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Extraction, preparation, and carboxymethyl of polysaccharide from *Lotus* root

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Abstract

In this study, we extracted *lotus* root polysaccharide (LRP) and synthesized carboxymethylated *lotus* root polysaccharide (CM-LRP) using response surface methodology (RSM). The monosaccharide component of LRP was analyzed by pre-column derivatization high performance liquid chromatography (PCD-HPLC). The polysaccharide was characterized by ultraviolet (UV) spectroscopy scan, Fourier transform infrared spectroscopy (FTIR), circular dichroism spectrometer (CD), and scanning electron microscopy (SEM). It was found that the main monosaccharide component of LRP was glucose, accounting for more than 98% of the total polysaccharide component. RSM analyses revealed that the optimal conditions for CM-LRP synthesis were a reaction duration of 2.22 h, the chloroacetic acid dosage is 2.02 g, and a temperature of 46.87 °C. Under these conditions, the predicted degree of substitution (DS) was determined to be 0.5101. The superoxide anion, ferrous ion and hydroxyl radical antioxidant system were constructed to study the antioxidant activity of LRP and CMLRP. The activity of CMLRP to remove ferrous ions and hydroxyl radicals were significantly stronger than that of LRP, and it also showed a certain concentration-dependent. LRP has a better scavenging ability than CMLRP in scavenging superoxide anion free radicals. Our data revealed that CM-LRP is a promising natural antioxidant with potential value as a food supplement.

Keywords: *Lotus* root; polysaccharide; carboxymethyl; antioxidant.

Practical Application: The work shows a complete study about the method of synthesised carboxymethyl lotus root polysaccharide, and their contribution to show the potential value of natural antioxidant with potential value as a food supplement.

1 Introduction

Lotus root (*Nelumbo nucifera Gaertn*) is the rhizome part of *Nymphaeaceae*, mainly distributed in Asian countries such as China and India (Wang et al., 2007). As a common plant, *lotus* root contains various nutrients, including polysaccharides, polyphenols, starches, proteins, fats, and vitamins (Hu & Skibsted, 2002; Yi et al., 2018; Zhang et al., 2016; Zhou et al., 2007). According to the relevant records in the Classic of Materia Medica, *lotus* root cleanses heat, stops bleeding, regulates the endocrine, nourishes, and calms the mind (Zhang et al., 2016; Yi et al., 2018). Referring to the Modern Pharmacopoeia, we can see that the raw use of *lotus* root can play the effects of clearing away heat, cooling blood, and hot rain, while familiar use has the effects of strengthening the spleen and stomach, tonifying blood, and promoting muscle and antidiarrheal effect (Yang et al., 2007). A large number of studies have shown that polysaccharides are an important component of *lotus* root active components, and polysaccharides have many biological activities, including antioxidant, anti-tumor, immune regulation, hypoglycemic and antiviral activities Yang (Chen et al., 2015; Chen et al., 2016; Jeddou et al., 2016; Liu et al., 2016; Wang et al., 2016; Yi et al., 2019; Zheng et al., 2016).

According to the current development level, the technical level of *lotus* root processing is relatively low, of which the extraction and development of polysaccharides may be a new way to develop and utilize its resources. Polysaccharide is closely related to the maintenance of life-related functions and is one of the basic substances that constitute human life (Leung et al., 2006; Liu et al., 2013; Yu et al., 2018; Zhang et al., 2019). Polysaccharides are a kind of important bioactive substance with a very large molecular weight, which can reach tens of thousands or even millions and make the molecular structure of polysaccharides very complex (Liu & Huang, 2019; Liu et al., 2019). There has a certain relationship between the molecular structure of polysaccharides and their biological activity. By modifying the molecular structure, the biological activity of polysaccharides can be significantly enhanced or produce new activities. Selecting a suitable modification method for the molecular structure of polysaccharides can improve the physical and chemical properties and biological activity of polysaccharides to a certain extent (Baba et al., 1988; Veena et al., 2007). Appropriate molecular modification of polysaccharides will also help study the relationship between structure and biological activity of polysaccharides. Therefore, in order to facilitate the discovery and study of the biological activity of polysaccharides, we can choose the way of molecular modification of polysaccharides (Chakka & Zhou, 2020). Polysaccharides' commonly used modification methods include carboxymethylation, phosphate esterification, selenylation, sulfate, alkylation, acetylation, etc. Carboxymethylation is relatively simple, the required reagents are cheap and easy to obtain, and the reaction process is easy to control. The toxicity of the obtained product is low, so it has

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become an important method for polysaccharide modification (Verraest et al., 1995).

At present, the modification method of carboxymethylation of polysaccharides is through the construction of NaOH-chloroacetic acid system, in which the NaOH master can etherify the polysaccharides and then react with chloroacetic acid under alkaline conditions to get carboxymethylated polysaccharides. According to the references, it is known that the carboxymethylation modification site of polysaccharides is usually the alcohol hydroxyl group on the sugar residue, which is replaced by-CH_2COO-group after carboxymethylation modification (Zhang et al., 2021). The biggest advantage of carboxymethylation modification of polysaccharides is that it can improve the water solubility of polysaccharides to some extent. In this study, using the response surface optimization design, *lotus* root polysaccharide was synthesized, and its antioxidant activities were studied before and after modification. This study will effectively support the utilization and development of functional foods and products related to *lotus* root and *lotus* root polysaccharides.

2 Materials and methods

2.1 Materials

Lotus root powder was purchased from the Hubei Province of China. All other chemicals and solvents were of analytical grade.

2.2 Extraction of lotus root polysaccharide (LRP) and preparation of carboxymethylated lotus root polysaccharide (CMLRP)

The LRP was extracted by hot water extraction, and the *lotus* root powder was extracted with *lotus* root powder as raw material. 10 g *lotus* root powder was put into 1000 mL flask, then added 500 mL of 75% ethanol (the ratio of material to liquid was 1:50), mixed evenly, and then put into a water bath and condensed at 50 °C, and refluxed for 2 hours to remove impurities such as lipids. The solution obtained by condensation reflux was centrifuged, and then ethanol was discarded, then 500 mL of ultra-pure water (the ratio of material to liquid was 1:50) was added, and polysaccharides were extracted by condensation refluxing for 2 hours in a water bath at 80 °C. The extract of *lotus* root polysaccharide was collected by 10000 r/min centrifugation and 10 min. The concentrated extract of *lotus* root polysaccharides and the same volume of Savage solution (the volume ratio of chloroform to n-butanol is 4:1) were mixed evenly in the funnel of 500 mL separation. Shake the 2~3 min gently. After the extract was delaminated, the supernatant was retained, and the others in the extract were removed. Transfer the supernatant to the 500 mL conical bottle, add 4 times the volume of 95% ethanol, mix evenly and then store at 4 °C for nights. Centrifuge to obtain *lotus* root crude polysaccharides. To improve the purity of LRP, the above alcohol-precipitated LRP was completely dissolved in ultra-pure water, condensed in a water bath at 50 °C, and refluxed for 2 hours to remove protein and ethanol precipitation again. The polysaccharides with high purity can be obtained by alcohol precipitation. The phenol-sulfuric acid method is widely used to determine the content of polysaccharides because of its simple operation and accurate determination (Chen et al., 2022). In this experiment, the phenol-sulfuric acid colorimetric method was used to determine the content of polysaccharides in the *lotus* root.

The preparation method of CMLRP and the determination of the degree of substitution of CMLRP refer to published (Cheng et al., 2020; Yu et al., 2022).

2.3 Optimisation of carboxymethylated lotus root synthesis and experimental design

DS% was the detection index.

Response surface methodology (RSM) was used to investigate the effects of the three variables. The levels and codes of DS% used in the Box-Behnken design (BBD) are shown in Table 1. The BBD and the results for PLP synthesis are shown in Table 2.

2.4 Characterisation of LRP and CMLRP

(1) Ultraviolet spectrum (UV) analysis

10 mg/mL aqueous solutions of LRP and CMLRP were prepared. Ultraviolet spectrum scan was carried out in the ultraviolet wavelength range of 190–600 nm.

(2) Fourier transform infrared spectroscopy (FTIR) analysis

20 mg dried LRP and CMLRP were fully mixed with 200 mg of KBr and uniformly ground, then the correct amount of fine powder was placed into a circular mold and pressed into a transparent circular sheet. The tableted powder was analyzed by Fourier transform infrared spectroscopy (FTIR; Spectrum400; PerkinElmer, USA) within the wavenumber range of 4000 to 500 cm^{-1}.

(3) Scanning electron microscopy (SEM) analysis

Bond the conductive film on the sample seat, evenly sprinkle a small amount of LRP and CMLRP powder, gently blow the

Table 1. Levels and code of variables used in Box-Behnken design.

Variable	Symbols	Coded levels		
	Coded	-1	0	1
Reaction time (h)	A	1	2	3
MCA (g)	B	1.5	2	2.5
Reaction temperature(°C)	C	40	45	50

Table 2. Experimental Design and Results of CM-LRP Box-Behnken.

Run	A	B	C	DS
1	3.00	2.00	40.00	0.4214
2	1.00	1.50	45.00	0.3087
3	1.00	2.00	50.00	0.3843
4	2.00	2.00	45.00	0.5137
5	1.00	2.50	45.00	0.3790
6	2.00	2.00	45.00	0.5112
7	2.00	2.00	45.00	0.5057
8	2.00	2.00	45.00	0.4976
9	3.00	1.50	45.00	0.3839
10	2.00	1.50	40.00	0.3680
11	2.00	2.00	45.00	0.5030
12	3.00	2.50	45.00	0.3786
13	1.00	2.00	40.00	0.4226
14	2.00	2.50	50.00	0.4202
15	3.00	2.00	50.00	0.4767
16	2.00	2.50	40.00	0.4365
17	2.00	1.50	50.00	0.4226

non adhered polysaccharide powder with an ear washing ball, and observe on the mirror after plating the conductive film.

(4) Determination of monosaccharide

The monosaccharide components of LRP were analyzed by the PCD-HPLC method used in reference (Yao et al., 2020). Galacturonic acid (GalUA), glucose (Glc), galactose (Gal), arabinose (Ara), mannose (Man), ribose (Rib), rhamnose (Rha), and glucuronic acid (GlcUA) were selected as monosaccharide standards. Then the above standard samples were prepared as 80 mg/mL solution. Then the 200 μL standard solution was precisely measured by the pipette and put into the EP tube of 10mL. After mixing evenly, the mixed standard solution was obtained. According to the method in the literature, the standard and mixed standard solution were treated, and then HPLC analysis was carried out. The solution of *lotus* root polysaccharides prepared into 10 mg/mL was derivatized by the method of in the literature, then treated according to the treatment of the standard, and then analyzed by HPLC.

The chromatographic conditions of HPLC were slightly modified according to the literature method. The chromatographic column was Agilent ZORBAX Extend-C18 (250 mm × 4.6 mm mine 5 μm); the mobile phase was 0.1 mol/L phosphate buffer (pH = 6.7), phase B was acetonitrile, the gradient elution ratio was 0, 25, 36, and 60 min, the ratio of A phase was 84%, 84%, 80%, and 84%, the injection volume was 20 μL, the flow rate was 1.0 mL, the column temperature was 30 °C, and the detection wavelength was 250 nm.

(5) Circular dichroism spectrometer (CD) analysis

LRP and CMLRP were scanned and determined by a circular dichroism spectrometer at room temperature. The scanning wavelength range was 190-300 nm, the scanning speed was 50 nm/min, the sensitivity was 2 (Mb/cm), and the sample concentration was 1 mg/mL.

2.5 Antioxidant activity of LRP and CMLRP

The superoxide anion, ferrous ion, and hydroxyl radical scavenging activity were investigated as described by Yan and Yu (Yan et al., 2022; Yu et al., 2022). The appropriate solutions of LRP and CMLRP were prepared at concentrations of 1, 2, 4, 8, and 10 mg/mL for analysis of antioxidant activities.

2.6 Statistical analyses

Data from triplicate assays were subjected to ANOVA to identify significant changes in response to treatments. Differences were considered obviously at $P < 0.05$ and highly significant at $P < 0.01$. Data are presented as the mean ± SEM unless otherwise stated.

3 Results and discussion

3.1 Extraction of LRP

The crude polysaccharides were purified, and the phenol-sulfuric acid colorimetric method was used to determine the purity of polysaccharides. The purity was 94.2%, which could be used for subsequent characterization analysis and antioxidant detection.

3.2 Analysis of monosaccharide components of polysaccharides from lotus root

The HPLC spectra of mixed standard samples are shown in Figure 1A, and the HPLC patterns of *lotus* root polysaccharides are shown in Figure 1B. According to the atlas of mixed standard samples, all standard samples can have peaks, and the corresponding peak times of monosaccharides are mannose 19.702 min, ribose 25.805 min, rhamnose 28.108 min, glucuronic acid 31.669 min, galacturonic acid 34.862 min, glucose 37.747 min, galactose 39.904 min, Ala beth sugar 41.550 min. According to the performance report of HPLC, the chromatographic peaks of all monosaccharides meet the requirements and can be separated effectively. According to the polysaccharide map of *lotus* root, according to peak area ratio, the ratio of mannose: glucuronic acid: galacturonic acid: glucose: galactose: arabinose = 0.12:0.28: 0.07: 98.9: 0.13:0.29. According to this, it can be known that the glucose content is the main component of *lotus* root polysaccharides, which is related to the extraction materials, extraction methods, derivatization treatment, and glycoside linkage of polysaccharides.

3.3 Response surface analysis of CMLRP

In the single factor experiment, the control variable method was used to investigate the effects of reaction time, amount of chloroacetic acid, and reaction temperature on the degree of substitution of *lotus* root polysaccharides modified by carboxymethyl, and the optimum values of three single factors were determined.

As shown in Figure 2A, the synthesis efficiency of carboxymethylated *lotus* root polysaccharide was the highest at the reaction time of 2 h. And then, the degree of substitution

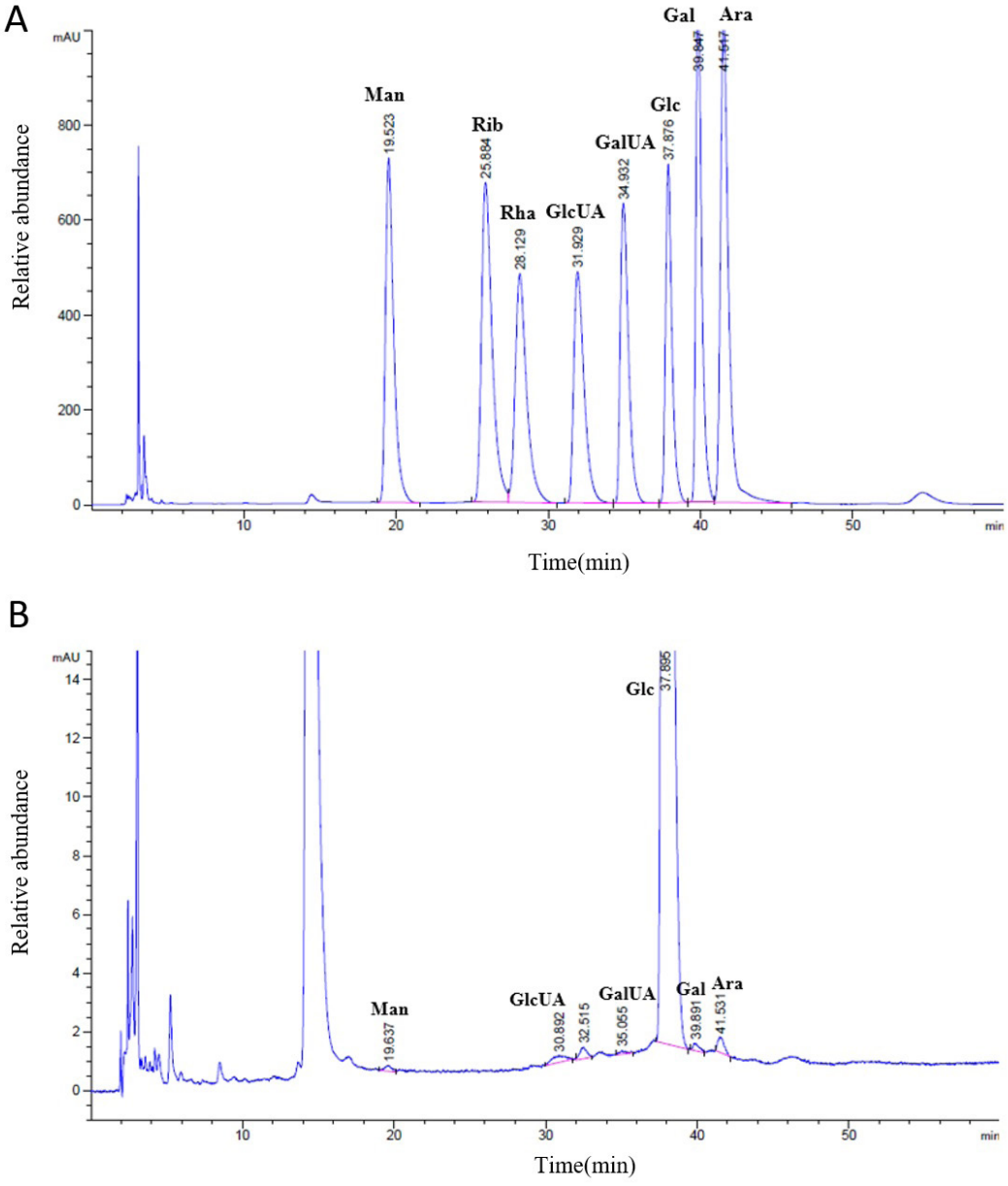

Figure 1. Analysis of the monosaccharide composition of LRP. (**A**) HPLC chromatogram of mixed standards. (**B**) HPLC chromatogram of LRP.

decreased gradually with the extension of reaction time, but the downward trend was not noticeable.

In Figure 2B, when the dosage of chloroacetic acid is 2 g, the degree of carboxymethylation substitution of polysaccharides is the highest. When the amount of chloroacetic acid is more than 2 g, the degree of carboxymethylation of polysaccharides decreases with the increase of the amount of chloroethyl acid, which indicates that the amount of chloroacetic acid in the process of carboxymethyl modification should be reasonable, not the more amount of chloroacetic acid, the better.

Also, in Figure 2C, when the reaction temperature is 45 °C, the carboxymethylation modification efficiency of *lotus* root polysaccharides is the highest. When the reaction temperature is higher than 45 °C, the degree of substitution decreases with the increase of reaction temperature. The ultra-high temperature may destroy the molecular structure of polysaccharides, resulting in the decrease of the degree of substitution.

As shown in Figure 3, the two-factor effect of the model was carried out. The results of 17 trial points tested in a random order based on the BBD design, including design and experimental values, are presented in Table 2. The predicted response (DS) for the CMLRP can be fitted into the following second-order polynomial Equation 1:

$$DS = -2.79853 + 0.14428A + 1.69234B + 0.061489C - 0.0378AB + 0.00468AC - 0.00709BC - 0.0646325A^2 - 0.31623B^2 - 0.0006143C^2. \quad (1)$$

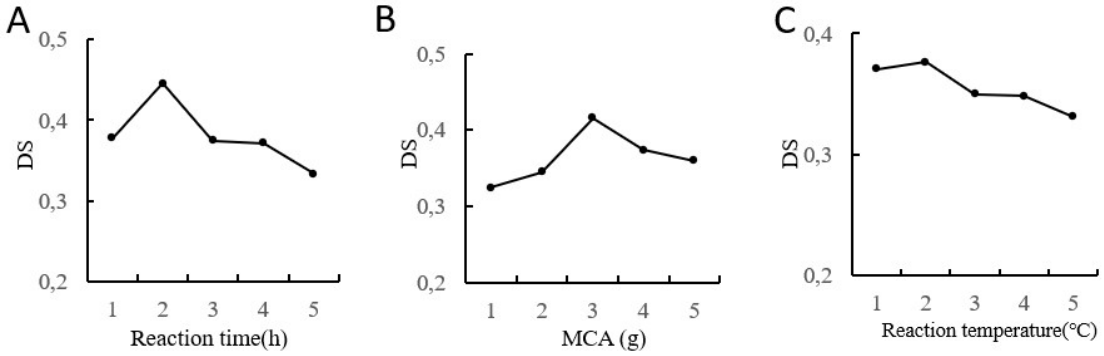

Figure 2. The effect of single factor on DS of CMLRP. (A) Reaction time single-factor experiment results. (B) Single-factor experiment results of chloroacetic acid dosage. **(C)** Reaction temperature single factor experiment results.

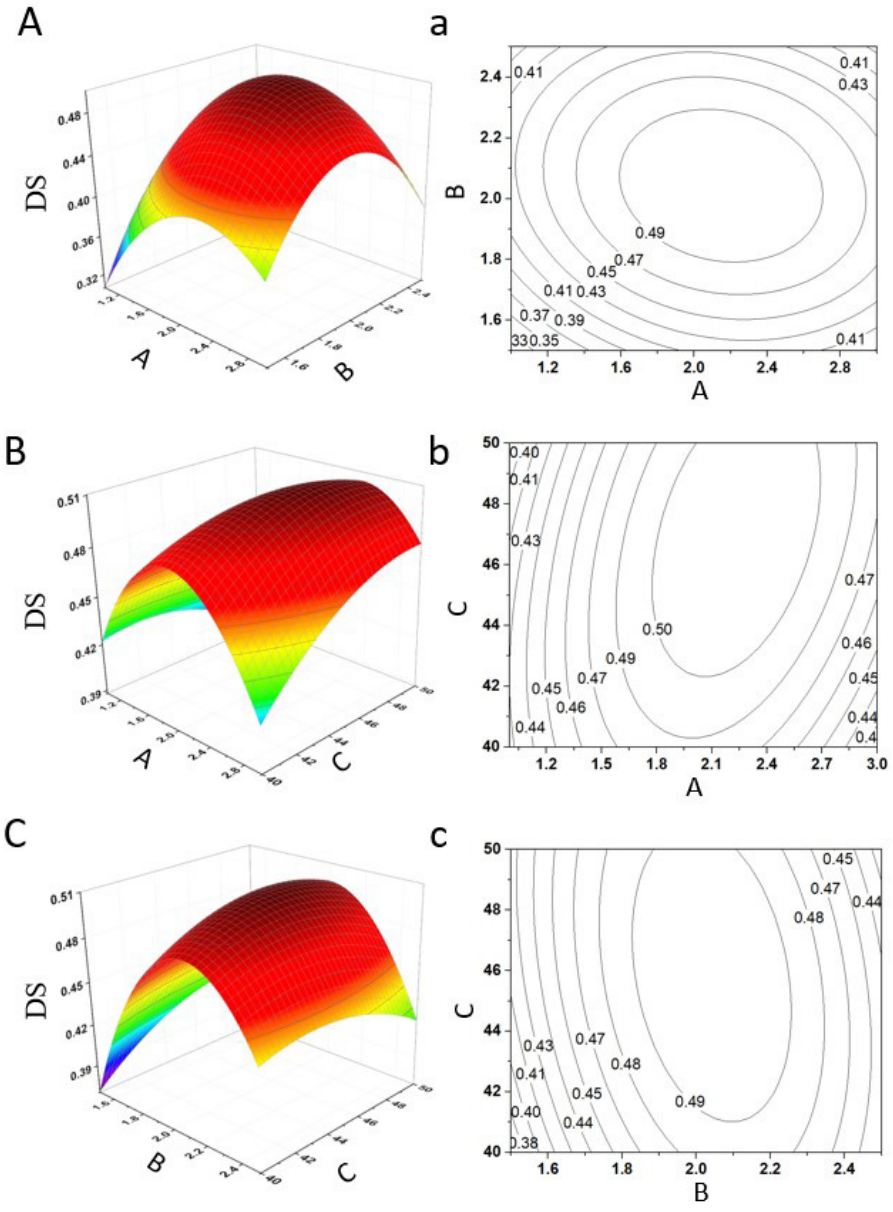

Figure 3. Response surface plots showing the effect of DS on CMLRP. (A, a) Response surface map and contour map of time (A) and MCA (B). **(B, b)** Response surface map and contour map of time (A) and temperature (C). **(C, c)** Response surface map and contour map of MCA (B) and temperature (C).

The results of the model were analyzed by variance analysis, and the results are shown in Table 3. The F value of the regression equation is 180.52, $P < 0.001$, which shows that the experimental model fitted by the response surface method is very significant. The analysis of variance in Table 3 showed that the reaction time and the amount of chloroacetic acid in response surface test combination had extremely significant effects on the degree of substitution of carboxymethyl *lotus* root polysacchlarides, and the reaction temperature was a significant factor. The greatest influence is the reaction time, the second is the amount of chloroacetic acid, and the least influence of reaction temperature.

3.3 Characterisation and analysis of LRP and CMLRP

The UV absorption spectra of LRP and CMLRP from 200 to 600 nm wavelength are shown in Figure 4A. The UV scanning results of LRP and CMLRP in the wavelength range of 200~600 nm were the same, indicating that the carboxymethylation modification method did not affect the structure of polysaccharides. At the same time, it was found that the absorbance of CMLRP was weaker than that of LRP in the wavelength range of 300~600 nm, indicating that carboxymethylation improved the solubility of *lotus* root polysaccharides to some extent.

The FTIR spectra of LPR and CMLRP are shown in Figure 4B. LRP and CMLRP have the characteristic absorption peaks of most polysaccharides in infrared spectra (the peak around 3400 cm^{-1} is the stretching vibration of OH or N-H, and the absorption peak near 2927 cm^{-1} is the stretching vibration of carbohydrate C-H). The strong absorption peak of CMLRP appeared at 1605.35 cm^{-1}, 1418.47 cm^{-1}, and 1030.38 cm^{-1}, the asymmetric absorption peak of carboxyl (-COOH) at 1605 cm^{-1}, and the vibration of C-H connected with C=O at 1418.47cm^{-1} (Ren et al., 2008). Compared with LRP, the new absorption peak of CMLRP at 1605 cm^{-1} and 1418.47 cm^{-1} indicated that CMLRP was synthesized successfully. In addition, by comparing the infrared spectra of LRP and CMLRP, other characteristic absorption peaks were not significantly changed, indicating that the carboxymethylation modification method used in this experiment successfully modified the *lotus* root with carboxymethylation without destroying the molecular structure of *lotus* root polysaccharides.

The CD spectra of LPR and CMLRP are shown in Figure 4C. After carboxymethyl modification, the positive peak of LRP around 200 and 230 nm weakens, similar to the physical modification of Inonotus obliquus polysaccharides by Zhang (Ren et al., 2008).

The SEM of LPR and CMLRP are shown in Figure 5. Figure 5 A1-A3 is 1000 times (200 μm), 500 times (100 μm) and 200 times (50 μm) of LRP, respectively, and Figure 5 B1-B3 is 1000 times (200 μm), 500 times (100 μm) and 200 times (50 μm) of CMLRP, respectively. According to the SEM image, it was observed that the morphology of polysaccharides and modified polysaccharides were fluffy, which accorded with the morphology of freeze-dried polysaccharides. Comparing the SEM images of LRP and CMLRP, we can find some differences in the solid morphology of the two, which is reflected in that the distribution of CMLRP is more uniform, and the particles are smaller than LRP. At the same time, it is also found that both LRP and CMLRP have different degrees of agglomeration, which affects the observed effect of the scanning electron microscope to some extent.

3.4 Antioxidant activities of LRP and CMLRP

As a prerequisite for other reactive oxygen species in organisms, superoxide anion can cause cell death, DNA degradation, and inactivation of other active substances. Both As shown in Figure 6A, LRP and CMLRP have the ability to scavenge superoxide ions to a certain extent, and the scavenging ability increases with the increase of sample concentration. However, according to the experimental results, the scavenging ability of LRP is stronger than that of CMLRP.

Table 3. Analysis of variance of the experimental results of the CM-LRP.

Variables	Sum of squares	df	Mean square	*F*-value	*p*-Value
model	0.059	9	6.606E-003	180.52	<0.001***
A	3.445E-003	1	3.445E-003	94.12	<0.001***
B	2.148E-003	1	2.148E-003	58.71	0.0001***
C	3.823E-004	1	3.823E-004	10.45	0.0144*
AB	1.429E-003	1	1.429E-003	39.04	0.0004***
AC	2.190E-003	1	2.190E-003	59.85	0.0001***
BC	1.257E-003	1	1.257E-003	34.34	0.0006***
A^2	0.018	1	0.018	480.62	<0.0001***
B^2	0.026	1	0.026	719.09	<0.0001***
C^2	9.931E-004	1	9.931E-004	27.14	<0.0012***
Residual	2.562E-004	7	3.660E-005		
Lack of fit	9.048E-005	3	3.016E-005		
Pure error	1.657E-004	4	4.142E-005	0.73	0.5864
Correlation total	0.060	16			
$R^2 = 0.9957$	$R^2_{Adj} = 0.9902$	$R^2_{Pred} = 0.9714$	*Adeq precision* = 43.047		

p: Significance test. F: F test. df: Degree of Freedom. * p<0.05. *** p<0.001.

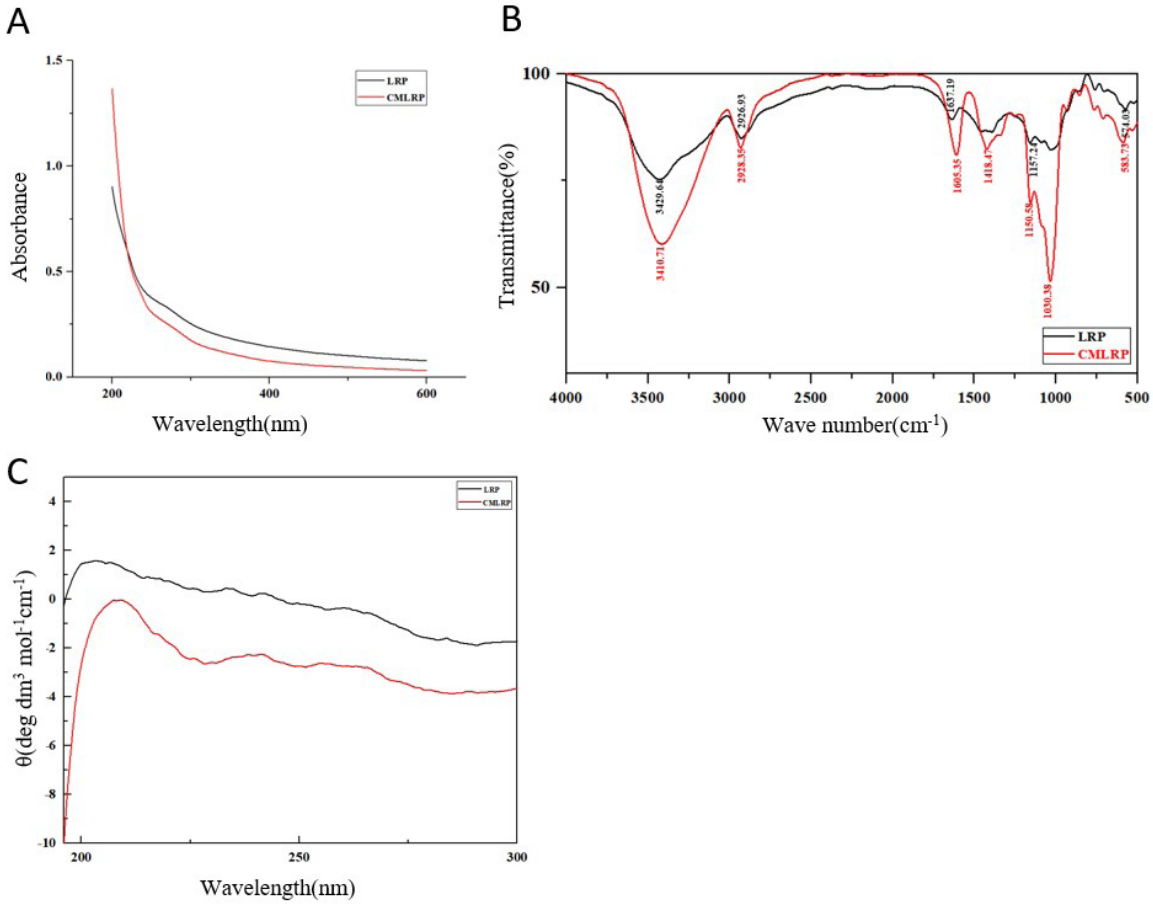

Figure 4. Characterization analysis of LRP and CMLRP. (**A**) Ultraviolet spectra of LRP and CMLRP. (**B**) FTIR spectra of LRP and CMLRP. (C). CD spectra of LRP and CMLRP.

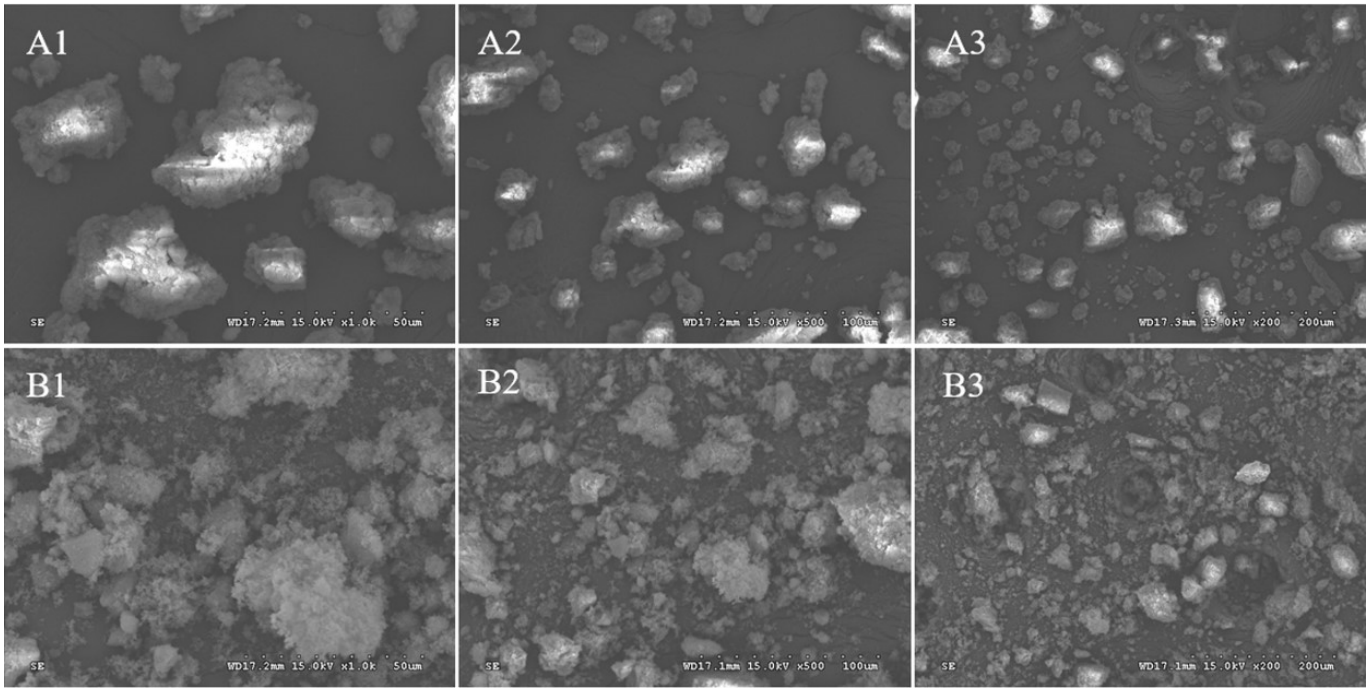

Figure 5. Morphology of LRP and CM-LRP observed by SEM at different magnifications. (**A1, A2**, and **A3**) SEM spectra of LRP. (**B1, B2,** and **B3**) SEM spectra of CMLRP.

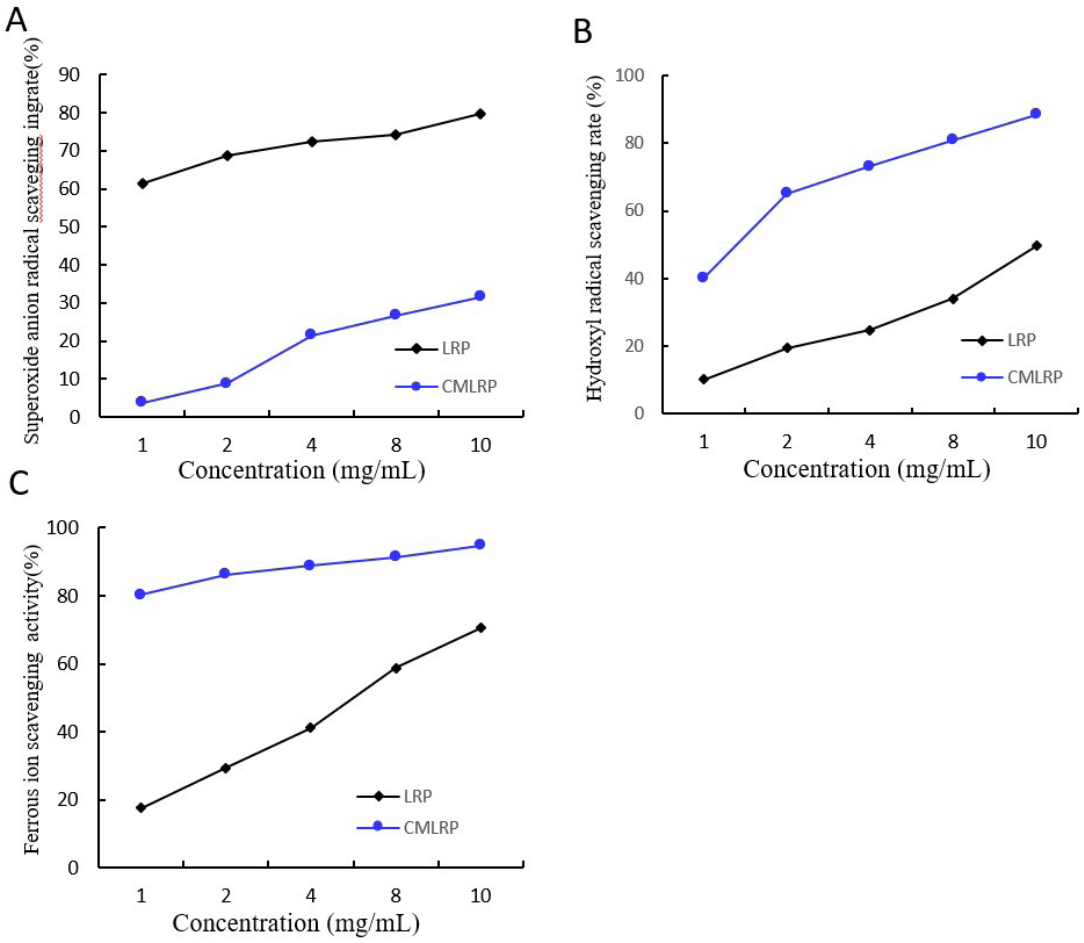

Figure 6. Antioxidant capacity of LRP and CM-LRP. (**A**) Scavenging of superoxide anions by LRP and CMLRP. (**B**) Scavenging of Hydroxyl radical by LRP and CMLRP. (**C**) The ferrous chelating activity of LRP and CMLRP.

Hydroxyl radicals can react with almost all cellular components, so it is a common method to study the ability to scavenge hydroxyl radicals. The scavenging effect of *lotus* root polysaccharides and carboxymethylated *lotus* root polysaccharides on hydroxyl radicals is shown in Figure 6B. According to the experimental results, with the increase of sample concentration, the scavenging efficiency is higher, and carboxymethylation modification can improve polysaccharides' hydroxyl radical scavenging ability.

Investigating the scavenging effect of ferrous ions is one of the methods widely used to evaluate antioxidant activity, which is fast, accurate, simple, and convenient. As shown in Figure 6C, LRP has a certain ability to scavenge ferrous ions. When the concentration of polysaccharide is 4 mg/mL, its scavenging ability is the strongest, but the scavenging efficiency decreases rapidly in the range of 4 mg/mL-10 mg/mL. The ferrous ion scavenging efficiency of CMLRP is relatively stable, and the scavenging efficiency is significantly stronger than that of LRP.

Carboxymethylation is a common modification method of polysaccharides, which can improve the biological activity of polysaccharides. Zhang et al. (2022) reported compared with unmodified polysaccharides, carboxymethyl polysaccharide of *Pholiota nameko* (CPPN) had significant antioxidant activity and water solubility. Liu et al. (2019) reported the carboxymethylated cushaw polysaccharide had better ability to scavenge superoxide anions and hydroxyl radicals. Also, carboxymethyl xylan polysaccharide shows better effects compared with xylan polysaccharide (Chen et al., 2021). Studies revealed that carboxymethylation of polysaccharides enhances the bioactivities and water solubility of native polysaccharides significantly, and provide structural diversity and even the addition of new bioactivities (Chakka & Zhou, 2020).

4 Conclusion

In this experiment, LRP was extracted and purified, and CMLRP was modified. The extraction method, content determination, monosaccharide component analysis, and preparation technology of CMLRP were studied, and the technological conditions of carboxymethylation modification of LRP were optimized by the response surface method. The traditional water extraction method is used in this paper. According to the analysis of monosaccharide components, glucose is the main component of LRP. Through the construction of a sodium hydroxide-chloroacetic acid reaction system, carboxymethylation modified products were synthesized by reacting chloroacetic acid with LRP. This single-

factor experiment revealed that the optimal CMLRP synthesis conditions established using RSM were a reaction duration of 2.22 h, the chloroacetic acid dosage is 2.02 g, and a temperature of 46.87 °C. Under these conditions, the predicted degree of substitution (DS) was determined to be 0.5101. The CMLRP was characterized by instrumental analysis, and there was a characteristic absorption peak of COOCH3. It was determined that the CMLRP were prepared successfully, and the degree of substitution of the CMLRP was 0.309- 0.514.

After carboxymethyl modification, the antioxidant activity of LRP was improved partly. The scavenging activity of CMLRP on hydroxyl radicals and ferrous ions was significantly stronger than that of LRP. The antioxidant activity of polysaccharides is related to its structural parameters, such as the degree of substitution of carboxymethylation and the type and position of substituents. As the structure of LRP was extremely complex, the antioxidant capacity of carboxymethylated polysaccharides varies in different antioxidant systems, and the mechanism is not completely clear, which needs to be further studied.

Conflict of interest

The authors have declared that no competing interest exists.

Availability of data and material

The data used to support the findings of this study are included in the article.

Author contributions

Dr. Zhang XF designed the study. Yan YY, Wang Q, Sun LH, and Zhang XF collected data. All authors agreed to the final version.

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