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Saki Kokubun [1], Madhav P. Yadav [2, 3], Robert A. Moreau[2], and Peter A. Williams [1]

[1]Centre for Water Soluble Polymers, Glyndwr University, Plas Coch, Mold Road, Wrexham LL11 2AW U.K.

[2]East Regional Research Center, Agricultural Research Service, US Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA

[3]Corresponding author: Telephone: 215-836-3783, Fax: 215-233-6406, E-mail: madhav.yadav@ars.usda.gov.

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Abstract

An emulsion was prepared using corn fibre gum (CFG) and the resulting oil and aqueous phases were separated by centrifugation. The material adsorbed onto the surface of the oil droplets in the oil phase was desorbed using surfactant. The desorbed CFG and the non adsorbed CFG that remained present in the aqueous phase were collected, precipitated using alcohol and freeze dried. Their sugar composition, phenolic acid, lipid and protein contents were determined. There was no consistent difference observed in the sugar composition, phenolic acid and lipid contents of the original material and the adsorbed and non adsorbed fractions. There was, however, a significant difference in the protein contents with the adsorbed fraction containing ~10.7% protein compared to 3.90% and 2.87% for the original and non adsorbed CFG samples respectively. The three samples were also found to have very similar molecular mass distributions and each showed the presence of two peaks using refractive index detection. The major peak, corresponding to ~95% of the total, had a molecular mass of ~ 650,000 g/mol and the minor peak corresponded to a molecular mass of ~ 90,000 g/mol. The corresponding UV elution profiles indicated that the minor peak contained a significant proportion of phenolic and/or proteinaceous material.

1.Introduction

Corn fibre gum (CFG) is producedobtained by an alkaline hydrogen peroxide extraction of corn fibre, which is a low value by-product of the wet or dry milling of corn (Yadav, Johnson & Hicks, 2007; Yadav, Moreau, Hotchkiss, & Hicks, 2012). It is an arabinoxylan consisting of a main chain of β –1,4 linked D-xylopyranose units with α -L- arabinofuranosyl units linked at the 2 or 3 positions. It also contains lesser amount of galactose, glucose, glucuronic acid and rhamnose residues. It has also been shown to contain a small proportion of phenolic acid residues, lipids and proteinaceous material which are present as an integral part of its structure (Yadav, Moreau & Hicks, 2007). The emulsification properties of CFG have been thoroughly studied and its structure function relationship has been reported (Yadav, Johnson

& Hicks, 2009; Yadav, Moreau, Hotchkiss, & Hicks, 2012). These investigatorsworkers prepared orange oil–in–water emulsions with various CFG extracts using a high pressure homogenizer and compared their stability with those prepared with gum Arabic which is the main gold standard emulsifier of choice for such application. The CFG samples were found to have very good emulsification properties, as determined from emulsion stability measurements and were as good or superior to gum Arabic. The emulsion stability, which was determined by turbidity measurements, showed a good correlation with the amount of protein present in the sample (Yadav, Johnson, Hotchkiss & Hicks, 2007). The CFG with higher protein content was a superior emulsifier than the CFG with a lower protein content. The role of protein present in polysaccharide materials in the stabilization of oil-in-water emulsions has recently been reviewed (Evans, Ratcliffe & Williams 2013). For polysaccharides such as gum arabic and pectin, it is generally recognized that the protein facilitates the adsorption of the polysaccharide molecules onto the surface of the oil droplets and that the hydrophilic carbohydrate component protrudes into the aqueous phase providing an electrosteric repulsion and barrier preventing droplet aggregation and coalescence (Randall, Phillips & Williams 1988; Akhtar, Dickinson, Mazoyer & Langendorff 2002; Chee Siew, Williams, Cui & Wang, 2008). The aim of theis present researchpaper is to isolate and characterize the functional groups associated with CFG adsorbed on oil droplets during emulsification process and gain a fundamental understanding of the mechanism by which CFG is able to stabilize oil-in-water emulsions.

2.Materials and Methods

2.1. Materials

(*R*)-(+)-Limonene 97%; sodium dodecyl sulphate (SDS), BioXtra, ≥99.0% (GC); sodium nitrate, ACS reagent; and sodium azide *ReagentPlus*®, ≥99.5%; were obtained from Sigma-Aldrich Chemie GmbH and were used as supplied. Isopropyl Alcohol (IPA), 70% v/v; was

obtained from Fisher Scientific Ltd. and was used as received. The deionised water was obtained from a Pur1 Te Select water purification system. The conductivity of the water was 18.2MΩ.

2.2 Isolation of corn fiber gum

CFG was isolated from de-oiled and de-starched corn fiber following the alkaline hydrogen peroxide technology of Yadav et al., (2007); with some modification. –De-oiled and de-starched corn fiber (50 g) was ~~mechanically~~ stirred using mechanical overhead propeller stirrer with blade (IKA RW 20) into water (1.0 l) and NaOH (12 g or 24 ml from 50% solution) and 42 ml of 30% H_2O_2 were carefully added in an open beaker in a fume hood. The mixture was boiled with efficient mechanical stirring for 1 h. During the reaction, its pH was kept at 11.5 by adding 50% NaOH as needed. After cooling the hot reaction mixture by stirring at room temp for an additional half an hour, it was centrifuged at 6000 × g for 20 min and the supernatant was separated from the residue by decantation. The pH of the alkaline H_2O_2 extract was then adjusted to 4.0-4.5 by adding Conc. HCl to precipitate Hemicellulose A (acid-insoluble arabinoxylan, "Hemi. A"), which was collected by centrifugation at 10,000 g for 30 min. Two volumes of ethanol (2.0 l) were gradually added to the supernatant (1.0 l) with stirring to precipitate the major arabinoxylan fraction, Hemicellulose B, or "Hemi. B", (CFG). The CFG was allowed to settle out as a white flocculent precipitate at the bottom of the beaker for 10-15 min. The clear alcohol/water mixture above the precipitate was removed by decantation. The white flocculent precipitate was transferred into another beaker, stirred in 100% ethanol and filtered under vacuum. The white residue obtained on the Buchner funnel was washed with 100% ethanol and dried in a vacuum oven at 50°C overnight.

2.3. Isolation of the adsorbed and non-adsorbed CFG fractions from its emulsions

30 ml of Limonene oil was added into 100 ml of a 5% solution of CFG (prepared by stirring overnight for making it homogeneous and complete hydration) and mixed for 30 minutes

using a high shear mixer (Silverson L4R, Chesham, UK) to form an emulsion. The adsorbed and non adsorbed CFG fractions from the emulsions were separated according to the method reported by Funami, et al., (2007). In brief, the emulsion was left overnight and centrifuged for 5 hours using a Heraeus Centrifuge Biofuge 28RS at ~~4800 rpm~~ 6,000 x g to separate the oil and aqueous layers. The aqueous layer was separated and poured slowly into 600 ml of IPA in order to precipitate the CFG which had not adsorbed onto the oil droplets. The precipitate was collected and dried at 40 ^{0}C for overnight. The amount of recovered non-adsorbed CFG material was weighed and found to be 64% of the original weight.

The oil phase containing CFG adsorbed onto the oil droplets was separated and added into 100 ml of 5% SDS and mixed for 15 minutes. The system was left overnight to enable the SDS to displace the oil from adsorbed CFG and centrifuged at ~~4800 rpm~~6,000 x g for 3 hours to separate the oil and aqueous layers. The CFG present in this aqueous layer (pH 5.03) was precipitated by pouring slowly into 500 ml of IPA~~isopropanol~~. The precipitate was collected and re-dissolved in 150 ml of distilled water, which was precipitated again by pouring slowly into 400 ml of IPA~~isopropanol~~ to remove any residual SDS present. The resulting precipitated material (CFG adsorbed on oil droplets) was dried at 40 ^{0}C for overnight. The amount of adsorbed CFG (recovered material) was 14% of the original weight of CFG, which was used for emulsification.

2.4. Determination of sugar composition

The sugar composition was determined by HPAEC-PAD using methanolysis combined with trifluoroacetic acid (TFA) hydrolysis (Yadav et al., 2007a) with some modification. In brief, the gum samples to be analyzed were first dissolved in de-ionized water (1 mg/ml). An aliquot of 100 nmoles myo-inositol (internal standard) was added to the gum solution and dried in a Teflon-lined screw cap glass vial by blowing with filtered nitrogen followed by drying in a vacuum oven at 50 °C for overnight. These samples were methanolyzed with 1.5

M methanolic HCl in the presence of 20% (v/v) methyl acetate for 16 h, cooled to room temperature and dried by blowing with filtered N_2 after adding five drops of t-butanol. The methanolyzed samples were hydrolyzed with 0.5 ml 2M-_TFA at 121°C for 1 h, evaporated by blowing with filtered N_2 at 50 °C and the residue was washed by sequential addition and evaporation of three aliquots (0.5 ml) of methanol. In four separate glass vials were placed 100, 300, 500 and 1000 nmoles of a mixture of standard sugars containing fucose, arabinose, rhamnose, galactose, glucose, xylose, glucuronic acid and galacuronic acid. Then, 100 nmoles of myo-inositol (internal standard) was added to each vial, evaporated and dried as above. These standard samples were also methanolyzed and hydrolyzed as described above and used for quantification.

Hydrolyzates were analyzed for neutral and acidic sugars by HPAEC-PAD using a Dionex DX-500 system that included a CarboPac PA20 column and guard column, a GP 50 gradient pump, an ED40 electrochemical detector utilizing the quadruple potential waveform (gold working electrode and pH reference electrode), an AS3500 autosampler with a thermal compartment (30EC column-heater), and a PC10 pneumatic controller post column addition system. The mobile phase consisted of isocratic 12 mM NaOH eluant for 10 min followed by 100 mM NaOH and 6 mM CH_3COONa for 3 min, 100 mM NaOH and 12 mM CH_3COONa for 17 minutes at a flow rate of 0.5 mL/min. at ambient temperature. The column wash with 1 M CH_3COONa for 0.10 min and 100 mM NaOH for 10 min followed by 30-min equilibration with 12 mM NaOH at a flow rate of 0.5 mL/min at ambient temperature was required to yield highly reproducible retention times for the monosaccharides. The total run time was ca. 70 min. In order to minimize baseline distortion due to change in pH of the eluant during monosaccharides detection by PAD, 730 mM NaOH was added to the postcolumn effluent via a mixing tee.

2.4. Phenolic acid and lipid content

CFG samples were extracted with hexane to remove non-covalently associated oils and/or lipids and the phenolic acids and hexane extractable contents were determined by HPLC as described previously (Yadav, Moreau, & Hicks, 2007b).

Defatted samples were placed in 50 ml screw cap glass tubes. 5 ml of 1.5M Methanolic KOH and 250 µL~~ul~~ H_2O were added to each sample. The tubes were capped and immersed in a 70°C water bath for 1 hour (removing to mix every 5 or 10 minutes). Then the samples were allowed to cool to room temperature.

11 mL of Methanol (totalling 16 ml MeOH) and 8 mL $CHCl_3$ were added. The samples were inverted 30 times to mix well. The samples were then filtered using vacuum filtration through a Buchner funnel with Whatman GF/A paper. Solvent was collected and any particulates seen were discarded. The mixture was acidified with 1.5M HCl to about pH2, and 2.75 ml of H_2O and 8 ml of $CHCl_3$ were added. The mixture was inverted 30 times to mix well. Then it was allowed to separate into phases by centrifuging at 70 x g for 10 minutes. The lower phase ($CHCl_3$) was removed, collected and dried under N_2~~2~~ and heat to obtain a rough dry weight of the hydrolyzed extract. The samples were then dissolved in 1 ml of 85:15 chloroform: methanol, filtered through a glass wool filter, and evaporated under a stream of N_2 to measure its mass accurately. After weighing, the lipid sample was redissolved in 85:15 chloroform: methanol and analyzed by HPLC with UV detection and Evaporative Light Scattering detection, as previously described (Yadav, Moreau and Hicks, 2007).

2.5. Protein content

The protein (N x 6.25) content was determined using "AACC Approved Method, 46-30" (AACC International, 2000).

2.6. Molecular mass distribution

178 The molecular mass distributions of the CFG samples were determined using gel
179 permeation chromatography (GPC). The GPC system consisted of Suprema column
180 (dimensions 300 mm x 8 mm; Polymer Standards Service GmbH) packed with 10 micron
181 beads of 3000 A pore size and protected by a Guard column containing 10 micron beads
182 (Polymer Standards Service GmbH~~: 10 microns~~).The eluent used was 0.05 M sodium nitrate
183 containing 0.005% sodium azide which was filtered with a GSWP 0.45 µm filter/ Millipore
184 filter and degassed using (v~~V~~acuum degasser (CS 1615/Cambridge Scientific Instrument,
185 Ltd) before use. The samples (0.15%) were dissolved in a 0.45 µm filtered aqueous solution
186 of 0.05 M sodium nitrate and left tumbling for overnight at 25 ^{o}C to fully dissolve. The flow
187 rate was set at 0.5 mL per minute using a Waters Corporation 515 HPLC pump and the
188 injection loop volume was 200 µL (Rheodyne model: 7.125). A Dawn® DSP Laser
189 Photometer (Wyatt Technology Corporation), OPTILAB DSP Interferometric Refractometer
190 (Wyatt Technology Corporation) and Agilent 1100 series (Agilent Technologies) UV
191 spectrometer (wavelength 280 nm) were used as detectors. The samples were passed
192 through a 0.45 micron pore size nylon syringe filter before being injected onto the columns.
193 Measurements were performed in duplicate. The molecular weight was determined using
194 Astra for Windows 4.90.08 QELSS 2.XX. The Berry model was used for evaluating all
195 analyses. A value of 0.135 ml/g was used for the refractive index increment (dn/dc).

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197 **3. Results and discussion**

198 The sugar composition and the phenolic acid, lipid, and protein contents of the original CFG,
199 the non adsorbed CFG and the adsorbed CFG are presented in Tables 1-4 respectively.
200 Table 1 shows that all three samples have similar sugar compositions consisting mainly ~~of~~
201 arabinose (~30%) and xylose (~47%), which is consistent with earlier findings (Yadav,
202 Johnston, Hotchkiss & Hicks, 2007c). Table 2 shows that all three samples contain ferulic
203 acid and a smaller amount of p-coumaric acid. The total amount~~content~~ of these phenolic

acids in the adsorbed and non adsorbed CFG is less than the original sample, probably due to loss of ~~because~~ some material ~~was lost~~. This loss of material might have ~~has probably~~ occurred during the separation and precipitation stages of their recovery process. The lipid content for the samples is presented in Table 3 and the data show that the total amount of lipid is lower in the adsorbed CFG compared to the original and non adsorbed samples. This suggests, therefore, that both phenolic acids and lipids may not play a major role in the adsorption process. However, it should also be noted that the total amount of lipid is very small (0.017 %, w/w) and although the samples were extracted using hexane to remove non covalently attached lipid material, we cannot rule out the presence of small amounts of SDS that was used to desorb the CFG from the surface of the oil droplets. The amount of protein in the samples is presented in Table 4. The amount present in the original and non adsorbed CFG was determined using the AACC approved Kkjeldahl method and the amount present in the adsorb CFG was determined by difference from the mass balance calculation. The higher amount of protein in the adsorbed material supports the previous experimental results reported for CFG samples, which showed that the emulsification stability increased with increasing protein content of CFG samples (Yadav, Johnston, Hotchkiss, & Hicks, 2007). It is also consistent with the investigations done ~~other work~~ on other polysaccharides, notably, gum aArabic and pectin, which have shown~~reported~~ that protein-rich fractions within the polysaccharides are responsible for their superior emulsification properties (Evans, Ratcliffe & Williams, 2013).

The sizes of the arabionxylan molecules in the three CFG samples, measured by GPC, are given in Figure 1A. It is evident that the molecular profiles of all three ~~for each~~ samples are very similar, each showing a main peak (which accounts for ~95% of the total material) with a peak maximum at an elution volume of ~8.5 mL and a much smaller minor peak with a peak maximum at an elution volume of ~12.5 mL. These peaks were found to correspond to Mw values of ~65,000 g/mol and ~ 90,000 g/mol respectively. But t~~T~~he UV elution profiles, which are presented in Figure 1B ~~and~~ show some differences. It is noted that for the original

CFG sample there is a large peak eluting at ~8.5 mL (labelled Peak 1) ~~which~~ correspondings to the major peak detected~~noted~~ by RI. However, there is ~~also~~ a significant peak eluting in the region 10-13 mL (labelled Peak 2) roughly corresponding to the minor RI detected peak noted above. It is assumed that material eluting in this region is rich in protein and/or phenolic components which have a high absorbance at the wavelength of the detector used. The magnitude of this minor peak is considerably reduced for the adsorbed and non adsorbed CFG samples and very clearly supports the comments made above that some material rich in phenolic compounds~~material~~ has been lost ~~on~~during the recovering process of these fractions. As reported previously, it is worthwhile mentioning that when the elution profiles of GPC is monitored by UV spectrophotometer, it does not give a quantitative assessment of the molecular mass distribution due to variation in the molecular absorptivities of the different chemical species present (Randall, Phillips & Williams, 1989b). The peak 2, which accounts for only about 5% of the total original material, looks rich in protein, when we compare its peak area in Figure 1A with UV detection peak in Figure 1B. ~~It is likely that the protein present in peak 1B contains more hydrophilic amino acids and less hydrophobic amino acids.~~ ~~But~~It is likely that protein present in peak 1A contains more hydrophobic amino acids in comparison to hydrophilic amino acids giv~~b~~ing these high molecular weight molecules more hydrophobicity than the low molecular weight molecules to adsorb on the oil droplets. The figure 1B also indicates that though the overall percent of protein in the high molecular weight peak 1 of all three samples is low in comparison to peak 2, the early eluting portion of the peak 1 in adsorbed CFG has more protein than the nonadsorbed and original CFG. It is very clear from this part of the figure that the early eluting, very high molecular weight molecules (which are eluting before the highest peak point of peak 1) in the adsorbed CFG have more protein than the later eluting lower molecular weight molecules in the same peak. This finding supports the earlier results, which showed that the CFG molecules with the highest molecular weight and rich in protein were superior emulsifier than the molecules with less protein (Yadav, Johnston, Hotchkiss & Hicks, 2007). Thus both the high molecular weight and protein content in CFG play an

259 important role during emulsification process. From all these results, it looks very obvious that
260 like gum arabic (Randall, Phillips & Williams, 1988) a few percent of very high molecular
261 weight CFG are rich in protein and they are very active emulsifiers.

262

263 **4. Conclusions**

264 CFG is very effective for stabilising limonene oil-in-water emulsions. The adsorbed CFG
265 fraction has been found to contain a significant amount of proteinaceous material and it is
266 believed that this facilitates the adsorption of the molecules onto the surface of the oil
267 droplets and is responsible for its emulsification properties. The high molecular weight CFG
268 molecules, which are rich in protein, are the most active emulsifier.

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Table 1 Sugar Composition of the CFG samples (Relative Mole %)

Samples	Fuc	Rha	Ara	Gal	Glc	Xyl	GalA	GlcA	Total
CFG (Original) Analysis # 1	0.09	1.55	31.22	5.94	4.08	47.56	3.31	6.25	100.00
CFG (Original)-Analysis # 2	0.11	1.43	32.10	5.75	3.96	47.57	2.86	6.23	100.00
CFG-Adsorbed, Analysis # 1	0.09	1.68	29.94	6.30	4.63	47.44	3.79	6.13	100.00
CFG-Adsorbed, Analysis # 2	0.09	1.45	32.68	5.29	3.90	47.58	3.34	5.67	100.00
CFG-Non-Adsorbed, Analysis #1	0.12	1.75	29.88	6.17	4.38	47.49	3.40	6.81	100.00
CFG-Non-Adsorbed, Analysis # 2	0.11	1.55	30.39	5.98	4.22	48.11	3.10	6.55	100.00

Abbreviations; Fuc, fucose; Rha, rhamnose: Ara, arabinose; Gal, galactose; Glc, glucose; Xyl, xylose; GalA, galacuronic acid; GlcA, glucuronic acid.

Table 2. Phenolic acid content of the CFG samples (µg/g) before and after saponification

Samples	Ferulic acid	p-Coumaric acid	Total Phenolic acids
CFG (Original) before saponification	0.02	0.02	0.04
CFG(Original) after saponification	45.38	3.81	49.19
CFG (Adsorbed) before saponification	0.01	0.01	0.02
CFG (Adsorbed) after saponification	13.99	3.07	17.06
CFG (Non-adsorbed) before saponification	0.01	0.01	0.02
CFG (Non-adsorbed) before saponification	4.02	1.22	5.24

Reported in µg/g of dry weight of sample

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355 **Table 3 . Lipid content of the CFG samples (µg/g) before and after saponification**

Samples					
	Palmitic/ Stearic Acids 3.2 min	Unknown 1 4.5 min	Unknown 2 6.5 min	Unknown 3 7.4 min	Total
CFG (Original) before saponification	13.70	0.63	0	1.36	15.69
CFG (Original) after saponification	112.98	30.9	7.57	16.80	168.33
CFG (Adsorbed) before saponification	15.66	3.59	0.18	2.87	22.30
CFG (Adsorbed) after saponification	61.87	13.17	0.55	20.62	92.12
CFG (Non-adsorbed) before saponification	15.98	0	0	0	15.98
CFG (Non-adsorbed) after saponification	105.59	15.08	2.02	17.59	140.08

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373 **Table 4 Protein content of the original CFG and its fractions**

Sample	Protein %w/w
CFG (Original)	3.90*
CFG (Adsorbed)	10.7**
CFG (Non-adsorbed)	2.87*

374 **determined by kjeldahl; **determined by mass balance calculation*

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Figure 1. GPC elution profiles of the three CFG samples using (A) RI detection for total mass and (B) UV detection at 280 nm to estimate phenolic acids and proteins.