (WS and SP). Starches were dispersed in water and dissolved by heating in boiling water bath for 20 min. After cooling to 40 °C, sodium acetate buffer was added for a final starch concentration of 10 g L^{-1} in a 0.05 M, pH 5 buffer solution, except for sample SP, which used 8 g L^{-1} OS waxy sorghum starch, with an additional 3 g L^{-1} csp (later measurements of Critical Aggregation Concentration (CAC) give the actual starch content). Three formulations using waxy maize were treated with β-amylase at 10 mg L^{-1} according to methods developed by Sweedman, Hasjim, et al. (2013), Sweedman, Tizzotti, et al. (2013) and another (PULL) was treated with pullulanase at 1.5 mL L^{-1}. These four formulations were kept at 40 °C for 10 (Bam1) and 30 (Bam2, in duplicate), and 40 (PULL) minutes, respectively, before being stopped in the following manner. All samples were subject to the enzyme-stopping procedure regardless of whether or not they were treated with enzyme. Samples were acidified with 15 mL 3 M HCl for one minute, before being returned to pH 5 with 15 mL 3 M NaOH, and then further adjusted to pH 7 before boiling again for 20 min. To prepare combined samples M2:B1 and M1:B2, replicates of waxy maize (WM) and β-amylase treated waxy maize (Bam2) formulations were used in ratios 2:1 and 1:2, respectively. This was designed to provide samples with properties related to the component formulations, with increased dispersity. After cooling, NaN3 was added as a preservative to each solution to a concentration of 0.04%.

2.2.5. Emulsion preparation and storage

Emulsions of β-carotene in canola oil in water were prepared in duplicate from the starch solutions according to methods published elsewhere (Sweedman et al., 2014). Beta-Carotene 2 w/w% was dissolved in Canola oil (food grade) by heating in boiling water bath for 10 min with agitation. The starch solution (pH 6.5–7.0) was allowed to cool, and sodium azide was added to a final concentration of 0.02 w/w%. The β-carotene in canola oil solution was added for a final concentration of 1.0 w/w%, giving a final, overall β-carotene content of 200 mg L^{-1}. The entire mixture was shaken, coarsely homogenized using an ultra-turrax T25 (IKA-Werke GmbH & Co. KG, Staufen, Germany) for 20 min at 9500 min^{-1} and finally homogenized using a TwinPanda400 two-stage valve homogenizer (GEA Niro-Soavi, Parma, Italy), with a two-stage pressure of 250 bar. In the case of the current study, only 3 passes were used for each emulsion, to limit degradation by shear scission. During preparation by HPH (high pressure homogenization), the temperatures for all samples did not exceed 40 °C. After HPH, 1 mL aliquots of each emulsion were stored at 55 °C, and 50 mL aliquots were stored in the dark at 55 °C, room temperature (rt, 22 ± 2 °C) and 4 °C.

2.3. Analytical methods I – structure

2.3.1. Size exclusion chromatography

Analytical SEC was performed using methods previously described (Vilaplana & Gilbert, 2010). The apparatus utilized an Agilent 1100 (PSS, Mainz, Germany) series with an isocratic pump, an autosampler injecting from a 100 μL piston without temperature control, an online degasser, calibrated to pullulan standards, with the column oven at 80 °C. For size separation, combined GRAM Pre-Column, 30 and 3000 analytical columns (PSS) at 0.3 mL min^{-1} were used. Data shown are from DRI (differential refractive index; RID-10A, Shimadzu, Kyoto, Japan) detection, operating at 635 nm and thermostated at 45 °C (i.e. this is the SEC weight distribution: the weight of particles as functions of size). All samples were fully dissolved in DMSO with 0.5% LiBr (w/w), thus providing the optimal conditions for separation. Data were processed using PSS WinGPC Unity (Build 5403; PSS, Mainz Germany).

2.3.2. Nuclear magnetic resonance

Samples were prepared and ^{1}H NMR spectra were obtained using methods previously described (Tizzotti, Sweedman, Tang, Schaefer, & Gilbert, 2011) with modifications to allow better stability of the OS group, as previously described (Sweedman et al., 2014). Spectra were recorded at 50 °C on a Bruker Avance NMR spectrometer operating at an observation frequency of 500.15 MHz for ^{1}H, equipped with a BBO5 probe (Bruker Biospin, Alexandria, New South Wales, Australia). Data were processed using Bruker TOPSPIN software (v2.1; Bruker Biospin). All spectra were manually phased and baseline-corrected. Values were taken from 3 technical replicates. A Lorentzian fit was used for spectral deconvolution.

2.3.3. Isolation of starch from emulsions

Starch was isolated from emulsions by first combining a 1:3:3 v/v mixture of emulsion:ethanol:hexane, followed by 6 or more sequential washes with water:ethanol:hexane in the same ratio (until a white pellet was retained), based on the method of (Mao et al., 2009). Separation at each stage was facilitated by thorough mixing, followed by centrifugation at 3000 × *g* for 2 min, after which the liquid layers were removed. Pellets were finally washed twice with pure ethanol and centrifugation, then dried overnight at 65 °C.

2.4. Analytical methods II – function

2.4.1. Critical aggregation concentration

CACs of OSA modified starches were determined using methods published previously by Tizzotti et al. (2013). Starch solutions of 18 concentrations from 0.01 to 10 g L^{-1} were produced by dilution of replicates of each formulation (excluding oil and β-carotene) in water containing 0.04% NaN3. Concentrations of 20 g L^{-1} were achieved by lyophilization to remove water from the dissolved surfactant, and dissolution in half the original volume of water. Samples were allowed to cool, and then pyrene in ethanol (40.5 mg L^{-1}) was added to a final pyrene concentration of 1×10^{-6} M. After storing in the dark overnight, these samples were analyzed in a quartz cuvette at room temperature (23 ± 2 °C) using a RF–5301 PC spectrofluorophotometer (Shimadzu). The emission wavelength and excitation/emission slit were at 390 and 5 nm, respectively. Intensity ratios were plotted against the log of concentration with a linear fit. The data points chosen for the super-CAC linear region reflect the point above which the R^2 value is at a maximum containing at least 4 points. The CAC in each case was the point where the super-CAC line reaches the $I327/I334$ (intensity ratio at the indicated wavelengths) equivalent to zero concentration.

2.4.2. Degradation of β-carotene

The color of intact emulsions was determined using a ChromaMeter CR-400 (Konica Minolta Sensing, Japan) calibrated with standard white tile and using an average from 3 measurements. Color evaluation used the L* (overall lightness), a* (redness and greenness), b* (yellowness and blueness) scale. Analysis used 10 mL samples stored at 55 °C in 15 mL tubes, taking 5 mL of emulsion for each analysis in a 6 mL glass beaker, shielded from ambient light, with a sample depth of 17 mm.

Residual β-carotene was determined using the method in Sweedman et al. (2014), which was based on that of Mao et al. (2009). β-Carotene was isolated from emulsions using 4 and 3 mL of hexane and ethanol, respectively, and diluted with hexane as appropriate to achieve concentrations within the linear standard curve of 0–2 mg L^{-1}. Standards were prepared from the same β-carotene in oil that was used for sample preparation. Samples were analyzed in triplicate by PharmaSpec UV-1700 Spectrophotometer (Shimadzu) at a wavelength of 453 nm in 4 mL PMMA cuvettes (www.Labco-online.com).

2.4.3. Determination of droplet size

Emulsion samples were consistently collected from approximately 20 mm below the emulsion surface after gentle inversion, and diluted to 10% of the original concentration to prevent multiple scattering effects. Analysis used 4 mL PMMA cuvettes in a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) at a fixed detector angle of 90°. Results were obtained for z-average size (nm) and polydispersity index (PDI).

2.4.4. Paste clarity

Before the addition of oil and β-carotene, each gelatinized starch was analyzed by spectrophotometer to determine *T%* at a wavelength of 650 nm, similar to the methods of Bello-Pérez, Agama-Acevedo, Sánchez-Hernández, and Paredes-López (1999).

2.5. Statistical analysis

All samples were performed in duplicate or triplicate, or in the case of CAC measurements were justified with high R^2 values for the linear components over 18 data points. Correlation analysis (Supplementary material, Table S1) and analysis of variance (ANOVA) were performed using Minitab 16 (State College, PA, USA). ANOVA used Fisher 95% individual confidence intervals from the mean values of replicate measurements.

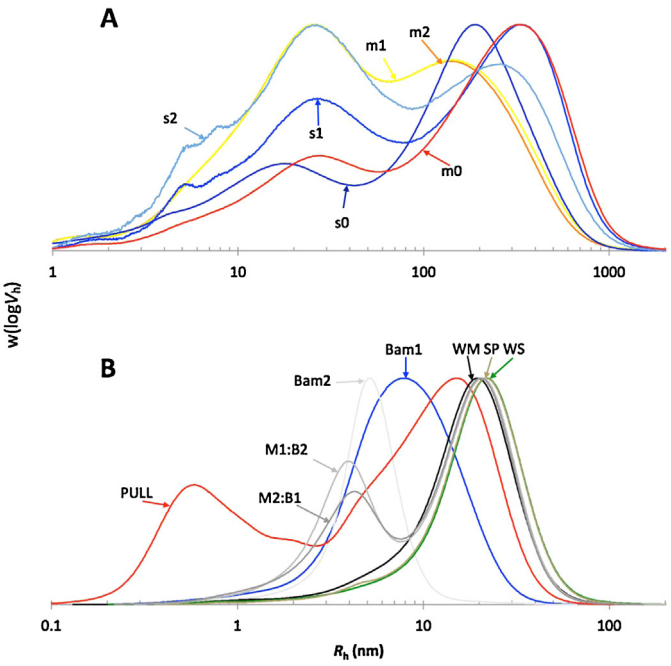

Fig. 2. SEC weight distributions (arbitrary units) of starches. In panel A: Before HPH; (m0) native waxy maize starch, (m1) acid hydrolyzed waxy maize starch, (m2) OS acid hydrolyzed waxy maize starch, (s0) waxy sorghum starch, (s1) acid hydrolyzed waxy sorghum starch, (s2) OS acid hydrolyzed waxy sorghum starch. In panel B: OS starches after preparation (post-HPH), labeled as named in text.

3. Results and discussion

3.1. Starch structural parameters in emulsion

Acid hydrolysis of granular starch is understood to affect α-(1 → 6) linkages to a greater extent than α-(1 → 4), an effect that is reversed when the starch is gelatinized (Bertolini, 2010). Bertolini's studies referred to acid hydrolysis in water, and the difference between granular and gelatinized starch was the result of the crystalline structure in granules. In the presence of alcohols in the current study, it can be expected that the crystalline effects are augmented by the low solubility of starch in alcohols. After acid hydrolysis and OSA modification, the DS and DB, respectively, were 0.0219% and 2.8% for waxy maize starch; and 0.0217% and 2.4% for waxy sorghum starch. This is slightly lower than the normal DB for waxy starches (Sweedman, Hasjim, et al., 2013; Sweedman, Tizzotti, et al., 2013), consistent with the theory from (Bertolini, 2010) on the effects of significant acid treatment during preparation.

It should be noted that the SEC apparatus used in this study is optimized for smaller molecules, as this study is most concerned with highly degraded starches (Vilaplana & Gilbert, 2010). The effects of acid hydrolysis and OSA modification can be seen in Fig. 2A. While the method of OSA modification is chosen to minimize starch damage, it does produce minor, inconsistent degradation (Sweedman, Hasjim, et al., 2013; Sweedman, Tizzotti, et al., 2013; Tizzotti et al., 2013). While the native structures of the two starches appear similar (peak $R_h \sim 300$–400 nm, Fig. 2A, m0 and s0), the waxy maize starch suffered greater degradation as a result of acid hydrolysis (Fig. 2A, m0 → m1) than did sorghum starch (Fig. 2A, s0 → s1). The acid hydrolysis of sorghum starch resulted in an apparent increase in the relative population of larger molecules, which was probably the result of smaller molecules being

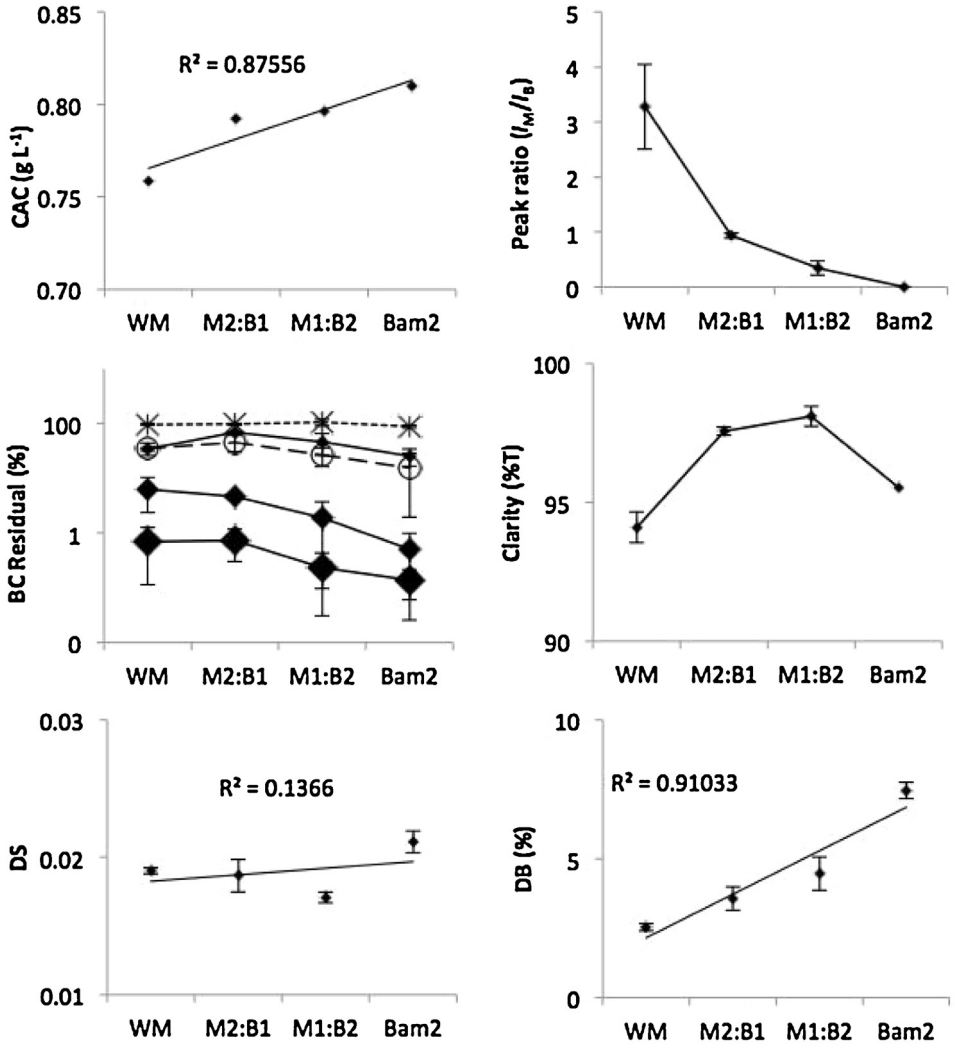

Fig. 3. Values resulting from combination of M and Bam2 at different ratios. CAC, paste clarity, DS, DB and peak ratio (I_M = intensity at peak R_h of WM; and I_B = intensity at peak R_h of Bam2); and β-carotene residual (%) at (—◆—) 1, (—◇—) 2 and (—◇—) 4 d after storage at 55 °C and 13 d at (- -✳- -) 4 °C and (—⊖—) room temperature.

selectively removed during acid hydrolysis. On the other hand, the waxy sorghum starch alone suffered some degradation during OSA modification (Fig. 2A, s1 → s2), which had the effect of bringing the peak R_h to around 25 nm, coincident with the waxy maize starch before and after OSA modification (Fig. 2A, m1 and m2). Hydrolysis during OSA modification can result from the harsh pH modifiers used in the OSA reaction if NaOH is added too quickly, for example; however, only the final structures are of interest here.

After the various enzyme treatments and HPH, all samples were degraded in size (Fig. 2B), whether it was a result of the pre-HPH treatments, or due to shear scission during HPH. The HPH degradation of OS starches of various structures has been examined in Sweedman et al. (2014), and these results have been used in the current study. The acid hydrolysis chosen for this study resulted in populations of molecules above the maximum size to which HPH degrades under the conditions used. As expected, the SEC weight distributions of sorghum samples were similar regardless of the original protein content. As a result, WM, SP and WS have very similar weight distributions after HPH, all peaking at $R_h \sim 20$ nm. PULL contained two peaks, the larger of which is only slightly smaller than the higher group. Pullulanase treatment resulted in a decrease to 12 nm for the main peak, with the addition of smaller peaks at 0.6 and 0.03 nm, respectively. The smaller peaks represent populations of essentially linear components after removal from the main molecules by pullulanase, while the main peak at 12 nm represents the remaining highly branched components. This is consistent with the long-held understanding that pullulanase acts on terminal (linear) branches (Bender & Wallenfels, 1966; Manners, 1997). Both Bam1 and Bam2 have successively smaller peak R_h, which is more attributable to the enzyme treatment than shear scission during HPH, due to being well below the upper size limit for shear scission. The results show that β-amylase treatment resulted in significant decrease from peak R_h around 20 nm (Fig. 2A, m2) down to about 2.5 nm for Bam2 and 8 nm for Bam1. Mixed samples M1:B2 and M2:B1 showed two peaks with height ratios that varied according to their constitutions of WM and Bam2 (Fig. 3). The relationship between peak ratios of Bam2 and WM components in M1:B2 and M2:B1 is not linear, and is dominated by the mass of WM. This is the result of β-amylolysis of Bam2, which actually reduces the total mass of the starch content as detected by SEC. Much of the mass of β-amylolyzed samples is removed when the maltose components are washed away during sample preparation, because ethanol does not precipitate the smaller dextrins. In the cases of CAC and emulsion analysis in this study, all breakdown products remain in the sample.

As β-amylase specifically targets α-(1 → 4) linkages from the non-reducing end of the starch molecule (Bernfeld, 1955), its action results in significantly increased DB (Table 1), though it may be that

Table 1
Experimental values.

Sample	Peak R_h (nm)	DB (%)	DS	Paste clarity (T%)	CAC (g L^{-1})
WM	23.17 ± 5.21^{ab}	2.53 ± 0.95^{d}	0.0190 ± 0.0068^{a}	94.1 ± 0.7^{c}	0.76 (R^2 = 0.93)
M2:B1	20.92 ± 0.97^{ab}	3.57 ± 2.75^{c}	0.0187 ± 0.0299^{a}	97.6 ± 0.3^{a}	0.79 (R^2 = 0.86)
M1:B2	23.77 ± 4.24^{ab}	4.46 ± 2.00^{b}	0.0171 ± 0.0084^{a}	98.1 ± 0.1^{a}	0.80 (R^2 = 0.92)
Bam2	5.07 ± 0.13^{c}	7.45 ± 0.46^{a}	0.0211 ± 0.0109^{a}	95.5 ± 0.6^{b}	0.81 (R^2 = 0.90)
Bam1	8.13 ± 0.34^{c}	4.80 ± 0.97^{b}	0.0197 ± 0.0056^{a}	95.8 ± 0.2^{b}	0.79 (R^2 = 0.92)
PULL	16.56 ± 1.80^{b}	1.81 ± 0.24^{e}	0.0167 ± 0.0050^{a}	89.8 ± 0.4^{d}	0.77 (R^2 = 0.74)
WS	25.83 ± 5.46^{a}	2.48 ± 0.57^{d}	0.0197 ± 0.0100^{a}	64.2 ± 0.3^{e}	0.72 (R^2 = 0.94)
SP	25.27 ± 4.81^{a}	2.65 ± 0.66^{d}	0.0121 ± 0.0089^{a}	3.8 ± 0.6^{f}	0.66 (R^2 = 0.99)

Means ± standard deviations.
Superscripts indicate ANOVA significant difference at $p < 0.05$.
CAC values correct to r^2 values calculated in excel.

many of these branch points where β-amylase halts are generally two or three monomers, meaning the structural impact of such a branch is limited. The results from NMR (Table 1; Supplementary material, Fig. S2) indicate that DS and DB have been predictably affected by enzyme treatments. The highest DB occurred in Bam2 (7.5%), which was the most significantly affected by β-amylase. Bam1 was the second highest (4.8%), but still significantly lower DB than Bam2, whereas PULL had the lowest DB of all (1.8%). Measurements of DS were not significantly varied between samples, so that differences in DS can be excluded as contributing to the results in these experiments. In the samples of various combinations of Bam2 and WM (Fig. 3), DB trended upwards (R^2 = 0.91), whereas DS was not significantly affected (R^2 = 0.14).

3.2. Critical aggregation concentrations

The ability of OS starch to self-aggregate (and in emulsion systems continuously layer upon the oil–water interface), as well as the rigidity of the molecules, determines its capacity to conform to the surface of droplets. Prochaska, Kedziora, Le Thanh, and Lewandowicz (2007) found that OS potato starch had an high capacity to lower the surface tension of solutions, but had low efficiency of adsorption. Assuming the low efficiency of adsorption is a property of OS starches in general, the stabilizing capacity is more likely a result of self-aggregation and rigidity.

The CACs of all samples (Table 1) were determined. The sample SP had the lowest CAC, followed closely by WS. The samples WM and PULL were closely matched, and there was a distinct increase in the CAC with β-amylolysis, though the increase was not proportional to the level of hydrolysis. The mixed samples varied in CAC, β-carotene degradation and droplet size as seen in Fig. 3. The results build on those seen in Tizzotti et al. (2013), who reported lower CACs for larger molecules where the branch structure was the same, but also for those where both size and amylopectin content were lower. These results are consistent with expectations from the way in which electrosteric stabilizers act, as discussed in Section 1.

Varona, Martin, and Cocero (2009) found CACs (called "critical micelle concentration" (CMC) in that paper) for OS starches between 4.3 and 7.2 g L^{-1}; however, using several different methods, Krstonosic, Dokic, and Milanovic (2011) reported much lower values of 0.41–0.88 g L^{-1}. The current study reports values between 0.65 and 0.81 g L^{-1}, which are more in line with the Krstonosic paper.

Comparisons between the samples used in this study show surprisingly little disparity between the highest and lowest CACs, considering that two of the starches resulted in emulsions that broke very quickly. Bam2 had the highest CAC of all (0.81 g L^{-1}), and its combinations with WM (CAC = 0.76 g L^{-1}) decreased in CAC proportionally, consistent with relationships established in Tizzotti et al. (2013). The lowest CAC was observed in WS (0.72 g L^{-1}) and SP (0.66 g L^{-1}). This indicates that the presence of protein may have had some effect on the CAC. Regardless, the low CAC for sample WS is an indication of good surface activity, comparable and perhaps superior to that of WM.

It is also notable that the CAC of the partially debranched sample PULL was not significantly different from that of WM, indicating that the removal of branches from WM did not result in an overall gain or loss of amphiphilic properties. As PULL contained two distinct size populations of molecules (Fig. 2B), one of which was similar to WM, the other much smaller, and presumably mostly linear, one might expect the influence of the larger population to be diminished by the smaller population, as is seen in the mixtures of WM and Bam2. As this is not the case, we conclude that the population of smaller molecules also plays an equally important role in aggregation, and that their diminished DB does not affect the aggregation of the population of larger molecules. While it is possible that the size difference affects kinetic factors of the aggregation process, there is no reason given in literature why it should affect the CAC.

3.3. Degradation of β-carotene

The reflected color of the intact emulsion showed dramatic changes over 8 d (Supplementary material, Fig. S3); the results across all samples demonstrated no significant differences. These results are consistent with results reported previously (Sweedman et al., 2014). Mao et al. (2010) investigated the effect of HPH on droplet size in nanoemulsions containing β-carotene, and found that higher pressures resulted in higher temperatures and smaller droplet size, as reported previously. In the same study, they determined the effects of surfactant (including one OS starch) on the degradation of β-carotene; unfortunately, they did not report a comparison between droplet size and degradation of β-carotene.

In the current study, the initial uptake of β-carotene in emulsions was recorded (Fig. 4) and subsequent measurements were taken as a percentage of these initial values. Experimental design meant that all emulsions were saturated with oil phase to give distinctions based on upper limits of the various surfactants' capabilities. For each preparation, the total β-carotene concentration was around 200 mg L^{-1}, and after HPH the suspended β-carotene was between 80 and 130 mg L^{-1}. Loss of β-carotene at this stage in processing is through the unstabilized oil phase (which floats to the top of emulsions) and heat damage during HPH; only the former of these is likely to be significantly different between samples. Bam2 and PULL showed the lowest uptake of oil (~80 mg L^{-1}), with Bam1 only slightly higher (~90 mg L^{-1}). In the case of the β-amylase treated samples, the low oil uptake can be attributed to a lower total mass of amphiphilic polymer in the solution, since the maltose released during β-amylolysis probably does not contain OS groups (Sweedman, Hasjim, et al., 2013; Sweedman, Tizzotti, et al., 2013), but it is also a result of the significantly weaker emulsion stability as discussed in the next section.

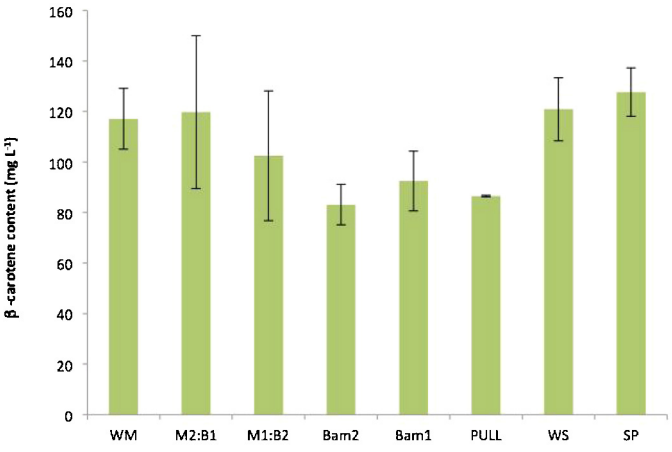

Fig. 4. Initial concentrations of β-carotene in emulsions.

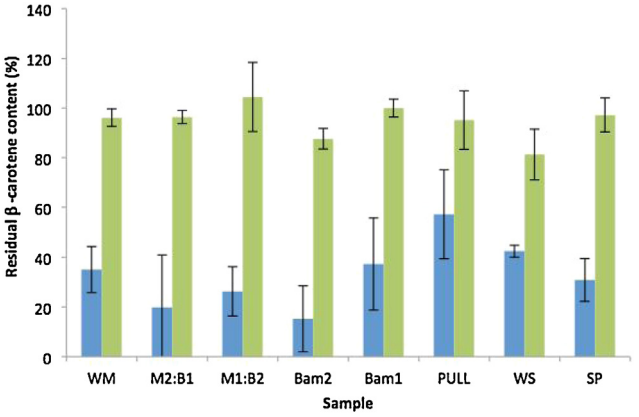

Fig. 6. Residual β-carotene in emulsions after storage for 13 d at room temp (left columns) and at 4 °C (right columns) as % of initial levels.

The degradation of β-carotene in emulsions was recorded over 13 d, and showed greater degradation than previously reported; however, these results are more similar to those of Mao et al. (2009) than Mao et al. (2010) (Fig. 5), between which there is some disagreement. The relationship between degradation and time in this study is also more logarithmic than the linear results presented in the two Mao papers. It is notable that one sample (WM) for this study was prepared by an equivalent method to one used in (Sweedman et al., 2014) ("W_{H23}"). The greater degradation is likely the result of the presence of salts within the buffer of the continuous phase, that being the key difference between the two methodologies. We excluded the number of cycles as a cause of this difference, as any effect from a change of HPH parameters would be noticeable as a difference in droplet size and the temperature reached during HPH. This presents a challenge, as Qian, Decker, Xiao, and McClements (2012) recently found that ionic strength does not affect the degradation of β-carotene to any significant extent. Salts are well known to destabilize emulsions (Klaus et al., 2012), particularly where the stabilizer has an electrostatic component; however, in the case of electrosteric OS starch, the primary stabilization mechanism is accepted to be steric (Tesch, Gerhards, & Schubert, 2002).

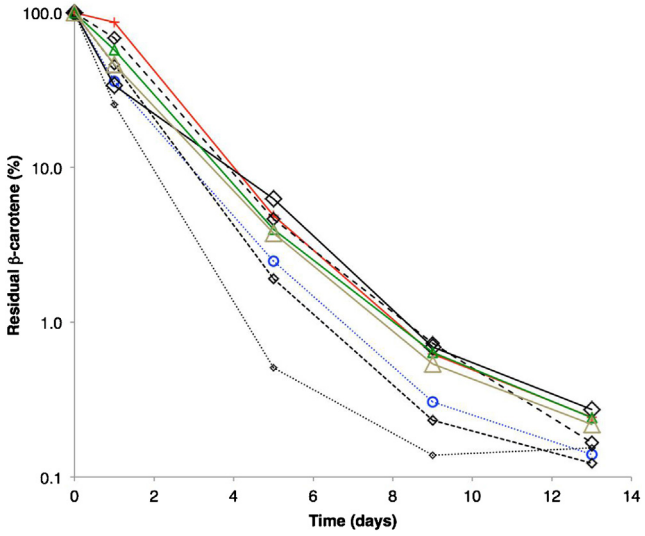

Fig. 5. Residual β-carotene content over 13 d at 55 °C. (—◇—) WM, (- - ◇ -) M2:B1, (---◇---) M1:B2, (·····◇·····) Bam2, (—○—) Bam1, (—+—) PULL, (—△—) WS, (—△—) SP.

By 13 d, all samples were almost completely depleted of β-carotene. However, at earlier times, where the difference between samples is greater, there was faster degradation at the higher levels of hydrolysis resulting from the β-amylase treatment. The sample WS was again comparable with WM and PULL, and the presence of protein in SP resulted in negligible decrease in β-carotene residue compared to WS. After 13 d, measurements were also taken for those samples stored at room temperature (23 ± 2 °C) and 4 °C (Fig. 6). Results for heat-stored samples are not shown on the same graph, due to being almost entirely depleted. The results indicate that under cooler conditions there is less breakdown of the β-carotene, but again the greatest decrease in β-carotene content was in Bam2, and to a lesser extent Bam1. Once again there is negligible difference between WM, WS and SP; however, PULL actually showed insignificantly superior β-carotene protective qualities, which is interesting as the size distribution (Fig. 2B) is significantly decreased from its parent (WM). Pullulanase treatment has resulted in a significant decrease in DB from 2.4% to 1.8% (Table 1), and the development of a significant population of smaller molecules. This is apparently in contradiction to the notion that stability of the emulsion relies on the highly rigid, densely branched structures alone. Considering that the emulsion containing PULL showed an initial β-carotene content of only slightly more than ¾ that of WM, it is possible that the similarities in residual β-carotene content are indicative of similar structure of the surface active components in the peak at higher R_h in PULL's weight distribution, which may imply that the linear components played little role in stabilizing the emulsion. However, when the proportion of the two populations of molecules in PULL are compared with the loss of oil uptake in emulsion, while considering as well the overall consistency in other emulsion properties like droplet size, it is clear that molecules in the smaller, less branched population are playing an important role.

From this study it is clear that β-amylase enzymatic modification is not ideal for the production of useful surfactant molecules of OS starch, despite previous indications that the greater DB should be advantageous, and also regardless of other advantages such as paste clarity and viscosity. The sample with decreased DB showed no strong change in either β-carotene protection or emulsion properties, supporting the conclusion that DB itself is less important than macromolecular architecture, branch length and overall size (R_h). These results indicate that the effect of botanical origin between sorghum and maize is largely inconsequential per se for emulsion properties, as is DB directly. This finding regarding DB is a refinement on our previous work (Sweedman et al., 2014), wherein the difference in DB was the result of differing amylose:amylopectin contents, rather than direct enzymatic alteration

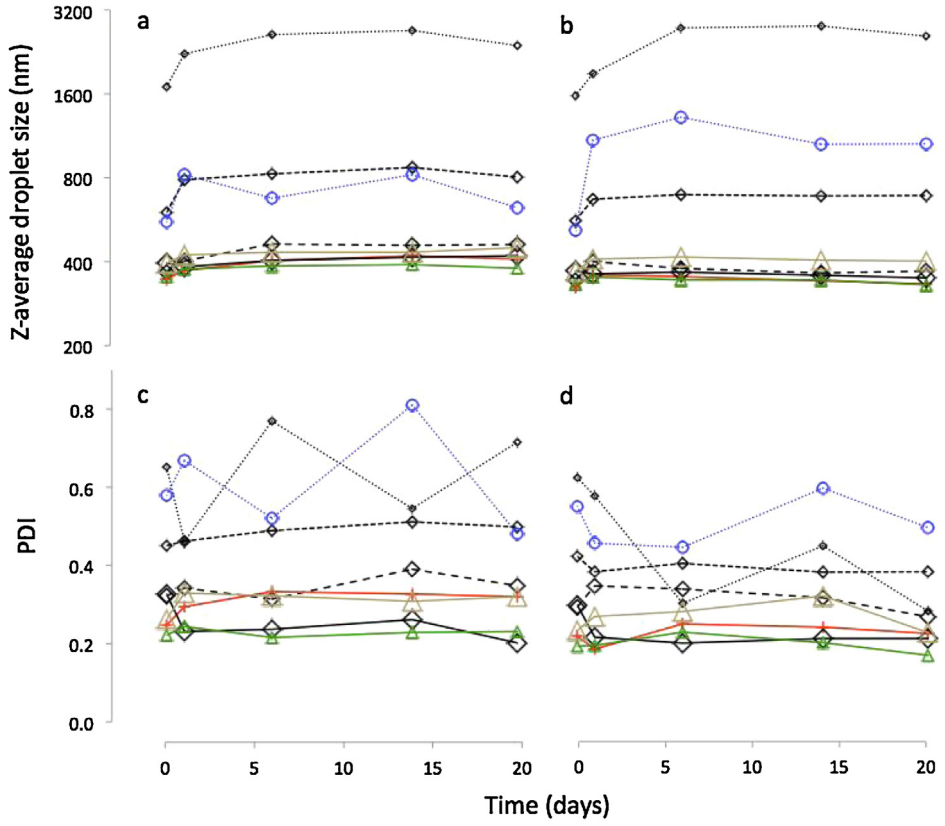

Fig. 7. Analysis of droplet size in emulsions stored at room temperature (a and c) and at 55 °C (b and d). (—◇—) WM, (- - ◇ -) M2:B1, (---◇---) M1:B2, (·····◇·····) Bam2, (—○—) Bam1, (—+—) PULL, (—△—) WS, (—△—) SP.

of the DB. The conclusion from this information is that other aspects of branching structure are influential, most importantly the length of detached linear components in pullulanase treated samples (PULL), which is also a significant structural difference between amylose and amylopectin.

3.4. Emulsion stability

The function of OS starches as steric stabilizers requires their hydrophilic components to be actively capable of preventing physical contact between oil droplets (Napper, 1983), thus preventing coalescence and the separation of oil and water phases. This steric hindrance is more effective when the bulk of polymer surfactants lie on the convex side of curved interfaces (i.e. outside the oil droplets), as is the case with OS starches (Dickinson, 2009) Within 24 h of forming the emulsions in this study, Bam1 and Bam2 emulsions were both observed to break, regardless of storage conditions; however, all other emulsions appeared to remain intact for the duration of the experiment 20 d. Droplet size (Fig. 7) is the best objective indicator of the physical stability of the emulsions used in this study (given that the emulsification conditions were the same). Contrasted with Sweedman et al. (2014), the emulsions stored under warm conditions (55 °C) showed no greater signs of instability than those stored at room temperature. Only one sample (Bam1) showed significant change between warm and room temperature storage, having consistently larger droplet size in the warmer samples. This, along with the clearly visible instability of the emulsion, may be accounted for by a tendency for smaller droplets to settle out or acquiesce in this sample. The $\log_{10}$ values for droplet size in room temperature and hot emulsions showed a positive correlation with DB ($p < 0.005$, $R^2 = 0.91$ and 0.93, respectively, Supplementary material, Fig. S4), meaning droplets were actually larger in emulsions containing OS starches of higher DB immediately after HPH. The size of the stabilized droplet would be the radius of the oil droplet, plus the thickness of any surfactant layer. Unfortunately the surfactant layer thickness is difficult to determine even though the peak R_h of the surfactant molecules is known to be up to 20 nm, because molecules that are fully dispersed are likely more compacted at the interface and not limited to a single layer (theoretical maximum at a single R_h equivalent) of the starch surfactant. Densely branched molecules (higher DB) generally have greater rigidity and will probably contribute to an increase in droplet size by providing a thicker adsorbed layer, though the extent to which this is relevant is probably insignificant given the relative size of the oil droplets.

The sample WS consistently resulted in smaller droplet sizes than all other samples only by an insignificant margin, and was comparable in droplet size to emulsions with WM, debranched waxy maize PULL and SP. Higher proportions of WM to Bam2 led to decreasing droplet size in the sequence, Bam2 > M1:B2 > M2:B1 > WM. Song et al. (2013) investigated the oil droplets in emulsions stabilized by four OS starches of differing amylopectin contents, and reported less dispersity in droplet size and smaller droplets. This was supported by finding positive effects of higher amylopectin content in a recent study (Sweedman et al., 2014). Keeping in mind that the emulsion containing Bam2 broke after 24 h, whether hot or cold, it is not surprising that that sample shows a large droplet size and significant fluctuations in droplet polydispersity index (PDI). The PDI of the droplet size became considerably lower at the higher temperature, possibly because the higher temperatures have an effect on the viscosity of oil itself, thus lowering the threshold for Ostwald ripening effects (Taylor, 1998).

In this study, only waxy starches were chosen, so the considerable differences between results can be deliberately linked to the

specific structural changes that have been performed. The almost complete breaking of emulsions stabilized by the OS starches treated by β-amylase (Bam1 and Bam2) indicated that DB alone is not a good predictor of surfactant quality, and this position is supported by the results from the PULL sample. For researchers considering starches in general, the relationship between DB and average branch length is almost a natural assumption, but in the case of samples like PULL, where there is an artificially high number of short, linear pieces of OS starch in the sample, the measurable DB is diminished, whereas the average branch length bears more resemblance to the parent amylopectin than to amylose of a similar DB. As such, the present results indicate that short linear components are probably just as effective as branched molecules in stabilizing emulsions. This suggests that the long history of empirical evidence supporting OS derivatives of amylopectin and phytoglycogen (Sweedman, Hasjim, et al., 2013; Sweedman, Tizzotti, et al., 2013) surfactant activity over amylose is almost certainly the result of having optimal branch lengths, rather than being explicitly related to DB. Furthermore, when one looks outside the realm of OS starch for branched surfactants, there is evidence that supports linear molecules over branched ones, at least when concerned with the hydrophobic region (Varadaraj, Bock, Valint, Zushma, & Brons, 1990; Wormuth & Zushma, 1991). Varadaraj et al. (1990) ascribed lower foam stability to weaker intermolecular cohesive forces in branched hydrophobes than unbranched ones; on the other hand, (Wormuth & Zushma, 1991) found linear surfactants to stabilize equal parts of oil in water more efficiently than branched ones, an effect that was also proportional to the level of branching. From this information it is possible that the successful emulsion from PULL is explained by the more linear molecules being less hindered intramolecularly than the branches in their intact counterpart WM, thereby allowing greater movement of individual molecules to areas where steric hindrance is useful at the interface. This advantage in pullulanase debranched molecules appears to be enough to counteract any disadvantage as the result of a loss of rigidity compared to the original molecules. Nilsson, Leeman, Wahlund, and Bergenståhl (2007) reported that higher molecular weight polymers adsorb preferentially to the oil–water interface over their low molecular weight counterparts, meaning PULL might be expected not to perform well considering its high content of smaller molecules; however, (Nilsson & Bergenstahl, 2006) also reported the role of kinetic factors in the colonization of oil droplets by surfactants, which may represent another advantage for smaller, more mobile molecules in the early stages of emulsion formation.

4. Conclusions

OSA modified starches of different structures resulting from enzyme treatments and different botanical origins have been tested for their capacity to maintain emulsions, and protect β-carotene in the oil phase against chemical stress. In all tests, samples containing larger molecules performed better in both emulsion stability and protection of the β-carotene from chemical stress, with waxy maize starch performing best overall, though both waxy sorghum starch tests showed very low CACs, perhaps influenced by residual protein. As well as average degree of branching, molecular size and branch-length fine structure are important in emulsification and protection against oxidation. Thus the common use of average branch length can be a misleading criterion for selection of emulsifier.

Waxy sorghum starch has similar properties to waxy maize starch, though the presence of high amounts of protein in the grain leads to either greater purification requirements or alternatively lower paste clarity.

It appears from these results that the presence of sorghum proteins does not significantly affect the emulsion properties, allowing for waxy sorghum to be used as a suitable substitute for waxy maize to produce modified starches in areas too dry for the latter. Overall, molecular size and branch length seem to be the greatest contributing factors in the OS starch stabilization of emulsions, consistent with the general precepts of electrosteric stabilization; however, it is interesting to see that the effects of shorter branch length extends to partially debranched samples. Further work may determine the effects of branch length distribution on the properties of OS starches and bring focus to the specific molecular structures that inhabit the interface of oil droplets.

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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.2014.04.088.

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