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Preparation and antibacterial activity studies of degraded polysaccharide selenide from *Enteromorpha prolifera*

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ABSTRACT

Polysaccharide extracted from *Enteromorpha prolifera* possessed excellent biological activities, but its molecular weight was greatly high which influenced the activity. Organic Se had higher biological activities and was safer than inorganic Se species. In the present study, degraded polysaccharide selenide (Se-LEP) was synthesized from sodium selenite and degraded polysaccharide (LEP) with the catalysis of nitric acid. The preparation conditions of LEP and Se-LEP were optimized by orthogonal experiments. The selenite ester group was formed, and the selenium content was 1335.27 μg/g. The thermal stability of Se-LEP decreased. LEP had less inhibitory effects on bacteria and plant pathogenic fungi. Se-LEP had stronger inhibitory effect on *Eschetichia coli*, and weaker inhibitory effect on *Staphylococcus aureus* than polysaccharide selenide (Se-EP). Se-LEP also had better inhibitory effects on plant pathogenic fungi.

1. Introduction

The essential trace element selenium (Se) was a key constituent of selenoproteins (Beckett & Arthur, 2005), and acted as an important antioxidant in human and animal bodies (Mukherjee et al., 1998). Se also participated in the synthesis of enzymes and protected biomembranes from overoxidation or damage. Se-deficiency could cause about 40 diseases, such as Keshan disease and Kashin-Beck disease (Ip et al., 2002; Ge & Chang, 1999; Tan et al., 2002). Since it was generally believed that organic selenium compounds were better and safer than inorganic selenium as dietary supplements, a variety of selenium-enriched biological products had been commercialized (Shang et al., 2009). The selenium supplementation using artificial chemical synthesis has received much attention in the past decade.

Enteromorpha prolifera was important edible and medicinal economic seaweeds, which was widely distributed along the coast of China, particularly in the eastern regions. Polysaccharide was one of the main biologically active substances in *Enteromorpha prolifera*. It possessed various bioactive functions, such as immunomodulation, hypoglycaemic, hypolipidemic, antitumor, anti-aging and antibacterial effects (Jiao et al., 2010; Xu et al., 2005; Wang & Chen, 1995). But, many studies have demonstrated that molecular weight distributions of polysaccharides had great influence on their biological activities (Yoshihiro et al., 1995). Polysaccharide selenide had the double effects of organic selenium and polysaccharide, with unique biological activity (Malinowska et al., 2009; Wang et al., 2011). Little information has been obtained regarding the degraded polysaccharide (Zhang et al., 2013). No study has been carried out on degraded polysaccharide selenide. In this paper, degraded polysaccharide (LEP) and degraded polysaccharide selenide (Se-LEP) were prepared to enhance the biological activity of polysaccharide from *Enteromorpha prolifera* (EP). Their antibacterial activities were also investigated. This study provided a scientific basis for developing new health products and food preservatives.

2. Materials and methods

2.1. Materials

Enteromorpha prolifera was obtained from Qingdao Huiquan Waters. Polysaccharide and polysaccharide selenide (Se-EP) were extracted and prepared by our laboratory, respectively. Trifluoroacetic acid (TFA), sodium selenite and other reagents were of analytical grade. Dialysis membranes were produced by Spectrum Co., and molecular weight was cut off at 3000 Da.

Staphlococcus aureau (*S. aureus*), *Eschetichia coli* (*E. coli*), *Colletotrichum capsici*, *Cytospora* sp., *Fusarium turcicum*, *Physalospora piricola*, *Gaeumannomyces graminis*, *Rhizoctonia solani*, *Coniothyrium diplodiella* and *Alternaria oleracea* were provided by Bionic Test Center in Qingdao Agricultural University.

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2.2. Analytical methods

Total sugar content was determined by phenol-sulfuric acid method using rhamnose as standard (Dubois et al., 1956). Selenium content analysis was performed by graphite furnace atomic absorption spectrometer WFX-210 (Deaker & Maher, 1997). Molecular weight was determined by PL aquagel-OH on a 1260 infinity GPC system with 2140 refractive index detector.

UV spectra were measured by shimadzu UV 2550. Fourier transform infrared (FTIR) was measured by a Nicolet Magna-Avatar 300 with KBr disks. Thermal stabilities were measured by thermogravimetry-differential thermal analyzer (TG-DTA), with the scanning temperature of 30–700 °C and the heating rate of 10 °C/min.

2.3. Preparation of degraded polysaccharide (LEP) and degraded polysaccharide selenide (Se-LEP)

The extraction and purification of polysaccharide from *Enteromorpha prolifera* were performed according to the method that we previously reported (Lü et al., 2013).

2.3.1. Optimizing preparation of LEP

Polysaccharides have shown many biological activities, such as anti-tumor, anti-oxidation, immune-stimulating, and neuroprotecting effects. Degradation of polysaccharide into low-molecular-weight polysaccharide (LEP) was expected to improve its biological activity. In this paper, the antioxidant activity of LEP was used to evaluate the effects of degradation. Since the antioxidant activity of the purified polysaccharide was often evaluated in vitro by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay, DPPH was chosen as evaluating the effects of degradation.

According to the single factor experiments, an orthogonal design $L_9(3^4)$ was adopted to select the optimum experimental conditions. 0.1 g polysaccharide was used to prepare LEP in each experiment. A combination of four factors (TFA concentration, reaction temperature, reaction time and solid–liquid ratio) with different levels was tested (shown in Table 1). The values for different variables were chosen by the single factor experiments. The potency of LEP in scavenging free radicals from DPPH was used to evaluate the effects of degradation.

Table 1
Orthogonal experimental results for the preparation of LEP.

No.	TFA concentration (mol/L)	Reaction temperature (°C)	Reaction time (h)	Solid–liquid ratio (g/mL)	Scavenging rate[a] (%)
1	0.5	70	3	1:100	43.08
2	0.5	80	4	1:200	53.35
3	0.5	85	5	1:300	55.96
4	0.7	70	4	1:300	42.24
5	0.7	80	5	1:100	54.50
6	0.7	85	3	1:200	54.85
7	1.0	70	5	1:200	50.27
8	1.0	80	3	1:300	55.37
9	1.0	85	4	1:100	52.12
k_1	50.80	45.20	51.10	49.90	
k_2	50.53	54.41	49.24	52.82	
k_3	52.59	54.31	53.58	51.19	
R	2.06	9.21	4.34	2.92	

[a] The potency of LEP in scavenging free radicals from DPPH was tested (Wang et al., 2012). $SE_{DPPH} = (A_c - A_s)/A_c \times 100\%$, where SE_{DPPH} was DPPH scavenging effect, A_c was the absorbance at 517 nm of the control reaction containing all reagents except the tested compound, and A_s was the absorbance at 517 nm of reactions containing the tested compound.

Table 2
Orthogonal experimental results for the preparation of Se-LEP.

No.	Raw material ratio	Reaction time (h)	Reaction temperature (°C)	Nitric acid volume fraction (%)	Selenium content (μg/g)
1	0.5	3	50	0.5	990.84
2	0.5	4	60	1.0	1219.48
3	0.5	5	70	1.5	1174.25
4	1.0	3	60	1.5	1131.23
5	1.0	4	70	0.5	1032.88
6	1.0	5	50	1.0	1241.56
7	1.5	3	70	1.0	1084.24
8	1.5	4	50	1.5	1193.44
9	1.5	5	60	0.5	1079.43
k_1	1128.19	1068.77	1141.95	1034.38	
k_2	1135.22	1148.60	1143.38	1181.76	
k_3	1119.04	1165.08	1097.12	1166.31	
R	16.18	96.31	46.26	147.38	

2.3.2. Optimizing preparation of Se-LEP

According to the single factor experiments, an orthogonal design $L_9(3^4)$ was adopted to select the optimum experimental conditions. 0.05 g LEP was used to prepare Se-LEP in each experiment. A combination of four factors (raw material ratio (LEP and Na_2SeO_3), reaction time, reaction temperature and nitric acid volume fraction) with different levels was tested (shown in Table 2). The values for different variables were chosen by the single factor experiments.

2.4. Antibacterial activity

2.4.1. Preparation of culture medium

The bacteria culture medium was composed of beef extract 3.0 g, peptone 10.0 g, sodium chloride 5.0 g, agar 15–20 g, and distilled water 1000 mL. The pH of the mixed solution was adjusted to 7.4–7.6 by adding 1.0 mol/L NaOH. Then, the medium was divided into 60-mL aliquots in 250-mL flasks, and sterilized under 121 °C for 20 min before storage.

The fungal culture medium (PDA) was composed of potato 200 g, glucose 20 g, agar 15–20 g, and distilled water 1000 mL, and maintained at its natural pH. Then, the medium was divided into 100-mL aliquots in 250-mL flasks, and sterilized under 121 °C for 20 min before storage.

2.4.2. Strains activation and preparation of bacterial suspension

Rings of bacteria, such as *Eschetichia coli* (*E. coli*) and *Staphlococcus aureau* (*S.aureus*), were purely cultured in the beef extract peptone medium under aseptic conditions. The original bacterial suspension was obtained by two rings of the activations, which was dispersed in 20 mL saline, about 10^5–10^6 cfu/mL. Then, the original bacterial suspension was diluted to $OD_{600\,nm} = 0.001$, and was used for the determination of minimal inhibitory concentration (MIC). After being cultured, the concentration of the bacterial suspension was determined by counting the number of colonies (30–300).

The right amount of PDA medium was added to a dish with a diameter of 6 cm. The tested fungi (*Colletotrichum capsici*, *Cytospora* sp., *Fusarium turcicum*, *Physalospora piricola*, *Gaeumannomyces graminis*, *Rhizoctonia solani*, *Coniothyrium diplodiella* and *Alternaria oleracea*) were punched to the colonies with diameters of 4 mm with a puncher. Then, they were inoculated on the medium to cultivate at 25 °C until the mycelium covered the entire dish.

2.4.3. Inhibitory effects on bacteria by filter paper method

Several pieces of circular filter papers (4 mm diameter), sterilized at 121 °C for 20 min, were immersed in LEP and Se-LEP with different concentrations to make them fully absorbed. 0.1 mL

original bacterial suspension was coated on the sterile solid flat surface of the medium to produce bacterial plates. The filter paper containing different sample solutions was placed at the center of the dishes, with one piece in each dish. Simultaneously, a piece of filter paper soaked in sterile water was used as the blank control. All dishes were cultured subsequently in the incubator at 37 °C, with *E. coli* for 24 h, and with *S. aureus* for 48 h. The inhibition zone diameter was used to indicate the inhibitory effect of the sample.

2.4.4. Minimum inhibitory concentration (MIC) on the bacteria (Speciale et al., 2002)

MIC was determined by the direct observation of the colonies. Samples were added to flasks containing 60 mL medium. Using double dilution method, the samples were diluted to different concentrations. After the medium was poured on each plate, 0.1 mL bacterial suspension was coated on the plate medium. Simultaneously, a piece of filter paper soaked in sterile water was used as the blank control. And all dishes were cultured subsequently in the incubator at 37 °C, with *E. coli* for 24 h, and with *S. aureus* for 48 h. The sample concentration, at which the bacteria did not grow completely, was regarded as the MIC.

2.4.5. Inhibitory effect on plant pathogenic fungi

The inhibitory effect was determined by the mycelial growth rate method. The blank control group (equivalent sterile water) and the tested samples were added into the PDA culture medium. After inoculation, they were placed in the incubator with a constant temperature of 25 °C until the control group bacteria mycelia covered the whole dish. The colony diameter was measured using crossing method. All the treatments were repeated three times. The stipe diameter was named D_0, the colony diameter of the control group was named D_1, and the treated colony diameter was named D_2. The inhibition rate was calculated using the following formula:

$$\text{Inhibition rate}(\%) = \frac{(D_1 - D_0) - (D_2 - D_0)}{D_1 - D_0} \times 100\%$$

Five sample concentrations with the inhibition rate of 15–85% were selected to conduct the virulence test. The mycelial growth inhibition rate, EC_{50} values and 95% confidence limits were calculated, respectively.

3. Results and discussion

3.1. Preparation of LEP

LEPs were obtained by varying TFA concentration, reaction temperature, reaction time and solid–liquid ratio (Table 1). The scavenging rate of the samples varied from 42.24% to 55.96%. According to the statistical analysis, the optimum conditions were 0.7 mol/L TFA, reaction temperature of 80 °C, reaction time of 5 h, and solid–liquid ratio of 1:200. After cooled, the supernatant was obtained by centrifugation. TFA in the supernatant was dialyzed against redistilled water. After freeze-dried, the degraded polysaccharide was obtained. Under these conditions, the scavenging rate was 61.83% (RSD = 0.65%), which was higher than those in Table 1. The yield of the degraded polysaccharide was 40.00%. The average molecular weights of EP and LEP were 11.9 kDa and 6.4 kDa, respectively.

3.2. Preparation of Se-LEP

Se-LEPs were obtained by varying the raw material ratio (LEP and Na_2SeO_3), reaction time, reaction temperature and nitric acid volume fraction in the reagent (Table 2). The Se content of the samples varied from 990.84 to 1241.56 μg/g. According to the statistical analysis, the optimum conditions were nitric acid volume fraction of 1.0%, reaction time of 5 h, reaction temperature of 60 °C and raw material ratio of 1:1 (LEP to Na_2SeO_3). After cooled to room temperature, the mixtures were centrifuged, and the supernatant was obtained. The residual sodium selenite in the supernatant was dialyzed against distilled water. After freeze-dried, the degraded polysaccharide selenide was obtained. The selenium content in Se-LEP could reach 1335.27 μg/g (RSD = 1.33%), and its yield was 52.40%, which was higher than those in Table 2. The average molecular weights of Se-EP and Se-LEP were 11.9 kDa and 6.4 kDa, respectively.

3.3. UV spectroscopic analysis

The UV spectra of LEP and Se-LEP were shown in Fig. 1. There was no absorption at 280 or 260 nm, which indicated proteins and nucleic acids in the sample were not observed and their contents were very low. Thus, the samples were of high purity. SeO_3^{2-} had strong absorption at 210 nm. There was no absorption appeared at 210 nm in Fig. 1, indicating the absence of SeO_3^{2-} in the samples. There was a strong absorption at 196.50 nm for Se-LEP, and no absorption at 196.50 for LEP. Thus, selenite ester groups might be formed in Se-LEP, which demonstrated further by IR.

3.4. IR spectroscopic analysis

IR spectra of LEP and Se-LEP were shown in Fig. 2. The absorption of Se-LEP at 730.25 cm^{-1} was due to bending vibration of Se—O; the absorption of Se-LEP at 1201.30 cm^{-1} was due to stretching vibration of Se═O. However, there was no absorption at 730.25 and 1201.30 cm^{-1} for the LEP sample, which indicated that a new bond was formed in Se-LEP. Since Se-LEP had similar spectral characteristics to LEP, the selenylation of LEP did not affect the main structure of LEP.

3.5. Thermal stability analysis

The DTA curves were presented in Fig. 3. The LEP curve could be divided into three stages. First, the endothermic peak appeared at 91.9 °C. Second, the exothermic peak appeared at 321.9 °C. Third, the endothermic peak appeared at 638.1 °C. After being combined with selenium, the endothermic peak and exothermic peak had undergone great changes. In the first stage, the endothermic peak moved from 91.9 °C to 92.6°C. In the second stage, the exothermic peaks appeared at 314.5 °C and 390.3 °C, and the endothermic peaks appeared at 208.3 °C, 360.0 °C and 450.3 °C, respectively. In the

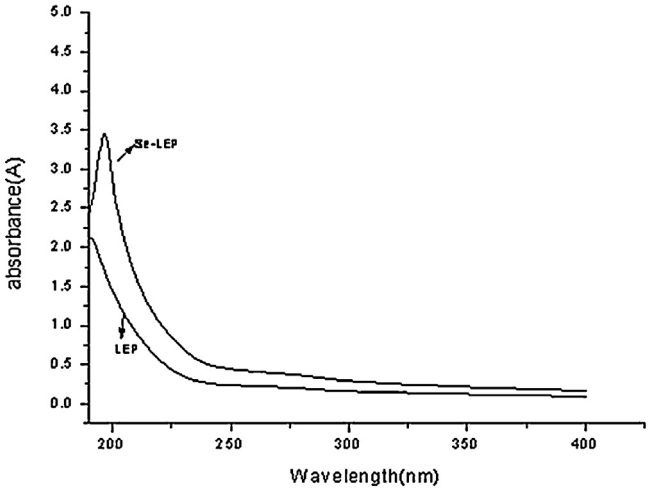

Fig. 1. UV spectra of LEP and Se-LEP.

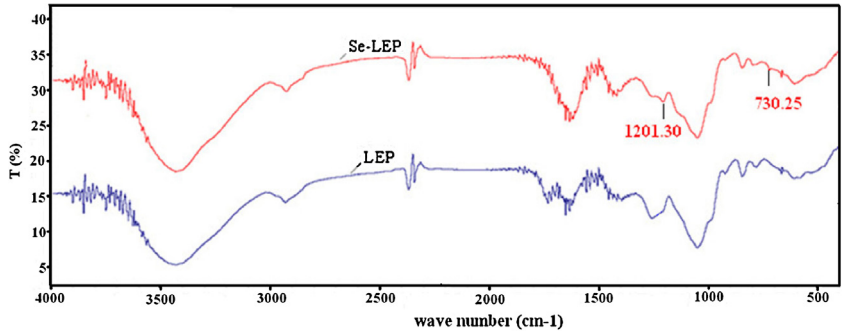

Fig. 2. IR spectra of LEP and Se-LEP.

third stage, the exothermic peak moved to 638.1 °C and 523.5 °C. Thus, the endothermic peak and exothermic peak of the samples changed greatly, and some peaks weakened or even disappeared.

Similar to the DTA curves, TG curves could also be divided into three stages. First, the weight loss of LEP at 19.23–200.66 °C was 17%, and the weight loss of Se-EP at 18.56–172.94 °C was 10.44%, which were mainly caused by dehydration. Second, the weight loss of LEP at 200.66–461.42 °C was 56.39%, and the weight loss of Se-EP at 172.94–465.04 °C was 64%, which was due to their own degradation. Third, the weight loss of LEP at 461.42–666.91 °C was 20.16%, and the weight loss of Se-LEP at 465.04–661.74 °C was 17.71%. From the TG-DTA curves, it was found that the thermal stability decreased after selenylation.

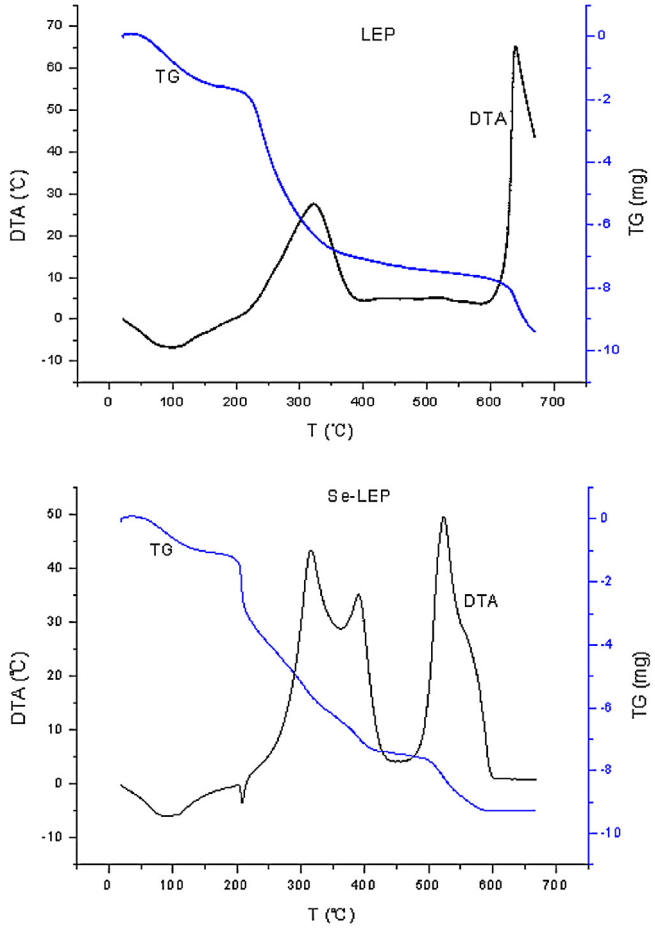

Fig. 3. TG-DTA curves of LEP and Se-LEP.

3.6. Inhibitory effects on bacteria

Under the experimental concentrations of 20, 40 and 80 mg/mL, the antibacterial effects of LEP were not obvious. However, Se-LEP showed better antibacterial properties. The inhibition zone diameter results of Se-LEP were 10.88, 12.39 and 14.50 mm for *E. coli,* 8.90, 9.20 and 12.13 mm for *S. aureus,* respectively. Compared with the polysaccharide selenide (Se-EP, previously prepared), the inhibitory effect of Se-LEP on *E. coli* was enhanced, but weakened on *S. aureus* (Gao & Lü, 2013).

3.7. MIC results

When the concentrations of Se-LEP were 2.92, 1.46, 0.73, 0.37, 0.19 mg/mL, the colony number of *E. coli* were 0, 0, 6, 54, and 66 (108 for the blank), and the colony number of *S. aureus* were 0, 29, 56, 84, and 112 (133 for the blank), respectively.

The MIC of Se-LEP on *E. coli* was 1.46 mg/mL, which was lower than that of Se-EP (1.70 mg/mL). The MIC of Se-LEP on *S. aureus* was 2.92 mg/mL, which was higher than that of Se-EP (1.70 mg/mL) (Gao & Lü, 2013). The results showed that the inhibitory effect of Se-LEP on *E. coli* increased, while decreased on *S. aureus*, which illustrated that the samples had selective effects on different bacteria.

3.8. Inhibitory effects on eight plant pathogenic fungi

In the experimental concentration range, it was found that LEP had less inhibitory effect on the tested plant pathogenic fungi. Se-LEP and Se-EP had the similar inhibitory effects on *Cytospora* sp., *Physalospora piricola*, *Coniothyrium diplodiella* and *Alternaria oleracea*. But Se-LEP had better effect on *Colletotrichum capsici*, *Fusarium turcicum*, *Gaeumannomyces graminis,* and *Rhizoctonia solani* (shown in Table 3).

The antimicrobial/antibacterial activities results established the antimicrobial/antibacterial potency of Se-LEP and Se-EP. However, the structures of Se-LEP and Se-EP were very complex, and their biological activities were usually not a function of one single factor but rather a combination of factors. To our knowledge, no paper had reported the relationship between their structures and biological

Table 3
Inhibitory effects of Se-LEP on four common plant pathogenic fungi (%).

Pathogenic fungi	Concentration of Se-LEP(mg/mL)			
	8.00	4.00	2.00	1.00
Fusarium turcicum	75.00	55.67	37.88	20.62
Gaeumannomyces graminis	79.00	57.73	19.59	10.31
Colletotrichum capsici	60.01	55.67	22.68	19.46
Rhizoctonia solani	80.21	67.29	46.04	26.04

Table 4
Toxicity of Se-LEP on four common plant pathogenic fungi.

Pathogenic fungi	Regression equation	Correlation coefficient	EC_{50}(mg/mL)	Confidence limit 95% (mg/mL)
Fusarium turcicum	$y = 1.6151x + 4.1965$	0.9994	3.14	0.98–9.46
Gaeumannomyces graminis	$y = 2.0987x + 3.8198$	0.9618	3.65	1.00–9.93
Colletotrichum capsici	$y = 1.3073x + 4.1211$	0.9220	4.70	1.36–22.81
Rhizoctonia solani	$y = 1.5487x + 4.4398$	0.9541	2.30	0.83–6.69

activities. The relationship between the structure of Se-LEP/Se-EP and antimicrobial/antibacterial mechanisms required further studies.

3.9. Toxicity of Se-LEP on fungi

As shown in Table 4, the order of the inhibitory effects of Se-LEP on plant pathogenic fungi was as following: *Rhizoctonia solani*>*Fusarium turcicum*>*Gaeumannomyces graminis*>*Colletotrichum capsici*. Se-LEP showed the best inhibitory effect on *Rhizoctonia solani*, with a EC_{50} of 2.30 mg/mL. Compared with Se-EP, the inhibition effect of Se-LEP had different degrees of increase.

4. Conclusions

In the present study, Se-LEP was synthesized with high Se content of 1335.27 μg/g. The UV and IR results indicated that selenylation of LEP had occurred. Se-LEP had stronger inhibitory effect on *E. coli*, and had improved the inhibitory effects on plant pathogenic fungi. Se-LEP could potentially serve as a dietary supplement of Se, new health products and food preservatives.

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