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Research Article

Selenylation Modification of *Atractylodes macrocephala* Polysaccharide and Evaluation of Antioxidant Activity

Ranran Hou,[1,2] Qiu Li,[1] Jie Liu,[2,3] and Yuanliang Hu[2]

[1]*Agr Biopharmaceut Lab, Qingdao Agr Univ, Qingdao 266000, China*
[2]*College of Traditional Chinese Veterinary Medicine, Nanjing Agr Univ, Nanjing 210095, China*
[3]*Feed Quality Inspection Institute of Shandong Province, Jinan 250000, China*

Correspondence should be addressed to Yuanliang Hu; ylhu@njau.edu.cn

Ranran Hou and Qiu Li contributed equally to this work.

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The *Atractylodes macrocephala* polysaccharide (AMP) was extracted by water extracting-alcohol precipitating method and further purified by DEAE column. After that, the polysaccharides were modified by nitric acid-sodium selenite method, and nine kinds of selenizing AMPs (sAMPs) were obtained, namely, from $sAMP_1$ to $sAMP_9$. AMP and sAMP were characterized using FTIR spectrometry. Then their antioxidant activities in vitro were measured by free radical-scavenging test. Among these, $sAMP_6$ presented the strongest antioxidant effect. For the test in vivo, the chickens at day 14 vaccinated with ND vaccine were repeatedly vaccinated at day 28. The chickens in sAMP and AMP were injected respectively with 1 mg of $sAMP_6$ and AMP and, in vaccination control (VC) and BC groups, injected with equal volume of normal saline. Respectively, after the first vaccine, on days 7, 14, 21, and 28, the serum GSH-Px and SOD activities and MDA content were determined. The results suggested that $sAMP_6$ could significantly promote GSH-Px and SOD activities and decrease MDA content. All these results indicated that selenylation modification could significantly enhance the antioxidant activity of AMP.

1. Introduction

Selenium is a trace element necessary for life [1] and an important food source of antioxidants. Selenium not only is the cofactor of a large number of selenium-dependent enzymes, such as glutathione peroxidase (GSH-Px) but also can prevent cells from oxidative free radicals [2], and it is extremely important antioxidant in many different cells [3]. Chinese medicine is derived from plant roots, stems, leaves, bark, seeds, and flowers [4]. Chinese medicine polysaccharide has antioxidant, immune enhancement, antitumor, antiradiation effect and so on. Because polysaccharide itself has low toxicity, wide range of sources make the polysaccharide study to become a research hotspot in recent years, and polysaccharide has a clear role in hydroxyl radical and oxygen free radicals [5].

Selenium-polysaccharide is organic selenium, as a compound of selenium combined with polysaccharides; it can play a dual function of selenium and polysaccharides [6] and effectively improve the bioavailability of selenium, significantly reducing toxicity and side effects more than inorganic selenium. Thus, it can be used as a safe, effective, healthy nutrient of selenium [7]. Natural selenium-polysaccharide normally exists in plants or microorganisms, but its content is lower even though the plants grow in high-selenium areas. Therefore, researchers have been exploring the chemical modification method in order to obtain more selenium-polysaccharide or selenizing polysaccharide [8].

Atractylodes macrocephala is a common medicine, possessing diuretic, antitumor, antidiabetic, and antiaging multiple roles [9]. According to national literature, the prescription including *Atractylodes macrocephala* up to 4755 treats as many as 938 kinds of diseases [10]. AMP as the great value of biologically active substances, with a clear antioxidation, enhances immune function, lowering blood sugar and any other effects [11].

Atractylodes macrocephala is used in traditional Chinese medicine, in which the polysaccharide has the function of antioxidation, but its effect is far from meeting the demands of the people. Up to now, however, little is known about that AMP could be modified by selenium and its selenizing polysaccharide could display stronger effects than its polysaccharide.

In this study, AMP was extracted by water extraction and alcohol precipitation method and further purified by DEAE cellulose. Then, we modified AMP using nitric acid-sodium selenite method; nine selenizing AMPs (sAMPs), named sAMP1-sAMP9, were obtained varying the amount of sodium selenite, reaction temperature, and reaction time, according to a L9 (3^4) orthogonal design. Further, we evaluated their antioxidation activities in vitro and in vivo with the unmodified AMP as control group. The aim of this study was to observe whether selenylation modification could enhance the antioxidation activity of *Atractylodes macrocephala* polysaccharide, so as to select the selenizing polysaccharide with strongest activity and provide the theoretical basis for the development of novel antioxidants.

2. Materials and Methods

2.1. Materials and Reagents. *Atractylodes macrocephala* was bought from Nanjing Jinling Dispensary. Sodium selenite bought from Shanghai Lingfeng Chemical Reagent Ltd. was dissolved into 100 mg·mL^{-1} with ultrapure water. Standardly selenium solution (100 ug·mL^{-1}) supplied by National Standard Substance Research Center was accurately diluted into 20, 10, 5, 2.5, and 1.25 μg·mL^{-1}. Nitric acid (HNO_3) was the product of Shanghai Lingfeng Chemical Reagent Ltd. Potassium persulfate, ferrous sulfate, hydrochloric acid, hydrogen peroxide, and other reagents were of analytical grade. Reagent kits of GSH-Px, SOD, and MDA were bought from Nanjing Jiancheng Bioengineering Institute.

Newcastle disease (ND) vaccine (La Sota strain, No. 100082007) was purchased from Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Science. Reagent kits of GSH-Px, SOD, and MDA were bought from Nanjing Jiancheng Bioengineering Institute.

2.2. Preparation of AMP and sAMP. AMP was extracted by water decoction and ethanol precipitation, purified by trichloroacetic acid method to eliminate protein, and eluted with distilled water through DEAE cellulose-52 column. Nine sAMPs, $sAMP_1$-$sAMP_9$, were obtained by nitric acid-sodium selenite method on the basis of L_9 (3^4) orthogonal design of three factors (dosage of Na_2SeO_3, reaction time, and temperature), respectively, at three levels, 500 mg of AMP reacting with Na_2SeO_3 at 200, 300, and 400 mg under 50, 70, and 90°C for 6, 8, and 10 h, respectively, according to our in-house protocol published before [12, 13]. The modification conditions, yield, carbohydrate content, and selenium content were listed in Table 1 (Supplementary material) [12].

2.3. Infrared Spectroscopy Analysis. 1 mg of sAMP or AMP was fully grinded with 100–200 mg of dried KBr in an agate mortar and then pressed into thin slices. FT-IR 920 Fourier transform infrared spectrometer (Tianjin Tuopu Instrument Co., Ltd.) determined the infrared spectroscopy of each polysaccharide in the wavenumber range of 4000–400 cm^{-1} [14].

2.4. In Vitro Test. sAMPs and AMP solutions diluted with distilled water to 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL were prepared for antioxidant test in vitro.

2.4.1. O_2^- Radical-Scavenging Ability. 0.2 mL of total AMP(AMP_t), protein-deleting AMP(AMP_d), purified AMP(AMP_p) at 2, 4, 8, and 16 mg/mL were, respectively, added in tubes with 1.8 mL of distilled water, 5 mL of Tris-Hcl (pH=8.2), and 0.5 mL of 3 mmol/L pyrogallol, shaked well, 4 repeats at each concentration. The compound was reacted under 25°C for 4 min. Distilled water was used instead of polysaccharide as the blank control group (A_0). A_0 and polysaccharide group (A_x) were determined at 325 nm after the reaction and 10 mmol/L HCl was used to zero setting. O_2^- radical-scavenging rate is calculated as follows: O_2^- radical-scavenging rate = $(1 - A_x/A_0) \times 100\%$.

2.4.2. Hydroxyl Radical-Scavenging Ability. Hydroxyl radical-scavenging method was referenced in the literature [15, 16]. 2 mL of sAMPs and AMP solutions prepared at 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL was added to tubes, four repeats per concentration. Then all testing tubes were added with 2 mL of 9 mmol/L $FeSO_4$ and 2 mL of 9 mmol/L salicylic acid-ethanol, as well as 2 mL of 8.8 mmol/L H_2O_2. Then 37°C water bath for 30 min took place, blank zero with distilled water, and with distilled water instead of polysaccharide as the blank control group (A_0). Finally, the absorbance of A_0 and polysaccharides test groups (A_x) was measured at 510 nm by microliter enzyme-linked immunosorbent assay reader (Model DG-3022, East China Vacuum Tube Manufacturer). Hydroxyl radical-scavenging rate is calculated as follows: hydroxyl radical-scavenging rate = $(1 - A_x/A_0) \times 100\%$.

2.4.3. DPPH Radical-Scavenging Ability. DPPH radical-scavenging method was referenced in the literature [16, 17]. Take 200 test tubes, 10 polysaccharide groups (1, 0.5, 0.25, 0.125, 0.0625 mg/mL in each group), four tubes per group, and polysaccharide prepared at each concentration was added 1 mL and 0.01% DPPH 2 mL, ethanol instead of polysaccharide as polysaccharides control groups (A_0), ethanol instead of DPPH as DPPH control groups (A_2). Shake the plate after finishing adding reagents and reacting 30 min in dark place. With ethanol make zero, polysaccharides test groups (A_1), A_2, and A_0 were measured by microliter enzyme-linked immunosorbent assay reader. DPPH radical-scavenging rate is calculated as follows: DPPH radical-scavenging rate = $[1-(A_1-A_2)/A_0] \times 100\%$.

2.4.4. ABTS Radical-Scavenging Ability. Preparing ABTS radical solution according to the literature [18], 7 mmol/L ABTS solution was mixed with 2.45 mmol/L potassium persulfate, reacted for 16 h in room temperature, and diluted with PBS (PH = 7) to the absorbance at 734 nm being 0.70±0.02. 10 μL of the diluted sample polysaccharide solution (1, 0.5,

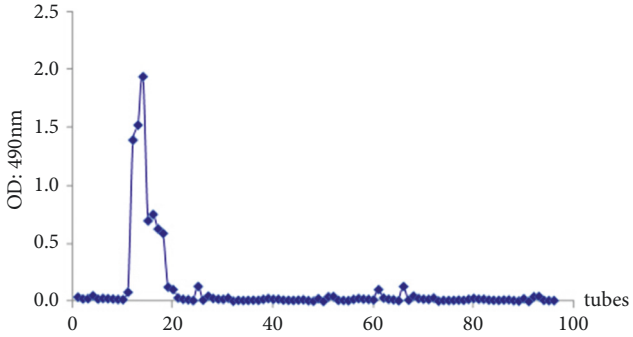

Figure 1: The elution curve of AMP purification.

0.25, 0.125, 0.0625 mg/mL) in each group was added with 2 mL ABTS and reacted for 6 min at room temperature, and absorbance at 734 nm by microliter enzyme-linked immunosorbent assay reader was measured, PBS instead of polysaccharide as control group. ABTS radical-scavenging rate is calculated as follows: ABTS radical-scavenging rate = $(1\text{-}A_{sample}/A_{control}) \times 100\%$.

2.5. In Vivo Test. $sAMP_6$ which was selected in vitro test and AMP were prepared with distilled water. 2 mg/mL of two polysaccharides were sterilized and then detected for endotoxin by pyrogen tests. The endotoxin amount of both was less than 0.5 EU·mL^{-1}, which is in accordance with the standard of Chinese Veterinary Pharmacopoeia [19], and they were stored at 4°C for the test.

One-day-old nonimmune health White Roman chicks (male) were purchased from Tangquan Poultry Farm and fed for 14 days with their maternal antibodies for 2.8 Log2, then 120 chicks were randomly assigned into four groups: sAMP, AMP, BC (blank control), and VC (vaccination control). sAMP, AMP, and VC group were vaccinated with Newcastle disease vaccine, and vaccination was repeated at day 28. sAMP and AMP group were intramuscularly injected with 0.5 mL of $sAMP_9$ and AMP (1 mg) and VC and BC group with equal volume of normal saline at the same time of each vaccination.

On days 7 (D_7), 14 (D_{14}), 21 (D_{21}), and 28 (D_{28}) after the first vaccination, the blood samples of six chickens randomly from wing vein in each group were collected for the determination of GSH-Px activity, SOD activity, and MDA content in serum. The GSH-Px and SOD activities and MDA content were assessed using commercially available assay kits in accordance with the respective manufacturer's instructions.

2.6. Statistical Analysis. Date was expressed as $\bar{x} \pm SE$, the Duncan's multiple range tests were used to analyze the difference among polysaccharides and control groups by SPSS 20.0 software, and the significant difference between means was considered at $p < 0.05$.

3. Results

3.1. Purification of AMP. The elution curve of AMP was illustrated in Figure 1. One polysaccharide peak was displayed from 10-21 tubes. The retention time in the chromatograph column (2.5 cm × 30 cm) was 46-105 min.

3.2. Antioxidant Activity Screening of AMP. The antioxidant activities of AMP_t, AMP_d, and AMP_p were compared respectively by hydroxyl radical-scavenging method and O_2^- radical-scavenging method at 2, 4, 8, and 16 mg/mL. AMP_p possessed the best antioxidant activity in there polysaccharides by two methods which were illustrated in Figure 2. Thus, we chose AMP_p as the target polysaccharide in the selenizing modification.

3.3. The Infrared Spectroscopy Characteristic of sAMP. The FTIR spectra of AMP and $sAMP_6$ in the ranges of 4000 – 400 cm^{-1} are illustrated in Figures 3(a) and 3(b). In the spectra of AMP and $sAMP_6$, there were two bands, one appeared in the region of 3600–3200 cm^{-1} corresponding to the O-H stretching vibration and another in the region of 1400–1000 cm^{-1} corresponding to C–O–C stretching vibration. This indicated that the AMP and $sAMP_6$ were polysaccharides. In comparison with the spectrum of AMP, the infrared spectrum of $sAMP_6$ presented vibration at 1037 cm^{-1}; this coincided with symmetrical O–Se–O stretching vibration (1040 – 1010 cm^{-1}), which is one indicative that sAMP might be modified by selenylation. The other selenizing AMPs also revealed stretching vibration absorptions in the region of 3600–3200 cm^{-1}, 1400–1000 cm^{-1}, and 1040–1010 cm^{-1}.

3.4. The Changes of Hydroxyl Radical-Scavenging Rate. The hydroxyl radical-scavenging rates in each group are illustrated in Figure 4(a). The hydroxyl radical-scavenging rates in $sAMP_6$ group (20.56%) and $sAMP_5$ (19.88%) were significantly higher than that in AMP group (6.12%) ($p < 0.05$). The hydroxyl radical-scavenging rates of other sAMPs groups were numerically higher than AMP group.

3.5. The Changes of DPPH Radical-Scavenging Rate. The DPPH radical-scavenging rates in each group are illustrated in Figure 4(b). The DPPH radical-scavenging rates in $sAMP_6$ group (46.70%) and $sAMP_4$ (46.62%) were the highest, significantly higher than that in AMP, $sAMP_3$, $sAMP_2$, and $sAMP_1$ ($p < 0.05$); the next were $sAMP_5$ (34.70%) and $sAMP_7$ (33.74%), in these two groups were significantly higher than that in AMP group (5.38%) ($p < 0.05$). The DPPH radical-scavenging rates of other sAMPs groups were numerically higher than AMP group.

3.6. The Changes of ABTS Radical-Scavenging Rate. The ABTS radical-scavenging rates in each group are illustrated in Figure 4(c). The ABTS radical-scavenging rate in $sAMP_6$ group (45.28%) was highest, significantly higher than that in AMP group (13.82%) and all other sAMPs groups except $sAMP_5$ and $sAMP_7$ ($p < 0.05$). The ABTS radical-scavenging rates of other sAMPs groups (except $sAMP_1$) were numerically higher than AMP group.

3.7. The Changes of Serum GSH-Px Activity. The serum GSH-Px activities in each group are listed in Figure 5(a). At

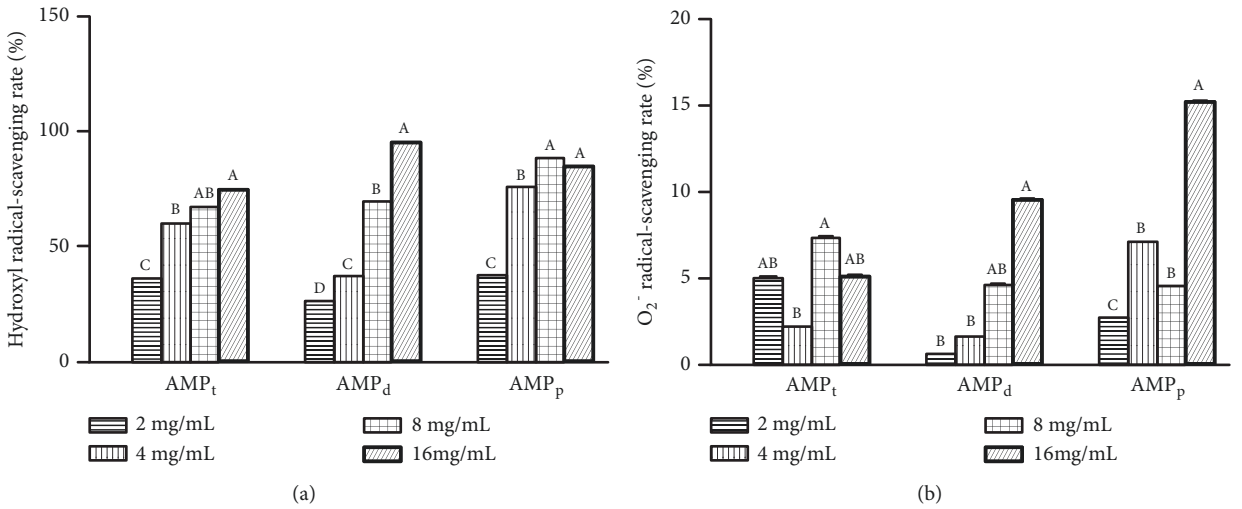

FIGURE 2: The antioxidant activity of AMP_t, AMP_d, and AMP_p at 2, 4, 8, and 16 mg/mL. AMP_t, total AMP; AMP_d, protein-deleting AMP; AMP_p, purified AMP. Bars without the same superscripts (A–D) differ significantly ($P < 0.05$).

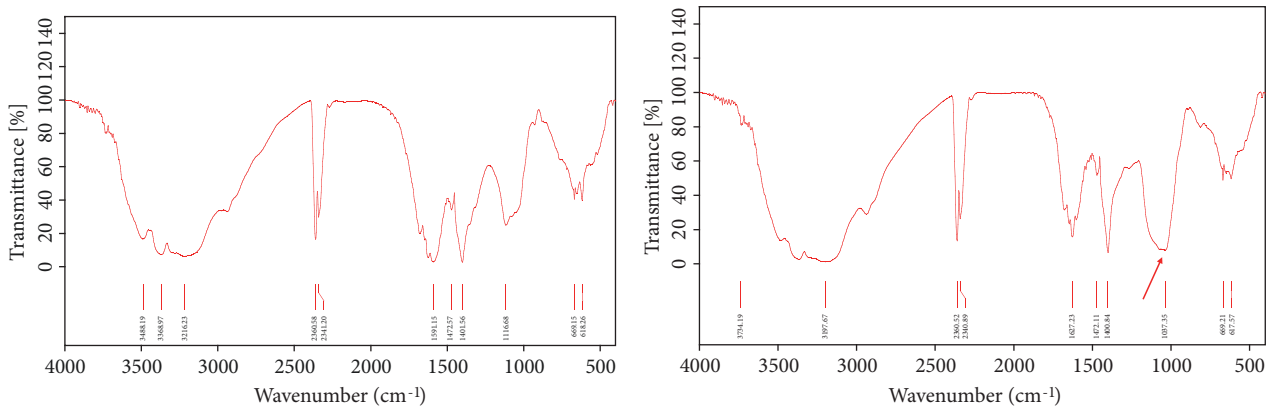

FIGURE 3: The infrared spectra of AMP (a) and $sAMP_6$ (b).

all-time points after injection, the serum GSH-Px activities in sAMP were highest, significantly higher than that in VC and BC groups, on D_{14}–D_{21} were significantly higher than that in AMP group ($p < 0.05$), on D_7, D_{14} were numerically higher than that in AMP group ($p > 0.05$); the serum GSH-Px activities in AMP group were significantly higher on D_{14}–D_{21}, numerically higher on D_7, D_{14} than that in VC and BC groups.

3.8. The Changes of Serum SOD Activity. The serum SOD activities in each group are listed in Figure 5(b). At all-time points after injection, the serum SOD activities in sAMP were significantly higher than that in VC and BC groups, on D_{21}–D_{28} were significantly higher than that in AMP group ($P < 0.05$); the serum SOD activities in AMP group were significantly higher than those in VC and BC groups ($P > 0.05$).

3.9. The Changes of Serum MDA Contents. The serum MDA contents in each group are listed in Figure 5(c). At all-time points after injection, the serum MDA contents in sAMP were significantly lower than that in VC and BC groups, on D_7–D_{21} were significantly lower than that in AMP group ($P < 0.05$); the serum MDA contents in AMP group were significantly lower on D_{14} ($P < 0.05$), and numerically lower than those in VC and BC groups at other time points.

4. Discussion

In the selenylation modification of polysaccharide, the nitric acid-sodium selenite method is one with most reports. It possesses a series of advantages such as simple process, less pollution and strong feasibility [20]. This method was chosen as the selenylation modification of AMP and the immune-enhancing activities of sAMP were compared in the published research in our previous study [12]. We had strong interest in the antioxidant activities of sAMP and AMP; therefore, a series of experiment were applied to prove the change between sAMP and AMP in antioxidant activities.

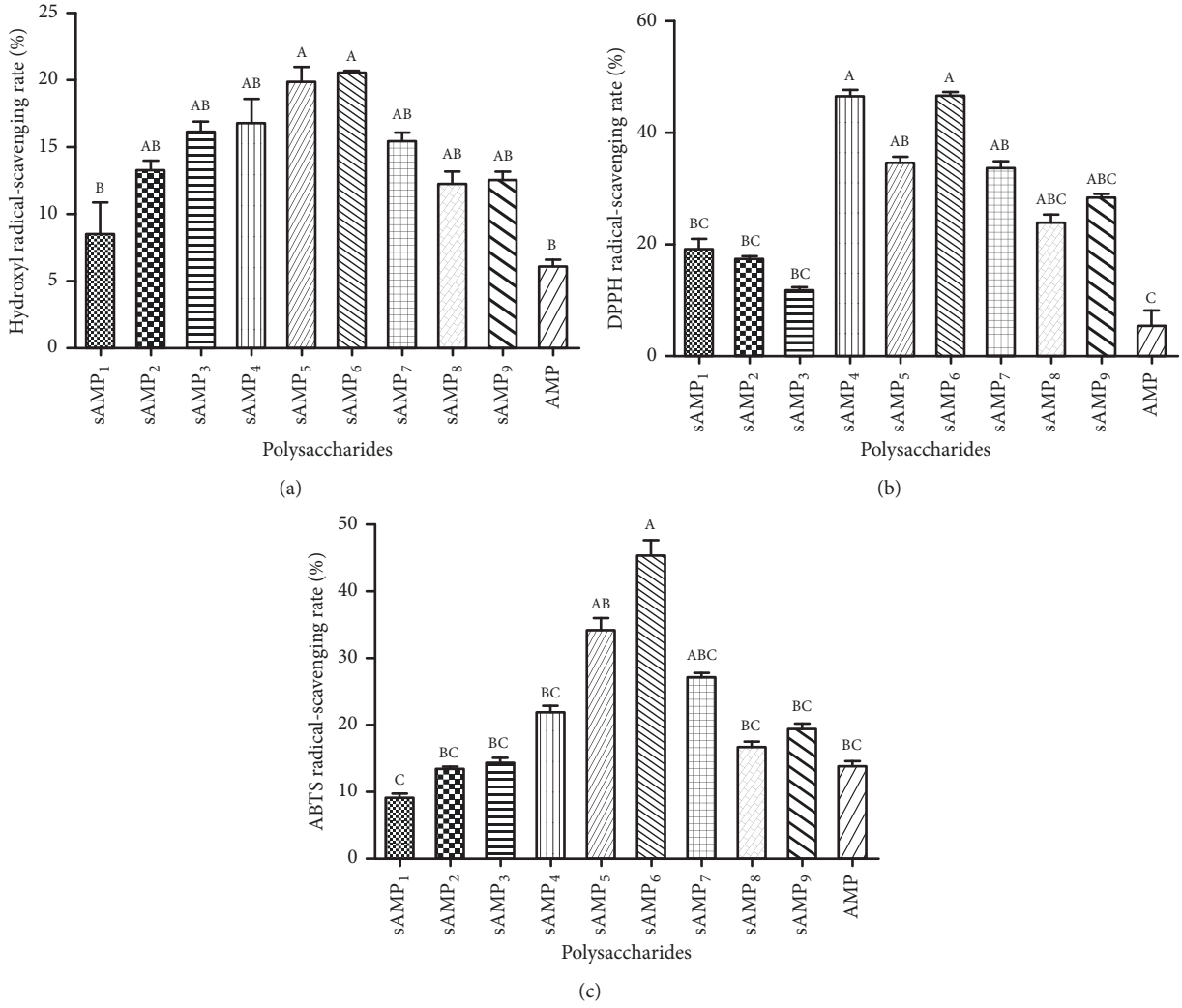

Figure 4: The radical-scavenging rate of every group in vitro test. (a) Hydroxyl radical-scavenging rate. (b) DPPH radical-scavenging rate. (c) ABTS radical-scavenging rate. Bars without the same superscripts (A–C) differ significantly ($p < 0.05$).

Hydroxyl radical is free radical having strong oxidizing power; it can make oxidation reaction of carbohydrates, proteins, nucleic acids, and other substances [21]. If it is not enough to remove the hydroxyl radical, it will trigger the body to produce many diseases. Salicylic acid detection method is currently the most widely used [22], the advantage of this method is that: it can simply simulate antioxidants scavenging hydroxyl radicals in tubes. The experimental results in vitro test showed that the hydroxyl radical-scavenging rates in all sAMPs groups were numerically or significantly higher than those in AMP group, in $sAMP_6$ were highest, and the next is $sAMP_5$; hydroxyl radical-scavenging rate of these two groups was significantly higher than that in AMP group. The results indicated that selenylation modification could significantly enhance the hydroxyl radical-scavenging activity of AMP, and action of $sAMP_6$ was the strongest.

DPPH radical-scavenging method is established in the 1950s [21], DPPH radical is very stable having strong absorption peak at 517 nm; the color fades when the antioxidant is present [23]. The advantage of DPPH radical method is simple, rapid, and sensitive. The experimental results in vitro test showed that the DPPH radical-scavenging rates in all sAMPs groups were numerically or significantly higher than those in AMP group, in $sAMP_6$ were highest, and the next were $sAMP_4$, $sAMP_5$, and $sAMP_7$ groups; in these groups they were significantly higher than that in AMP group. The results indicated that selenylation modification could significantly enhance the DPPH radical-scavenging activity of AMP, and action of $sAMP_6$ was the strongest.

ABTS method, also known as TEAC method, was established by Miller in 1993 [24]. It is a common method of detecting antioxidant capacity. Antioxidant capacity of the sample is stronger, the color fading reaction will be more obvious and the absorbance at 734 nm wavelength will be lower. This method has been applied by seeram, Liu and Du et al. [25–27] to measure antioxidant activity of U.S. polyphenol-rich beverages, lychee flower extract, and kiwi. The experimental results in vitro test showed that the ABTS radical-scavenging rates in all sAMPs (except $sAMP_1$) groups were numerically or significantly higher than those in AMP

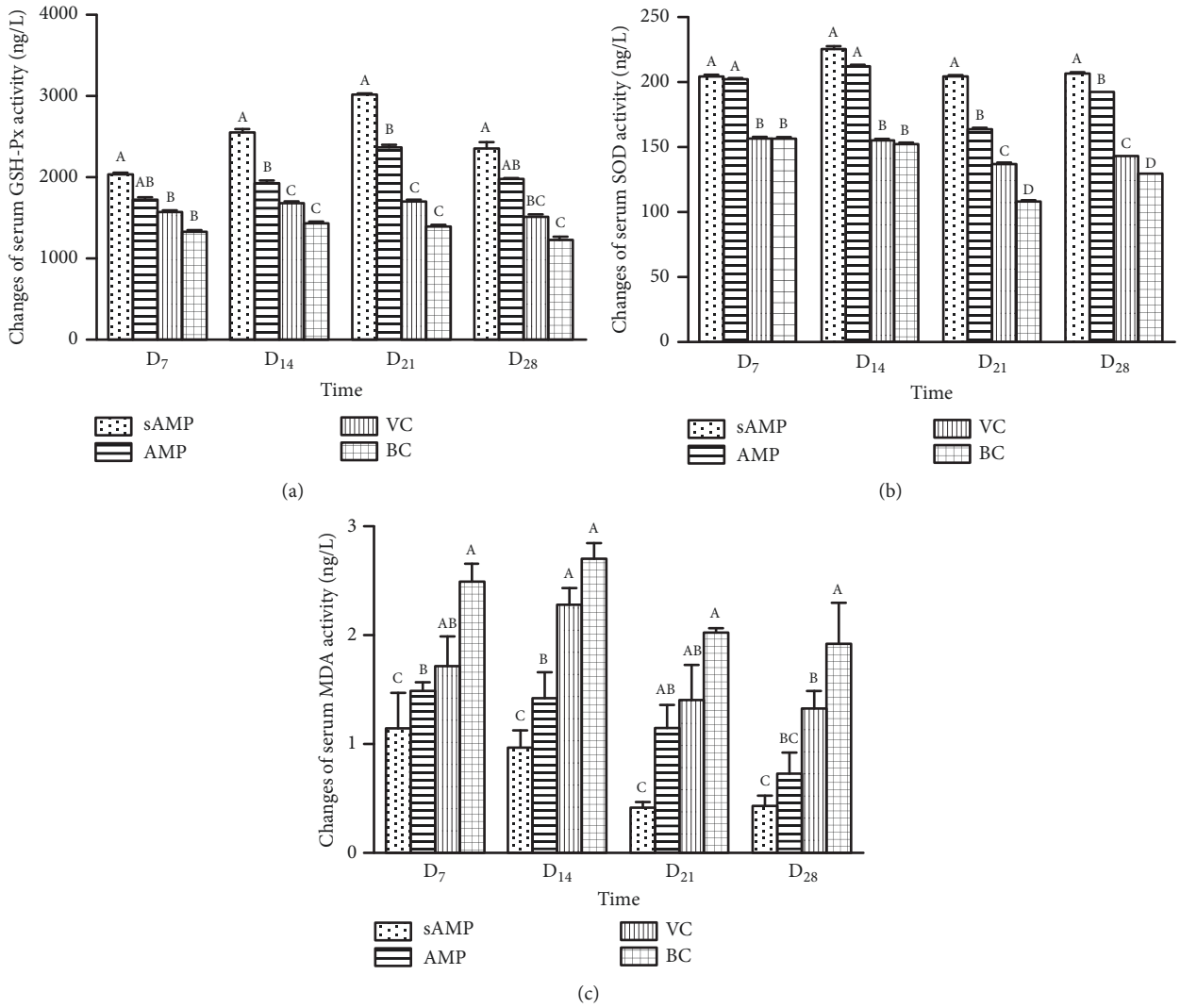

Figure 5: The changes of serum GSH-Px activity (a), SOD activity (b), and MDA content (c) in vivo; bars without the same superscripts (A–D) differ significantly ($p < 0.05$).

group; in $sAMP_6$ was highest and significantly higher than that in AMP group.

$sAMP_6$ was picked out according to the results of vitro test to further compare with AMP the change effects of chicken GSH-Px, SOD, MDA contents. There exist effective free radical-scavenging antioxidant enzyme systems in animal body, such as GSH-Px, SOD, and CAT. MDA is the final product of lipid peroxidation (LPO) that may cause damage to body cells. Detection of these indicators can be integrated to determine antioxidant activity of living organisms [4].

GSH-Px and SOD are the body's antioxidant enzymes, their main role is to remove free radical and prevent free radical damaging cell structures. GSH-Px and SOD contents will reduce when degenerative diseases occur [28]. GSH-PX is a necessary enzyme to catalyze hydrogen peroxide decomposition which is widespread in body; it can specifically have catalytic reduction reaction on H_2O_2, protecting the structure and function of the cell membrane integrity to play the role in antioxidation [4]. SOD eliminates superoxide anion radicals, protecting cells from damage [29]. The experimental results in vivo test showed that at all-time points after injection, GSH-Px and SOD activities in sAMP group were significantly higher than that in VC and BC groups, numerically or significantly higher than that in AMP group.

MDA is the end product of LPO oxide formation; its content reflects the severity level of the body's cells by free radicals attack. MDA production which is inhibited could cause direct activation of the enzymes or binding of metal ions which are necessary for organisms to produce free radical [30]. The MDA content in sAMP group was significantly lower than that in BC and VC groups, numerically or significantly lower than that in AMP group. The result showed that antioxidant activity of sAMP is significantly stronger than AMP; selenylation modification could significantly enhance the antioxidant activity of AMP in vivo.

In conclusion, selenylation modification could significantly enhance the antioxidant activity of *Atractylodes macrocephala* polysaccharide.

Abbreviations

AMP: *Atractylodes macrocephala* polysaccharide
sAMP: Selenizing *Atractylodes macrocephala* polysaccharide
BC: Blank control
VC: Vaccination control
PBS: Phosphate buffered saline
DPPH: 1,1-Diphenyl-2-picryl-hydrazyl
ABTS: 2,2′-Azino-bis-(3-ethyl benzthiazoline-6-sulphonate)
GSH-Px: Glutathione peroxidase
SOD: Superoxide Dismutase
MDA: Malondialdehyde.

Data Availability

The corresponding author has the original data.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Ranran Hou and Qiu Li contributed equally.

Acknowledgments

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Supplementary Materials

The modification conditions, yields, contents of carbohydrate and selenium of nine sAMPs. *(Supplementary Materials)*

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