Unsaturated guluronate oligosaccharide used as a stabilizer of oil-in-water nanoemulsions loaded with bioactive nutrients

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ABSTRACT

Unsaturated guluronate oligosaccharide (GOS) is generated via alginate-derived polyguluronate (PG) degradation by alginate lyase, followed by formation of a double bond between C-4 and C-5 at the nonreducing end. In this study, GOS was first used as a stabilizer to fabricate O/W nanoemulsions loaded with resveratrol (GOS-RES). Our results revealed that both the GOS-RES and normal O/W resveratrol nanoemulsions (water-RES) showed small droplet sizes and narrow size distributions under certain experimental conditions. However, the particle size and stability of the GOS-RES were slightly greater than those of the water-RES in acidic and neutral environments and at high temperatures. Furthermore, the GOS-RES exhibited a better sustained release effect for resveratrol than the water-RES. Moreover, the GOS-RES showed a significant superoxide radical scavenging effect. All these results demonstrated that GOS has good prospects for preparing nanoemulsions to encapsulate hydrophobic nutrients, which could be applied as food-grade components in beverages and other foods.

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene), a natural food compound found in grapes, can prevent or slow the development of a number of diseases, including cardiovascular disease and cancer, as well as increase resilience to stress and increase lifespans (Baur & Sinclair, 2006). However, resveratrol has low water solubility and can be metabolized and degraded rapidly in vivo, thus leading to a reduction in the dissolution rate and limited cell absorption and oral bioavailability (Sessa et al., 2014). As nutrient delivery systems, nanoemulsions have attracted widespread attention in the food industry because they cannot only prevent the degradation of bioactive compounds, such as resveratrol, with low chemical stability but also improve their bioavailability (Silva, Cerqueira, & Vicente, 2015). An oil-in-water (O/W) nanoemulsion normally is composed of small oil droplets distributed in the aqueous phase and surrounded by surfactant molecules, with particle sizes ranging from 10 to 200 nm; these nanoemulsions can be easily prepared by homogenization of food-grade ingredients (Flores-Andrade, Allende-Baltazar, Sandoval-Gonzalez, Jiménez-Fernández, Beristain, & Pascual-Pineda, 2021).

Alginates is a linear acidic polysaccharide consisting of alternating β-D-mannuronic acid and α-L-guluronic acid units with 1,4-glycosidic linkages (Bi, Yang, Lu, & Xu, 2022; Bi et al., 2017). Due to its advantages, such as low cost, eco-friendliness, and pH sensitivity, it has been widely investigated in the food industry for its ability to encapsulate nutrients to improve their stability during food processing and storage (Lin, Kelly, Maidannyk, & Miao, 2021). However, due to the high molecular weight and viscosity of alginate, emulsions prepared with alginate generally have a large particle size.Degradation of alginate to oligosaccharides can reduce the molecular weight and viscosity (Bi, Xiao, et al., 2021; Bi, Yao, et al., 2021). Jiang et al. reported that alginate-derived oligosaccharides can significantly stabilize zein to prepare complex nanoparticles for the delivery of curcumin (Jiang, Yang, Wang, Ying, Ling, & Ouyang, 2021). Unsaturated guluronate oligosaccharide (GOS) is formed via alginate-derived polyguluronate (PG) degradation by alginate lyase, followed by formation of a double bond between C-4 and C-5 at the nonreducing end (Li et al., 2021). It has been reported that GOS exhibits various physiological activities, such as...
2. Materials and methods

2.1. Materials

Resveratrol, Tween 80, and anhydrous ethanol were purchased from Macklin (Shanghai, China). Olive oil was obtained from local supermarkets. GOS was prepared from alginic-derived PG using alginate lyase, which was purified from *Pseudoalteromonas* sp. strain 272 (Li et al., 2021).

2.2. Preparation of a nanoemulsion

A nanoemulsion was prepared according to a previous study with some modifications (Moghadassi, Housaindokht, Darroudi, Bozorgmehr, & Sadeghi, 2018). In brief, 100 μL of an ethanol solution of resveratrol (50 mg/mL) was added to 5 mL of olive oil, and the mixture was stirred using a magnetic stirrer to obtain the oil phase (O). Then, different quantities of Tween 80 were added to the oil phase. After stirring for 5 min, a mixture of the resveratrol-enriched oil phase and aqueous phase (with/without 1 mg/mL GOS) (1:3, v/v) was stirred for another 30 min to prepare a O/W crude emulsion. Then, the crude emulsion was homogenized by a high-speed homogenizer (FSH-2; Jintan Co., Jiangsu, China) at 11,000 rpm for 90 s.

2.3. Determination of the encapsulation efficiency (EE)

The EE of the resveratrol nanoemulsion was determined based on the ultraviolet absorption of resveratrol at 310 nm. In brief, a dialysis bag (molecular weight cutoff ≥500 Da) containing 1 mL of nanoemulsion was immersed in 20 mL of PBS without shaking. After 24 h, the content of resveratrol in PBS was obtained according to the standard curve.

2.4. Measurement of particle size

The diameter and polydispersity index (PDI) of the resveratrol nanoemulsion were determined by means of a particle size analyzer (Beckman Coulter, California, USA) after five dilutions based on our previous study (Li et al., 2021).

2.5. Morphological analysis

After ten dilutions, the resveratrol nanoemulsion was deposited on Formvar-carbon-coated copper grids and dried overnight at room temperature (RT). Then, the morphology of the nanoemulsion was observed by transmission electron microscopy (TEM) (HT7700, Hitachi, Tokyo, Japan).

2.6. Stability analysis

2.6.1. pH stability

One milliliter of freshly prepared resveratrol nanoemulsion was added to 3 mL of 0.01 M PBS, and then the pH value of the mixture was adjusted to 3.0, 5.0, 7.0 or 9.0 using 1 M HCl or 1 M NaOH. After 2 h of incubation at RT in the dark, the particle size of the nanoemulsion was determined.

2.6.2. Temperature stability

Freshly prepared resveratrol nanoemulsions were incubated at 4, 25, 37, or 55 °C in the dark for one week. Then, the particle size of the nanoemulsions was determined.

2.6.3. Storage stability

Freshly prepared resveratrol nanoemulsions were stored at 4 °C in the dark. After 15, 30, 45, and 60 days of storage, the particle size of the nanoemulsions was determined.

2.7. Investigation of release in vitro

*In vitro* release of the resveratrol nanoemulsion was assessed by investigating the changes in the free resveratrol concentration in solution. Dialysis bags (molecular weight cutoff ≥500 Da) containing 1 mL of a resveratrol ethanol solution (RES) or resveratrol nanoemulsion, both of which contained equal amounts of resveratrol, were immersed in 20 mL of release medium (15% ethanol solution) and shaken at 37 °C. At predetermined time intervals, 200 μL of the release medium was withdrawn, and an equal volume of release medium was added. The amount of free resveratrol in the release medium was detected, and the release kinetics were evaluated using Origin software (OriginLab Corp. Northampton, MA, USA).

2.8. Antioxidant capacity analysis

The antioxidant properties of resveratrol and the resveratrol nanoemulsions were determined by a superoxide anion scavenging capacity assay kit (Solarbio, Beijing, China). In brief, 10 μL of freshly prepared RES or resveratrol nanoemulsion, both of which contained equal amounts of resveratrol, was added to a 96-well plate and incubated with reaction solution for 50 min at RT. Then, the absorbance was measured at 530 nm using a microplate reader (BioTek, Winooski, VT, USA).

2.9. Statistical analysis

All the experiments were conducted at least three times, and the results are presented as the mean ± standard deviation. A t test was used to determine the significant differences by GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). *P* values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Optimization of the preparation conditions of the O/W resveratrol nanoemulsion

High-energy emulsification methods, including high-pressure homogenization, high-speed homogenization, and ultrasonic methods, have been widely applied in the preparation of nanoemulsions (Wang, Jiang, Wang, Huang, Ho, & Huang, 2008). In this study, the effect of high-speed homogenization on the particle size distribution of the nanoemulsion was determined. As shown in Fig. 1A, the particle size...
The distribution of the unhomogenized nanoemulsion was bimodal and concentrated in the ranges of 6–75 nm and 75–1,000 nm. After homogenization, the average particle size of the nanoemulsion was distributed in the range of 7–14 nm (Fig. 1A and Fig. 2A), which was much smaller and narrower than that of the unhomogenized nanoemulsion. Furthermore, the PDI of the homogenized nanoemulsion was <0.3, which also indicates a narrow size distribution of the homogenized nanoemulsion. This is mainly because when the emulsion was homogenized by high-speed homogenizer, high shear stress, cavitation and other forces cooperate to deform the droplet, and then make the droplet crack into a small and uniform droplet (Li, Zhao, Zu, & Zhang, 2015). These results are similar to those of previous studies, leading to the conclusion that homogenization can effectively reduce the particle size and distribution of nanoemulsions (Kotta, Khan, Ansari, Sharma, & Ali, 2015; Qian & McClements, 2011). Both homogenized and unhomogenized nanoemulsions were light yellow and transparent in appearance, and there was no obvious difference between them (Fig. 1A).

During nanoemulsion preparation, the surfactant is distributed at the surface of the oil droplet and provides repulsive forces to prevent droplet aggregation (Silva et al., 2015). The effect of the ratio of oil to Tween 80 on the state of the resveratrol nanoemulsion was determined. When the ratio of oil to Tween 80 was 1:9 (w/w), the nanoemulsion was light yellow and transparent, and the average diameter of the nanoemulsion was near 10 nm (Fig. 1A and Fig. 2A). However, as the surfactant dose decreased, the nanoemulsion appeared turbid and stratified, and at oil/Tween 80 ratios of 2:8, 3:7, and 4:6, the average diameter of the nanoemulsion was more than 200 nm, which was much higher than that at an oil phase/Tween 80 ratio of 1:9 (Fig. 1B). This trend is consistent with previous research, in which Moghaddasi found that when the ratio...
of oil to surfactant was increased from 1:9 to 1.5:8.5, the particle size of the nanoeulsion was dramatically increased from 25.0 nm to 647.6 nm (Moghaddasi et al., 2018). These phenomena may be due to the decrease in surfactant interfacial area and interfacial tension resulting from a reduction in surfactant dose, which decreases the stability of the oil–water interface of the nanoeulsion, resulting in aggregation of the oil droplets and stratification of the nanoeulsion (Moghaddasi et al., 2018; Tadros, Izquierdo, Esquena, & Solans, 2004). Thus, the preparation conditions of the resveratrol nanoeulsion were selected as homogenization at 11,000 rpm for 90 s and an oil/Tween 80 ratio of 1:9.

### 3.2. Effects of GOS on the stability of resveratrol nanoeulsions

Alginate, the only polysaccharide that naturally contains carboxyl groups in each constituent residue, can be adsorbed on the surface of oil droplets to enhance the mechanical strength and steric repulsion of the interfacial layers around oil droplets (Yan, Liang, Ma, McClements, Liu, & Liu, 2021). Additionally, as reported, alginate oligosaccharides can significantly stabilize zein to prepare complex nanoparticles for the delivery of curcumin (Jiang et al., 2021). GOS is an oligosaccharide prepared from alginate-derived polyguluronate via alginate lyase-mediated depolymerization, with an average molecular weight of 1.5 kDa (Li et al., 2021). In this study, whether GOS can be used in the preparation of O/W nanoeulsions to improve the stability of the nanoeulsion was investigated. First, the particle size and EE of GOS-RES were determined. As shown in Fig. 2A and B, the diameters of the water-RES and GOS-RES were similar, and both were near 11 nm. The low molecular weight of GOS might be the primary reason why the particle size distribution and diameter of GOS-RES were almost the same as those of water-RES. Additionally, the morphology of the nanoeulsion was observed by TEM. The TEM images revealed that droplets of both samples were nearly uniform and spherical with no adhesion (Fig. 2C and D). Regarding EE, there were also no significant differences between the two nanoeulsions, and the values of both samples reached 93%. Our experimental results also confirmed that GOS cannot be used as an emulsifier alone (data not shown) but can be used only in combination with surfactants such as Tween 80 when preparing nanoeulsions.

### 3.3. The stabilities of the resveratrol nanoeulsion

Then, the stabilities of the two nanoeulsions in different environments were compared based on the changes in the average particle size, which is usually used to evaluate the physical stability of a nanoeulsion (Li et al., 2021). When the stability of a nanoeulsion is lost, the nanoeulsion tends to coalesce, which leads to a larger particle size (Tadros et al., 2004).

Normally, food and beverages have a certain pH value, so it is necessary to measure the influence of pH on the stability of nanoeulsions. Fig. 3A shows that both water-RES and GOS-RES had the smallest particle sizes at neutral pH. However, the droplet size of the two nanoeulsions did not show an obvious change at pH values from 3.0 to 9.0. Additionally, the appearance of the nanoeulsions at different pH values did not change significantly, with both maintaining their light yellow and transparent liquid forms, indicating the good pH stability of the resveratrol nanoeulsion. The surfactant Tween 80 may have played a role because it is a nonionic surfactant that does not react with the buffer used to adjust the pH of the nanoeulsion directly, so the particle size of the nanoeulsion does not change dramatically with the pH. However, the average particle size of GOS-RES was slightly smaller than that of water-RES at pH values from 3 to 7 (Fig. 3A). This might be because GOS was negatively charged and could adsorb around the oil droplets, enhancing the electrostatic repulsion between the oil droplets.

When the ambient temperature of the nanoeulsions increased, the collision frequency between droplets in the emulsion increased, which could reduce the repulsive force between the droplets, thus promoting agglomeration of the droplets (McClements, 2004). Fig. 3B shows that the particle size of the resveratrol nanoeulsion did not change significantly when stored at 4 °C and 25 °C for one week. However, the particle size of the water-RES and GOS-RES increased significantly, both reaching 400 nm, when stored at 37 °C for one week (Fig. 3B). When the temperature reached 55 °C, the water-RES and GOS-RES became cloudy, and their particle sizes were approximately 2,300 nm and 1,100 nm, respectively (Fig. 3B), indicating that GOS in the water phase has a certain temperature stability effect on the nanoeulsion. Thus, resveratrol nanoeulsions should be stored at low temperature or RT.

As reported, nanoeulsions can lose their transparency with time as a result of an increase in droplet size (Tadros et al., 2004). However, both water-RES and GOS-RES were still light yellow and transparent liquids when stored at 4 °C for 60 days. Additionally, their particle size...
increased from 11 nm to only approximately 12 nm (Fig. 3C), indicating that both nanoemulsions could be stored at 4 °C for at least two months. Furthermore, the average particle size of GOS-RES was always less than that of water-RES, which might also be caused by the electrostatic repulsive effect of GOS adsorbed around the oil droplets.

3.4. In vitro release of resveratrol from the nanoemulsion

The in vitro release behavior of nanoemulsions is an important index to evaluate the properties of nanoemulsions. In this study, the in vitro release profile of the resveratrol nanoemulsion was assessed in a 15% ethanol solution. As shown in Fig. 4A, the time for RES, water-RES and GOS-RES to reach equilibrium was 8 h, 24 h and 24 h, respectively, and the release rate of resveratrol from the water-RES and GOS-RES was much lower than that of RES, suggesting that water-RES and GOS-RES had a better sustained release effect for resveratrol than RES. This sustained release effect might be due to transport through the interfacial barrier acting as a rate-limiting step for drug release from the nanoemulsion system in vitro (Li et al., 2021). However, the release of resveratrol from water-RES was much faster in the first six hours than that from GOS-RES (Fig. 4A), indicating that GOS-RES may have a better sustained release effect for resveratrol than water-RES. In addition, GOS is negatively charged and could adsorb around oil droplets, which might act a significant role in reducing the transport rate of the active material through the interface barrier.

Then, the release mechanism of resveratrol from the GOS-RES was evaluated, and three release kinetics models, namely, the zero-order, first-order and Higuchi models, were used for fitting. As presented in Table 1, the first-order model afforded the highest $R^2$ value ($R^2 = 0.988$), indicating that the release of resveratrol from the GOS-RES might follow the first-order model (Fig. 4B). This model clarified that the release rate of resveratrol was proportional to the amount of resveratrol remaining in the GOS-RES and reduced over time. Taken together, these results revealed that GOS, as a stabilizer of O/W nanoemulsions loaded with bioactive nutrients, could have good application potential in the field of health food.

3.5. Antioxidant activity of resveratrol and the resveratrol nanoemulsion

Resveratrol has been reported to be a scavenger of free radicals (Gülçin, 2010), which was also confirmed by our results (Fig. 5). However, water-RES, which contained the same amount of resveratrol as the RES group, showed less superoxide radical scavenging activity than RES and GOS-RES (Fig. 5). This might be because the freshly prepared water-RES did not release all the resveratrol in time because the resveratrol released from the emulsion system needs to be transported across the interfacial barrier, leading to low levels of total resveratrol involved in the reaction. Interestingly, GOS-RES showed much more significant superoxide radical scavenging activity than RES (Fig. 5), which is the expected effect of GOS in the emulsion system. GOS was prepared from alginate-derived polyguluronate via alginate lyase-mediated depolymerization, followed by double bond formation between C-4 and C-5 (Li et al., 2021), endowing GOS with significant antioxidant activity (Falkeborg et al., 2014). Therefore, although resveratrol cannot be completely released from GOS-RES in time, the antioxidant activity of GOS-RES makes the overall superoxide radical scavenging effect of GOS-RES particularly high. In summary, we confirmed our hypothesis that GOS, as a water-soluble antioxidant with large negative charges, could be used as a stabilizer in nanoemulsion preparation and endowed the nanoemulsion system with stronger resistance to free radical scavenging. This nanoemulsion could encapsulate hydrophobic and unstable nutrients and be used as a food-grade component in beverages and other foods.

4. Conclusion

In summary, GOS was used as a stabilizer in the fabrication of O/W nanoemulsions loaded with resveratrol, and its properties were compared with those of water-RES. The results revealed that both the water-RES and GOS-RES prepared in this study showed small droplet

Table 1

| Kinetic model    | Release mechanism     | Equation                   | $R^2$ |
|------------------|-----------------------|----------------------------|-------|
| Zero-order       | Constant-speed release | $Q = Q_0 + kt$             | 0.772 |
| First-order      | First release         | $\ln(1-Q) = -kt$           | 0.988 |
| Higuchi          | Fick release          | $Q = kt^{1/2}$             | 0.946 |

Fig. 5. Antioxidant activity of RES, water-RES and GOS-RES. ***$P < 0.001$. 

Fig. 4. Release profiles of resveratrol from RES, water-RES and GOS-RES (A) and fitting of the release kinetic equation $[\ln(1-Q) = -kt]$ to data for RES release from the GOS-RES in vitro (B).
sizes and narrow size distributions. However, the particle size of GOS-RES was slightly smaller than that of water-RES in acidic and neutral environments. Additionally, both resveratrol nanoemulsions should be stored at low temperature, but the temperature stability of GOS-RES was slightly better than that of water-GOS. Our experimental results confirmed that adding surfactants such as Tween 80 can improve the stability of the nanoemulsions to a certain extent. Furthermore, GOS-RES had a better sustained release effect for resveratrol than water-RES, and the release of resveratrol from the GOS-RES might follow a first-order model. Moreover, GOS-RES showed a significant superoxide radical scavenging effect. All these results demonstrate that GOS has good prospects for application in nanoemulsion preparation to encapsulate easily destroyed nutrients and that nanoemulsions could be used in beverages and other foods.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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