The study on antioxidant activity of ulvan-calcium derivative

Xiaoqian Wang¹, Yuzhou Wan¹, Lin Liu¹, Haiting Xu², Junying Zou¹, Siming Liang¹, Chen Yang¹ and Huimin Qi¹*

¹Weifang Medical University, No. 7166 Baotong Road, Weifang 261053, PR China.  
²Weifang People’s Hospital, No. 151 Guangwen Road, Kuiwen District, Weifang 261000, PR China.

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Ulvan is a water-soluble polysaccharide that is extracted from the algae Ulva pertusa (Chlorophyta). In this study, ulvan-calcium derivative (U-calcium) was prepared and calcium content was 5.2%. Ulvan-calcium could effectively scavenge on 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazolie-6-sulfonic acid) (ABTS), hydroxyl, and superoxide radicals. The inhibition rate on superoxide radicals (•O₂⁻) was 83.14% at 0.2 mg/mL, and the scavenging activity of ABTS and hydroxyl radical (•OH) at 3.0 mg/mL was 58.45 and 43.65%, and that of DPPH radicals at 8.0 mg/mL was 57.37%. All results showed that the antioxidant activity of ulvan-calcium was stronger than that of ulvan. The ulvan-calcium might be used as an antioxidant to remove ROS and resist excessive oxidative stress.

Key words: Ulvan-calcium, Ulva pertusa, antioxidant activity.

INTRODUCTION

Scientists have found that almost all the free radicals that damage human health were related to the more active substances containing oxygen. The free radicals that combine with these substances were called reactive oxygen species (ROS). These free radicals were particularly important in biology. Oxygen-centered radicals include peroxyl radicals (•ROO⁻), nitric oxide (NO⁻), superoxide radical anion (•O₂⁻), hydroxyl radical (•OH), and non-radical molecules (Kostyuk and Potapovich, 2009). Overproduction of ROS can cause DNA damage, protein modification, lipid peroxidation and other effects, leading to oxidative damage and cellular death (Jiang et al., 2020). It has been reported that overproduction of free radicals played a significant role in related diseases such as Alzheimer’s disease (AD) (Chen et al., 2019), cardiovascular (Valko et al., 2006), hypertension (Reckelhoff et al., 2019) and cancer (Thanh et al., 2016). As a natural biomolecule, plant polysaccharides could promote health and have excellent therapeutic effects in many diseases. Also, due to its high safety, less adverse reactions and good biodegradability, in the past decades, a variety of bioactive plant polysaccharides have been widely studied, such as polysaccharides from jujube (Ziziphus jujuba Mill.) fruit, a polysaccharide from mulberry fruit, polysaccharides from alfalfa roots, etc (Gao et al., 2020; Ji et al., 2020; Wang et al., 2020). Ulvan, a polysaccharide present in the cytoderm of the green Ulva pertusa, has various biological...
activities and has a great potential activity to be developed. \[\beta\text{-D-GlcP\text{\textalpha}{\text{1\textrightarrow\text{4}}}}\text{-L-Rhap3s}\] and \[\alpha\text{-L-IdoP\text{\textalpha}{\text{1\textrightarrow\text{4}}}}\text{-L-Rhap3s}\] were the two major disaccharide units in ulvan. It was mainly constituted of sulfated, rhamnose, glucose, xylose, and glucuronic acid with smaller amounts of iduronic acid (IdoA) (Lahaye and Robic, 2007; Robic et al., 2009). Ulvan had obvious scavenging activity of \(\mathrm{O}_2\). Beyond that, low molecular weight algae polysaccharides had higher water solubility, stability, and easy organism absorption, and showed the strongest antioxidant activity (Qi et al., 2005). Also, in vitro and in vivo studies showed the anticoagulant, antiproliferative, antiviral, antibacterial, antihyperlipidemic, and immune-modulatory activities of the polysaccharides from \(U.\ pertusa\) (Fedorov et al., 2013; Ngo et al., 2011; Wijesekara et al., 2011). The antioxidant activity of polysaccharides was closely related to its main chain, branch chain mechanism, type of substituents, molecular weight, and so on (Ferreira et al., 2014; Seedevi et al., 2017). At present, there have been many studies on modifying the structure of polysaccharides to change their biological activities and reduce their toxicity. Modification of polysaccharides included sulfation, phosphorylation, acetylation, benzoylation, etc (Chen et al., 2019b).

Metal complexes played an important role in living organisms and medicines (Habala and Valentová, 2018). Calcium was an essential mineral element in the human body. Calcium can promote the activity of certain enzymes in the body and regulate the activity of enzymes, participate in nerve and muscle activities, release neurotransmitters, and regulate the secretion of hormones (Straub, 2007). The calcium absorption efficiency of organic calcium was higher than inorganic calcium carbonate (Adluri et al., 2010); commonly used supplements were calcium carbonate and calcium citrate. In this study, ulvan-calcium was prepared successfully. Also, the antioxidant activity in vitro of ulvan-calcium was determined.

**MATERIALS AND METHODS**

\(U.\ pertusa\), harvested from the coast of Qingdao in September 2018, was dried and preserved. Ulvan were extracted from \(U.\ pertusa\) by water extraction and alcohol precipitation method of Qi et al. (2005). Dialysis membrane of molecular weights was cutoff 3,500 Da, nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide-reduced (NADH), ethylene diamine tetra-acetic acid (EDTA), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Solarbio Chemical Co (Beijing, China). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Shanghai TCI Chemical Industry Development Co., Ltd (Shanghai, China). The other chemicals used in this experiment were of analytical grade.

**Preparation of polysaccharides-calcium complex**

A small amount of ulvan (2 g) was dissolved in distilled water (100 mL). The 2 M calcium chloride solution (10 mL) was added to the polysaccharide solution slowly, stirred for 1 h under temperature 60°C at pH 11. The reaction solution was dialyzed with a 3,500 Da dialysis bag to remove the free calcium, and lyophilized, and then ulvan-calcium derivative was obtained (Cui et al., 2015).

**Determination of physicochemical properties of ulvan and ulvan-calcium derivative**

Calcium content was determined by the method of titration with EDTA (McCormick, 1973). The total sugar of polysaccharide was analyzed by phenol-sulfuric acid colorimetric method (Dubois et al., 1956). The uronic acid and sulfate contents of polysaccharide were analyzed by sulfuric acid carbazole and barium chloride-gelatin methods (Dodgson and Price, 1962; Yu et al., 2003). Monosaccharide contents were analyzed by using HPLC (Agilent 1260 Infinity, USA) (Zhang et al., 2013). The information of organic functional groups present in the sample were analyzed by FT-IR ( Nicolet 6700) (Chen et al., 2019a).

**Antioxidant activity of ulvan and ulvan-calcium derivative in vitro**

**Superoxide radical-scavenging determination**

Superoxide radicals were generated using the PMS-NADH system (Canadan, 2003). The reaction system (3 mL) includes Tris-HCl buffer (16 mM, pH 8.0), NADH (338 μM), NBT (72 μM), PMS (30 μM), and polysaccharide solutions of gradient concentrations (0.01-0.2 mg/mL), then keep for 5 min at room temperature. The color reaction of superoxide and NBT was measured by the spectrophotometric method at 560 nm. The ascorbic acid dissolved in Tris-HCl buffer was used to replace polysaccharide solution. The scavenging abilities of \(\mathrm{O}_2\) was calculated as follows: scavenging ability (\%) = \(\left(1 - \frac{A_{\text{sample}}}{A_{\text{blank}}}\right) \times 100\%\).

**DPPH radical-scavenging determination**

DPPH is a stable free radical. Based on the literature (Marinova et al., 2011), the detection method was minor modifications. Aqueous solution of different concentrations of the sample (1 mL) was blended with 0.1 mM DPPH ethanolic solution (2 mL). After 30 min at room temperature, absorbance was measured at 517 nm. The positive control was aqueous solution of ascorbic acid. The clearance rate was calculated as follows:

\[
\text{Clearance rate (\%)} = \left(1 - \frac{A_1}{A_0}\right) \times 100\%
\]

where \(A_0\) represents the absorbance of distilled water, \(A_1\) represents the absorbance of the tested sample, and \(A_2\) represents the sample ethanol solution absorbance.

**ABTS radical-scavenging determination**

The ABTS free radicals scavenging activity of ulvan and ulvan-calcium were determined by the ABTS radical cation decolorization technique (Miller et al., 1996). In short, ABTS solution (7 mM) was mixed with potassium persulfate (2.45 mM) and was oxidized at room temperature for 12 h in the dark, then ABTS\(^{+}\) was obtained. Before use, the ABTS\(^{+}\) solution was diluted with 0.15 M phosphate buffered saline (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. Then different concentrations sample solution (300 μL) was added to the obtained ABTS\(^{+}\) solution (3 mL). After 6 min, the absorbance at 734 nm was measured. The reference substance was phosphate buffered saline of ascorbic acid. The ABTS free radical scavenging effect was calculated by the following equation:
ABTS radicals scavenging activity (%) = \left(1 - \frac{\text{Abs1}}{\text{Abs0}}\right) \times 100\%

where \text{Abs0} is the absorbance of the blank (phosphate buffered saline instead of the sample), \text{Abs1} is the absorbance of the sample or positive control.

**Hydroxyl radical-scavenging determination**

Hydroxyl radicals can specifically make discoloration reaction of crocus, therefore, the content of \text{•OH} could be measured by a colorimetric method. The literature method with a modification (Yang et al., 2010) was used to measure the ability of scavenging \text{•OH} of the sample. The final reaction volume was maintained at 4.5 mL, which contained 150 mM sodium phosphate buffer (pH = 7.4, 1 mL), crocus (360 μg/mL, 1 mL), EDTA-Fe (2 mM, 0.5 mL), sample solutions of different concentrations (0.5-3.0 mg/mL, 1.0 mL), and 3% H₂O₂ (1 mL). The reaction system was shaken in a test tube, was incubated in 37 ºC water bath for 30 min, and measured the absorbance at 520 nm. The ascorbic acid dissolved in sodium phosphate buffer was used as a reference substance. The ability of scavenging \text{•OH} of samples was computed with the following formula:

\[
\text{Hydroxyl radical scavenging rate (\%) = } \frac{A_{\text{sample}} - A_0}{A_1 - A_0} \times 100\%
\]

where \text{A₀} is the absorbance of the blank group (distilled water replaces the test sample). \text{A₁} is the absorbance of the control group (distilled water replaces the test sample and sodium phosphate buffer solution replaces H₂O₂).

**Statistical analysis**

All data were obtained by three parallel experiments and expressed as mean ± SD. Graphpad prism 8.3 was used for statistical analysis, and \(P<0.05\) was defined as statistically significant.

**RESULTS**

**Physicochemical properties of ulvan and ulvan-calcium derivative**

As shown in Table 1, the ulvan-calcium derivative was successfully prepared by using calcium chloride with a yield of 29.5%. The calcium content of the derivative (5.2%) was higher than that of ulvan (undetected). Compared with ulvan, we obtained ulvan-calcium derivative with higher contents of total sugar and uronic acid. The total sugar content of ulvan-calcium (65.28%) was higher than that of ulvan (56.29%), and the content of uronic acid (16.10%) was also higher than that of ulvan (11.86%), but the sulfate content of ulvan-calcium (23.5%) was lower than that of ulvan (24.9%).

| Table 1. Synthesis and physicochemical properties of ulvan and ulvan-calcium. |
|-------------------------------------------------|
| **Physicochemical properties** | **Ulvan** | **Ulvan-calcium** |
|-----------------------------|---------|----------------|
| Yield (%)                   |         | 29.5          |
| Calcium (%)                 |         | *             |
| Total sugar (w/w %)         | 56.29   | 65.28         |
| Uronic acid (w/w %)         | 11.86   | 16.10         |
| Sulfate (w/w %)             | 24.9    | 23.5          |

*Not detected.

FT-IR analysis of ulvan and ulvan-calcium

Figure 2 displayed the structural changes of ulvan and ulvan-calcium with FT-IR spectra. Compared with ulvan, the absorption peak of ulvan-calcium became weak. The characteristic peaks at 3409.44 and 2939.04 cm⁻¹ in the ulvan indicate the presence of O-H and C-H groups, respectively. Compared with ulvan, the peak of ulvan-calcium at 3409.44 cm⁻¹ moved to 3401.58 cm⁻¹ and the peak shape became blunt, indicating the association of O-H bonds. In addition, the peaks at 1643.11 and 1416.47 cm⁻¹ (asymmetric stretching vibration and symmetrical stretching vibration of C-O-O) and 1054.64 cm⁻¹ (stretching vibration of C-O) became weak significantly. Moreover, the peak of 1416.47 cm⁻¹ shifted to 1425.18 cm⁻¹, indicating that calcium may be mainly complexed by C-O bonds. The main absorption peak of the ulvan-calcium has not changed, and it can be considered that the preparation was successful.

**Radical-scavenging analysis of ulvan and ulvan-calcium**

**Superoxide radical-scavenging analysis**

The PMS-NADH system produces •O₂⁻, which can generate purple substances with NBT. If •O₂⁻ is
Figure 1. Combined chromatograms of ulvan, ulvan-calcium and standard substance (1: Man; 2: GlcN; 3: Rha; 4: GlcA; 5: GalA; 6: GalN; 7: Glc; 8: Gal; 9: Xyl; 10: Ara; 11: Fuc).

Figure 2. FT-IR spectra of ulvan and ulvan-calcium.
consumed by antioxidants, it may cause a certain degree of discoloration. Therefore, the absorbance can be measured spectrophotometrically at 560 nm (Parejo et al., 2002). The results showed that the ability of ulvan and ulvan-calcium to scavenge •O$_2^-$ becomes stronger as the concentration increases (Figure 3). When the polysaccharide concentration was under 0.05 mg/mL, the clearing effect increased fast. Similarly, the scavenging effect of ascorbic acid and polysaccharides on the •O$_2^-$ decrease in the following order: ulvan-calcium > ulvan > ascorbic acid. The clearances respectively were 83.14, 71.62, and 44.29% at concentrations of 0.2 mg/mL, which confirmed its ability to scavenge •O$_2^-$, and was significantly better than ascorbic acid ($P < 0.01$).

**DPPH radical-scavenging analysis**

The results showed that as the sample concentration increased, the DPPH radicals-scavenging activity also increased. Ascorbic acid had significant scavenging activity for DPPH radicals. As shown in Figure 4a, the scavenging effect of ascorbic acid at a concentration of 0.2 mM was more than 90%. As shown in Figure 4b, EC$_{50}$ values of ulvan-calcium were 6.7 mg/mL. When the concentration of ulvan and ulvan-calcium was 8 mg/mL, the scavenging efficiency was 34.99 and 57.37%, respectively. In addition, ulvan-calcium is more effective than ulvan in removing DPPH radicals.

**ABTS radical-scavenging analysis**

ABTS reacts with potassium persulfate to directly generate stable blue-green ABTS•$^+$ free radicals. The radical has maximum absorption at 734 nm, so the absorbance is detected by spectrophotometry at 734 nm to calculate its concentration. All tested samples showed effective ABTS radicals scavenging ability and were concentration dependent in manner. The EC$_{50}$ value of ascorbic acid scavenging ABTS radicals was 0.09 mg/mL (Figure 5a). The EC$_{50}$ values of ulvan and ulvan-calcium were 3.66 and 2.53 mg/mL, respectively. When the concentration of the ulvan and ulvan-calcium was 3 mg/mL, the clearance rates were 40.14 and 58.45%, respectively, and ulvan-calcium was greater than ulvan’s clearance effect on ABTS radical cation (Figure 5b).

**Hydroxyl radical-scavenging analysis**

Figure 6 shows the •OH scavenging effect of ascorbic acid, ulvan, and ulvan-calcium. In each test group, the sample concentration increased, the ability to scavenge •OH also increased. When the concentration of ascorbic acid, ulvan and ulvan-calcium was 3 mg/mL, the clearance rates were 17.00, 20.40, and 43.65%, respectively, and the •OH scavenging effect of ulvan-calcium was better than that of ulvan and ascorbic acid at the test concentrations.
In order to generally analyze the antioxidant capacity of ulvan-calcium, according to the literature (Terashima et al., 2010), we use radar-chart method to further comprehensively compare the antioxidant capacity of polysaccharides. The 3-axe radar-chart of ulvan and ulvan-calcium (3.0 mg/mL) is as shown in Figure 7. It can be clearly seen from Figure 7 that the area covered by ulvan-calcium is larger, indicating that ulvan-calcium has a certain antioxidant activity.

**DISCUSSION**

Table 1 shows the physicochemical properties of ulvan and ulvan-calcium. According to the literature, basically, all the ulvans were mainly composed of Rha and GlcA,
contents of Xyl and Glc vary, while the content of Gal was small. In this study, calcium substituted some active groups or interacted with the hydroxyl of ulvan, which changed the monosaccharide content and caused the variation of molar ratio of monosaccharide (Xian et al., 2018; Zhang et al., 2013).

High levels of ROS may exceed cellular tolerance, causing inevitable oxidative damage to DNA, protein modification and lipid. There have been literatures that prove that ulvan has several therapeutic effects, including anti-inflammatory and antioxidant properties (Abd-Ellatef et al., 2017). In this study, we studied the antioxidant effect of ulvan and ulvan-calcium. We chose to study the effects of this ulvan and ulvan-calcium on •O$_2^-$, DPPH, ABTS and •OH, because they are generally used to analyze the antioxidant ability of biological samples and compounds. In this study, ulvan and ulvan-calcium demonstrated strong specific •O$_2^-$ scavenging activity. Ulvan and ulvan-calcium may effectively donate electron to DPPH free radicals, which brings a more stable

Figure 5. Percentage of inhibition of ABTS radical by ascorbic acid, ulvan and ulvan-calcium.
As shown in Figure 5, ulvan and ulvan-calcium act as antioxidants to reverse the formation of ABTS•+. Figure 6 shows that both polysaccharides have •OH scavenging activity, and the ulvan-calcium scavenging rate increases significantly with concentration. In this study, the antioxidant ability of ulvan-calcium was dose-dependent. Compared with ulvan, ulvan-calcium could effectively improve the situation of ROS and reduce •O₂, DPPH, ABTS and •OH levels. The ulvan-calcium at concentrations of 0.2 mg/mL was significantly increased by 11.52% than ulvan (P < 0.01). Compared with ulvan, the effect of ulvan-calcium (8 mg/mL) on DPPH was the best, significantly increased by 22.38% (P < 0.01). However, ulvan-calcium (3 mg/mL) showed higher activity on ABTS and •OH, significantly increased by 18.31 and 23.25% (P < 0.01). Some studies have shown that the addition of metal calcium ions can enhance the scavenging effect of chitosan on •O₂. Therefore, the better scavenging effect of ulvan-calcium on •O₂ may be related to the divalent metal calcium ion

Figure 6. Hydroxyl scavenging effect of ascorbic acid, ulvan and ulvan-calcium (0.5-3.0 mg/mL).

Figure 7. A comprehensive comparison of antioxidant capacity.
(Liu et al., 2008). Compared with ulvan, the ulvan-calcium can contribute more hydrogen atoms and has a stronger scavenging capacity of DPPH, which is due to the positive charge. For example, at 1.6 mg/mL, the scavenging DPPH radical ability of chitosan was lower than that of quaternized chitosan derivatives (Wei et al., 2018). Most reported polysaccharides with antioxidant capacity were crude polysaccharides and contain uronic acid. Chen et al. (2004) determined that uronic acid was the critical factor in the tea polysaccharides antioxidant activity. The uronic acid content of ulvan-calcium is higher than that of ulvan. According to the literature, the scavenging ability of antioxidants was mainly due to the formation of a stable structure of polysaccharide molecules and •OH, which terminates the radical chain reaction (Chang et al., 2010). The monosaccharide composition, molecular weight and physiochemical properties of polysaccharides could affect the •OH scavenging ability. In conclusions, polysaccharides obtained through structural modification of calcium have higher antioxidant ability than ulvan.

At present, there have been many researches on the antioxidant capacity of metal ion complexes. Such as rice bran polysaccharides (RBP)-Ca have a strong scavenging effects on DPPH radicals; while RBP-Ca, RBP-Cu and RBP-Fe show strong scavenging capacity for •O₂⁻. However, when the concentration of the RBP-Ca was 4 mg/mL, the scavenging effects of •O₂⁻ and •OH were 85.19 ± 1.31% and 20.76 ± 1.01%, respectively, which are lower than ulvan-calcium in this study (Pan et al., 2019). Copper (II) complexes and their ligands also have the ability to scavenge DPPH radicals (Esmaeili et al., 2019). Sulfated polysaccharide iron (III) complex (SPIC) showed a strong effect of scavenging •OH in vitro (Shi et al., 2013; Xian et al., 2018). Wulangerile et al. (2016) reported that scavenging effects on hydroxy radical of Narenmandula polysaccharide calcium was between 0.125 and 2.0 mg/mL, which was higher than ulvan-calcium in this present study. Ulvan-calcium can reduce free radical levels, which is in keeping with those observed in polysaccharides through structural modification of sulfation (Qi et al., 2005). This showed that ulvan-calcium can be used to enhance the natural defense of cells and/or directly scavenge free radicals, and to reduce oxidative damage to tissues, which achieve the purpose of resisting disease and delaying aging. Ulvan-calcium needs further chemical studies to determine the specific mechanisms involved in antioxidant ability.

Conclusion

This study showed that ulvan-calcium was successfully prepared, and used as an antioxidant to remove ROS and resist excessive oxidative stress. Ulvan-calcium derivative showed stronger antioxidant activity than ulvan, but the antioxidant mechanism of ulvan-calcium needed further study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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