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RAPID COMMUNICATION

Effect of heat stress on production and in-vitro antioxidant activity of polysaccharides in *Ganoderma lucidum*

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Abstract *Ganoderma lucidum* is a traditional Chinese medicine, and its polysaccharides possess diverse and significant pharmacological activities. This study aimed to investigate the polysaccharide production, molecular characteristics and in-vitro antioxidant activity of *G. lucidum* fruiting body after the mushroom was harvested and treated with heat stress (HS). HS enhanced the production of polysaccharides after harvest and treatment of 42 °C HS for 2 h, and that resulted in the highest polysaccharide yield of 10.50%, which was 45.63% higher than that of the control, while 37, 45 °C HS had no significant effect on the production. In terms of molecular characteristics, 42 °C HS significantly changed monosaccharide ratio of polysaccharides, but no apparent molecular weight and functional group changes were found in polysaccharides after HS treatment. The results of in-vitro antioxidant activity assay revealed that 42 °C HS significantly improved the antioxidant activities of polysaccharides at the concentration of 2 mg/mL. In conclusion, this study provides a promising strategy to improve the production of *G. lucidum* fruiting body polysaccharides.

Keywords *Ganoderma lucidum* polysaccharides · Heat stress · Production · Molecular characteristics · In-vitro antioxidant activity

Introduction

Ganoderma lucidum, known as Lingzhi in Chinese, has been widely consumed throughout China and Southeast Asia as a precious medicinal mushroom [1]. Polysaccharides extracted from *G. lucidum* have been reported to have several physiological functions and health benefits, such as anti-tumour, anti-ageing, immunomodulatory, hypoglycaemic, antiviral and antibacterial effects [1–3]. However, it usually takes several months to cultivate and plant the fruiting bodies and the process is complex and easily influenced by the environment. And the polysaccharide content of fruiting body is quite low. Therefore, there is a technical demand in improving the polysaccharide content in *G. lucidum*. Many attempts, including manipulation of culture conditions, addition of inducer, and manipulating the expression levels of the biosynthetic genes, have been made to enhance mycelium polysaccharide production [4–7]. But few reports aimed to improve the polysaccharide production in fruiting bodies.

For many years, researchers have been investigating the effect of heat stress (HS) on fungi. The fungal stress response often involves the production of various protective substances, one of which known to be accumulated during heat stress response is carbohydrate [8]. Liu et al. [9] found that heat shock pretreatment induced the gene expression of *trehalose-6-phosphate synthase* and trehalose accumulation

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in a biocontrol yeast *Metschnikowia fructicola* and thus enhanced its stress tolerance. Feofilova et al. [10] reported that under hyperthermic conditions, a significant increase in the sugar content occurred. Therefore, as a main functional component of carbohydrate in fungi, polysaccharides may be also involved in responding to HS, and the biosynthesis of polysaccharides could be regulated by HS. In previous study on *G. lucidum*, Zhang et al. [11] have found that 42 °C HS induced the accumulation of ganoderic acid in *G. lucidum* mycelium, which is also a functional component of *G. lucidum*. However, no study focused on the effect of HS on polysaccharide production in *G. lucidum*, especially in fruit-body stage.

The objective of this work was to investigate the production of polysaccharides responding to HS in *G. lucidum* fruiting bodies. At the same time, we also analysed the changes of molecular weight, monosaccharide composition, functional groups and *in-vitro* antioxidant activities of polysaccharides after HS treatment. These results provide a useful and convenient strategy for increasing polysaccharide production, which will bring value to the *Ganoderma* industry.

Materials and methods

Materials

The fruiting bodies of *G. lucidum* (GIMCC No. GIM5.250) were cultivated on cut-log and harvested after about 3 months in Longquan city in Zhejiang province. Standard dextrans of different molecular weights were purchased from National Institutes for Food and Drug Control. Standard monosaccharides (D-glucose, D-mannose, L-rhamnose, D-galactose, D-xylose, L-arabinose, L-fucose and D-ribose) and bovine serum albumin (BSA) were purchased from Sigma Co. (USA). All other chemicals and reagents were of grade AR.

Heat treatment and polysaccharide preparation

The fruiting bodies of *G. lucidum* were harvested and placed at room temperature 28 °C (as control), or exposed to 37, 42 or 45 °C immediately in a temperature-controlled chamber (DLHR-Q200, HDL, China) for 1–4 h, then dried to a constant weight at 60 °C and pulverized into powder by using a mechanical disintegrator (500A, Hongtaiyang, China); the powders smaller than 60 mesh were collected as material. Powders were defatted by ethyl acetate and then acetone. 0.5 g of samples was immersed in 50 mL of distilled water by refluxing for 2 h on a water bath at 100 °C. The extract was filtered and then concentrated to 25 mL by rotary evaporator (SHB-IIIS, Hengyan, China). The concentrated extracts were precipitated with fourfold volume of ethanol overnight at 4 °C. After centrifugation (4000 rpm, 15 min), the precipitate was collected, deproteinated by Savag method [12] and finally dried at 60 °C to get polysaccharides.

Determination of polysaccharide and protein content

The extracted polysaccharides were redissolved in 10 mL of distilled water and the content was determined by the phenol–sulfuric acid method, using D-glucose as the standard [13]. Per cent yield was calculated with the following equation:

$$Y(\%) = c \times v/w \times 100, \quad (1)$$

where Y is the polysaccharide percentage, c is the concentration of polysaccharides in the sample solution (mg/mL), v is the volume of the sample solution (mL) and w is the mass of the fresh sample (mg).

Protein content was measured by the method of Bradford using BSA as the standard [14].

Monosaccharide composition analysis of polysaccharides

The monosaccharide composition of polysaccharides was analysed using reversed-phase high-performance liquid chromatography (HPLC) after pre-column derivatization according to the method reported in the previous literature [14]. 10 mg of polysaccharides was first hydrolyzed with 2 M trifluoroacetic acid (TFA) in 105 °C for 5 h, and then dried under 105 °C. Excessive acid was removed by co-distillation with methanol after hydrolysis. The dry hydrolysate was then dissolved in 3 mL distilled water for the subsequent derivatization. 1 mL of the hydrolyzed polysaccharide samples or monosaccharide standards was mixed with 600 μL each of 1-phenyl-3-methyl-5-pyrazoline (PMP) solution (0.5 M in methanol) and 0.3 M NaOH, and incubated at 70 °C for 2 h, with vortexing every 15 min. After cooling, the mixtures were neutralized by the addition of 600 μL of 0.3 M HCl and then added to 1 mL of trichloromethane, thoroughly mixed by vortexing for 10 s. This extraction process was repeated five times to remove the excess reagents. Finally, the supernatant was filtered through a 0.22-μm PES membrane and stored at 4 °C before HPLC analysis.

Chromatography analysis was performed using an Agilent 1200 HPLC system, equipped with an Agilent C18 column operated at 30 °C with a flow rate of 1.0 mL/min and the mobile phase was 20 mM phosphate buffer solution (pH 6.7) and acetonitrile (v/v, 82:18). PMP-labelled monosaccharides were detected at UV detection wavelength of 250 nm.

Molecular weight analysis

The molecular weight of polysaccharides was identified by high-performance gel-permeation chromatography (HPGPC) on an Agilent 1200 HPLC system equipped with an Agilent G1362A refractive index detector (RID). Ultrahydrogel column PL aquagel-OH MIXED-H (300 × 7.5 mm) was eluted with distilled water at a flow rate of 1 mL/min and column temperature of 30 °C. The HPLC system was pre-calibrated with D-series dextran standards (180, 9750, 36,800, 300,600, 2,000,000 Da). Calibration curve of Log Mw (molecular weight) of standard dextrans against their retention time (Rt) was acquired ($\mathrm{Log}\ M_w = -1.243R_t + 14.62$, $R^2 = 0.9964$).

FT-IR spectral analysis

The polysaccharides were identified by using KBr pressed-disk method on a Fourier Transform Infrared Spectrophotometer (Shimadzu, Japan), according to a method described by Chang et al. [15]. 4 mg of each dried sample was ground with 200 mg dried KBr powder and then pressed into 1 mm pellets for FT-IR spectrum measurement in the frequency range of 4000–400 cm^{-1}.

Antioxidant activity assays

Hydroxyl radical scavenging activity

An improved Fenton-type reaction was used to determine hydroxyl radical scavenging activity of polysaccharides [16]. 250 μL of sample solution (0.1–2.0 mg/mL) was mixed with 75 μL of $FeSO_4$ solution (2 mg/mL), 250 μL of H_2O_2 (1%), and 250 μL of salicylic acid solution (1.5 mg/mL in ethanol), shaken vigorously, and then the reaction mixture was incubated in a water bath at 37 °C for 1 h. The reaction absorbance at 526 nm was promptly determined and recorded. Hydroxyl radical scavenging activity was calculated according to the following formula:

$$\text{Scavenging activity (\%)} = \left[1 - (A1 - A2/A0)\right] \times 100 \quad (2)$$

where A1, A2 and A0 are the absorbance value of the sample, a control reaction with 100% ethanol instead of salicylic acid/ethanol solution, and a blank control.

DPPH scavenging assay

The scavenging activity of the DPPH free radical was assayed according to the method of Hu et al. [17] with slight modifications. 500 μL of sample solution (0.1–2.0 mg/mL) was added to 500 μL of DPPH ethanol solution (0.2 mg/mL), the reaction mixture was shaken vigorously, and then incubated in the dark at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm. The ability of scavenging the DPPH radicals was calculated as follows:

$$\text{Scavenging efficiency (\%)} = \left[1 - (B1 - B2)/B0\right] \times 100, \quad (3)$$

where B1, B2 and B0 are the absorbance value of the DPPH solution alone, sample/DPPH mixtures, and a control reaction with water instead of DPPH solution, respectively.

Measurement of ferric reducing antioxidant power

The reducing power of polysaccharides was determined based on the method described by Cheng et al. [3] with minor modifications. 200 μL of polysaccharides solution (0.1–2.0 mg/mL), 500 μL of phosphate buffer (0.2 M, pH 6.6) and 500 μL of potassium ferricyanide (1:100, w/v) were mixed and incubated at 50 °C for 20 min, followed by addition of 500 μL trichloroacetic acid in water (1:10, w/v). Afterwards, the mixture was centrifuged at 10,000 rpm for 10 min. The upper layer (600 μL) was mixed with 600 μL of distilled water and 120 μL of ferric chloride in water (1:1000, w/v) and allowed to react for 10 min at room temperature. Absorbance was then mearsured at 700 nm against as the blank (water instead of the sample solution). Antioxidant activity was calculated using the following equation:

$$\text{Reducing power} = C1 - C0, \quad (4)$$

where C1 and C0 are the absorbance value of the sample or a control reaction with water instead of ferric chloride, respectively.

Total antioxidant capacity analysis

The total antioxidant capacity (T-AOC) of polysaccharides was measured according to the instruction of T-AOC Assay Kit (Jiancheng, Nanjing, China). Absorbance of reaction mixture was measured at 520 nm. T-AOC was calculated as follows:

$$\text{T-AOC (U/mL)} = (D1 - D0) \times 3.7/0.03, \quad (5)$$

where D1 and D0 are the absorbance value of the sample and a blank control.

Results and discussion

42 °C HS increased polysaccharide production

When responding to temperature stress, organisms will synthesize protective substances, among which carbohydrate

is the most important one [10]. Therefore, polysaccharide content may also be increased in the HS reaction. Usually *G. lucidum* fruiting bodies are dried under a relatively high temperature of approximate 60–70 °C without any pretreatment. In this study, fruiting bodies of *G. lucidum* were pretreated at 28 °C (marked as CK), 37, 42, 45 °C, respectively and then dried to constant weight at 60 °C. The powders pulverized and passed through a 60-mesh screen were used as samples. Figure 1 showed that the polysaccharide content increased significantly under 42 °C treatment and the yield increased to 10.50% after fruiting bodies were treated under 42 °C for 2 h. Specifically, the 2-h 42 °C HS treatment caused a significant increase in the polysaccharide content by 45.63% compared to CK (7.21%). However, 37 and 45 °C HS had no significant impact on the polysaccharide production, even led to a slight decrease in the first 2 hours (5.23 and 6.16%, respectively). While it seemed that the production increased gradually under 45 °C HS (from 5.54 to 7.03%), we could suppose that 45 °C HS might also improve the polysaccharide content after a long-term HS treatment. These results suggested that proper HS could promote the synthesis of *G. lucidum* fruiting body polysaccharides, thereby increasing the polysaccharide production yield. To further analyse the effects of 42 °C HS on polysaccharides, polysaccharides treated with or without 42 °C HS for 2 h were selected as samples for the subsequent molecular characteristic analysis and *in-vitro* antioxidant activity assays in our study.

Molecular characteristic analysis

Since the polysaccharide biosynthesis was supposed to be affected by HS, the molecular characteristics of polysaccharides were analysed. Results in Table 1 showed that polysaccharides treated by 42 °C HS or not were both composed of four fractions: 0.6×10^4, 52.3×10^4, 143.3×10^4 and 833.4×10^4 Da for HS-untreated polysaccharides and 0.5×10^4, 56.4×10^4, 133.5×10^4 and 855.2×10^4 Da for HS-treated ones. And HS almost had no effect on the molecular weight of polysaccharides. In addition, the functional groups present in polysaccharides' structure were not affected by HS either (Fig. 2). The main peaks were similar among the two polysaccharides. The absorption bands at 1640 cm^{-1} were assigned to the stretching vibrations of the CHO and C=O bonds, indicating acidic polysaccharides. Several weak and specific bands in the 1085–1044 cm^{-1} region were attributed to the existence of pyranose and the weak absorption bands at 888.88 cm^{-1} were related to the C–H deforming vibrations in β-pyran ring [16].

Table 1 Molecular weight of polysaccharides

Sample	Molecular weight ($\times10^4$ Da)			
	Peak 1	Peak 2	Peak 3	Peak 4
FCK	0.6	52.3	143.3	833.4
FHS	0.5	56.4	133.5	855.2

FCK fruiting body polysaccharides without 42 °C HS treatment, *FHS* fruiting body polysaccharides treated with 42 °C HS for 2 h, – not detectable

Monosaccharide composition analysis (Table 2) indicated that HS-treated and untreated polysaccharides were composed of the same six monosaccharides, which were mannose, rhamnose, ribose, glucose, xylose and arabinose, and glucose was the main component. However, HS changed the ratio of different monosaccharides. After 42 °C HS treatment, the content of glucose decreased by 22.73%, while the content of the other five monosaccharides increased to different extent (indicated in Table 2). This was in accordance with other fungi like *Cunninghamella japonica, Myceliophthora thermophila* and *Pleurotus ostreatus* reported by Feofilova et al. [10]. Thus, it could be assumed that HS may affect the transformation between different monosaccharides during the biosynthesis of *G. lucidum* polysaccharides.

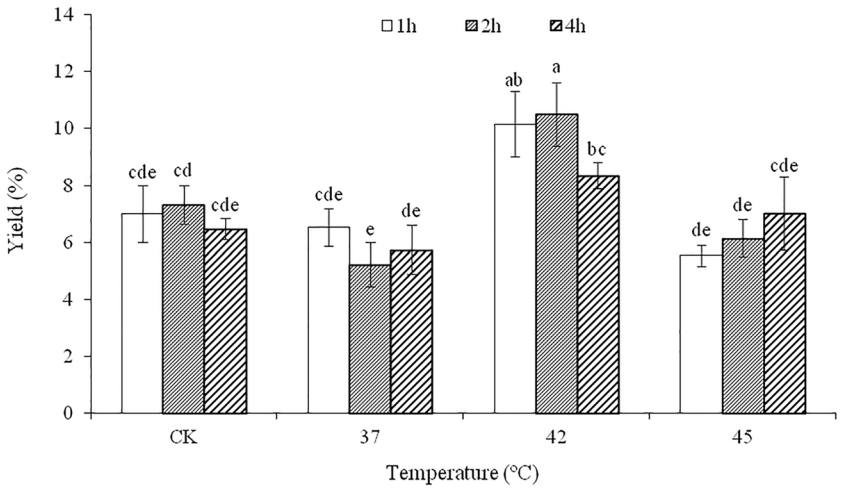

Fig. 1 Effect of heat stress on polysaccharide production. Values are means ± SD ($n=5$). Different letters in the same bar chart indicate significant differences ($P<0.05$)

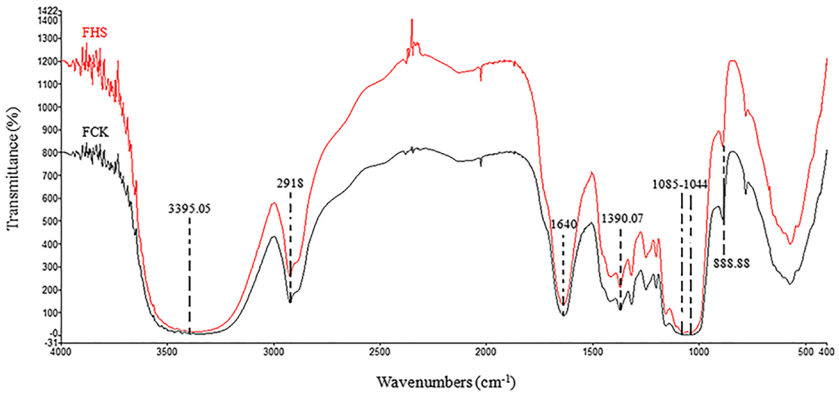

Fig. 2 FT-IR spectroscopy of polysaccharides between 4000 and 400 cm^{-1}. FCK, fruiting body polysaccharides without 42 °C HS treatment; FHS, fruiting body polysaccharides treated with 42 °C HS for 2 h

Table 2 Monosaccharide composition of polysaccharides

Sample	Monosaccharide content in polysaccharides (%)							
	Man	Rha	Rib	Glu	Xyl	Gal	Ara	Fuc
FCK	2.01	3.61	3.31	78.12	6.04	–	6.90	–
FHS	3.28	9.16	7.62	56.85	8.01	–	15.07	–

FCK fruiting body polysaccharides without 42 °C HS treatment, *FHS* fruiting body polysaccharides treated with 42 °C HS for 2 h, – not detectable, *Man* mannose, *Rha* rhamnose, *Rib* ribose, *Glu* glucose, *Xyl* xylose, *Gal* galactose, *Ara* arabinose, *Fuc* fucose

In-vitro antioxidant activity assay

Among the reactive oxygen species, hydroxyl radical is the most active free radical that attacks all the biological molecules [16]. In the hydroxyl radical scavenging assay, the scavenging ability of 42 °C HS-untreated and -treated polysaccharides increased to 31.91 and 41.05% at 2 mg/mL, respectively, and HS apparently increased the polysaccharide hydroxyl radical scavenging ability (Fig. 3a).

DPPH is a stable free radical and the DPPH radical scavenging activity is one of the most important functional properties for bioactive compounds [18]. Figure 3b shows that the scavenging ability of polysaccharides with 42 °C HS significantly increased by 14.87% at the concentration of 2 mg/mL.

In the ferric reducing power assay, the presence of antioxidants in the samples would result in the reduction of the Fe^{3+}–Fe^{2+} transformation by donating an electron [19]. Similarly, Fig. 3c showed that polysaccharides with 42 °C HS showed much stronger ferric reducing power at the concentration of 2 mg/mL.

In the total antioxidant capacity (T-AOC) assay, markedly, after 42 °C HS treatment, the total antioxidant capacity of polysaccharides increased to 0.8 U/mL, higher than that of control (0.7 U/mL), indicating a greater antioxidant activity (Fig. 3d).

Based on the four in-vitro antioxidant activity assays, we could determine that 42 °C HS for 2 h could improve the in-vitro antioxidant activity of polysaccharides in *G. lucidum* fruiting bodies, which was supposed to be closely related to its pharmacological functions.

Conclusions

In conclusion, 42 °C heat stress (HS) significantly increased *G. lucidum* fruiting body polysaccharide production yield. The highest yield of polysaccharide (10.50%) was observed after 2 h of exposure to 42 °C HS, which was 45.63% higher than that without HS. Molecular characteristic analysis revealed that 42 °C HS pretreatment changed the ratio of monosaccharides, indicating HS may affect the transformation between different monosaccharides during the biosynthesis of polysaccharides. In terms of molecular weight and functional group composition, there was no significant change after 42 °C HS. Notably, the antioxidant activities of 42 °C HS-treated polysaccharides improved significantly, probably due to the changes in its monosaccharide composition. Therefore, HS may serve as a promising bioprocess to improve polysaccharide production of *G. lucidum*. The future challenge is to explore the mechanism of HS promoting polysaccharide biosynthesis.

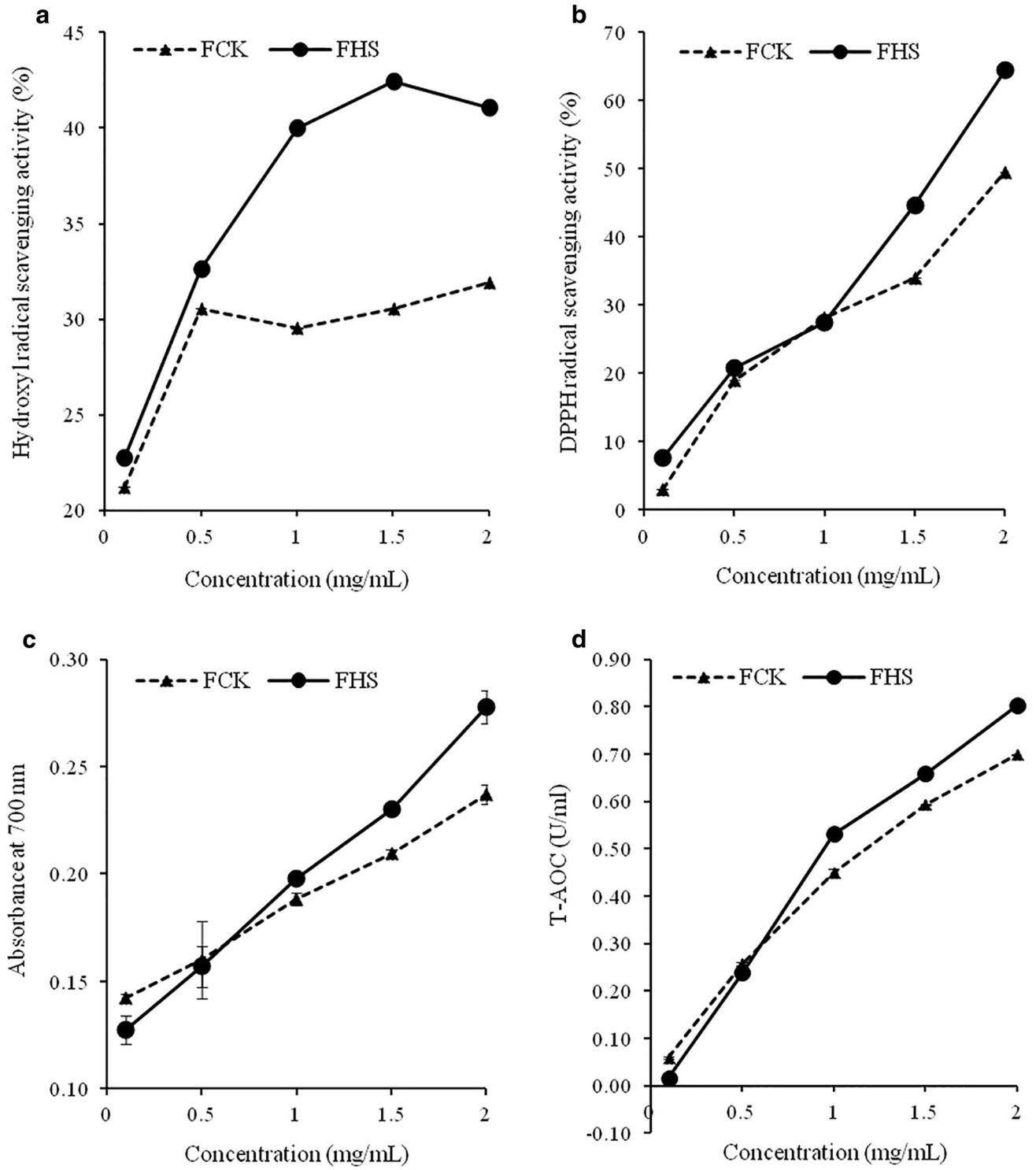

Fig. 3 Antioxidant effects of polysaccharides at different concentrations as determined by hydroxyl radical (**a**) and DPPH free radical (**b**) scavenging activities, ferric reducing antioxidant power (**c**) and total antioxidant capacity (**d**). Values are means ± SD ($n = 3$). *FCK* fruiting body polysaccharides without 42 °C HS treatment, *FHS* fruiting body polysaccharides treated with 42 °C HS for 2 h

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Compliance with ethical standards

Conflict of interest The authors declare that they do not have any conflict of interest.

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