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In vitro and *in vivo* antioxidant activities of polysaccharide purified from aloe vera (*Aloe barbadensis*) gel

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ABSTRACT

The *in vitro* and *in vivo* antioxidant potentials of a polysaccharide isolated from aloe vera gel were investigated. Enzymatic extracts were prepared from aloe vera gel by using ten digestive enzymes including five carbohydrases and five proteases. Among them, the highest yield was obtained with the Viscozyme extract and the same extract showed the best radical scavenging activity. An active polysaccharide was purified from the Viscozyme extract using ethanol-added separation and anion exchange chromatography. Purified aloe vera polysaccharide (APS) strongly scavenged radicals including DPPH, hydroxyl and alkyl radicals. In addition, APS showed a protective effect against AAPH-induced oxidative stress and cell death in Vero cells as well as in the *in vivo* zebrafish model. In this study, it is proved that both the *in vitro* and *in vivo* antioxidant potentials of APS could be further utilized in relevant industrial applications.

1. Introduction

Free radicals play an important role in living organisms and comprise superoxide (O^{2-}), peroxyl (ROO•), alkoxyl (RO•), hydroxyl (HO•), and nitric oxide (NO•) (Han, Zhang, & Skibsted, 2012). Excessive free radicals induce various harmful effects in the human body such as cancer, liver injury, skin damage and aging (Sarma, Mallick, & Ghosh, 2010). It is known that oxidative stress is caused by increasing the reactive oxygen species (ROS) which cause an imbalance with natural antioxidants that influences cell death and lipid peroxidation (Gill & Tuteja, 2010). Generally, synthetic antioxidants including butylated hydroxyl anisole (BHA), butylated hydroxyltoluene (BHT) and propyl gallate (PG) have been used for industrial purposes (Kang et al., 2012). However, recent research has been focusing on the extraction of natural antioxidants from various plants such as green tea, seaweeds and *Camellia sinensis* (Chen, Zhang, & Xie, 2005; Hongmei, 2011; Kang et al., 2011).

Over the past few years, aloe vera gel has been the most widely used healthcare product in the world. According to some previous studies, the major active components of aloe vera gel include minerals, amino acids, polyphenols and polysaccharides (Hamman, 2008; Mazzulla et al., 2012). Glucose and mannose are the main polysaccharides in aloe vera gel (Nema, Shrivastava, & Mitra, 2012). Aloe vera gel has been reported to possess various biological activities such as hepato-protective effects, immunity effects and anti-diabetes effect (Nandal and Bhardwaj, 2012; Saki et al., 2011; Talmadge et al., 2004; Yagi, Hegazy, Kabbash, & Wahab, 2009; Yu, Jin, Xin, & JianMin, 2009). However, the antioxidant potentials of polysaccharides isolated from aloe vera have not been reported yet. Taken together, the objective of the present study was to evaluate the *in vitro* and *in vivo* antioxidant effect of a polysaccharide which was purified from aloe vera gel.

2. Materials and methods

2.1. Chemicals and reagents

The cells of an African green monkey kidney (Vero) cell line were purchased from the Korea Cell Line Bank (KCLB, Seoul, Korea). Five carbohydrate digestive enzymes including Viscozyme L. Celluclast 1.5 L FG, AMG 300 L, Termamyl 120 L, Ultraflo L, and the five proteases including Protamex, Kojizyme

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500 MG, Neutrase 0.8 L, Flabourzyme 500 MG, Alcalase 2.4 L FG were obtained from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin and trypsine-EDTA were purchased from Gibco/BRL (Burlington, Ont, Canada). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, Dihydroethidium (DE), 2,7-dichlorofluorescein diacetate (DCF-DA), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). All other chemicals and reagents were analytical grade.

2.2. Plant material and extraction

Aloe vera was collected along Jeju Island of Korea during the period from March to May 2012. Sand and epiphytes were removed using tap water. Then, aloe vera sample was rinsed carefully with fresh water and freeze-dried. Dried aloe gel sample was ground and sieved through a 50-mesh standard testing sieve. The preparation of enzyme-assistant extract was done according to a previously reported method (Athukorala, Jung, Vasanthan, & Jeon, 2006; Heo, Jeon, Lee, Kim, & Lee, 2003). In this study, the enzyme-assisted extracts of aloe gel was obtained *via* enzymatic hydrolysis using the five carbohydrases including Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo and the five proteases including Kojizyme, Alcalase, Protamex, Flabourzyme, and Neutase. The yields and radical scavenging activities of these extracts were determined. A 50 g of aloe gel sample was homogenized with water (2 L), and mixed with 500 μL of enzyme. Each reactant was adjusted to be within the optimum pH and temperature range of the respective enzymes, and enzymatic reactions were performed for 24 h. After the extraction, the reactant was boiled for 10 min at 100 °C to inactivate the enzyme. Then, samples were clarified by centrifugation (3000 rpm, for 20 min at 4 °C) to remove the residue. This extracts were adjusted to pH 7.0 and designated to as enzymatic extract. The sample was kept in −20 °C for further experiments.

2.3. Crude polysaccharide separation (CPS)

The extract sample was (750 mL) mixed well with 1.5 L of 99.5% ethanol. Then, the mixture was allowed to stand for 30 min at room temperature and the crude polysaccharide fraction was collected by centrifugation at 10,000 × *g* for 20 min at 4 °C (Kuda, Taniguchi, Nishizawa, & Araki, 2002; Matubara, Matsuura, Hori, & Miyazawa, 2000).

2.4. Anion-exchange chromatography

The crude polysaccharide from aloe vera gel (500 mg) obtained by using the procedure described above was applied to a DEAE-cellulose column (17 cm × 2.5 cm) equilibrated in 50 mM sodium acetate (pH 5.0) and washed with the same buffer containing 50 mM NaCl. Elution was carried out at a flow rate 15 mL/h with a linear gradient of 50–250 mM NaCl containing 50 mM sodium acetate (pH 5.0). Fractions of 5 mL were collected and measured for polysaccharide by phenol–H_2SO_4 assay. According to the bioactivity guided fractionation, elution was collected which shows antioxidant effect against AAPH damage. These fractions were pooled and concentrated by rotary evaporator under reduced pressure below 30 °C. Then, samples were subjected to gel filtration chromatography after dialysis and freeze-drying.

2.5. Neutral sugar analysis

The extract and the purified polysaccharide isolated from aloe vera gel were hydrolyzed in a sealed glass tube with 4 M of trifluoroacetic acid for 4 h at 100 °C to analyze neutral sugars. In order to analyze the monosaccharide, the samples were digested using 6 N of HCl for 4 h. Then, the sample was separately applied to CarboPac PA1 cartridge (4.5 mm × 50 mm) column to analyze neutral and amino sugar respectively. The column was eluted using 16 mM of NaOH at 1.0 mL/min flow rate. Each sugar of the samples was detected by using ED50 Dionex electrochemical detector and data were analyzed by Peack Net on-line software.

2.6. DPPH radical scavenging assay

The DPPH radical scavenging activity was measured using the method described by Nanjo et al. (1996). An ethanol solution of 60 μL each sample at the concentration of 0.25, 0.5, 1, 2, and 4 mg/mL (or ethanol itself as a control) was added to 60 μL of DPPH (60 μmol L^{-1}) in ethanol solution. After mixing vigorously for 10 s, the solutions was then transferred to a 100 μL Teflon capillary tube which was fitted to the cavity of the electron spin resonance spectrometer (JEOL, Tokyo, Japan). The spin adduct was measured on an electron spin resonance (ESR) spectrometer exactly 2 min later. Measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, temperature 298 K.

2.7. Hydroxyl radical scavenging assay

Hydroxyl radicals were generated by Fenton reaction, and reacted rapidly with nitrone spin trap DMPO. The resultant DMPO-OH adducts was detectable with an ESR spectrometer. The ESR spectrum was recorded 2.5 min after mixing in a phosphate buffer solution (pH 7.4) with 0.3 M DMPO 0.2 mL, 10 mM $FeSO_4$ 0.2 mL, and 10 mM H_2O_2 0.2 mL using a JES-FA electron spin resonance spectrometer (JEOL) set at the following conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 1 mW, gain 6.3×10^5, temperature 298 K (Kang, Yokozawa, Yamabe, Kim, & Park, 2007).

2.8. Alkyl radical scavenging assay

Alkyl radicals were generated by AAPH, a PBS (pH 7.4) reaction mixture containing 10 mmol/L AAPH, 10 mmol/L 4-POBN and indicated concentrations of tested samples, was incubated at 37 °C in a water bath for 30 min (Hiramoto, Johkoh, Sako, & Kikugawa, 1993), and then transferred to a 100 μL Teflon capillary tube. The spin adduct was recorded on JES-FA ESR spectrometer. Measurement conditions include: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW, gain 6.3×10^5, temperature 298 K.

2.9. Cell culture

The cells of an African green monkey kidney (Vero) were maintained at 37 °C in an incubator, under a humidified atmosphere containing 5% CO_2. The cells were cultured in DMEM containing 10% heat-inactivated FBS, streptomycin (100 μg/mL), penicillin (100 unit mL^{-1}), and sodium pyruvate (110 mg L^{-1}).

2.10. Intracellular AAPH scavenging assay by DCF-DA

For the detection of intracellular AAPH (10 mM), the Vero cells were seeded in 96-well plates at a concentration of 1.0×10^5 cells mL^{-1}. After 16 h, the cells were treated with samples (10 μL) and incubated at 37 °C under a humidified atmosphere. After 30 min, AAPH was added at a concentration of 10 mM, and then the cells were incubated for an additional 30 min at 37 °C. Finally, DCF-DA (5 μg/mL) was introduced to the cells, and

2′,7′-dichlorodihydrofluorescein fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a Perkin-Elmer LS-5B spectrofluorometer (Waltham, MA, USA).

2.11. Origin and maintenance of zebrafish

Adult zebrafish were obtained from a commercial dealer (Seoul Aquarium, Korea) and 10 fish were kept in a 3-L acrylic tank under the following conditions: 28.5 °C, with a 14/10 h light/dark cycle. Fish were fed three times a day, 6 days a week, with Tetramin flake food supplemented with live brine shrimps (*Artemia salina*). Embryos were obtained from natural spawning that was induced in the morning by turning on the light. Collection of embryos was completed within 30 min.

2.12. Waterborne exposure of embryos to polysaccharide and AAPH

From approximately 7–9 h post-fertilization (7–9 hpf), embryos (group = 25 embryos) were transferred to individual wells of a 24-well plate and maintained in embryo medium containing 1 mL of vehicle (0.1% DMSO) or 50, 100 and 200 μg/mL polysaccharide for 1 h. Then embryos were treated with 25 mM AAPH or co-treated with AAPH and polysaccharide for up to 24 h post-fertilization (24 hpf).

2.13. Estimation of intracellular ROS generation and image analysis

Generation of ROS in zebrafish embryos was analyzed using an oxidation-sensitive fluorescent probe dye, 2,7-dichlorofluorescein diacetate (DCF-DA). DCF-DA was deacetylated intracellularly by nonspecific esterase, which was further oxidized to the highly fluorescent compound dichlorouorscein (DCF) in the presence of cellular peroxides (Rosenkranz et al., 1992). At 3–4 hpf, the embryos were treated with 50, 100 and 200 μg/mL polysaccharide and 1 h later, 25 mM AAPH was added to the plate. After incubating with 25 mM AAPH for 24 h, the embryo media was changed and the embryos developed up to 1 dpf. The embryos were transferred into 96 well plate and treated with DCF-DA solution (20 μg/mL), and the plates were incubated for 1 h in the dark at 28.5 °C. After incubation, the embryos were rinsed in fresh embryo media and anesthesized before visualization. Individual embryo fluorescence intensity was quantified using a spectrofluorometer (Perkin–Elmer LS-5B, Austria) and the image of stained embryos was observed using a fluorescent microscope, which was equipped with a CoolSNAP-Pro color digital camera (Olympus, Japan).

2.14. Measurement of oxidative stress-induced cell death in zebrafish embryo

Cell death was detected in live embryos using acridine orange staining. Acridine orange stain cells with disturbed plasma membrane permeability, therefore, it preferentially stains necrotic or very late apoptotic cells. At 3 dpf, a zebrafish larva was transferred to one well of 96-well plates, treated with acridine orange solution (7 μg/mL) and incubated for 30 min under the dark at 28.5 ± 1 °C. After the incubation, the zebrafish larvae were rinsed by fresh embryo media and anaesthetized by 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan). A fluorescence intensity of individual zebrafish larva was quantified using the image J program.

Table 1
Yields and scavenging activities of the enzymatic extracts from aloe vera gel against DPPH, hydroxyl and alkyl radical.

mg/mL	DW	U	C	A	P	T	AMG	V	K	F	N
Yield (%)	9.63 ± 0.05	9.86 ± 0.49	13.3 ± 0.06	13.5 ± 022	11.63 ± 0.58	10.7 ± 0.09	9.73 ± 0.48	14.86 ± 0.44	10.63 ± 0.53	9.83 ± 0.44	9.96 ± 0.49
DPPH(ICso)[a]	7.76 ± 8.05	2.50 ± 0.35	2.45 ± 035	2.27 ± 0.07	2.44 ± 0.04	1.15 ± 0.08	1.94 ± 0.13	0.55 ± 0.06	2.04 ± 0.07	2.33 ± 039	2.26 ± 0.09
Hydro xyl(ICso)[b]	1.19 ± 0.68	0.86 ± 0.12	1.00 ± 0.09	0.70 ± 0.05	2.06 ± 0.03	1.14 ± 0.15	2.08 ± 0.07	0.59 ± 0.14	1.27 ± 0.16	1.52 ± 0.45	1.57 ± 0.14
Alkyl (ICso)[c]	3.64 ± 0.45	1.08 ± 0.09	0.54 ± 0.12	1.07 ± 0.14	1.07 ± 0.08	1.14 ± 0.07	0.93 ± 0.04	0.22 ± 0.02	0.67 ± 0.08	0.90 ± 0.07	0.78 ± 0.12

[a] DPPH radical scavenging activity.
[b] Hydroxyl radical scavenging activity.
[c] Alkyl radical scavenging activity.

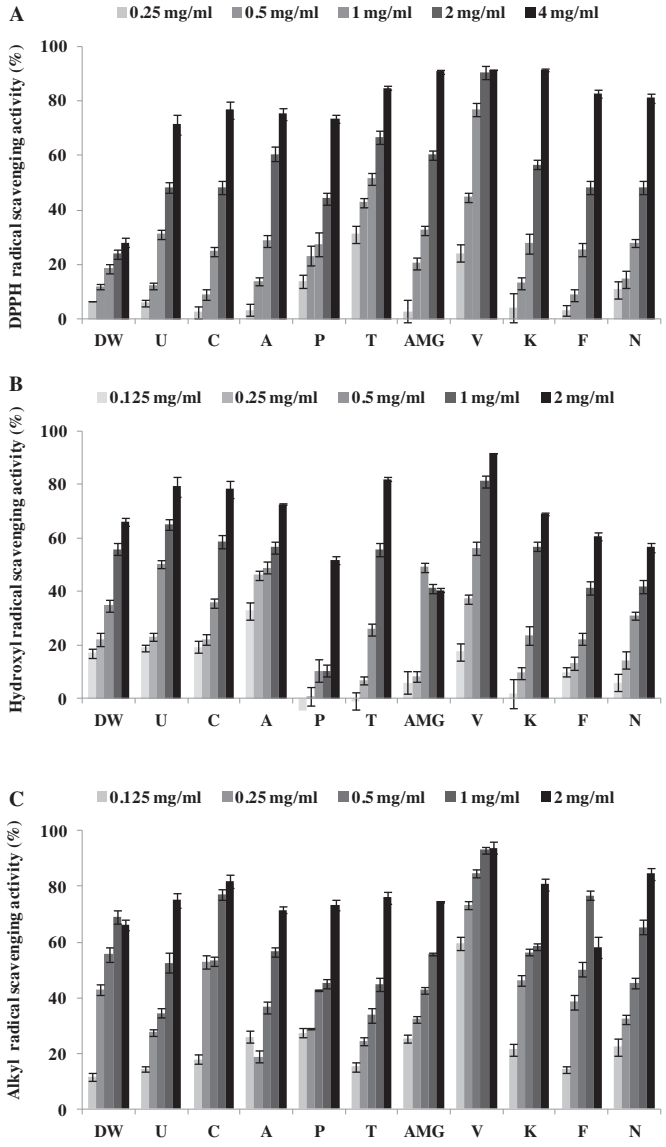
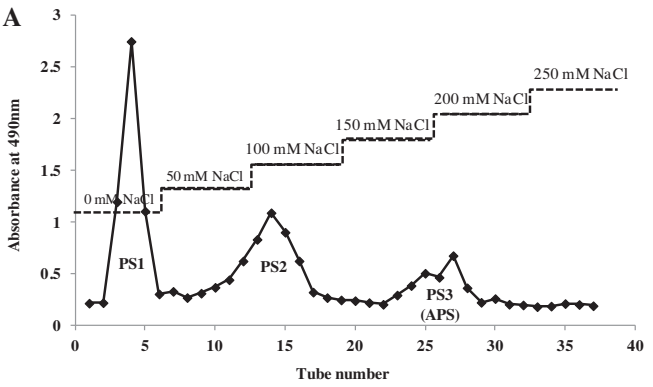


Fig. 1. Radical scavenging effects of enzymatic extract of aloe vera gel on the DPPH (A), hydroxyl (B), and alkyl (C) radical (U, Ultraflo extract; C, Celluclast extract; A, Alcalase extract; P, Protamex extract; T, Termamyl extract; AMG, AMG extract; V, Viscozyme extract; K, Kojizyme extract; F, Flavourzyme extract; N, Nutrase extract).



B

(mg/ml)	Radical scavenging activity (IC_{50}) PS1	PS2	PS3 (APS)
DPPH	1.21±0.05	0.73±0.04	0.67±0.09
Hydroxyl	4.57±0.07	0.98±0.09	0.66±0.04
Alkyl	0.50±0.03	0.29±0.02	0.15± 0.03

Fig. 2. DEAE-cellulose chromatogram of the polysaccharide separated from Viscozyme extract of aloe vera gel (A) and scavenging activities of the PS1, PS2 and PS3 fraction against DPPH, hydroxyl and alkyl radical.

2.15. Statistical analysis

All the measurements were made in triplicate and all the values were represented as mean ± SE. The results were subjected to an analysis of the variance using the Tukey test to analyze the difference. $P < 0.05$ and $P < 0.01$ were considered significant.

3. Results and discussion

3.1. Purification of a polysaccharide with radical scavenging activities

Oxidative stress increases with accumulation of free radical species and decreases with the defense provided by antioxidants. Free radicals including hydroxyl and alkyl are thought to increase oxidative stresses in living organisms (Lu, Lin, Yao, & Chen, 2010). Oxidative stresses induce cellular damage through distortion of biochemical components such as enzymes, lipids, proteins, and DNA (Sharma, Jha, Dubey, & Pessarakli, 2012). Enzyme hydrolysis for preparations of enzymatic extracts from various plant materials brings some advantages including higher yields and production of active components (Heo, Park, Lee, & Jeon, 2005; Ko et al., 2012). In addition, many researchers have employed enzyme-assisted extraction to improve extraction efficiency of the active polysaccharides from plant sources (Lee et al., 2011).

Among the enzymatic extracts, Viscozyme-assisted extract (VAE) showed the highest yield and the strongest scavenging activities against DPPH, hydroxyl and alkyl radicals (Fig. 1 and Table 1). Therefore, the crude polysaccharide from VAE, which was obtained by adding alcohol to VAE, was subsequently introduced to a DEAE-cellulose column with a NaCl gradient (0–250 mM) to separate out the antioxidative polysaccharide. The separated fractionations on the chromatogram were labeled as three peaks, PS1, PS2 and PS3 (Fig. 2A and B). The PS3 showed the strongest scavenging activity of DPPH, hydroxyl and alkyl radicals compared to the other fractions. Therefore PS3 was considered as an antioxidant polysaccharide (APS) purified from aloe gel.

Table 2
Monosaccharide compositions of polysaccharide fractions isolated from aloe vera gel.

Sugar(%)	VAE[a]	CPSFr[b]	APS[c]
Fucose	2.98	4.52	4.62
Rhamnose	0.79	10.96	48.55
Galactose	2.35	14.55	13.85
Glucose	32.66	26.92	11.41
Mannose	60.51	36.16	7.35
Xylose	–	1.04	3.45
Arabinose	0.69	5.81	10.73

[a] Viscozyme extract.
[b] Crude polysaccharide.
[c] The purified aloe polysaccharide.

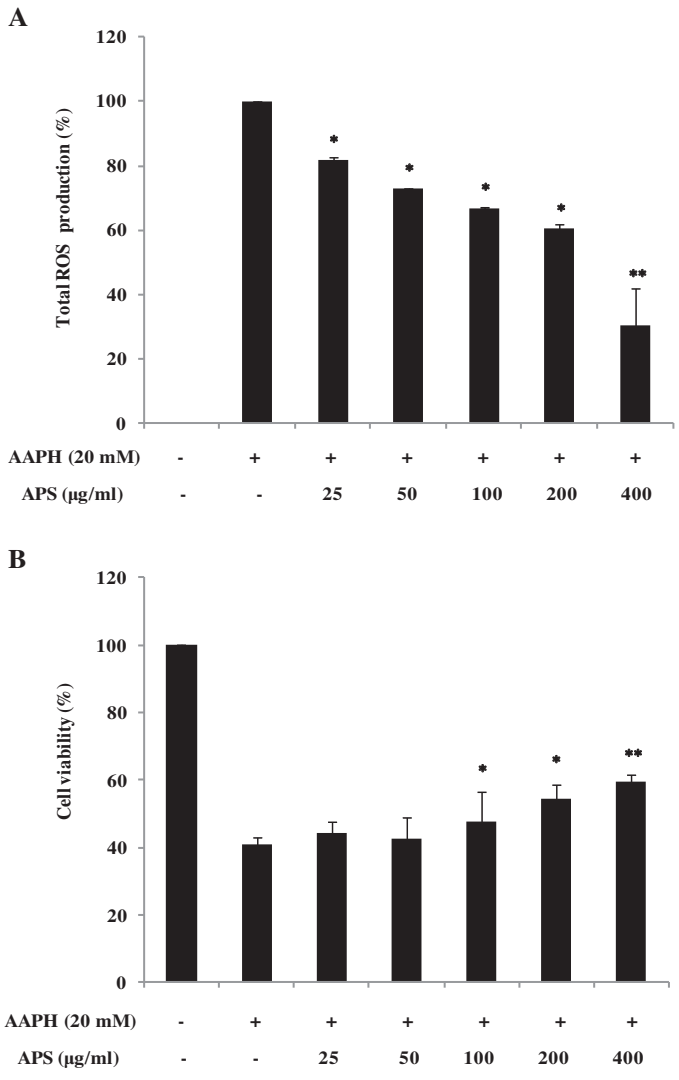


Fig. 3. Intracellular reactive oxygen species (ROS) scavenging activities of APS against AAPH-induced oxidative stress in Vero cells (A) and protective effects of APS against AAPH-induced cell death in Vero cells (B). Experiments were performed in triplicate and the data are expressed as mean ± SE. $^{*}P < 0.05$ and $^{**}P < 0.01$.

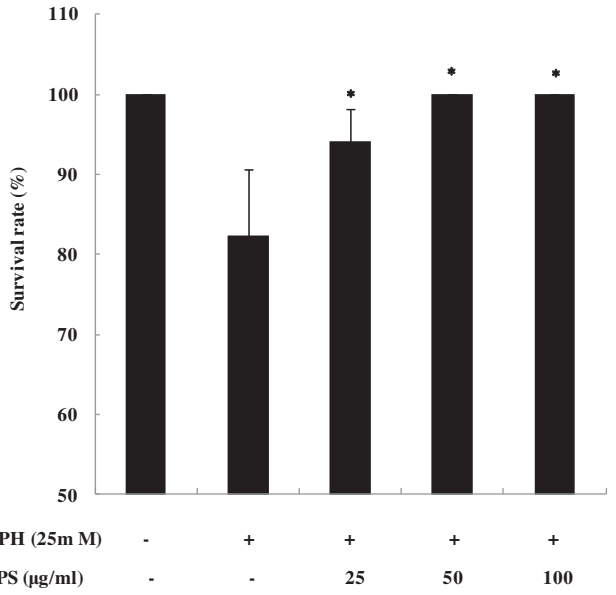


Fig. 4. Survival rates after treatment with AAPH or co-treatment with APS. The embryos were treated with 25 Mm AAPH and co-treated with APS. Experiments were performed in triplicate and the data are expressed as mean ± SE. $^{*}P < 0.05$ and $^{**}P < 0.01$.

3.2. Analysis of monosaccharide composition and sulfate contents

Interestingly, it was observed that rhamnose and arabinose increased and mannose and glucose decreased with the purification. Some reports explain that rhamnose and arabinose are associated with the antioxidant activities of polysaccharides isolated from mushrooms and *Hovenia dulcis* (He, Ru, Dong, & Sun, 2012; Wang et al., 2012). Also, previous studies reported that aloe vera gel was composed mainly of mannose (60.9%), glucose (13.1%) and galactose (1.5%) (Ni, Turner, Yates, & Tizard, 2004). These results demonstrate that increasing rhamnose and arabinose in the monosaccharide composition might lead to strong antioxidant activity (Table 2). This is the first report regarding the possible antioxidant effect of APS, the polysaccharide isolated from aloe gel.

3.3. Antioxidant activity of APS in intracellular ROS inhibition and protective effect

The DCFH-DA assay is a rapid and accurate fluorescence method for detection and measurement of ROS production (Kang et al., 2012; Ko, Lee, Samarakoon, Kim, & Jeon, 2013). Thus, we used the DCFH-DA assay to measure the protective effects of oxidative stress in Vero cells. The cells were treated with APS prior to AAPH treatment, and a dramatic reduction in the levels of ROS was observed (Fig. 3A). The cell viabilities treated with AAPH or co-treated with APS are shown in Fig. 3B. The cell viability of non-treated cells was assigned as 100%, and the cell viability was reduced to at least 40% in the AAPH-treated cells. On the other hand, cell viabilities were increased with increased concentrations of APS in a dose-dependent manner, and significant increases were observed in the range of 100–400 μg/mL. This result indicates that APS can improve cell viability and has a protective effect against ROS oxidation stress in cells *in vitro*.

3.4. Protective effect of APS against AAPH-induced oxidative stress and cell death in the in vivo zebrafish model

The zebrafish model possesses many advantages in modern biotechnology such as a large clutch size, transparent embryos, low-cost, and easy handling. Recently, zebrafish as an *in vivo* model has been used to study vertebrate development and for modeling human diseases and processes such as oxidative stress, liver damage and inflammation (Kang et al., 2013; Yang et al., 2012). Oxygen free radicals are known to be one of the most important biological indicators in the evaluation of oxidative damage (Shukla, Bhatnagar, & Khurana, 2012). AAPH-induced oxidative stress leads to cell damage and eventually cell death. Some reports have shown that the zebrafish model has attracted a lot of interest because it is low cost, is of small size, and the zebrafish has physiological similarity to mammals (Choi et al., 2007; Kang et al., 2013; Yang et al., 2012). In this study, we investigated the antioxidant effect of APS on the AAPH-induced oxidative stressed zebrafish model. The survival rates of the experiment were also determined in the zebrafish model. In the non-treated group, the survival rate was 100% during the experimental period. As shown in Fig. 4, when the zebrafish was treated with AAPH (25 mM) in the positive group, the survival rate was reduced to 82%. In the case of the APS-treated group (25, 50 and 100 μg/mL), the survival rates were 82%, 100% and 100%, respectively. The antioxidant effects of APS on the intensity of ROS (DCF-DA) and cell deaths (Acridin orange) are shown in Fig. 5. From

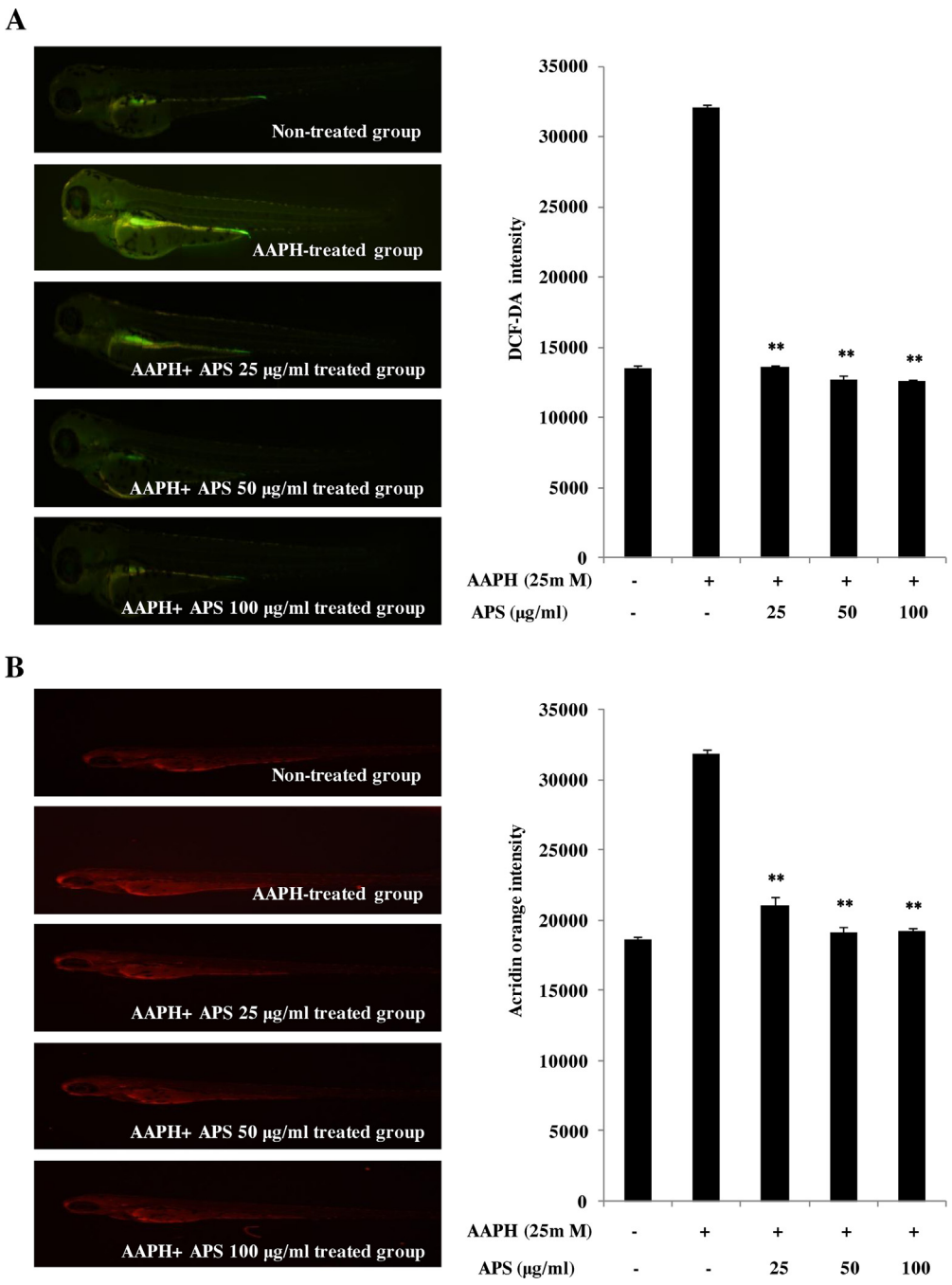


Fig. 5. Effects of APS against AAPH-induced oxidative stress (ROS level-A) and cell deaths (B) in the zebrafish model. Experiments were performed in triplicate and the data are expressed as mean ± SE. $^{*}P < 0.05$ and $^{**}P < 0.01$.

the results, significant decreases of ROS levels and cell deaths were dose-dependently observed in the APS-treated groups.

4. Conclusion

In conclusion, the crude polysaccharide isolated from aloe vera can be considered as materials with natural antioxidant and natural cosmetic applications. In addition, the active polysaccharide and its composition were identified in this study. Based on the results, rhamnose and arabinose are the effective monosaccharides in the polysaccharide structure. The isolated polysaccharide needs to be further examined for its potential functions to be unveiled.

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