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Gum Arabic that is Enzymatically Modified with Arabidopsis Beta-Glucuronosyltransferase Can Make Smaller and more Stable Oil-in-Water Emulsions

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Abstract: A plant glycosyltransferase was utilized to modify the properties of gum arabic as an oil-in-water emulsifier. We previously reported that recombinant beta-glucuronosyltransferase (AtGlcAT14A) from *Arabidopsis thaliana* produced in *Pichia pastoris* possesses glucuronosyltransferase activity to transfer glucuronic acid (GlcA) from UDP-GlcA to beta-1,3-galactan main chain and beta-1,6-galactan side chains of type II arabinogalactan. In this paper, we report that AtGlcAT14A can also transfer GlcA from UDP-GlcA to gum arabic at the optimal pH value of 5 in the absence of dicationic ion. In the modified gum Arabic, GlcA was primarily incorporated into the beta-1,6-galactans. The oil-in-water emulsions created by the modified gum arabic were smaller, less flocculated and threefold more stable than that produced by the unmodified gum arabic. It is conceivable that the additional GlcA on the surface of gum arabic prevents flocculation by increasing surface electrostatic repulsion, which leads to more stable oil-in-water emulsions. Our study implicates the structure-function relationship and provides a potential method for the enzyme-based manipulation of gum arabic.

Key words: Gum arabic, arabinogalactan-protein, glucuronosyltransferase, glycosyltransferase, oil-in-water emulsions, enzymatic modification.

1. Introduction

Gum arabic (Acacia gum, E414) is an exudate plant gum that is obtained from the stems and branches of *Acacia senegal* and *A. seyal* trees upon wounding [1]. Gum arabic is one of the food hydrocolloids that are commonly used as an emulsifier, stabilizer, thickener and flavor encapsulator, and its use is particularly common in soft drinks and confectionaries [2, 3]. Specifically, gum arabic is superior to other plant gums as an emulsifier for producing oil-in-water emulsions, particularly in beverages because of its highly soluble properties and the viscosity of its solution (< 40% (w/v)) increase significantly[1, 4]. In recent years, gum arabic has attracted even more attention because it serves as a prebiotic and a dietary fiber that helps to reduce caloric intake [2, 5]. Furthermore, gum arabic is also widely used in the non-food industries including cosmetics, textiles, ceramics and lithography [6].

The structure of gum arabic is a heterogeneous mixture comprised of three different fractions; i.e., highly branched polysaccharides (type II arabinogalactans, type II AGs), AGP (arabinogalactan-protein), and a glycoprotein fraction, which contribute approximately 88.4%, 10.4% and 1.2% of the total weight of the gum, respectively [7-9]. Type II AG is commonly composed of a β-1,3-galactan main chain substituted at the *O*6 position with β-1,6-galactan side chains, which can then be further substituted by arabinose and its oligosaccharides and less frequently with other sugars; i.e., (4-*O*-methyl)-glucuronic acid and rhamnose [10-12]. The protein portion of the AGP is rich in hydroxyproline in which the β-1,3-galactan main chain of AG is attached, via an *O*-glycosidic linkage between reducing end of β-1,3-galactan and

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thehydroxyl group of hydroxyproline.

The AGP is considered to be the component that responsible for the oil-in-water emulsifying properties of gum arabic because the amount of protein adsorbed to the surface of the oil droplets is equal to the amount of AGP present in gum arabic and because proteolytic degradation abolishes the emulsifier function of gum Arabic [13]. To function as an emulsifier, the hydrophobic protein and hydrophilic carbohydrate parts are required to adsorb oil droplets to the surface and to block protrusion into the solution, respectively [13]. The carbohydrate component is believed to provide a strong steric barrier against flocculation and coalescence [1, 14]. Several physical and chemical approaches have been applied to improve the emulsion properties of gum Arabic [15-20]; however, only one enzymatic approach, which improved the oil-in-water emulsifying function of the gum via treatment with β-galactosidase, has been reported [21].

We recently reported a recombinant AtGlcAT14A (Arabidopsis β-glucuronosyltransferase) that was expressed in *Pichia pastoris* [22]. AtGlcAT14A belongs to the Carbohydrate Active Enzyme GT (glycosyltransferase) family 14 [23] and catalyzes the transfer of GlcA (glucuronic acid) to both β-1,3-galactan main chains and β-1,6-galactan side chains in type II AG [22]. In this paper, we reported that the treatment of gum arabic with recombinant AtGlcAT14A in the presence of UDP-GlcA improved the emulsifier properties of gum arabic in the production of oil-in-water emulsions under the tested condition. We discussed probable structural changes and mechanism of functional improvement.

2. Experiments

2.1 Materials

UDP-GlcA (Uridine diphosphate-α-D-glucuronic acid), β-glucuronidase type II from *Helix Pomatia*, gum arabic from the Acacia tree, and anti-FLAG agarose were purchased from Sigma Aldrich (St. Louis, MO). UDP-α-D-[^{14}C]-GlcA was purchased from PerkinElmer (Boston, MA). Arabinogalactan from Larch wood and α-arabinofuranosidase were purchased from Megazyme (Wicklow, Ireland). Orange oil (*Citrus sinensis*) was purchased from Urtegaarden (Allingåbro, Denmark). Exo-β-1,3-galactanase from *Phanerochaete chrysosporium* [24] and endo-β-1,6-galactanase from *Streptomyces avermitilis* [25] were kind gifts from Dr. Satoshi Kaneko of the National Food Research Institute, Tsukuba, Japan.

2.2 Recombinant Enzyme Preparation

The cloning, production, purification, and protein analyses of the recombinant AtGlcAT14A in *Pichia pastoris* have been described previously [22].

2.3 Radioactive Enzymatic Activity Assay

To evaluate the use of gum arabic and Larch arabinogalactan as acceptor substrates (total volume: 25 μl), the reaction was performed in the presence of 0.1 mM UDP-GlcA (containing 296 Bq UDP-[^{14}C]-GlcA), 28 mM McIlvaine buffer [26], pH 5.0, 5 μl affinity-purified AtGlcAT14A on the anti-FLAG agarose slurry and 7.5 μl of 100 mg/ml gum arabic or Larch arabinogalactan (22 ^{o}C, 900 rpm, overnight). The [^{14}C-]-GlcA incorporated polysaccharide products were precipitated in 70% ethanol by the addition of 1 ml cold 70% ethanol and maintained at −20 ^{o}C prior to centrifuge at 15,000g for 12 min. The pellets were collected and washed three times with 70% ethanol prior to scintillation counting.

Analyses of the pH activity optima of AtGlcAT14A toward gum arabic and Larch arabinogalactan were determined for the pH range of 3-10 in 56 mM Britton-Robinson buffers [27] without $MnCl_2$ using the condition described above.

2.4 Product Analysis via Hydrolase Treatment

To evaluate the incorporation of [^{14}C]-GlcA into gum arabic, the ethanol-precipitated [^{14}C]-GlcA incorporated gum arabic produced by the reaction of AtGlcAT14A was incubated with 0.02 U

exo-β-1,3-galactanase, 0.0022 U endo-β-1,6-galactanase, 0.08 U α-arabinofuranosidase, and/or 3.4 U β-glucuronidase either as a single enzyme or combined enzyme mixture (as indicated in Fig. 2) in 80 mM McIlvaine buffer [26], pH 5.0 (900 rpm, 37 ^{o}C, overnight). The hydrolyzed products were filtered (Ultrafree-MC Centrifugal Filter Units Durapore PVDF membrane 0.2 μm, Millipore, Billerica, MA) and applied to a Superdex peptide HR 10/30 (GE Healthcare) equilibrated with 50 mM ammonium formate (flow rate: 0.4 ml/min, 2 min/fraction). The radioactivity eluted in each fraction was evaluated by scintillation counting.

2.5 Modified Gum Arabic Preparation

The reaction was performed in the presence of 0.8 mM UDP-GlcA (without UDP-[^{14}C]-GlcA), 28 mM McIlvaine buffer, pH 5.0, 10-20 μl affinity-purified AtGlcAT14A on the anti-FLAG agarose slurry [22] and 15-30 μl of 100 mg/ml gum arabic or Larch arabinogalactan (22 ^{o}C, 900 rpm, overnight) in a total volume of 50-100 μl. To remove the agarose slurry, the reaction was set for 5 min at room temperature, and the modified gum arabic solution in the supernatant was collected by decantation and sedimented with 70% ethanol precipitation. The pellets were collected and stored at -20 ^{o}C prior to emulsion preparation. For the unmodified gum arabic, the solution was precipitated with 70% ethanol and stored in the same conditions.

2.6 Preparation of Oil-in-Water Emulsions

The emulsion preparations were performed according to [21] with some modifications. For the stock solutions, native or AtGlcAT14A-modified gum arabic (24%, w/v) was prepared by dissolving in deionized water and mixing until the solution was homogenously dissolved. For the emulsion preparations, 850 μl 24% modified or unmodified gum arabic solution, 145 μl orange oil, 5 μl 50% citric acid and 2 μl 10% sodium azide (as a antimicrobial and not as a food additive) were mixed by vortexing in a 2 ml Eppendorf tube followed by homogenization using an Analog 250 ultrasonic homogenizer (Branson Ultrasonics, Danbury, CT) equipped with a 1/8" Tapered Microtip. The homogenizer was set to a power level of 3 with 30% duty cycle dial and performed for 20 s three times with 15 s pauses on ice (these parameters were designed for small-scale emulsion testing and not for comprehensive evaluation).

2.7 Microscopy and Particle Size Distribution

Microscopic images of the emulsions were studied using an Olympus BX41 Microscope (Tokyo, Japan) equipped with a Colorview Soft Imaging System (Münster, Germany). The images of the freshly prepared emulsions (day 0) were taken within 15 min of the preparation and subsequently every 24 h following storage at room temperature. Approximately 50 μl of the solution containing the emulsions was diluted ten to twenty fold (five-fold for the fourth day) and placed on the microscope slide. A coverslip was placed on the sample and, after ensuring that no air bubbles were trapped between the sample and the coverslip, the sample was examined with a 60X objective via the collection of 10-20 images. [28-30].

The particle size distributions of the emulsions were analyzed using ImageJ software [31]. The images were converted to 8-bit prior to background subtraction (with a 10-pixel rolling ball radius), brightness and contrast adjustments, and thresholding. The signals with circularities between 0.60 and 1.0 were defined using the "analyze particles" and "area distribution" functions available in ImageJ software. The inside areas of particles were recorded for the 10-18 images.

3. Experimental Results

3.1 Glucuronosyltransferase Activity of AtGlcAT14A towards Gum Arabic

We previously reported that the soluble catalytic domain of Arabidopsis β-glucuronosyltransferase (AtGlcAT14A) expressed in *P. pastoris* catalyzes the transfer of GlcA from UDP-GlcA to both β-1,3- and

β-1,6-galactooligosaccharides of various lengths that are present in AGP [22]. In this paper, we tested the potential of AtGlcAT14A to transfer GlcA to the commercial AGP, i.e., gum arabic. To distinguish between the existing and the additional GlcAs on the AGP molecule, we used UDP-[^{14}C]-GlcA as donor substrate in the reaction. Approximately 15 pmol of [^{14}C]-GlcA was incorporated into gum arabic by the recombinant AtGlcAT14A at the optimal pH of 5 (Fig. 1). In these conditions, we used 0.1 mM UDP-GlcA to estimate that approximately 3 mol of GlcA were incorporated onto each molecule of gum arabic (based on the assumption that the number average molecular weight of gum arabic is 250,000 g/mol, Sigma Aldrich). AtGlcAT14A also catalyzed the transfer of GlcA to another commercial arabinogalactan (Larch arabinogalactan) at a slightly lower level when the same amount was used (Fig. S1).

To confirm the incorporation and investigate the possible sites of GlcA incorporation into the gum arabic, the [^{14}C]-GlcA-incorporated gum arabic was treated with the AG-specific hydrolases and analyzed using size exclusion chromatography (Fig 2(a)). The exo-β-1,3-galactanase from *Phanerochaete chrysosporium* can cleave the β-1,3-galactan backbone and bypass the side-chain substitutions [24], while the endo-β-1,6-galactanase from *Streptomyces avermitilis* can hydrolyze β-1,6-linked galactooligosaccharides with DP (degrees of polymerization) above 3 [25]. Because the substitution of arabinose on β-1,6-linked galactooligosaccharides sterically hinders the action of endo-β-1,6-galactanase, the analysis of the β-1,6-linked galactooligosaccharides was performed via co-treatments of α-arabinofuranosidase and endo-β-1,6-galactanase. The exo-β-glucuronidase from *Helix pomatia* hydrolyzes β-linked GlcA in an exo fashion from the non-reducing end, and this process was used to confirm the β-linkage formed by Glucuronosyltransferase. The expected sites of action of the hydrolases used in the present study are shown in Fig. 2(b). Treatment of the [^{14}C]-GlcA-incorporated

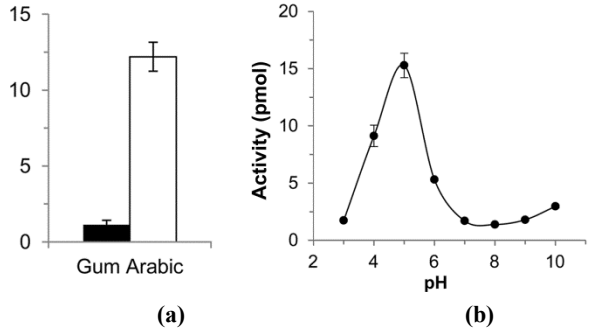

Fig. 1 Glucuronosyltransferase activity of AtGlcAT14A toward gum arabic. (a) Comparison of the activities of AtGlcAT14A (□; 38.5 ng protein) and the empty pPICZαA vector (●) in the presence of UDP-[^{14}C-]-GlcA, the error bars show the standard deviations (n = 4); (b) glucuronosyltransferase activities of AtGlcAT14A at different pH values. The error bars show the standard deviations (n = 2).

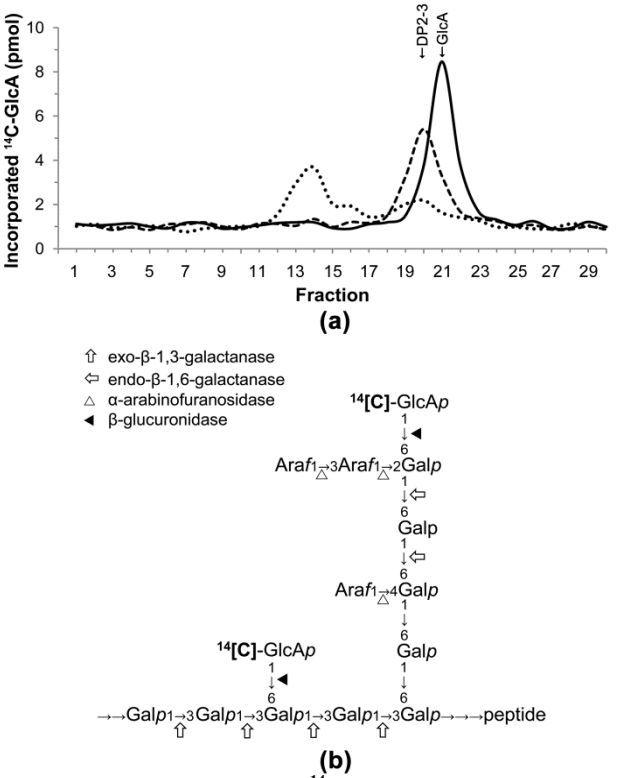

Fig. 2 Product analysis of [^{14}C]-GlcA incorporation into gum arabic by size exclusion chromatography and a simplified model of the AGP with the sites of action of the AG-specific hydrolases. (a) Treatments with exo-β-1,3-galactanase (···), endo-β-1,6-galactanase + α-arabinofuranosidase (- - -), and exo-β-1,3-galactanase + endo-β-1,6-galactanase + α-arabinofuranosidase + β-glucuronidase (—); (b) a simplified model of the AGP illustrating the sites of action of the AG-specific hydrolases used in (a), modified from Fig. 7 in [32].

gum arabic with endo-β-1,6-galactanase and α-arabinofuranosidase released short [^{14}C]-labeled oligosaccharides of DP2-3 with a peak at fraction 20 (Fig. 2(a), dashed line), which indicates that [^{14}C]-GlcA was incorporated into the β-1,6-galactan. The products detected at fraction 20 were likely either [^{14}C]-GlcA-Gal or [^{14}C]-GlcA-Gal-Gal. Treatment of the [^{14}C]-GlcA-incorporated gum arabic with exo-β-1,3-galactanase produced approximately three peaks (Fig. 2(a), dotted line). The small peak at fraction 20 was composed of short oligosaccharides (DP2-3), which indicates that [^{14}C]-GlcA was incorporated in the β-1,3-Gal, whereas the two peaks at fractions 14 and 16 were oligosaccharides with greater molecular masses that possibly represented the incorporation of [^{14}C]-GlcA into β-1,6-galactan (side chains) and/or undigested molecules. These findings also indicated that the majority of [^{14}C]-GlcA was incorporated into the β-1,6-galactan as illustrated by the larger peaks at fractions 14-16 relative to the peak at fraction 20. Treatment with a mixture of all of the hydrolases used in this study (i.e., exo-β-glucuronidase, exo-β-1,3-galactanase, endo-β-1,6-galactanase and α-arabinofuranosidase) released high levels of [^{14}C]-GlcA as shown by the peak at fraction 21 (Fig. 2(a), solid line), which confirms the incorporation of [^{14}C]-GlcA into gum arabic via β-glycosidic linkage.

3.2 Preliminary Analyses of the Oil-in-Water Emulsion Properties of the Modified Gum Arabic

Because gum arabic is one of the most important naturally occurring oil-in-water emulsifiers and because its properties are unique among other plant polysaccharides, we preliminarily investigated the emulsifying properties of modified gum arabic. As a proof of this concept, we first evaluated the emulsifying properties in a small scale. The emulsifying properties were evaluated via analyses of the sizes and distributions of the emulsion particles created by the homogenization of orange oil and water in the presence of modified gum arabic at different time points using the methods described in [21]. For this study, we avoided the use of UDP-[^{14}C]-GlcA in the reaction and instead increased the amount of non-radioactive UDP-GlcA (from 0.1 to 0.8 mM) to increase the amount of incorporated GlcA. In this condition, approximately 25 molecules of GlcA were incorporated into each molecule of gum arabic. The emulsion particles were photographed under a light microscope, and the distributions of particle size were analyzed using the particle analysis option of the ImageJ software [31]. The oil-in-water emulsion particles created with the modified gum arabic (Fig. 3(a)-(e)) were 19%-60% smaller and much less flocculated than those created with unmodified gum arabic over the time period of 4 days (Figs. 3(f)-(j)). The rate of the particle size expansion in the oil-in-water emulsion prepared with the modified gum arabic was three times slower than that of the unmodified emulsion (Fig. 3(k)), and the particle size distributions were also narrower, which indicates better emulsion stability of the modified gum arabic emulsion. We further treated the emulsion with heat (90 ^{o}C, 30 min) [21] to accelerate the coalescence (Fig. 3(l)) and found that the particle sizes of the heat-treated emulation made with the modified gum arabic were still two times smaller than those of the emulsion made with the unmodified gum arabic without heat-treatment. These findings support the superior stabilities of the modified gum arabic emulsions. These results indicate that the increase in GlcA content in gum arabic possibly improved the stability of the emulsifying properties of the gum arabic. Because the majority of the additional GlcA was incorporated into the β-1,6-galactan side chain of the gum arabic molecule (Fig. 2(a)), it is conceivable that increase in the surface charges of emulsions could prevent flocculation via electrostatic repulsion (Fig. 4), even though this assumption needs to be further investigated. It has previously been reported that β-galactosidase treatment improves the performance of gum arabic as an oil-in-water emulsifier [21]. We have not tested the

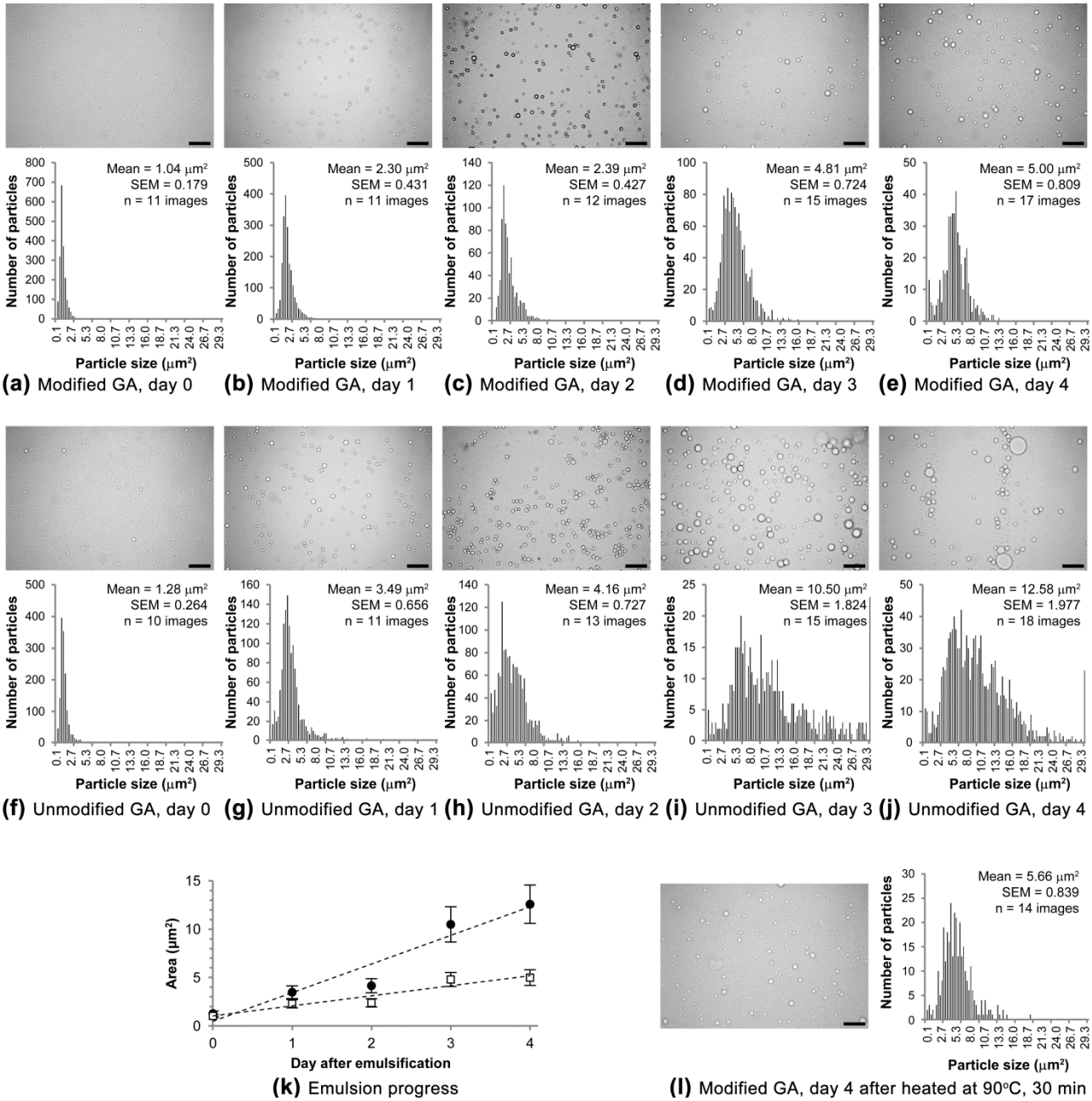

Fig. 3 Micrograph images and distribution histograms of the oil-in-water emulsions made with modified (a)-(e) and unmodified gum arabic (f)-(j). The images were taken immediately after preparation (day 0) and every 24 hours thereafter until day 4. (k) The particle areas of the emulsions made with modified gum arabic (□) and with unmodified gum arabic (●) were calculated and are plotted for each time point. (l) Micrograph image and distribution histogram of the oil-in-water emulsions made with modified gum arabic, day 4, after heated at 90 °C for 30 min. Mean: mean inside area of the analyzed particles (μm²); SEM: standard error of mean; n: number of analyzed images.

effects of double treatments using β-galactosidase (or other related hydrolases) and AtGlcAT14A on the properties of gum arabic, and such issues would be interesting subjects for further investigation.

Our results showed that AtGlcAT14A can be used to modify commercial arabinogalactans, and at the tested condition, the modified gum arabic showed improved emulsifying properties. However, because of the low enzyme production and the relatively low enzyme stability, it does not permit us to produce the modified

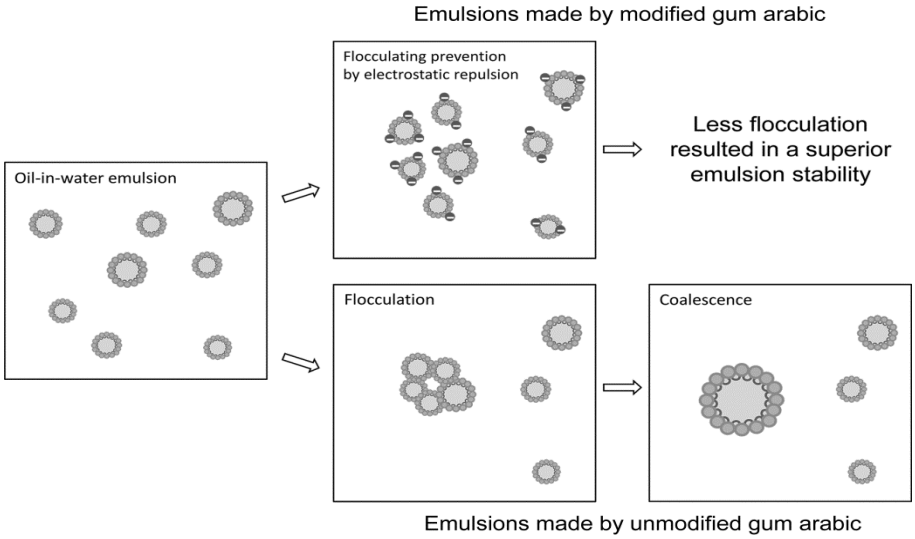

Fig. 4 Proposed mechanism of the prevention of flocculation in the oil-in-water emulsions made with modified gum arabic, modified from Fig. 2 in [14].

gum arabic in the larger scale. Future work will focus on: 1) improve the production and the stability of AtGlcAT14A; 2) optimize the donor (UDP-GlcA) and acceptor (gum arabic) ratio to increase the production yield of modified gum arabic; 3) further evaluate the emulsion stability, e.g., influences of oil and water ratio, and pH of aqueous phase; 4) analyze the zeta potential of modified gum arabic to confirm that the properties changed of modified gum arabic was caused by addition of GlcA. The obtained knowledge offers a potential method for the enzyme-based manipulation of gum arabic.

4. Conclusions

We reported the use of plant β-glucuronosyltransferase (AtGlcAT14A) to improve the properties of gum arabic as an emulsifier in oil-in-water emulsions. The modified gum arabic emulsions were more stable and less flocculated than the unmodified emulsions were. This modified gum arabic should be useful for the beverage, confectionery and cosmetic industries.

Acknowledgments

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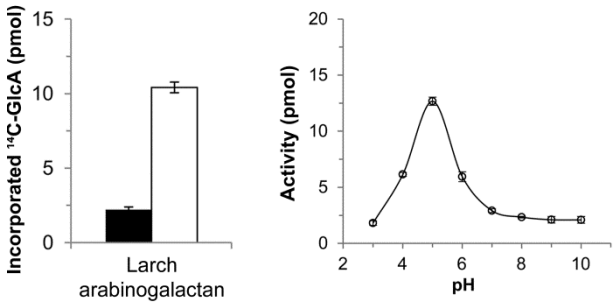
Supporting information



(a) **(b)**

Fig. S1 Glucuronosyltransferase activity of AtGlcAT14A towards Larch Arabinogalactan. (a) Comparison of the activities of AtGlcAT14A (□; 38.5 ng protein) and the empty pPICZαA vector (●) in the presence of UDP-[^{14}C-]-GlcA. The error bars show the standard deviations (n = 4). (b) Glucuronosyltransferase activities of AtGlcAT14A at different pH. The error bars show the standard deviations (n = 2).