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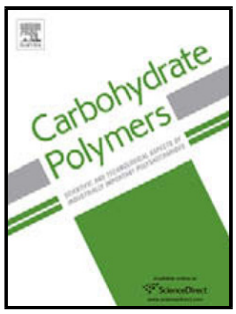

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Aggregate and emulsion properties of enzymatically-modified octenylsuccinylated waxy starches.

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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.

Keywords

octenylsuccinic anhydride starch; structural modification; emulsion stability; critical aggregation concentration; chemical degradation

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, Tizzotti, Schäfer & Gilbert, 2013), since the original conception of the process by Caldwell & 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, submitted). 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 activity (Mao, Xu, Yang, Yuan, Gao & Zhao, 2009; Mao, Yang, Xu, Yuan & Gao, 2010). In one set of studies (Mao, Xu, Yang, Yuan, Gao & Zhao, 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, Yang, Xu, Yuan & Gao, 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

79 effects: the enthalpic repulsion caused by the charged groups, and the entropic repulsion
80 caused by the difficulty in compressing the water-soluble moieties when colloidal entities
81 come too close. These precepts are useful in understanding the observations in this study.

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

101 As pointed out by a reviewer, the heterogeneous nature of the modification process means
102 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 towards the surface of the granule. Now, starch molecular structure does not vary strongly with location in the granule (Angellier-Coussy, Putaux, Molina-Boisseau, Dufresne, Bertoft & Perez, 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 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. 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, Tizzotti, Schäfer & Gilbert, 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, Tizzotti, Schäfer & Gilbert, 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 analysed 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.

148 *2.2.2. Acid hydrolysis in alcohol*

149 Acid hydrolysis was based on methods given by (Tizzotti, Sweedman, Schäfer & Gilbert,
150 2013), which were slightly modified using information in (Ma & Robyt, 1987). The solvent
151 system was chosen to provide molecular size distributions of degraded starch that are larger
152 than those obtained using other alcohols and with less dispersity than systems containing
153 significant amounts of water (Supplementary material, Figure S1). The solvent used was 39.5,
154 59.5 and 1.0 % methanol, isopropanol and HCl (saturated, 37%), respectively. Granular starch
155 was suspended in an equivalent weight of solvent and allowed to sit at room temperature with
156 gentle stirring for 5 d. Upon completion, starch was recovered using a centrifuge (3000 *g*, 1
157 min) and the sample washed three times with tricine buffer (pH 7.5, 250 mM), then washed
158 twice by suspending it in ethanol, followed by centrifugation, before being allowed to dry
159 overnight at 65 °C.

160 *2.2.3. OSA modification of starches*

161 OSA modification was performed based on methods previously published (Song, He, Ruan
162 & Chen, 2006). OSA (4.5 g) was dissolved in ethanol (22.5 g), as this ratio has been found
163 optimal for other starches (Shi & He, 2012). Starch (150 g) was suspended in water (450 mL)
164 at 35 °C. The pH was continually maintained at 8.5 with 0.2 M NaOH over a 3 h period as the
165 OSA mixture was added drop-wise during the first 2 h. Samples were neutralized with 0.02 M
166 HCl, washed twice with ethanol and centrifugation (3000 *g*, 3 min), and dried overnight at 65
167 °C.

168 *2.2.4. Stabilizer preparation*

169 Emulsions were prepared according to the schema in Figure 1; in eight formulations using
170 OS starch, six using a derivative of waxy maize starch (WM, Bam1, Bam2, M2:B1, M1:B2

171 and PULL), and two using a derivative of waxy sorghum starch (WS and SP). Starches were
172 dispersed in water and dissolved by heating in boiling water bath for 20 min. After cooling to
173 40 °C, sodium acetate buffer was added for a final starch concentration of 10 g L^{-1} in a 0.05
174 M, pH 5 buffer solution, except for sample SP, which used 8 g L^{-1} OS waxy sorghum starch,
175 with an additional 3 g L^{-1} csp (later measurements of Critical Aggregation Concentration
176 (CAC) give the actual starch content). Three formulations using waxy maize were treated
177 with β-amylase at 10 mg L^{-1} according to methods developed by (Sweedman, Hasjim,
178 Tizzotti, Schäfer & Gilbert, 2013) and another (PULL) was treated with pullulanase at 1.5 mL
179 L^{-1}. These four formulations were kept at 40 °C for 10 (Bam1) and 30 (Bam2, in duplicate),
180 and 40 (PULL) minutes, respectively, before being stopped in the following manner. All
181 samples were subject to the enzyme-stopping procedure regardless of whether or not they
182 were treated with enzyme. Samples were acidified with 15 mL 3 M HCl for one minute,
183 before being returned to pH 5 with 15 mL 3 M NaOH, and then further adjusted to pH 7
184 before boiling again for 20 min. To prepare combined samples M2:B1 and M1:B2, replicates
185 of waxy maize (WM) and β-amylase treated waxy maize (Bam2) formulations were used in
186 ratios 2:1 and 1:2, respectively. This was designed to provide samples with properties related
187 to the component formulations, with increased dispersity. After cooling, NaN_3 was added as a
188 preservative to each solution to a concentration of 0.04%.

189 *2.2.5. Emulsion preparation and storage*

190 Emulsions of β-carotene in canola oil in water were prepared in duplicate from the starch
191 solutions according to methods published elsewhere (Sweedman, Hasjim, Schaefer & Gilbert,
192 submitted). Beta-Carotene 2% w/w was dissolved in Canola oil (food grade) by heating in
193 boiling water bath for 10 min with agitation. The starch solution (pH 6.5 – 7.0) was allowed
194 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).

216 *2.3.2. Nuclear magnetic resonance*

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

226 *2.3.3. Isolation of starch from emulsions*

227 Starch was isolated from emulsions by first combining a 1:3:3 v/v mixture of
228 emulsion:ethanol:hexane, followed by 6 or more sequential washes with water:ethanol:hexane
229 in the same ratio (until a white pellet was retained), based on the method of (Mao, Xu, Yang,
230 Yuan, Gao & Zhao, 2009). Separation at each stage was facilitated by thorough mixing,
231 followed by centrifugation at 3000 *g* for 2 min, after which the liquid layers were removed.
232 Pellets were finally washed twice with pure ethanol and centrifugation, then dried overnight at
233 65 °C.

234 ***2.4. Analytical methods II- Function***

235 *2.4.1. Critical aggregation concentration*

236 CACs of OSA modified starches were determined using methods published previously by
237 (Tizzotti, Sweedman, Schäfer & Gilbert, 2013). Starch solutions of 18 concentrations from
238 0.01 to 10 g L^{-1} were produced by dilution of replicates of each formulation (excluding oil

239 and β-carotene) in water containing 0.04% NaN_3. Concentrations of 20 g L^{-1} were achieved
240 by lyophilization to remove water from the dissolved surfactant, and dissolution in half the
241 original volume of water. Samples were allowed to cool, and then pyrene in ethanol (40.5 mg
242 L^{-1}) was added to a final pyrene concentration of 1×10^{-6} M. After storing in the dark
243 overnight, these samples were analysed in a quartz cuvette at room temperature (23 ± 2 °C)
244 using a RF–5301 PC spectrofluorophotometer (Shimadzu). The emission wavelength and
245 excitation/emission slit were at 390 and 5 nm, respectively. Intensity ratios were plotted
246 against the log of concentration with a linear fit. The data points chosen for the super-CAC
247 linear region reflect the point above which the R^2 value is at a maximum containing at least 4
248 points. The CAC in each case was the point where the super-CAC line reaches the I_{327}/I_{334}
249 (intensity ratio at the indicated wavelengths) equivalent to zero concentration.

250 *2.4.2. Degradation of β-carotene*

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

257 Residual β-carotene was determined using the method in (Sweedman, Hasjim, Schaefer &
258 Gilbert, submitted), which was based on that of (Mao, Xu, Yang, Yuan, Gao & Zhao, 2009).
259 β-Carotene was isolated from emulsions using 4 and 3 mL of hexane and ethanol,
260 respectively, and diluted with hexane as appropriate to achieve concentrations within the
261 linear standard curve of 0 to 2 mg L^{-1}. Standards were prepared from the same β-carotene in
262 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 analysed by spectrophotometer to determine %T at a wavelength of 650 nm, similar to the methods of (Bello-Pérez, Agama-Acevedo, Sánchez-Hernández & 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.

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 gelatinised 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, Tizzotti, Schäfer & Gilbert, 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 Figure 2A. While the method of OSA modification is chosen to minimize starch damage, it does

297 produce minor, inconsistent degradation (Sweedman, Hasjim, Tizzotti, Schäfer & Gilbert,
298 2013; Sweedman, Tizzotti, Schäfer & Gilbert, 2013; Tizzotti, Sweedman, Schäfer & Gilbert,
299 2013). While the native structures of the two starches appear similar (peak R_h ~ 300 – 400
300 nm, Figure 2A, m0 and s0), the waxy maize starch suffered greater degradation as a result of
301 acid hydrolysis (Figure 2A, m0⟶m1) than did sorghum starch (Figure 2A, s0⟶s1). The acid
302 hydrolysis of sorghum starch resulted in an apparent increase in the relative population of
303 larger molecules, which was probably the result of smaller molecules being selectively
304 removed during acid hydrolysis. On the other hand, the waxy sorghum starch alone suffered
305 some degradation during OSA modification (Figure 2A, s1⟶s2), which had the effect of
306 bringing the peak R_h to around 25 nm, coincident with the waxy maize starch before and after
307 OSA modification (Figure 2A, m1 and m2). Hydrolysis during OSA modification can result
308 from the harsh pH modifiers used in the OSA reaction if NaOH is added too quickly, for
309 example; however, only the final structures are of interest here.

310 After the various enzyme treatments and HPH, all samples were degraded in size (Figure
311 2B), whether it was a result of the pre-HPH treatments, or due to shear scission during HPH.
312 The HPH degradation of OS starches of various structures has been examined in (Sweedman,
313 Hasjim, Schaefer & Gilbert, submitted), 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 ~ 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 (Figure 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 (Figure 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 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, Figure 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 (Figure 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 & 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 Figure 3. The results build on those seen in (Tizzotti, Sweedman, Schäfer & Gilbert, 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 the Introduction.

(Varona, Martin & 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 & Milanovic, 2011) reported much lower values of 0.41 to 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, Sweedman, Schäfer & Gilbert, 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 (Figure 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, Figure S3); the results across all samples demonstrated no significant differences. These results are consistent with results reported previously (Sweedman, Hasjim, Schaefer & Gilbert, submitted). (Mao, Yang, Xu, Yuan & Gao, 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 (Figure 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

405 80 and 130 mg L^{-1}. Loss of β-carotene at this stage in processing is through the unstabilized
406 oil phase (which floats to the top of emulsions) and heat damage during HPH; only the former
407 of these is likely to be significantly different between samples. Bam2 and PULL showed the
408 lowest uptake of oil (~80 mg L^{-1}), with Bam1 only slightly higher (~90 mg L^{-1}). In the case
409 of the β-amylase treated samples, the low oil uptake can be attributed to a lower total mass of
410 amphiphilic polymer in the solution, since the maltose released during β-amylolysis probably
411 does not contain OS groups (Sweedman, Hasjim, Tizzotti, Schäfer & Gilbert, 2013), but it is
412 also a result of the significantly weaker emulsion stability as discussed in the next section.

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

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 (Figure 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 (Figure 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, Hasjim, Schaefer & Gilbert, submitted), wherein the difference in DB was the result of differing amylose:amylopectin contents, rather than direct enzymatic alteration 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 (Figure 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, Hasjim, Schaefer & Gilbert, submitted), 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, Figure 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, Zhao, Li, Fu & Dong, 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

503 (Sweedman, Hasjim, Schaefer & Gilbert, submitted). Keeping in mind that the emulsion
504 containing Bam2 broke after 24 h, whether hot or cold, it is not surprising that that sample
505 shows a large droplet size and significant fluctuations in droplet polydispersity index (PDI).
506 The PDI of the droplet size became considerably lower at the higher temperature, possibly
507 because the higher temperatures have an effect on the viscosity of oil itself, thus lowering the
508 threshold for Ostwald ripening effects (Taylor, 1998).

509 In this study, only waxy starches were chosen, so the considerable differences between
510 results can be deliberately linked to the specific structural changes that have been performed.
511 The almost complete breaking of emulsions stabilized by the OS starches treated by β-
512 amylase (Bam1 and Bam2) indicated that DB alone is not a good predictor of surfactant
513 quality, and this position is supported by the results from the PULL sample. For researchers
514 considering starches in general, the relationship between DB and average branch length is
515 almost a natural assumption, but in the case of samples like PULL, where there is an
516 artificially high number of short, linear pieces of OS starch in the sample, the measurable DB
517 is diminished, whereas the average branch length bears more resemblance to the parent
518 amylopectin than to amylose of a similar DB. As such, the present results indicate that short
519 linear components are probably just as effective as branched molecules in stabilizing
520 emulsions. This suggests that the long history of empirical evidence supporting OS
521 derivatives of amylopectin and phytoglycogen (Scheffler, Huang, Bi & Yao, 2010; Scheffler,
522 Wang, Huang, San-Martin Gonzalez & Yao, 2010) surfactant activity over amylose is almost
523 certainly the result of having optimal branch lengths, rather than being explicitly related to
524 DB. Furthermore, when one looks outside the realm of OS starch for branched surfactants,
525 there is evidence that supports linear molecules over branched ones, at least when concerned
526 with the hydrophobic region (Varadaraj, Bock, Valint Jr, Zushma & Brons, 1990; Wormuth &
527 Zushma, 1991). (Varadaraj, Bock, Valint Jr, Zushma & Brons, 1990) ascribed lower foam

528 stability to weaker intermolecular cohesive forces in branched hydrophobes than unbranched
529 ones; on the other hand, (Wormuth & Zushma, 1991) found linear surfactants to stabilize
530 equal parts of oil in water more efficiently than branched ones, an effect that was also
531 proportional to the level of branching. From this information it is possible that the successful
532 emulsion from PULL is explained by the more linear molecules being less hindered
533 intramolecularly than the branches in their intact counterpart WM, thereby allowing greater
534 movement of individual molecules to areas where steric hindrance is useful at the interface.
535 This advantage in pullulanase debranched molecules appears to be enough to counteract any
536 disadvantage as the result of a loss of rigidity compared to the original molecules. (Nilsson,
537 Leeman, Wahlund & Bergenståhl, 2007) reported that higher molecular weight polymers
538 adsorb preferentially to the oil-water interface over their low molecular weight counterparts,
539 meaning PULL might be expected not to perform well considering its high content of smaller
540 molecules; however, (Nilsson & Bergenstahl, 2006) also reported the role of kinetic factors in
541 the colonization of oil droplets by surfactants, which may represent another advantage for
542 smaller, more mobile molecules in the early stages of emulsion formation.

543 # 4. Conclusions

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

551 in emulsification and protection against oxidation. Thus the common use of average branch
552 length can be a misleading criterion for selection of emulsifier.

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

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

565 ## Acknowledgements

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569 the provision of the waxy sorghum grain. This paper is dedicated James Tusitala Sweedman,
570 who was born during experimental work.

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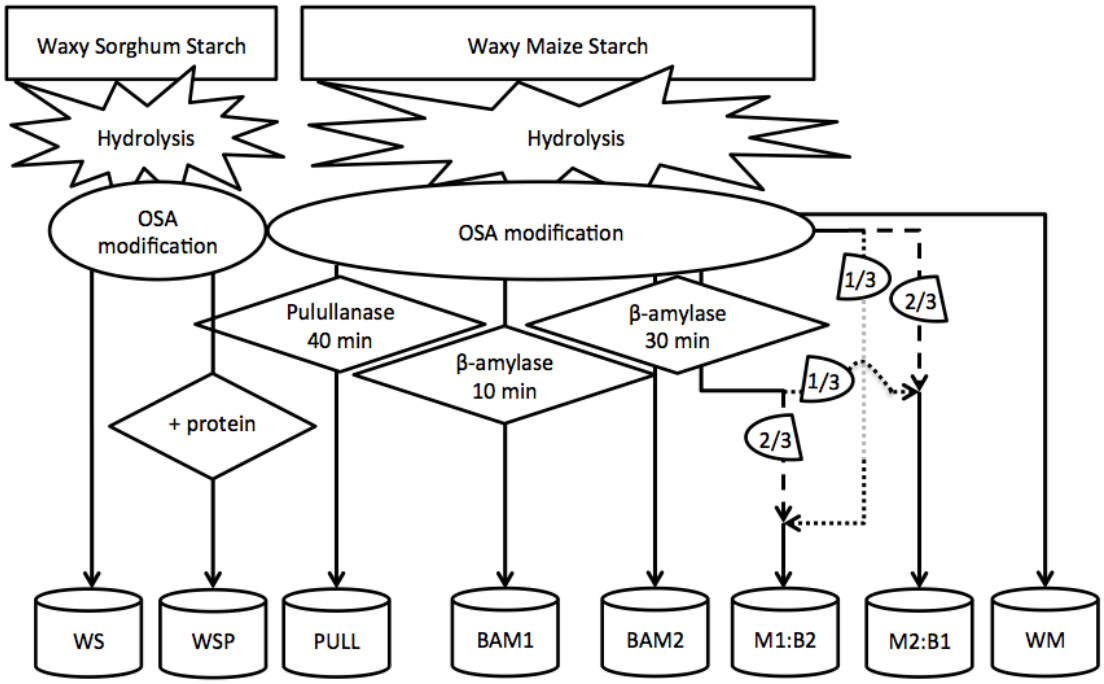
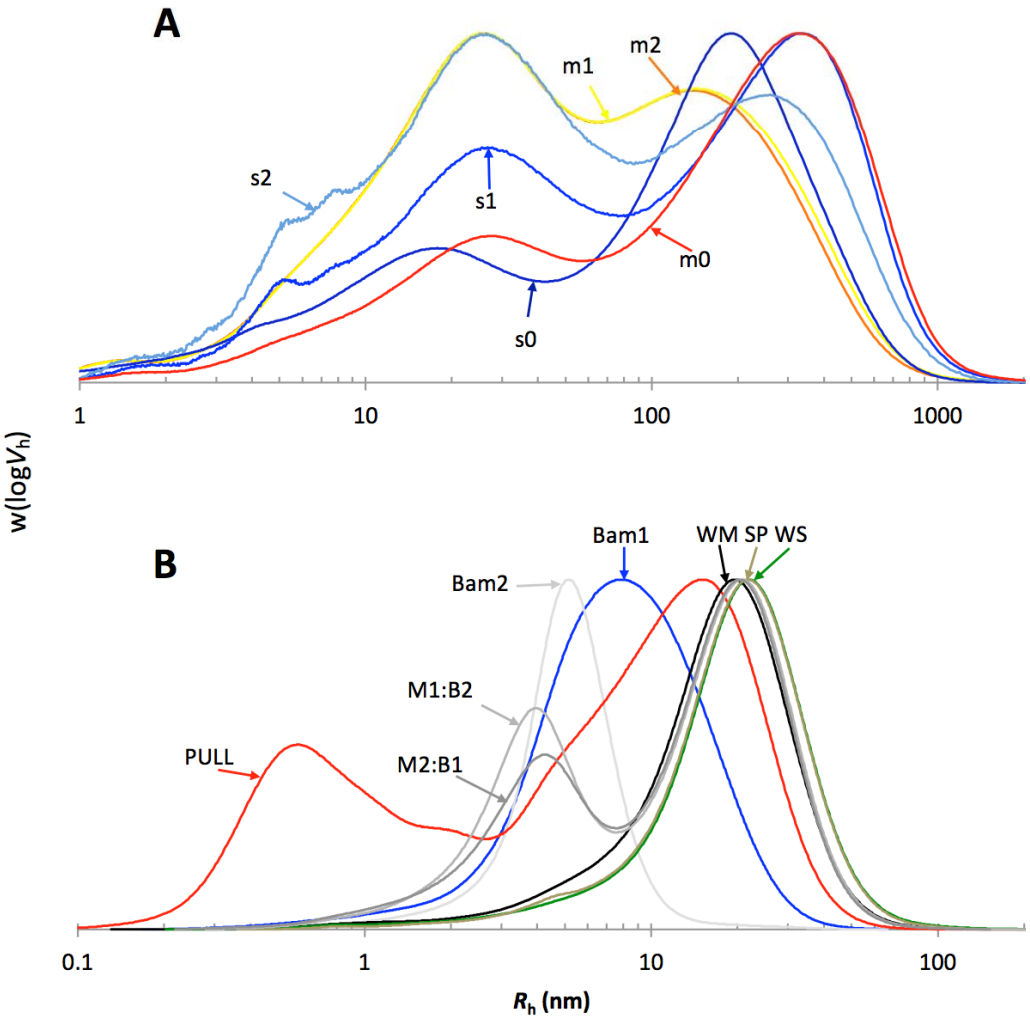
662 Figures



663

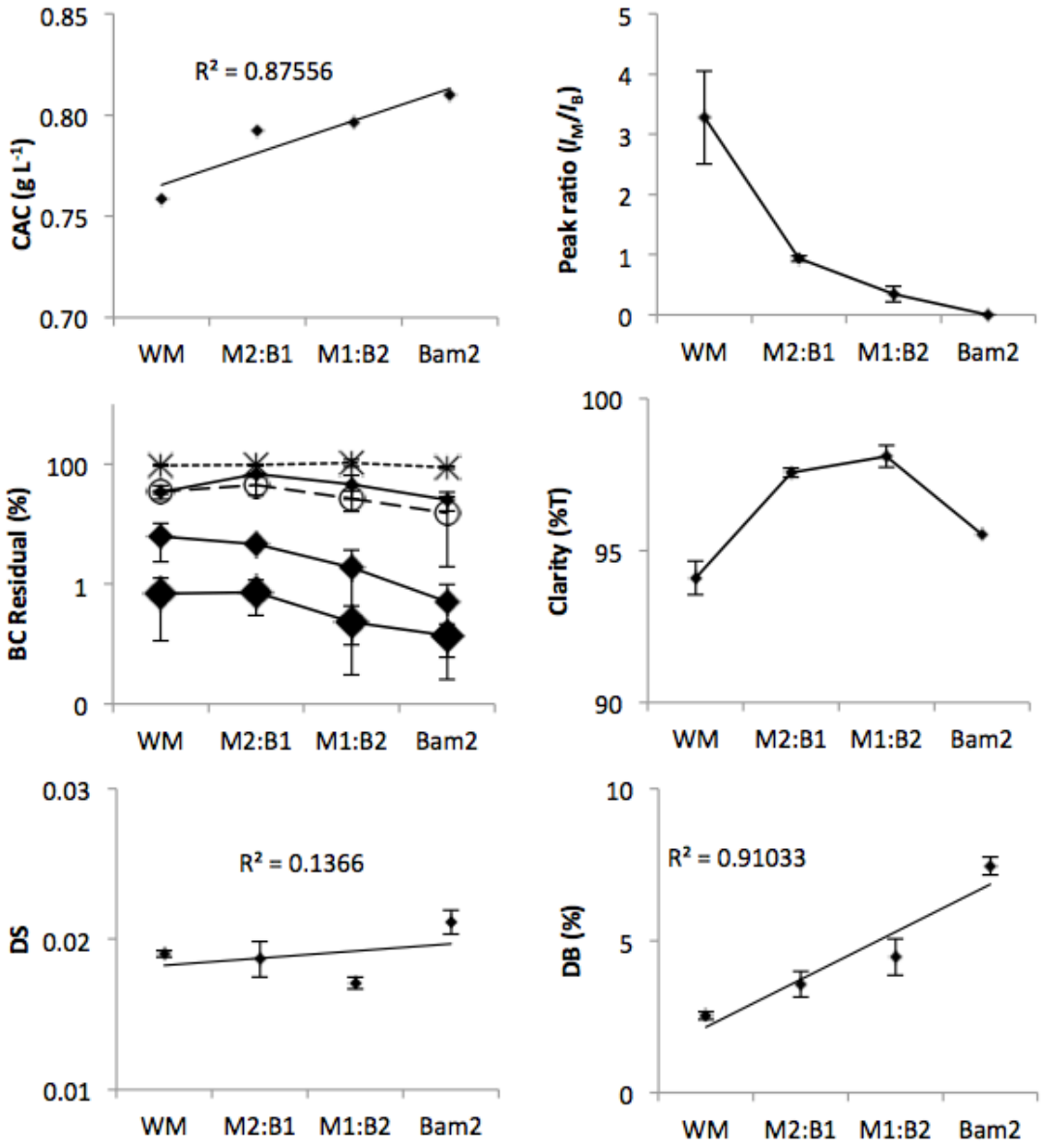
664 Figure 1. Schema of experimental design outlining the 8 surfactant formulations.



665

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

671



672

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

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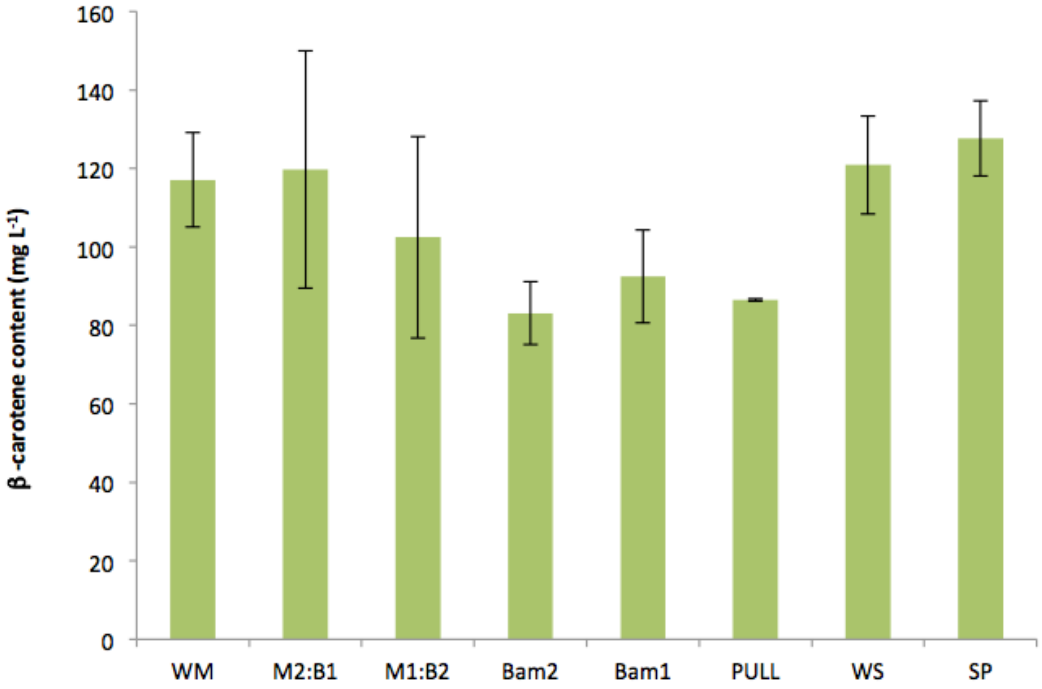

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679 Figure 4. Initial concentrations of β-carotene in emulsions.

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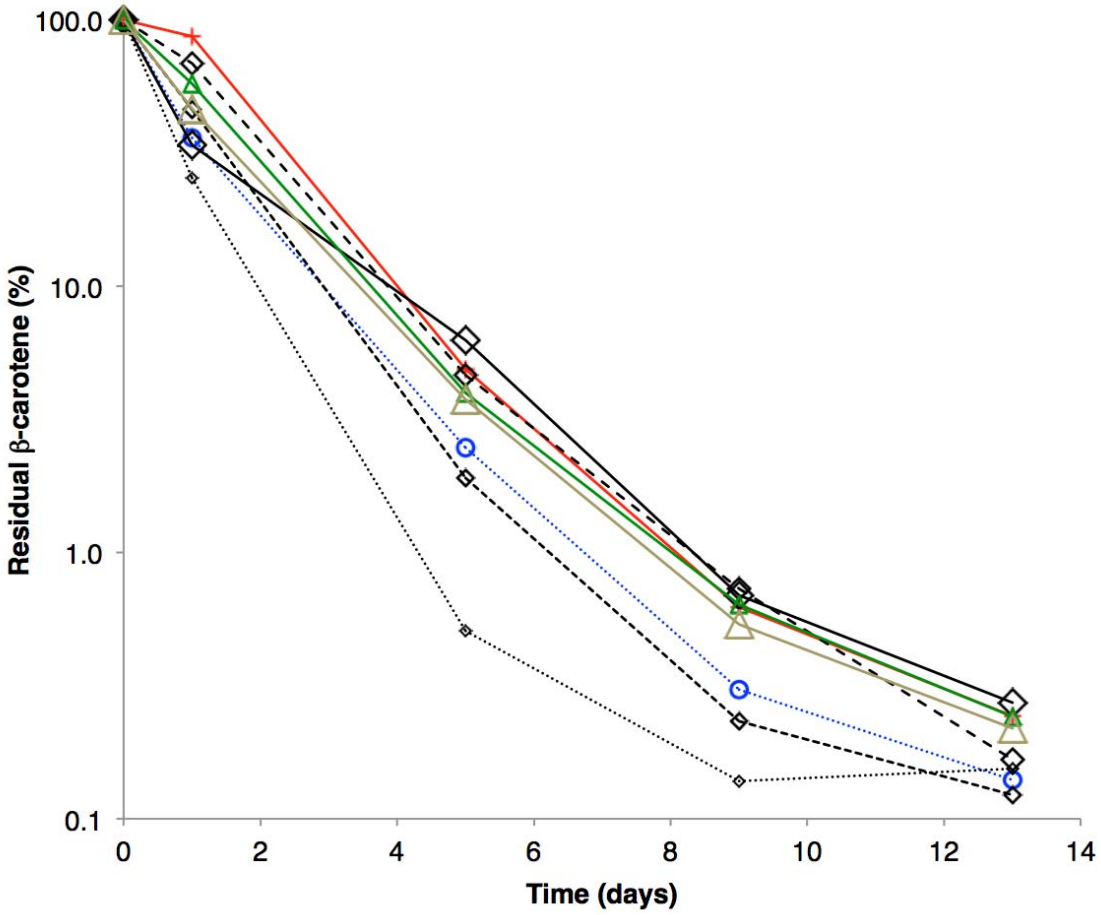

681

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

684

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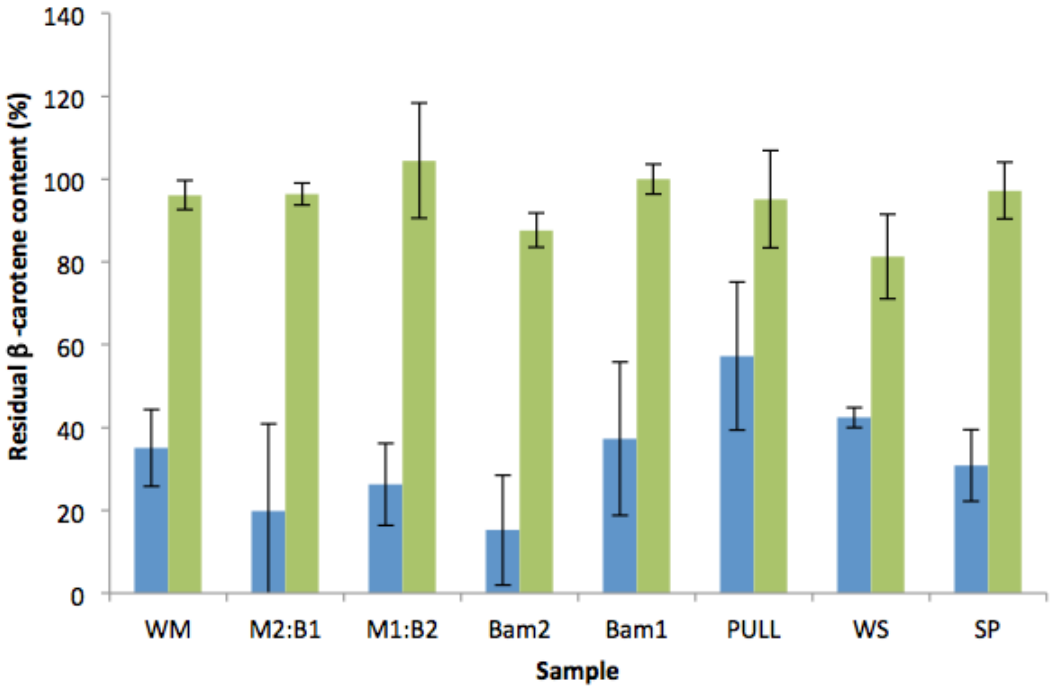
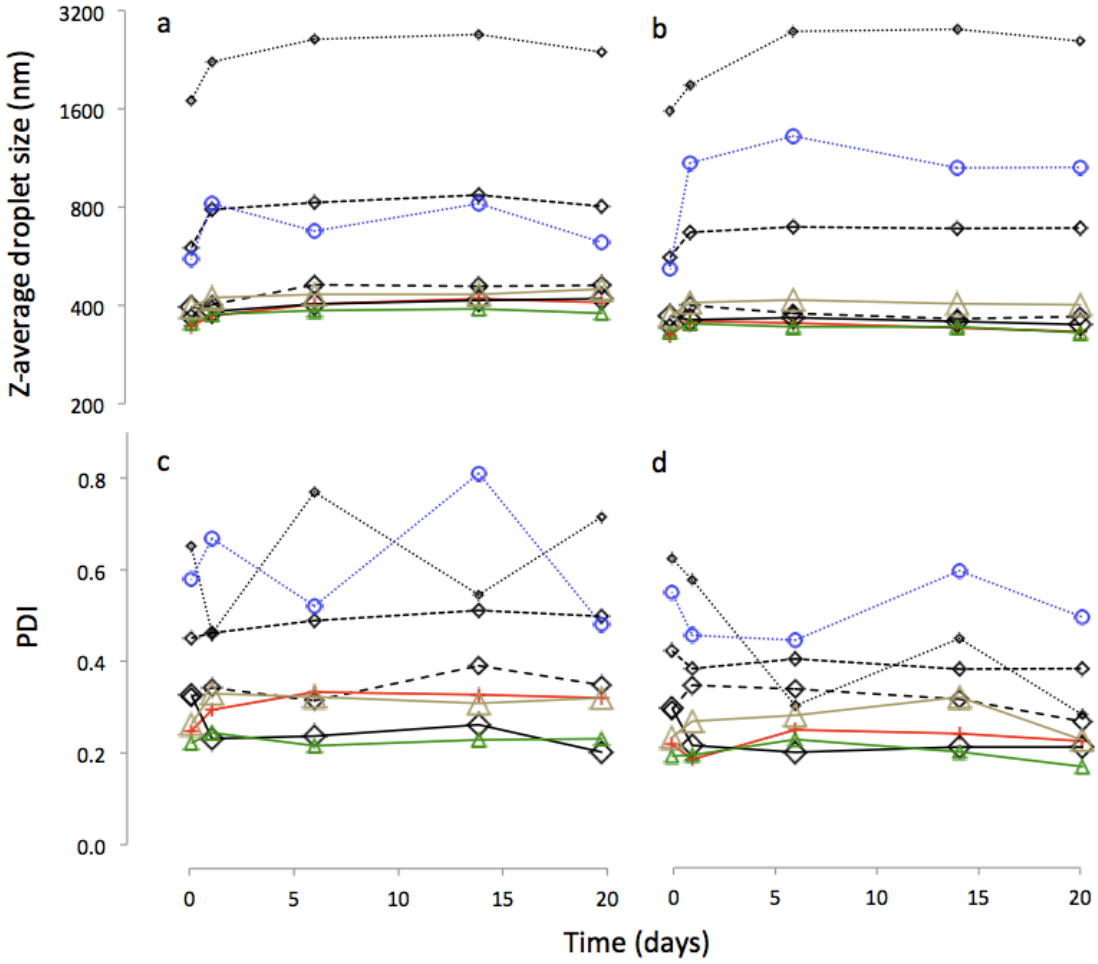


685

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



688

689 Figure 7. Analysis of droplet size in emulsions stored at room temperature (a, c) and at 55
690 °C (b, d). (◇) WM, (◇) M2:B1, (◇) M1:B2, (◇) Bam2, (○) Bam1, (+) PULL, (△) WS, (△) SP.

692

693

694 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)

695 Means ± standard deviations.
696 Superscripts indicate ANOVA significant difference at $p < 0.05$.
697 CAC values correct to r^2 values calculated in excel.
698

698 Highlights:

699 Emulsification properties of OSA starches were examined

700 A range of molecular structures of these starches was used

701 Waxy sorghum starch performs as well as market leading waxy maize

702 Molecular size and chain-length fine structure are important

703 Use of average degree of branching alone can be a misleading criterion

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