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Carboxymethylation of Qingke β-glucans and their physicochemical properties and biological activities

Huan Guo [a,1], Kang-Lin Feng [a,1], Jia Zhou [a], Lu Liu [a], Si-Yu Wei [a], Li Zhao [a], Wen Qin [a], Ren-You Gan [b,*], Ding-Tao Wu [a,*]

[a] Institute of Food Processing and Safety, College of Food Science, Sichuan Agricultural University, Ya'an 625014, Sichuan, China
[b] Institute of Urban Agriculture, Chinese Academy of Agricultural Sciences, Chengdu 610213, China

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ABSTRACT

In this study, the response surface methodology was used to optimize the reaction conditions of carboxymethylated modification of Qingke β-glucans (QG), and effects of different degrees of carboxymethylation (high, medium, and low) on their structural characteristics, *in vitro* antioxidant activities, and *in vitro* hypolipidemic activities were studied. The optimal reaction conditions of carboxymethylated Qingke β-glucans (QG-Cs) with high degree of carboxymethylation (DC = 0.90) were as follows: reaction temperature of 62.1 °C, reaction time of 3.29 h, and concentration of chloroacetic acid of 1.75 mol/L. Results demonstrated that the carboxymethylated modification significantly affected the solubilities, molar ratios of constituent monosaccharides, molecular weights, and apparent viscosities of QG. Indeed, the QG-Cs exhibited much higher antioxidant activities (reducing powers, NO, and DPPH radical scavenging activities), *in vitro* binding properties (fat, bile acid, and cholesterol binding capacities), and pancreatic lipase inhibition activities than that of QG. Furthermore, results showed that bioactivities of QG-Cs were closely correlated to their carboxymethyl groups. Results suggested that the carboxymethylated modification could be an efficient method for enhancing bioactivities of QG, and QG-Cs had good potential applications in bio-pharmaceutical industry.

1. Introduction

Qingke (Tibetan hull-less barley, *Hordeum vulgare* L.) is a cultivar of hull-less barley grown in the Qinghai-Tibet Plateau in China, and is used as an important food crop, a brewing raw material, and an indispensable source of feed for Tibetans [1,2]. Given special nutrients, such as high dietary fiber content, high soluble β-glucan content, and high feeding quality, researchers have paid increasing attention on Qingke. Studies have revealed that the long-term consumption of Qingke can reduce the risk of some diseases, such as hypertension, diabetes, colon cancer, atherosclerosis, and hyperlipidemia [1,3]. β-glucans, the major nutrient components of Qingke, have been implicated in reducing glycaemic index, improving lipid metabolism, and lowering plasma cholesterol [4–8]. In fact, β-glucans possess different bioactivities, such as cellular antiproliferative activity, immunomodulation effect, prebiotic activity, hypolipidemic effect, and antioxidant activity [9–11]. In general, the functional group, molecular weight, and rheological property of polysaccharides contribute to their multiple bioactivities, and chemical modification is an important method to improve the physicochemical characteristics and enhance the bioactivities of polysaccharides.

Modifications, such as chemical modifications, physical modifications, and biological modifications, are identified as effective methods to change the physicochemical characteristics and improve the bioactivities of polysaccharides [12,13]. Recently, the chemical modifications, including partial acid hydrolysis and sulfated modification, have been utilized to improve the water solubility, decrease the molecular weight, and reduce the viscosity of Qingke β-glucans, as well as change their *in vitro* antioxidant activity and hypolipidemic effect [10,14]. Both molecular weight and sulfated group play important roles in the bioactivities of Qingke β-glucans. Furthermore, it has been demonstrated that the carboxymethylated modification is an effective, speedy, and simple method to improve physicochemical characteristics and bioactivities of polysaccharides [12,15,16]. Carboxymethylated modification has been used to modify the structure of polysaccharides to provide diverse physiological functions including antioxidant, anti-proliferation, and antitumor activities [17]. However, as far as we know, the carboxymethylated modification of Qingke β-glucans is rarely reported, and studies on the correlations of the carboxymethylated modification to the physicochemical properties and bioactivities of Qingke β-glucans are restricted.

* Corresponding authors.
E-mail addresses: ganrenyou@caas.cn (R.-Y. Gan), DT_Wu@sicau.edu.cn (D.-T. Wu).
[1] These authors contributed equally to this work.

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Therefore, for further understanding of the structure-function relationships of Qingke β-glucans (QG) and expand the application of Qingke resources, effects of carboxymethylated modification on the physicochemical characteristics (molecular weights, viscosities, water solubilities, constituent monosaccharides, and carboxymethyl groups) and bioactivities (*in vitro* antioxidant and hypolipidemic activities) of QG were investigated.

2. Material and methods

2.1. Material and chemicals

Qingke (*Dinqing* Qingke) was collected from Changdu (altitude >4000 m), Tibet, China.

Dimethyl sulfoxide (DMSO), chloroacetic acid, 2,7-dihydroxynaphthalene, bovine serum albumin, trifluoroacetic acid, arabinose (Ara), xylose (Xyl), glucose (Glc), mannose (Man), galactose (Gal), sodium cholate, sodium deoxycholate, sodium glycocholate, sodium taurocholate, cholesterol, carboxymethyl cellulose, oleic acid, griess reagent, sodium nitroprusside (SNP), 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox), and 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The mixed-linkage β-glucan assay kit was obtained from Megazyme (Wicklow, Ireland). Cholestyramine and orlistat were obtained from a local pharmacy in Ya'an. Pancreatin (4000 U/g), heat-stable α-amylase (40,000 U/g), pancreatic lipase (4000 U/g), and amyloglucosidase (1000 U/g), and a free cholesterol assay kit was purchased from Solarbio (Beijing, China). All other chemicals and reagents used were of analytical grade.

2.2. Synthesis of carboxymethylated Qingke β-glucans (QG-Cs)

2.2.1. Extraction of Qingke β-glucans (QG)

Qingke β-glucans were extracted and prepared based on the previously reported method [11]. After extraction, QG were obtained by freeze drying, and the content of total polysaccharides in QG was estimated by the phenol-sulfuric acid method. In addition, the purity of β-glucans in QG was estimated by the mixed-linkage β-glucan assay kit, and the contents of proteins in QG were measured by Bradford's method [18].

2.2.2. Optimization of the carboxymethylated modification of QG

Carboxymethylated β-glucans were obtained based on a previously reported method with slight adjustments [19], and both single-factor experimental design and Box-Behnken experimental design were applied to obtain carboxymethylated QG with high degree of carboxymethylation (DC). Firstly, the effects of reaction temperature (40, 50, 60, 70, and 80 °C), reaction time (1.0, 2.0, 3.0, 4.0, and 5.0 h), and concentration of chloroacetic acid (1.0, 1.5, 2.0, 2.5, and 3.0 mol/L) on the DC of QG were investigated using a single-factor experimental design. Briefly, 30.0 mg of QG were dissolved in 12.5 mL of anhydrous DMSO, then add 5 mL of NaOH (20%, w/v) to the mixture, and stirred at 40 °C for 3 h. Subsequently, the chloroacetic acid was added, which dissolved in a mixed solvent of DMSO (12.5 mL) and NaOH (20%, 5 mL). The mixture was heated to a definite temperature and held for a definite time. When studying the temperature parameters, the concentration of chloroacetic acid was fixed at 2 mg/mL, and the reaction time was fixed at 3 h. It was worth noting that the three single-factor experiments were completed in turn, and the optimal reaction condition of the previous single-factor was used to conduct the next single-factor experiment. After the reaction, the mixture was cooled to 25 °C in an ice-water bath and neutralized to pH 7.0 with 0.5 M HCl. The solution was then transferred into a dialysis membrane (molecular weight cut off: 3.5 kDa) and dialyzed at 40 °C to accelerate the movement of small molecules. The carboxymethylated Qingke β-glucans (QG-Cs) were obtained after lyophilization.

Furthermore, based on the results of the single-factor tests, a Box-Behnken design (BBD) was applied to optimize the conditions of the carboxymethylation reaction [20]. Table 1 showed the result of a total of 17 randomised experiments, including 5 zero point and 12 factorial tests. The Design Expert software 8.0.5 (Stat-Ease Inc., Minneapolis, MN, USA) was applied for data analysis. The second-order polynomial model as follows was applied for the explanation of the experimental data from BBD [20],

$$Y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X^2{}_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} A_{ij} X_i X_j$$

where Y is the predicted response; X_i and X_j are different variables ($i \neq j$); A_0, A_i, A_{ii}, and A_{ij}, are regression coefficients for intercept, linearity, square, and interaction, respectively.

2.2.3. Preparation of QG-Cs with different degrees of carboxymethylation

Three QG-Cs with different DCs (high, medium, and low) were prepared for further analysis, which coded as QG-C3 (high DC), QG-C2 (medium DC), and QG-C1 (low DC), respectively. The reaction conditions for the preparation of QG-C1 were as follows: reaction temperature of 50 °C, reaction time of 3.0 h, and the concentration of chloroacetic acid of 2.0 mol/L. Then the reaction conditions for the preparation of QG-C2 were as follows: reaction temperature of 60 °C, reaction time of 3.0 h, and the concentration of chloroacetic acid of 2.0 mol/L. Finally, QG-C3 was obtained using optimal reaction conditions.

2.3. Physicochemical characterization of QG and QG-Cs

2.3.1. Determination of degree of carboxymethylation (DC)

The DCs of QG-Cs were estimated according to a colorimetric method [21]. In brief, disperse the carboxymethylated derivative (1.0 mg/mL) with 1.0 mL of 50% H_2SO_4 for 30 min to break the chemical bonds between the polysaccharides and the carboxymethyl groups, thereby releasing the carboxymethyl groups. Add 2.0 mL of the naphthol reagent (0.1 g of 2,7-dihydroxynaphthalene in 1000.0 mL of concentrated H_2SO_4), and heat the mixture for 20 min in a boiling water bath. The mixture was cooled to room temperature and the absorbance was measured at 530 nm. The DCs of QG-Cs were determined by using

Table 1
Box-Behnken design with independent variables and observed values.

Runs	Levels of independent factors[a]			Degrees of carboxymethylation
	X_1 (°C)	X_2 (h)	X_3 (mol/L)	
1	−1 (50)	1 (4.0)	0 (1.5)	0.68
2	1 (70)	0 (3.0)	1 (2.0)	0.84
3	0 (60)	0 (3.0)	0 (1.5)	0.87
4	0 (60)	0 (3.0)	0 (1.5)	0.87
5	1 (70)	0 (3.0)	−1 (1.0)	0.65
6	1 (70)	1 (4.0)	0 (1.5)	0.79
7	−1 (50)	−1 (2.0)	0 (1.5)	0.65
8	1 (70)	−1 (2.0)	0 (1.5)	0.68
9	−1 (50)	0 (3.0)	−1 (1.0)	0.67
10	0 (60)	0 (3.0)	0 (1.5)	0.88
11	0 (60)	0 (3.0)	0 (1.5)	0.88
12	0 (60)	−1 (2.0)	−1 (1.0)	0.65
13	0 (60)	−1 (2.0)	1 (2.0)	0.74
14	0 (60)	1 (4.0)	−1 (1.0)	0.72
15	0 (60)	0 (3.0)	0 (1.5)	0.89
16	0 (60)	1 (4.0)	1 (2.0)	0.83
17	−1 (50)	0 (3.0)	1 (2.0)	0.74

[a] X_1, reaction temperature (°C); X_2, reaction time (h); X_3, concentration of chloroacetic acid (mol/L).

glycolic acid as a standard, which was calculated according to the following formula [9],

$$DC = \frac{162 \times C\%}{24 - 58 \times C\%}$$

where, C% was the mass fraction of C atom; 162 was the molar mass of one anhydrous glucose residue (162 g/mol); 24 was the molar mass of two carbon atoms (24 g/mol); 58 was the molar mass of carboxymethyl groups (58 g/mol); DC was the degree of carboxymethylation.

2.3.2. Fourier transform infrared (FT-IR) spectroscopy analysis

The FT-IR spectroscopy analysis of QG and QG-Cs were conducted by a Nicolet iS10 FT-IR (ThermoFisher scientific, Waltham, MA, USA) at a frequency range of 4000–400 cm^{-1} based on the previously reported method [20].

2.3.3. Determination of water solubility and apparent viscosity

The water solubility of each sample was measured based on the previously reported method [22]. The water solubility of each sample was displayed as the weight of polysaccharide per mL of water (mg/mL).

The apparent viscosities of QG and QG-Cs were determined by a Discovery Hybrid Rheometer-1 (DHR-1) (TA instruments, New Castle DE, USA) equipped with a parallel steel plate (40 mm diameter, 1.0 mm gap) based on the previously reported method [23–25]. Each sample was measured over the range of 0.1–100 s^{-1} at 25 °C. The apparent viscosity of each sample was determined at 15.0 mg/mL.

2.3.4. Determination of molecular weights and constituent monosaccharides

The weight-average molecular weights (M_w) and polydispersities (M_w/M_n) of QG and QG-Cs were estimated by using a high performance size exclusion chromatography coupled with multi angle laser light scattering and refractive index detector (HPSEC-MALLS-RID, Wyatt Technology Co., Santa Barbara, CA, USA) based on the previously reported method [25].

Constituent monosaccharides of QG and QG-Cs were also determined by GC–MS analysis based on the previously reported methods [11,26]. The derivatives were determined by using an Agilent 7890A gas chromatography instrument coupled with an Agilent 5975C mass spectrometer (Agilent Technologies, Palo Alto, CA, USA).

2.4. Evaluation of antioxidant activities of QG and QG-Cs

The reducing powers, nitric oxide (NO) radical scavenging activities, and DPPH radical scavenging activities of QG and QG-Cs were measured by the previously reported methods [20,27].

2.5. Evaluation of in vitro hypolipidemic activities of QG and QG-Cs

2.5.1. In vitro binding properties

The *in vitro* binding properties (fat binding capacity, bile acid binding capacity, and cholesterol binding capacity) of QG and QG-Cs were also determined by the previously reported methods [11].

2.5.2. In vitro pancreatic lipase inhibition activities

The *in vitro* inhibition activities on pancreatic lipase of QG and QG-Cs were also determined by the previously reported methods [11]. The orlistat was used as the positive control. The inhibition activities on pancreatic lipase of QG and QG-Cs were determined at different concentrations (2.0, 4.0, 6.0, and 8.0 mg/mL), and a logarithmic regression curve was established to calculate IC_{50} values (mg/mL).

2.6. Statistical analysis

All experiments were conducted in triplicate, and dates were expressed in means ± standard deviations. Origin 9.0 software (OriginLab Corporation, Northampton, Mass., USA) was applied for statistical analysis. Statistical significances were carried out by one-way analysis of variance (ANOVA), taking a level of $p < 0.05$ as significant to Duncan's multiple range test.

3. Results and discussions

3.1. Optimization of reaction conditions for carboxymethylated Qingke β-glucans

The single-factor experimental results (reaction temperature, reaction time, and concentration of chloroacetic acid) of carboxymethylated modification were shown in Fig. 1. Briefly, when the reaction time (3.0 h) and the concentration of chloroacetic acid (2.0 mg/mL) were fixed, DCs increased with the increase of reaction temperature until 60 °C (Fig. 1A). However, DCs decreased significantly with the increase of reaction temperature over 60 °C. This condition might be that the carboxymethylated reaction was an exothermic reaction, and the equilibrium direction of the carboxymethylated reaction was changed due to the high temperature. At the fixed concentration of chloroacetic acid (2.0 mg/mL) and the reaction temperature (60 °C), DCs increased significantly with the increase of reaction time until 3.0 h, and then DCs decreased (Fig. 1B). Furthermore, at the fixed reaction time (3.0 h) and the reaction temperature (60 °C), the DCs increased with the increase of concentration of chloroacetic acid until 1.5 mol/L (Fig. 1C), after which DCs increased slightly. However, the DCs decreased significantly over 2.0 mol/L. In short, results demonstrated that the optimal reaction temperature, the optimal reaction time, and the optimal concentration of chloroacetic acid were determined to be 60 °C, 3.0 h, and 1.5 mol/L, respectively.

In addition, Table 1 showed the 17 sets of BBD experimental results for further optimization of the reaction conditions of carboxymethyl modification. The final equation with coded factors was as follows,

$$DC = +0.88 + 0.028X_1 + 0.037X_2 + 0.057X_3 + 0.02X_1X_2 + 0.03X_1X_3 + 0.005X_2X_3 - 0.094X_1^2 - 0.084X_2^2 - 0.059X_3^2$$

where DC was the degree of carboxymethylation; X_1, X_2, and X_3 were reaction temperature, reaction time, and concentration of chloroacetic acid, respectively.

As shown in Table 2, the low p-value ($p < 0.0001$) and high F-value (88.37) of the quadratic regression model showed that the fitness was very significant [20]. The high p-value ($p > 0.05$) and the low lack of fit F-value (4.52) showed that the quadratic regression model equation was enough to predict the DCs. Furthermore, the adeq. precision (22.916) and coefficient variation (*C.V.*, 1.73%) showed that the quadratic regression model possessed adequate accuracy and general applicability [20]. It is noticeable that the linear coefficients (X_1, X_2, X_3), the quadratic terms (X_1X_1, X_2X_2, X_3X_3), and interaction coefficients (X_1X_2, X_1X_3) significantly affected the DCs, while the interaction coefficient (X_2X_3) had no significant effect ($p > 0.05$).

As shown in Fig. 2, it was obvious that the interactions between the concentration of chloroacetic acid and the reaction temperature (X_1X_3), and the reaction time and the reaction temperature (X_1X_2) were significant (Table 2). However, the interplay of the reaction time and the concentration of chloroacetic acid (X_2X_3) was not significant (Table 2). Besides, the maximum DC (0.90) could be obtained under the following optimal reaction conditions: reaction temperature of 62.1 °C, reaction time of 3.29 h, and concentration of chloroacetic acid of 1.75 mol/L. According to the

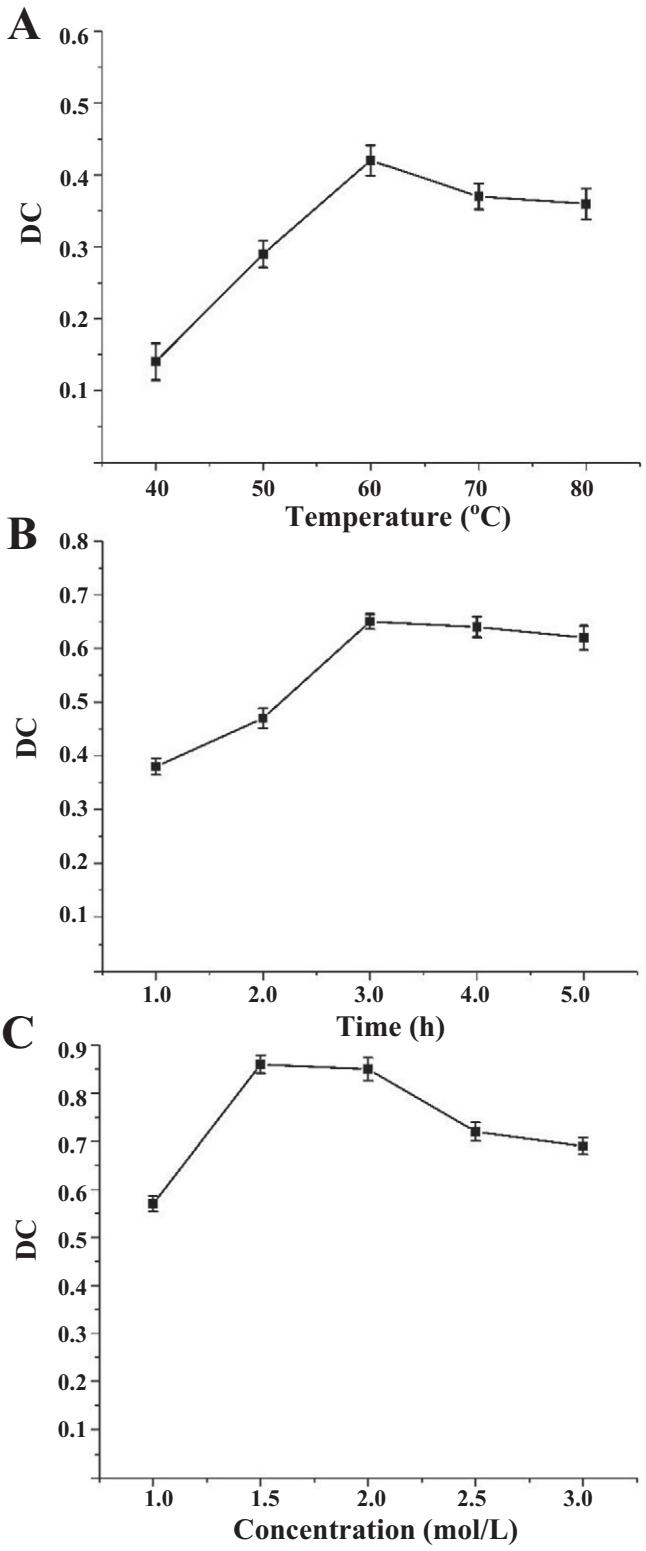

Fig. 1. Effects of different reaction conditions on the degree of carboxymethylation of Qingke β-glucans.

feasibility of practical application, the conditions of verification experiment were as follows: reaction temperature of 62 °C, reaction time of 3.3 h, and the concentration of chloroacetic acid of 1.75 mol/L. The actual DC of carboxymethylated derivatives was 0.89 ± 0.03 (n = 3), which was in accordance with the predicted value.

Table 2
Analysis of variance of regression equation and coefficients.

Source[a]	Carboxymethylated Qingke β-glucans				
	Sum of squares	df[b]	Mean square	*F*-value	*p*-Value[c]
Model	0.14	9	0.016	88.37	<0.0001**
X_1	6.050 E−003	1	6.050 E−003	34.43	0.0006**
X_2	0.011	1	0.011	64.02	<0.0001**
X_3	0.026	1	0.026	150.53	<0.0001**
X_1X_2	1.600E−003	1	1.600E−003	9.11	0.0195*
X_1X_3	3.600E−003	1	3.600E−003	20.49	0.0027**
X_2X_3	1.000 E−004	1	1.000 E−004	0.57	0.4752
X_1^2	0.037	1	0.037	211.73	<0.0001**
X_2^2	0.030	1	0.030	169.08	<0.0001**
X_3^2	0.015	1	0.015	83.41	<0.0001**
Residual error	1.230E−003	7	1.757E−004		
Lack of fit	9.500E−004	3	3.167E−004	4.52	0.0894
Pure error	2.800E−004	4	7.000E−005		
Correlation total	0.14	16			

R^2 = 0.9913, R^2_{adj} = 0.9801, coefficient of variation = 1.73%, adeq. precision = 22.916.
[a] X_1, reaction temperature (°C); X_2, reaction time (h); X_3, concentration of chloroacetic acid (mol/L).
[b] *df*, the degree of freedom.
[c] *Significantly different (p < 0.05), **Extremely significantly different (p < 0.01).

3.2. Physicochemical characteristics of QG and QG-Cs

3.2.1. Chemical compositions

The DCs and yields of QG-Cs were displayed in Table 3. The DCs of QG-C1, QG-C2, and QG-C3 were measured to be 0.32 ± 0.02, 0.65 ± 0.03, and 0.88 ± 0.03, respectively. In other words, QG-Cs with different DCs were successfully obtained. The yields of QG-C1, QG-C2, and QG-C3 were estimated by weighing methods, and the estimated values were 70.91%, 73.64%, and 79.15%, respectively. In addition, the content of total polysaccharides (92.24 ± 1.83%) in QG was significantly (p < 0.05) higher than those of QG-Cs, and the contents of total polysaccharides in QG-C1, QG-C2, and QG-C3 were detected to be 85.07 ± 1.60%, 81.85 ± 1.72%, and 79.62 ± 1.79%, respectively. Similarly, the β-glucan purity (87.36 ± 1.49%) of QG was also significantly (p < 0.05) higher than those of QG-Cs, and the β-glucan contents of QG-C1, QG-C2, and QG-C3 were detected to be 78.11 ± 1.72%, 75.42 ± 1.33%, and 72.09 ± 1.55%, respectively. A small number of proteins were determined in QG-C1, QG-C2, QG-C3, and QG, which were lower than 4%. Furthermore, the water solubilities of QG and QG-Cs were also summarized in Table 3. The solubility of QG (23.69 ± 0.52 mg/mL) was significantly (p < 0.05) lower than those of QG-Cs, and the solubilities of QG-C1, QG-C2, and QG-C3 were detected to be 26.49 ± 0.79 mg/mL, 30.72 ± 0.99 mg/mL, and 38.58 ± 1.12 mg/mL, respectively. Results showed that the carboxymethylated modification could improve the solubility of QG in water, which was in accordance with the previous reports [9,28]. Indeed, the solubility of polysaccharides was associated with their molecular chain and molecular weight. Carboxymethylated modification disrupted the molecular chain of polysaccharides, and increased the solubility of carboxymethylated derivatives.

3.2.2. FT-IR spectra

The FT-IR spectra of QG-C1, QG-C2, QG-C3, and QG were presented in Fig. 3A. As shown in Fig. 3A, 3416 cm^{-1} and 2922 cm^{-1} are the broad peaks caused by the stretching vibration of hydroxyl group and the C—H asymmetric stretching vibration [29,30]. Compared with QG, the new absorption peak at around 1332 cm^{-1} suggested a symmetrical C = O stretching vibration. Similar absorption peaks were found in the carboxymethylated derivatives of polysaccharides extracted from *Morchella angusticepes* Peck [31]. In addition, compared with QG, carboxyl groups of QG-Cs were revealed by two new absorption peaks in the 1605 cm^{-1} and 1425 cm^{-1} [17]. The intensity of the two peaks increased with increasing degree of carboxymethylation, indicating that

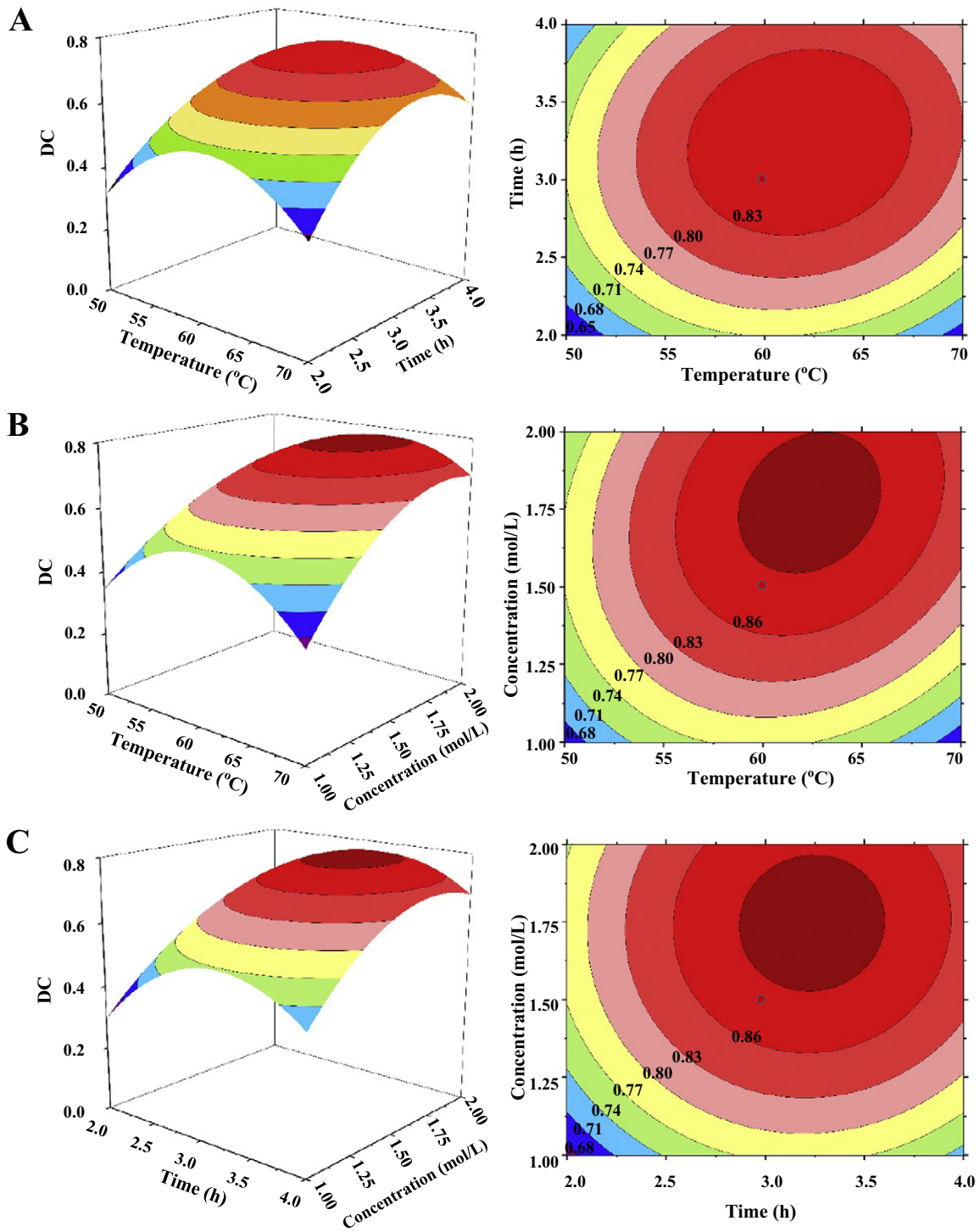

Fig. 2. Three-dimensional response surface (left) and two-dimensional contour plots (right).

more and more carboxymethyl groups were attached to β-glucans from QG-C1 to QG-C3. In short, results further indicated that carboxymethylated Qingke β-glucans were successfully prepared.

3.2.3. Constituent monosaccharides, molecular weights, and apparent viscosities

It is generally believed that the biological activities of natural polysaccharides are closely related to their constituent monosaccharides, molecular weights, and viscosities [32,33]. Thus, the constituent monosaccharides, molecular weights, and apparent viscosities of QG and QG-Cs were measured. Fig. 3B indicated that compositional monosaccharides of QG-C1, QG-C2, QG-C3, and QG were similar, which were determined as Ara, Xyl, Glc, and Gal. The major constituent monosaccharides in QG and QG-Cs were consistent with previous studies [11]. Molar ratios of Ara, Xyl, Glc, and Gal in QG, QG-C1, QG-C2, and QG-C3 were summarized in Table 3, which indicated that Glc was the dominant monosaccharide in QG and QG-Cs. Results showed that different degrees of carboxymethylation had no effects on the types of constituent monosaccharides in QG, but significantly affected their molar ratios. Similar studies have also demonstrated that carboxymethylated modification can influence constituent monosaccharides of polysaccharides [16].

Table 3
Degree of carboxymethylation (DC), yield, β-glucan purity, solubility, molecular weight (M_w), polydispersity (M_w/M_n), and compositional monosaccharides of QG, QG-C1, QG-C2, and QG-C3.

Sample	QG	QG-C1	QG-C2	QG-C3
DC	–	0.32 ± 0.02^c	0.65 ± 0.03^b	0.88 ± 0.03^a
Yield (%)	–	70.19 ± 2.93^c	73.64 ± 2.68^b	79.15 ± 3.09^a
β-glucan purity (%)	87.36 ± 1.49^a	78.11 ± 1.72^b	75.42 ± 1.33^c	72.09 ± 1.55^d
Total polysaccharides (%)	92.24 ± 1.83^a	85.07 ± 1.60^b	81.85 ± 1.72^c	79.62 ± 1.79^d
Protein content (%)	3.09 ± 0.16^a	2.63 ± 0.14^b	2.12 ± 0.15^c	1.58 ± 0.11^d
Solubility (mg/mL)	23.69 ± 0.52^d	26.49 ± 0.79^c	30.72 ± 0.99^b	38.58 ± 1.12^a
$M_w \times 10^4$ (g/mol)	17.38 (± 0.53%)^a	10.25 (± 0.18%)^b	8.87 (± 0.52%)^c	3.25 (± 0.38%)^d
M_w/M_n	1.64 (± 0.84%)	1.77 (± 0.76%)	1.87 (± 0.57%)	2.11 (± 0.69%)
Monosaccharides and molar ratios				
Arabinose	1.00	1.00	1.00	1.00
Xylose	1.25	1.46	1.83	2.30
Glucose	16.96	17.49	24.00	33.25
Galactose	0.60	0.30	0.43	0.18

QG, β-glucans from Qingke; QG-C1, QG-C2, and QG-C3, carboxymethylated β-glucans with low, medium, and high degrees of carboxymethylation, respectively; The error bars are standard deviations; Values represent mean ± standard deviation, and superscripts a–d differ significantly ($p < 0.05$) column wise between different degrees of carboxymethylation. Statistical significances were carried out by ANOVA and Ducan's test.

Furthermore, Fig. 3C showed the HPSEC-RID chromatograms of QG, QG-C1, QG-C2, and QG-C3. Molecular weights of QG, QG-C1, QG-C2, and QG-C3 were also summarized in Table 3, which were measured to be 1.738×10^5 Da, 1.025×10^5 Da, 8.87×10^4 Da, and 3.25×10^4 Da, respectively. The molecular weights of carboxymethylated derivatives have obvious degradations during the carboxymethylated reactions. Results were similar to previous studies, which indicated that the degradation might occur in the process of carboxymethylated modification of polysaccharides [34]. Moreover, the polydispersities of QG, QG-C1, QG-C2, and QG-C3 ranged from 1.64 to 2.11, which were consistent with their HPSEC chromatograms.

Moreover, Fig. 3D showed the effects of shear rate on the apparent viscosities of QG, QG-C1, QG-C2, and QG-C3. Results showed that the apparent viscosities of QG, QG-C1, QG-C2, and QG-C3 decreased with the increasing shear rate (0.1–100 s^{-1}), respectively. The order of the viscosities of QG and QG-Cs was as follows: QG > QG-C1 > QG-C2 > QG-

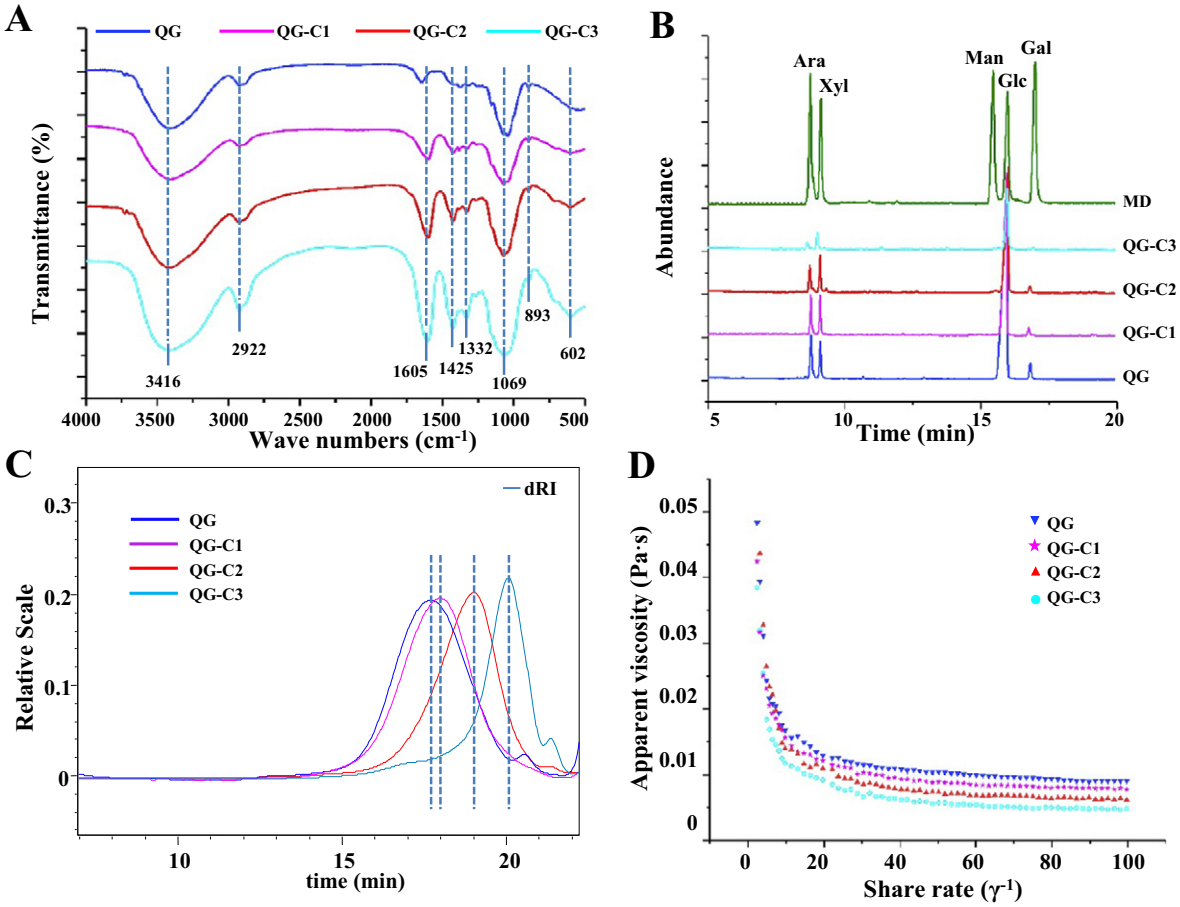

Fig. 3. FT-IR spectra (A), HPLC profiles (B), HPSEC chromatograms (C), apparent viscosities (D) of QG, QG-C1, QG-C2, and QG-C3.

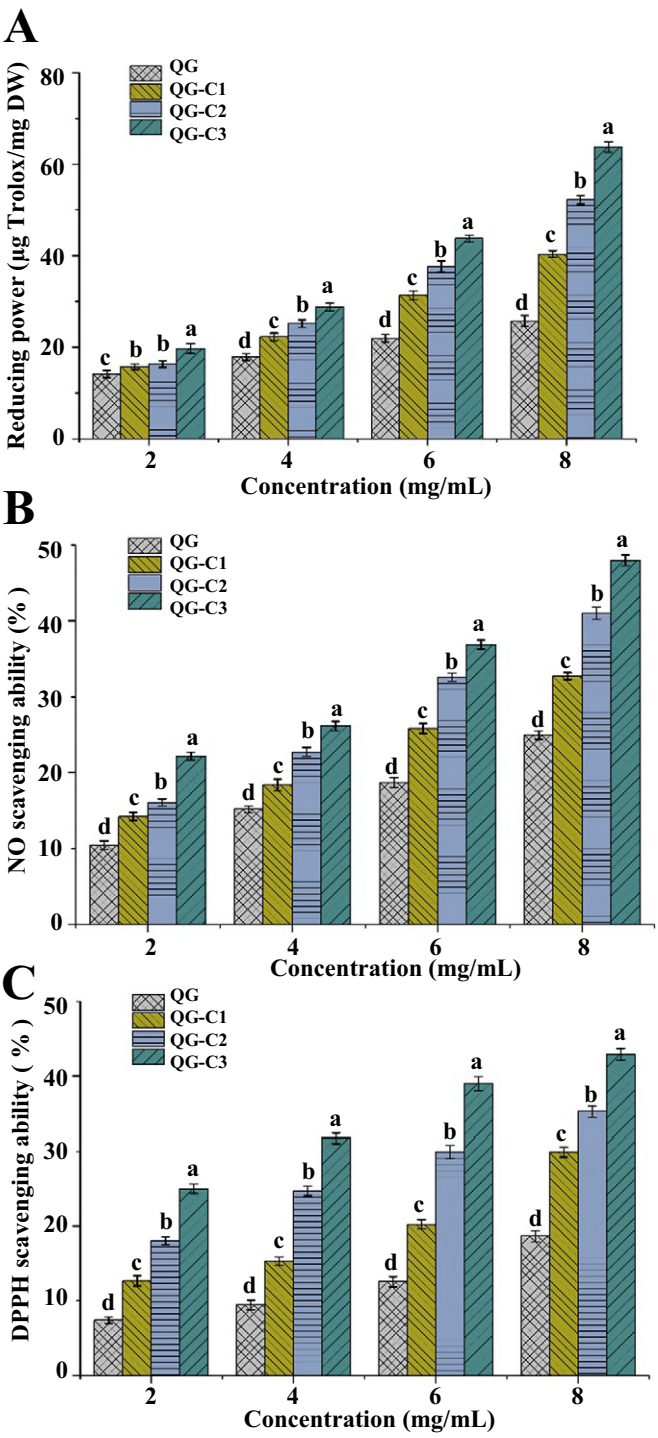

Fig. 4. Reducing power (A), nitric oxide (NO) radical scavenging activity (B), and DPPH radical scavenging activity (C) of QG, QG-C1, QG-C2, and QG-C3.

C3. In general, the lower the molecular weight, the lower apparent viscosity and higher solubility it becomes, and *vice versa*. QG-C3 had the lowest molecular weight and equipped with the highest solubility and lowest apparent viscosity. Results demonstrated that the carboxymethylated modification could reduce the apparent viscosity and improve the water solubility of QG, which was in accordance with the results of polysaccharides from *Ganoderma lucidum* [12]. These results suggested that the apparent viscosities of QG, QG-C1, QG-C2, and QG-C3 have close relationship with their molecular weights, which were in accordance with previous studies [12,35,36].

3.3. Antioxidant activities of QG and QG-Cs

Previous study has demonstrated that Qingke β-glucans exert moderate antioxidant activities [27]. In fact, carboxymethylated modification can enhance the antioxidant activity of polysaccharides [9,12,15,37]. Thus, the antioxidant activities of QG, QG-C1, QG-C2, and QG-C3 were measured. The reducing powers, NO and DPPH radical scavenging activities of QG, QG-C1, QG-C2, and QG-C3 were summarized in Fig. 4. QG, QG-C1, QG-C2, and QG-C3 exhibited obvious reducing powers, NO and DPPH radical scavenging activities with a dose-dependent manner, respectively. Results demonstrated that the antioxidant activities of QG were significantly ($p < 0.05$) lower than that of QG-Cs. Especially, at the concentration of 8.0 mg/mL, the reducing powers of QG, QG-C1, QG-C2, and QG-C3 were determined to be 25.72 ± 1.21 μg Trolox/mg, 40.35 ± 0.76 μg Trolox/mg, 52.23 ± 0.97 μg Trolox/mg, and 63.78 ± 1.16 μg Trolox/mg, respectively; The NO radical scavenging activities of QG, QG-C1, QG-C2, and QG-C3 were determined to be 24.94 ± 0.55%, 32.72 ± 0.45%, 41.02 ± 0.77%, and 47.98 ± 0.71%, respectively; The DPPH radical scavenging activities of QG, QG-C1, QG-C2, and QG-C3 were determined to be 18.68 ± 0.75%, 29.93 ± 0.67%, 35.25 ± 0.76%, and 48.45 ± 0.81%, respectively. Results indicated that the introduction of carboxymethyl groups could enhanced the ability of QG to scavenge NO and DPPH radical, and the higher DCs, the better scavenging effects [15]. The significant differences among QG, QG-C1, QG-C2, and QG-C3 implied that the antioxidant activities were dependent on the DCs. QG-Cs with higher DC exhibited stronger antioxidant activities, which were in accordance with previous studies [12]. In short, compared with QG, the antioxidant activities of QG-Cs were improved owing to the introduction of the carboxymethyl groups. This condition may be due to the introduction of carboxymethyl groups in QG to increase the electron cloud density of the active hydroxyl groups, and increase the electron-withdrawing activity of QG, thereby terminating free radical-mediated oxidative chain reaction [12,15].

3.4. In vitro hypolipidemic activities of QG and QG-Cs

3.4.1. In vitro binding capacities

Excessive intake of fat and bile acids can lead to obesity and some other diseases, such as cardiovascular disease, diabetes, and cancers [38]. β-glucans exhibit obvious abilities to lower blood fat and cholesterol, which may contribute to their hypolipidemic activities [14,39,40]. The *in vitro* fat binding capacities, bile acid binding capacities, and cholesterol binding capacities of QG, QG-C1, QG-C2, and QG-C3 were summarized in Fig. 5. The fat binding capacities (Fig. 5A), bile acid binding capacities (Fig. 5B), and cholesterol binding capacities (Fig. 5C) of QG, QG-C1, QG-C2, and QG-C3 ranged from 2.31 ± 0.06 to 4.02 ± 0.06 g/g, from 19.23 ± 0.42% to 26.92 ± 0.59%, and from 39.55 ± 0.62 to 48.27 ± 0.77 mg/g, respectively. Furthermore, the significantly highest fat binding capacity, bile acid binding capacity, and cholesterol binding capacity were measured in QG-C3, followed by lower in QG-C2 and QG-C1, and the lowest in QG. Results revealed that the QG-Cs exerted stronger binding capacities than that of QG. In fact, the *in vitro* binding properties of QG-Cs were closely connected with DCs. The higher DC of QG-Cs, the stronger binding properties of QG-Cs were observed. In general, the relatively high bioactivity of carboxymethylated polysaccharides is often connected with their carboxymethyl groups, which can interact with positively charged biomacromolecules [41]. Results showed that the carboxymethyl groups of QG-Cs might be the key factor for enhancing their binding capacities. In addition, both QG and QG-Cs exerted significantly higher fat binding and cholesterol binding capacities than that of the positive control (carboxymethyl cellulose). Results demonstrated that the carboxymethylated β-glucans had good potential for the prevention of obesity and hypercholesterolemia.

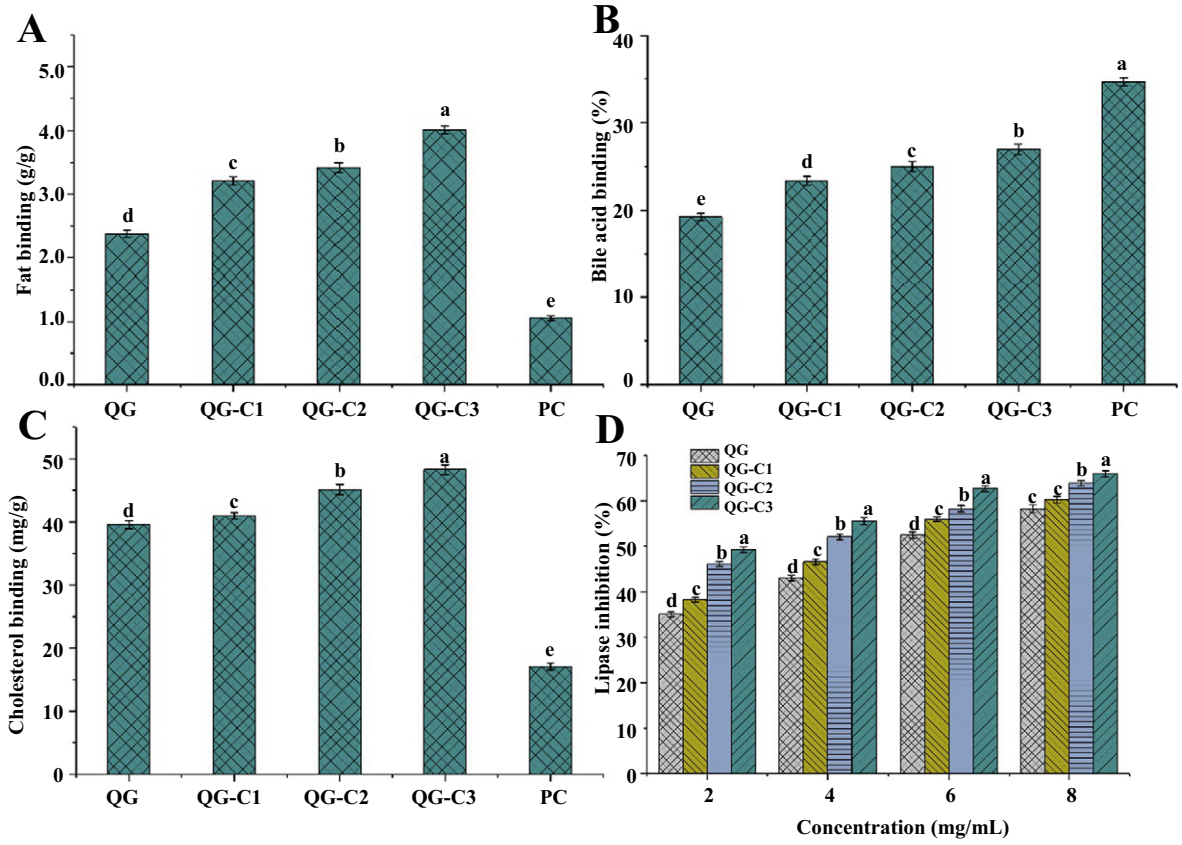

Fig. 5. Fat binding (A), bile acid binding (B), cholesterol binding (C) capacities, and *in vitro* inhibitory activities on the pancreatic lipase (D) of QG, QG-C1, QG-C2, and QG-C3.

3.4.2. Inhibitory effects on the pancreatic lipase

Pancreatic lipase belongs to the most essential enzymes that involve in the digestion of triglyceride. Generally, the inhibition of lipase activity is an important way for the regulation of the obesity and hyperlipidemia. Previous studies have indicated that β-glucans exhibit obvious inhibition effects on the pancreatic lipase [11,14]. Thus, effect of carboxymethylated modification on the inhibition activities of QG and QG-Cs on the pancreatic lipase was studied. The results of inhibition activities of QG and QG-Cs on the pancreatic lipase were displayed in Fig. 5D. QG, QG-C1, QG-C2, and QG-C3 exhibited obvious inhibition effects against pancreatic lipase with a dose-dependent manner. At the concentrations from 2.0 to 8.0 mg/mL, the pancreatic lipase inhibition effects of QG-C1, QG-C2, and QG-C3 were significantly higher than that of QG, which were similar with the previous studies [42]. Indeed, at the concentration of 8.0 mg/mL, the significantly lowest inhibition effect was measured in QG (58.36 ± 0.86%), followed by higher in QG-C1 (60.29 ± 0.82%) and QG-C2 (63.14 ± 0.75%), and the highest in QG-C3 (66.97 ± 0.78%). According to the DCs, apparent viscosities, and M_w of QG, QG-C1, QG-C2, and QG-C3 as measured above, results suggested that the pancreatic lipase inhibition effects of QG-C might be associated with their DCs, apparent viscosities, and M_w. Results demonstrated that the introduction of carboxymethyl groups could improve the inhibitory activity on pancreatic lipase. Besides, the IC_{50} values of pancreatic lipase inhibition effects of QG, QG-C1, QG-C2, and QG-C3 were measured to be 5.724 mg/mL, 5.218 mg/mL, 3.873 mg/mL, and 2.109 mg/mL, respectively. Compared with the positive control (orlistat, IC_{50} = 3.816 mg/mL), QG-C3 exerted strong pancreatic lipase inhibition effects. Indeed, the pancreatic lipase inhibition effect of QG-C3 was also significantly higher than that of sulfated Qingke β-glucans [14]. Results suggested that carboxymethylated β-glucans could be further applied for the prevention of obesity.

4. Conclusions

In this study, results indicated that carboxymethylated modification was significantly affected solubilities, molar ratios of constituent monosaccharides, molecular weights, and apparent viscosities of QG. In addition, the QG-Cs exhibited much better antioxidant activities, *in vitro* binding capacities, and inhibitory effects on pancreatic lipase than that of QG. Results suggested that the carboxymethylated modification could be an efficient method for enhancing bioactivities of QG, and QG-Cs could be further explored in the bio-pharmaceutical industry.

CRediT authorship contribution statement

Huan Guo: Data curation, Formal analysis, Investigation, Resources, Software, Writing - original draft.**Kang-Lin Feng:**Formal analysis, Investigation, Validation, Resources, Software, Writing - original draft.**Jia Zhou:**Formal analysis, Investigation.**Lu Liu:**Formal analysis, Investigation.**Si-Yu Wei:**Formal analysis, Investigation.**Li Zhao:**Formal analysis, Investigation, Software.**Wen Qin:**Resources, Software.**Ren-You Gan:** Formal analysis, Funding acquisition, Methodology, Supervision.**Ding-Tao Wu:**Data curation, Formal analysis, Funding acquisition, Methodology, Supervision, Project administration, Writing - review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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