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Sulphation can enhance the antioxidant activity of polysaccharides produced by *Enterobacter cloacae* Z0206

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ABSTRACT

The protective effects of sulfated polysaccharide derivatives produced by *Enterobacter cloacae* Z0206 against H_2O_2-induced oxidative damage in RAW264.7 murine macrophages as well as the possible mechanisms governing the protective effects were studied. Sulfated polysaccharides protected RAW264.7 cells from oxidative damage and apoptosis induced by H_2O_2 by protecting the cellular structure; improving the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px); and inhibiting caspase-3 activation and DNA fragmentation. In addition, the sulfated polysaccharides conferred higher levels of protection from H_2O_2-induced oxidative damage in RAW264.7 murine macrophages compared to the native polysaccharide lacking sulfation. These results indicated that sulfated modifications might be an effective approach to enhance the antioxidant activity of polysaccharides produced by *E. cloacae* Z0206, and the sulfated derivatives of these polysaccharides may act as potent antioxidant agents.

1. Introduction

The uncontrolled generation of free radicals and reactive oxygen species (ROS) is involved in the pathogenesis of more than 50 human diseases, such as cancer, diabetes, aging, and atherosclerosis (Jin, Zhao, Huang, Xu, & Shang, 2012b; Moskovitz, Yim, & Chock, 2002). Antioxidants can protect the cells in the body from free radicals and ROS and can prevent the oxidation of biomacromolecules, including DNA, membrane lipids and proteins (Cho, Kim, Ahn, & Je, 2011). Some synthetic antioxidants have been developed and extensively used, including butylated hydroxytoluene and butylated hydroxyanisole (Krishnaiah, Sarbatly, & Nithyanandam, 2011). However, these synthetic agents have certain drawbacks, including adverse side effects, which may cause liver damage and carcinogenesis (Krishnaiah, Sarbatly, & Bono, 2007). Therefore, there has recently been increased interest in the exploration of safer and more effective naturally occurring antioxidants to inhibit oxidative damage (Zhao et al., 2012).

Polysaccharides are a group of naturally occurring macromolecules present in many organisms (Wang et al., 2010b) that have been extensively studied in medicine due to their various biological activities (Jin, Lu, Huang, Wang, & Wang, 2012a). In particular, polysaccharides have recently received considerable attention due to their function as antioxidants (Chen, Ma, Liu, Liao, & Zhao, 2012; Ye & Huang, 2012). The biological activity of polysaccharides might be enhanced by chemical modifications and structural improvements (Liu et al., 2009; Wang et al., 2010a). Sulfated polysaccharides are polysaccharides in which the hydroxyl groups are partially replaced by sulfate groups, and they possess different or stronger biological activity compared to non-sulfated polysaccharides (Jin, Lu, Huang, Wang, & Wang, 2011; Wang et al., 2010b). Many studies have demonstrated that the antioxidant activity of polysaccharides is strikingly improved by sulfated modification (Wang et al., 2010a; Zhang et al., 2011). Therefore, sulfated modification may be used to improve the antioxidant activity of some polysaccharides and to generate naturally occurring antioxidants.

The bacterial strain *Enterobacter cloacae* Z0206 can produce large amounts of exopolysaccharides. In our previous study, we extracted and purified the major exopolysaccharide (EPS) produced by *E. cloacae* Z0206 (Jin et al., 2010). The structural analysis indicated that the EPS is composed of L-fucose, D-glucose, D-galactose, D-glucuronic acid and pyruvic acid in the approximate molar ratio of 2:1:3:1:1 with an average molecular weight of approximately 1.1×10^6 Da. A combination of chemical analysis coupled with electrospray ionization mass spectrometry and nuclear magnetic resonance spectroscopy showed that the EPS comprises a heptasaccharide repeating unit (Wang, Yang, & Wang, 2013). The administration of EPS at the dose of 200 mg/kg body weight to

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cyclophosohamide-exposed mice resulted in a significant increase and recovery of B lymphocyte proliferation, tumor necrosis factor α production, and the activity of antioxidant enzymes (Jin et al., 2010). In another study, nine sulfated derivatives of EPS with different degrees of substitution (DS) were prepared using the chlorosulfonic acid-pyridine (CSA-Pyr) method with an orthogonal array design. Sulfated derivatives of EPS showed noticeable effects on the scavenging of superoxide radicals and hydroxyl radicals *in vitro* compared to the native EPS lacking sulfation, which indicated that sulfated modification might be an effective approach to enhance the antioxidant activities of EPS *in vitro* (Jin et al., 2011). However, to the best of our knowledge, there is limited literature about the antioxidant activity and mechanism of sulfated EPS derivatives in cells.

In the present study, the protective effect of the sulfated derivative of EPS (SEPS-4) from H_2O_2-induced oxidative damage in RAW264.7 murine macrophages and the possible mechanism of this protection were determined. The purpose of this research is to validate the likelihood of improving the antioxidant activity of EPS through the sulfated modification. In addition, it may provide a basic understanding of the relationship between structure and the bioactivity of polysaccharides.

2. Materials and methods

2.1. Materials and reagents

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Gibco (Grand Island, NY, USA). All other chemical reagents were of analytical reagent grade.

EPS was prepared and purified in our laboratory through fermentation, ethanol precipitation, deproteination, dialysis and lyophilization according to our previous report (Jin et al., 2010). The sulfated derivative of EPS, named SEPS-4, was prepared using the CSA-Pyr method (Jin et al., 2011). The ratio of CSA to Pyr was 1:2, the reaction temperature was 50 °C, and the reaction time was 2 h.

The contents of polysaccharide in EPS and SEPS-4 determined using the phenol-sulfuric acid method (Dubois, Gilles, Harmilton, Rebers, & Smith, 1956) were 97.24% and 55.89%, respectively. Both of the samples showed a negative response to the Bradford test (Bradford, 1976). The sulfur contents of EPS and SEPS-4 determined using the barium chloride-gelatin method (Wang, Li, & Chen, 2009) were 0% and 3.09%, respectively. The chemical structure of SEPS-4 determined from a Fourier transform infrared spectrum (FT-IR, AVATAR 370, Thermo Nicolet, USA) using the KBr-disk method indicated that sulfation of EPS was successful (Jin et al., 2011).

2.2. Cells

RAW264.7, a mouse macrophage cell line, was obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 IU/ml) and streptomycin (100 mg/l) in a humidified 5% CO_2 atmosphere at 37 °C.

2.3. Experimental design

Exponentially growing RAW264.7 cells were seeded into 96-well microplates or 6-well culture plates (Corning, NY, USA) at a density of 1×10^6 cells/ml in DMEM. After incubation for 12 h, the EPS and SEPS-4 groups were treated with EPS and SEPS-4 (0.125, 0.5 or 2.0 μg/ml) for 24 h, whereas for the control and H_2O_2 groups, an equal volume of DMEM was added. After 24 h incubation, cells in the experimental groups were treated with 0.658 mM H_2O_2 for 12 h. After cell cultivation, assays for cell viability, cell morphology, antioxidant enzyme activity, caspase-3 activity, and DNA fragmentation were performed.

2.4. Assessment of cell viability

The cell viability was determined using an MTT assay as previously described (Jin et al., 2010). Briefly, MTT (5 mg/ml) was added to each well of a 96-well microplate, followed by incubation for 4 h, and then 100 μl of DMSO was added to each well to dissolve the precipitated material completely. The light absorbance was measured at 570 nm with an Enzyme-linked Immunosorbent Assay Reader (Model BIO-RAD-550, USA). The relative survival rate was calculated using the following equation: survival rate (%) = (absorbance of experimental group cells/absorbance of untreated control cells) × 100% (Feng et al., 2012).

2.5. Analysis of morphological changes

The morphology of RAW264.7 cells was observed by transmission electron microscopy according to the method described by Zhao, Liu, Zhou, and Liu (2010). Briefly, the cells were fixed with 2.5% glutaraldehyde in phosphate buffer (pH = 7.0) for 12 h. Then, the samples were rinsed with the same buffer, postfixed with 1% osmium tetroxide for 2 h, and washed 3 times in the phosphate buffer. After dehydrating the cells through a graded alcohol series and infiltrating the cells with Spurr resin, ultrathin sections were obtained with an ultra-microtome (Ultracut E, Reichert-Jung). The specimen sections were stained with uranyl acetate and alkaline lead citrate for 15 min and observed with transmission electron microscopy (JEM-1230, JEOL).

2.6. Assays for antioxidant enzyme activity

Cells from a 6-well culture plate were washed with PBS, and the cells were collected and lysed with ultrasonic wave treatment on ice. After centrifugation at 1000 × *g* for 10 min at 4 °C, the supernatant fractions were collected and the enzymatic activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) was determined by spectrophotometric methods using corresponding diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7. Measurement of caspase-3 activity

The activation of caspase-3 was determined with a colorimetric kit (Nanjing KeyGEN Biotech. Co., Ltd., China) according to the manufacturer's instructions. Briefly, RAW264.7 cells ($3–5 \times 10^6$) were harvested and washed twice with PBS. After the cells were lysed, 50 μl of 2 × reaction buffer was added, followed by the addition of 5 μl of caspase-3 substrate. The mixture was incubated in a 96-well plate at 37 °C for 4 h. The plate was then read using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm.

2.8. DNA fragmentation assay

DNA fragmentation was assayed qualitatively with agarose gel electrophoresis. The cells in a 6-well plate were harvested and washed twice with PBS. An Apoptotic DNA Ladder Detection Kit (Nanjing KeyGEN Biotech. Co., Ltd., China) was used to selectively extract DNA fragments according to the provided protocol. The

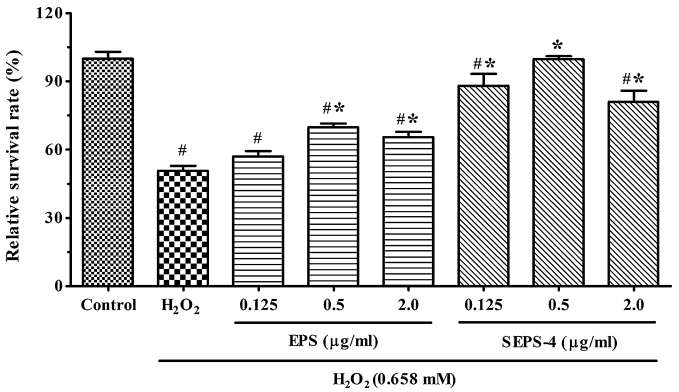

Fig. 1. Effects of EPS and its sulfated derivative SEPS-4 on the viability of RAW264.7 cells treated with H_2O_2. Data are representative of three independent experiments and presented as means ± S.E. $^{\#}P < 0.05$, compared with the control group. $^{*}P < 0.05$, compared with the H_2O_2 group.

DNA fragments were separated on a 1.5% agarose gel with ethidium bromide and visualized with ultraviolet light using a gel imaging system (Image Master VDS, Amersham Pharmacia Biotech, Uppsala, Sweden).

2.9. Statistical analysis

Data were analyzed with one-way analysis of variance (ANOVA), followed by a Dunnett's *t*-test. The results were presented as means ± S.E. Differences between groups were considered statistically significant at the 5% ($P < 0.05$) level.

3. Results and discussion

3.1. Effect of EPS and SEPS-4 on the viability of H_2O_2-treated RAW264.7 cells

Hydrogen peroxide, a major source of ROS, can rapidly penetrate the cell membrane and react with intracellular metal ions, such as iron or copper, to form highly toxic hydroxyl radicals, which cause oxidative stress (Gao, Huang, & Xu, 2001; Gille & Joenje, 1992). Macrophages are vital for the recognition and elimination of microbial pathogens in the host defense system, and many studies have indicated that the virulence of some bacteria is due to their ability to trigger the death of activated macrophages *via* stimulation of ROS production (Lin, Shen, Lin, Yang, & Chen, 2007). RAW264.7, a murine macrophage cell line initially derived from BALB/c mice infected with Abelson leukemia virus, has many of the properties of normal macrophages (Fong et al., 2007) and has become the most commonly used macrophage cell line in medical research (Hartley et al., 2008). Therefore, oxidative damage induced by H_2O_2 in RAW264.7 murine macrophages was used to establish an experimental model to investigate the antioxidant activity of sulfated derivative of EPS in the present study.

The effect of EPS and its sulfated derivate SEPS-4 on the viability of H_2O_2-treated RAW264.7 cells at different concentrations from 0.125 μg/ml to 2.0 μg/ml were evaluated using the MTT assay (Fig. 1). Compared with the normal control group, treatment with 0.658 mM H_2O_2 for 12 h alone significantly decreased the viability of RAW264.7 cells ($P < 0.05$). However, when the cells were pre-incubated with EPS at 0.5 and 2.0 μg/ml or SEPS-4 at 0.125, 0.5 and 2.0 μg/ml for 24 h, the decreased cell viability induced by H_2O_2 was significantly attenuated ($P < 0.05$). Pre-treatment with SEPS-4 significantly increased the cell viability up to 80% at all concentrations. Moreover, the relative survival rate recovered to the normal level when the cells were pretreated with 0.5 μg/ml SEPS-4 compared to normal control cells. In addition, SEPS-4 was more effective than EPS in protecting RAW264.7 macrophages against H_2O_2-induced oxidative damage.

3.2. Effect of EPS and SEPS-4 on the cell morphology of H_2O_2-treated RAW264.7 cells

Apoptosis, which is a controlled physiological process leading to cell death characterized by various features such as membrane blebbing, cell shrinkage, nuclear condensation, and DNA fragmentation can be induced in a variety of cells with H_2O_2 (Li et al., 2010b; Suematsu, Hosoda, & Fujimori, 2011). The effect of EPS and SEPS-4 pre-incubation on the cell morphology of H_2O_2-treated RAW264.7 cells were examined by transmission electron microscopy. As shown in Fig. 2A, the cells in the normal control group showed a round nucleus with very few small vacuoles in the cytoplasm. The cell membrane was well demarcated and the organelles in the cytoplasm were intact under normal conditions. After treatment with 0.658 mM H_2O_2 for 12 h, the cell membrane was less prominent and the cytoplasm was highly vacuolated, with obviously distended areas. Meanwhile, an irregular and shrunken nucleus with an ill-defined edge was observed (Fig. 2B), indicating that the cells were apparently undergoing apoptosis. However, pre-incubation of cells with 0.5 μg/ml EPS or SEPS-4 significantly protected the cells from H_2O_2-induced damage (Fig. 2C and D). EPS and SEPS-4 prevented the formation of an irregular and shrunken nucleus and decreased the presence of cytoplasmic vacuoles significantly, thus protecting the cells from H_2O_2-induced apoptosis. In addition, there were more tiny cytoplasmic vacuoles in EPS-pretreated cells compared to the normal control cells. In this case, SEPS-4 exhibited superior anti-apoptotic protection from H_2O_2 than EPS.

3.3. Effect of EPS and SEPS-4 on antioxidant enzyme activity in H_2O_2-treated RAW264.7 cells

Several antioxidant enzymes, such as SOD and GSH-Px, are important buffers in the interception and degradation of superoxide anion and hydrogen peroxide (Jin et al., 2010). SOD is the only enzyme that disrupts superoxide radicals, and it can convert superoxide to hydrogen peroxide and maintain low superoxide concentrations (Jiang et al., 2008). GSH-Px is an equally important antioxidant that is able to react with hydrogen peroxide, thus preventing intracellular damage (Jin, Xu, Lu, Huang, & Wang, 2009). As shown in Fig. 3A and B, treatment of RAW264.7 cells with 0.658 mM H_2O_2 for 12 h caused a decrease in the activity of SOD and GSH-Px by 13.42 ± 1.6% and 46.51 ± 11.0% ($P < 0.05$), respectively. However, pre-incubation with EPS (0.5 μg/ml) significantly attenuated the decrease of GSH-Px activity ($P < 0.05$) caused by H_2O_2, and pre-treatment with SEPS-4 (0.5 and 2.0 μg/ml) dramatically increased SOD and GSH-Px activity compared to H_2O_2-treated cells ($P < 0.05$). At 0.5 μg/ml of SEPS-4, the H_2O_2-induced decrease in SOD and GSH-Px activity was reversed, reaching approximately 109.07 ± 6.1% and 93.41 ± 7.7%, respectively, of the activity in control cells. This activity was the highest of the SEPS-4 concentrations tested and was higher than the antioxidant recovery caused by EPS at 0.5 μg/ml (100.93 ± 2.8% and 90.69 ± 21.9%, respectively). According to the present study, SEPS-4 promotes the activity of SOD and GSH-Px, indicating that this molecule might improve the synthesis of essential antioxidant enzymes, which play an important role in lowering the pathological concentrations of oxygen radicals induced by H_2O_2.

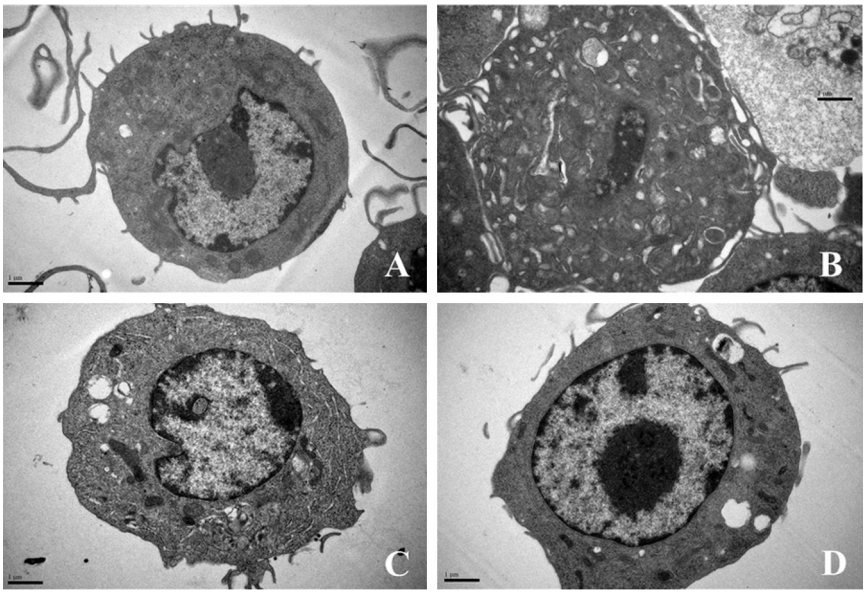

Fig. 2. Effects of EPS and its sulfated derivative SEPS-4 on the cell morphology of RAW264.7 cells treated with H_2O_2. (A) Normal control group; (B) H_2O_2-treated alone group; (C) 0.5 μg/ml EPS pre-treatment group; (D) 0.5 μg/ml SEPS-4 pre-treatment group. Bars represent 1 μm.

3.4. Effect of EPS and SEPS-4 on caspase-3 activity in H_2O_2-treated RAW264.7 cells

Caspases, a family of cysteine-containing aspartate-specific proteases, play a pivotal role in the execution phase of apoptosis by transducing the apoptotic signal cascade (Li et al., 2010a; Matsura, Kai, Fujii, Ito, & Yamada, 1999). There are at least three apoptotic pathways that have been found to date: the mitochondrial pathway, the death receptor pathway and the endoplasmic reticulum pathway (Budihardjo, Oliver, Lutter, Luo, & Wang, 1999; Zheng et al., 2012). Caspase-3, one of the key effectors, is initiated by caspase-9 and involved in the mitochondria-mediated pathway (Li et al., 2010a). Many studies have indicated that the oxidative stress induced by H_2O_2 leads to the activation of caspase-3 in cell lines (Jiang, Liu, Bao, & An, 2003; Maheshwari, Misro, Aggarwal, Sharma, & Nandan, 2009; Park et al., 2007). Consistent with these results, a significant increase of caspase-3 activity was observed in RAW264.7 cells in response to H_2O_2 treatment compared to the control group cells in this study. However, pre-treatment of RAW264.7 cells with EPS at 2.0 μg/ml or with SEPS-4 at 0.125, 0.5 and 2.0 μg/ml for 24 h decreased caspase-3 activity as shown in Fig. 4. Remarkably, pre-incubation of cells with 0.5 μg/ml SEPS-4 maintained caspase-3 activity at a normal level compared to H_2O_2-treated cells ($P < 0.05$). In this case, SEPS-4 exhibited a superior effect on the reduction of caspase-3 activity than EPS.

3.5. Effect of EPS and SEPS-4 on DNA fragmentation in H_2O_2-treated RAW264.7 cells

During the initiation of apoptosis, poly ADP-ribose polymerase (PARP), which functions in DNA repair and genetic integrity, is cleaved by caspase-3 at Asp216-Gly217 into two fragments (Enari et al., 1998). The two DNA-binding zinc finger domains in PARP are separated from the C-terminal catalytic domain and normal function is lost (Zheng et al., 2012). As a result, Ca^{2+}/Mg^{2+}-dependent endonucleases, which are negatively regulated by PARP, increase in activity and cleave the DNA between nucleosomes, producing characteristic DNA fragments and inducing apoptosis (Ivana Scovassi & Diederich, 2004). As shown in Fig. 5, after treatment with 0.658 mM H_2O_2 for 12 h, DNA electrophoresis of RAW264.7 cells showed the

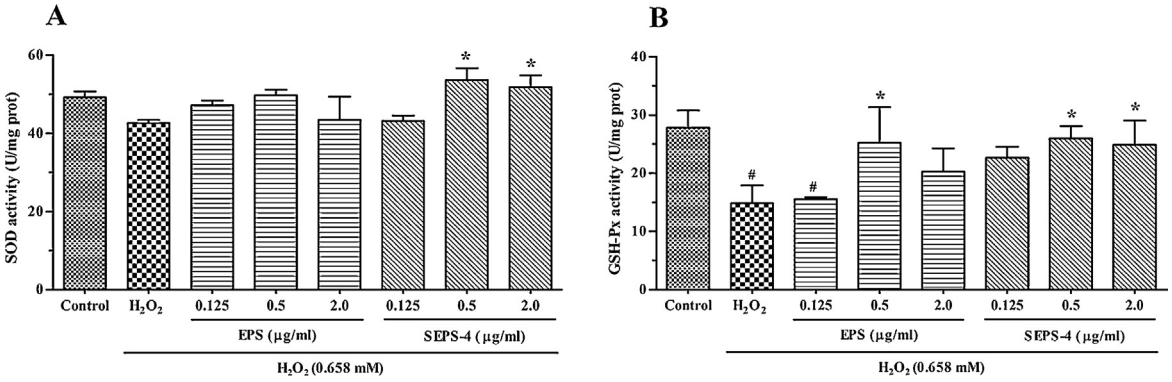

Fig. 3. Effects of EPS and its sulfated derivative SEPS-4 on SOD and GSH-Px activities in RAW264.7 cells treated with H_2O_2. (A) SOD activity; (B) GSH-Px activity. Data are representative of three independent experiments and presented as means ± S.E. $^*P < 0.05$, compared with the H_2O_2 group.

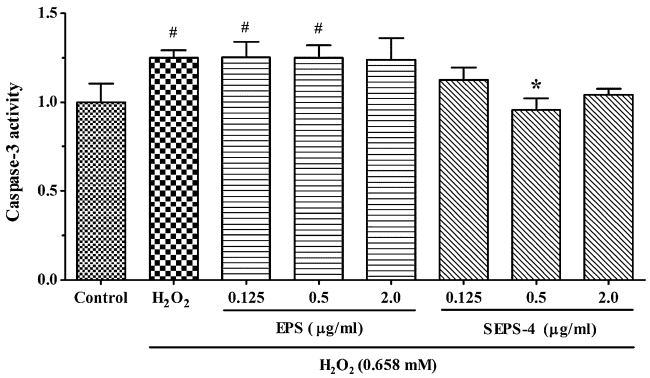

Fig. 4. Effects of EPS and its sulfated derivative SEPS-4 on caspase-3 activity in RAW264.7 cells treated with H_2O_2. Data are representative of three independent experiments and presented as means ± S.E. $^{\#}P < 0.05$, compared with the control group. $^{*}P < 0.05$, compared with the H_2O_2 group.

DNA ladder characteristic of apoptosis. At all tested concentrations, changes to the DNA fragmentation pattern were not obvious in H_2O_2-treated cells that were pre-incubated with EPS. On the contrary, the degree of DNA fragmentation was significantly suppressed by pre-treatment with SEPS-4 at 0.5 μg/ml and 2.0 μg/ml.

In addition, we found that pre-incubated with EPS and SEPS-4 could increase the cell relative survival rate, and improve the activities of SOD and GSH-Px in RAW264.7 cells treated with H_2O_2. However, the relative survival rate, and activities of the above two antioxidant enzymes did not increase linearly with the increase of EPS and SEPS-4 concentrations. The relative survival rate of RAW264.7 cells treated by 2.0 μg/ml EPS and SEPS-4 was less than that treated by 0.5 μg/ml, and so as the antioxidant enzymes activities. As the oxidation and antioxidant in the cells is a process of homeostasis, the high concentration of polysaccharides (2.0 μg/ml) could interfere with this process which may affect the cells and induce the negative feedback control on the antioxidant process compared with 0.5 μg/ml. The similar result has been reported by Qin, Huang, and Xu (2002).

The bioactivity of polysaccharides mainly depends on physiochemical and molecular structural features, such as the sugar unit and the glycosidic bond in the backbone, the type and polymerization degree of the branch, and the flexibility and spatial configuration of the chains (Alban, Schauerte, & Franz, 2007; Lu, Wang, Hu, Huang, & Wang, 2008). Many studies have demonstrated that the sulfation of many natural polysaccharides can not only enhance water solubility but also change the physicochemical characterization and chain conformation, resulting in an improvement of their bioactivity (Chaidedgumjorn et al., 2002). In our previous study, sulfated modification significantly improved the scavenging abilities of EPS on superoxide radicals and hydroxyl radicals *in vitro* (Jin et al., 2011). The present research indicated that the sulfated derivative of EPS showed noticeable protective effects against H_2O_2-induced oxidative damage in RAW264.7 murine macrophage compared with native polysaccharide. The improved antioxidant activity of sulfated polysaccharides may be attributed to the presence of the sulfate group. Theoretically, the presence of more electron-withdrawing groups, such as sulfate and carboxyl groups, in the polysaccharide results in a weaker dissociation energy of the O-H bond (Chang, Hsu, & Chen, 2010). Therefore, sulfated polysaccharides possess a greater capacity to donate hydrogen to the superoxide anion because of the weaker dissociation energy of O—H bond. In addition, substitution of an —OH with $-OSO_3H$ group would enhance the scavenging activity of hydroxyl radicals (Wang et al., 2010a). Examination of the detailed mechanism of antioxidant action and the structural characteristics of the sulfated derivative of EPS is currently in progress in our lab, which may promote the understanding of the structure-function relationship in sulfated polysaccharides.

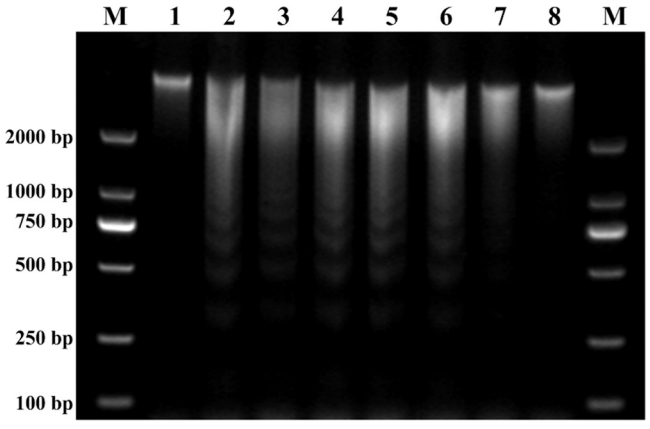

Fig. 5. Effects of EPS and its sulfated derivative SEPS-4 on DNA fragmentation in RAW264.7 cells treated with H_2O_2. Lane M, standard DNA marker; lane 1, normal control group; lane 2, H_2O_2-treated alone group; lane 3–5, 0.125, 0.5 and 2.0 μg/ml EPS pre-treatment group; lane 6–8, 0.125, 0.5 and 2.0 μg/ml SEPS-4 pre-treatment group.

4. Conclusions

In this study, the protective effects of a sulfated derivative of polysaccharides produced by *E. cloacae* Z0206 against H_2O_2-induced oxidative damage in RAW264.7 macrophage were investigated. The results confirmed that sulfated modification improves the antioxidant activity of the polysaccharides and that SEPS-4 protects RAW264.7 cells against oxidative damage and apoptosis induced by H_2O_2 through protection of the cell structure, improvement of antioxidant enzyme activity, and inhibition of caspase-3 activation and DNA fragmentation. Therefore, sulfated modification might be an effective approach to enhance the antioxidant activity of natural polysaccharides. These data increase our knowledge of the relationship between the structure and biological activity of polysaccharides.

Acknowledgements

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