Improvement of antioxidant activity of *Morchella esculenta* protein hydrolysate by optimized glycosylation reaction

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**ABSTRACT**

*Morchella esculenta* (MPH) is produced from an edible and medicinal fungus, *Morchella esculenta* (L.), and exhibits antioxidant activity. In order to further improve the antioxidant activity of MPH, glycosylation of MPH was conducted by applying response surface methodology via the Maillard reaction (MR). Optimum glycosylation conditions were confirmed as: xylose/MPH mass ratio of 3.7:1, pH 11.80, and reaction time 60 min. The glycosylation of MPH under these optimized conditions was characterized by Fourier transform infrared spectroscopy, fluorescence spectroscopy, and scanning electron microscopy. *In vitro* antioxidant activity tests showed that glycosylated MPH had significantly enhanced antioxidant activities compared to the native MPH, heat-treated MPH, or a MPH-xylose mixture. Collectively, these results indicate that glycosylation by the MR can improve the antioxidant activity of MPH, making it a novel source of natural antioxidants with excellent developmental prospects as a functional food.

1. Introduction

*Morchella* (*Morchella esculenta*) is a medicinal, edible, delicious, and sought-after wild mushroom rich in various nutrients, such as polysaccharides, proteins, vitamins, and trace elements. It can enhance the functioning of the immune system, and also contains anti-fatigue, antiviral, antioxidant, and antitumor growth properties (Ajmal, Akram, Ara, Akhund, & Nayyar, 2015; Meng et al., 2010). The protein content of *Morchella* is 32.7% (dry weight), comprising eight essential amino acids, making it a valuable source of protein (García-Pascual, Sanjuán, Melis, & Mulet, 2006; LeDuy, Kosaric, & Zajic, 1974). With the rapid advancement of submerged fermentation technology, the development of functional foods from *Morchella* has gained significant commercial interest. However, most of the existing studies have focused on utilization of the crude extract, such as aqueous-ethanol extracts (Nitha & Janardhanan, 2008), methanol extracts (Nitha, Meera, & Janardhanan, 2007), or polysaccharides (Li et al., 2013). Research on *Morchella* proteins is lacking.

In recent years, the antioxidant peptides from the enzymatic hydrolysis of proteins have received significant research attention. Studies have shown that these have a simple structure, possess good stability, are non-immunogenic, and are safe and healthy compounds with broad application potential in functional foods, food additives, pharmaceuticals, and cosmetics (Sarmadi & Ismail, 2010). However, the antioxidant activities of these peptides are not as high as those of synthetic antioxidants due to restrictions presented by the raw protein’s primary structure. Therefore, it is desirable to make further modifications to the native peptides in order to increase their antioxidant activity.

Due to its simplicity, low cost, and mild reaction conditions, the Maillard reaction (MR) is considered to be a method with significant potential for modifying proteins

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or peptides in the food industry (Liu, Ru, & Ding, 2012; Moisés, Corzomartinez, Villamiel, Javier, & Sanz, 2011). The MR is a widely used nonenzymatic browning reaction that occurs when foods are heated, and refers primarily to the complex reactions between carbonyl compounds (reducing saccharides) and amino compounds (amino acids, peptides, and proteins), which occur during food processing. It gives food a particular aroma, taste, and color, and plays an important role in the sensory quality of foods (Sun, Zhao, Cui, Zhao, & Bao, 2010; Zeng, Zhang, Guan, Zhang, & Sun, 2013). Maillard reaction products (MRPs) have antioxidant, anti-allergic, and anti-inflammatory properties (Chen & Kitts, 2011). In particular, their antioxidant activities make them potential substitutes for synthetic antioxidants (Vhangani & Van Wyk, 2016). The MR is influenced by many factors such as temperature, pH, reaction time, and the concentration and type of substrates (Yin, Sun, Zhang, & Hao, 2014). Among these, substrate type plays a leading role in the occurrence of MR and the characteristics of the resulting products. Protein hydrolysates, as a crude extract, are nourishing, nontoxic, and cost-effective. They possess diverse biological activities, are nonallergenic, and are easily digested and absorbed (Rao, Kamdar, & Labuza, 2013). They are suitable substrates for the MR; however, the MR of the saccharide–protein hydrolysate system has largely not been examined (Chawla, Chander, & Sharma, 2009).

Morchella protein hydrolysate (MPH) represents a potential source of protein and peptides that can undergo MR. It was anticipated that copolymerization of MPH with reducing saccharides would produce a functional product with high antioxidant activity, which could have wide application in the production of health-promoting foods. To address this, the present study sought to optimize the glycosylation of MPH in the MR using response surface methodology (RSM), and to examine the molecular characteristics of the glycosylated Morchella protein hydrolysates (MPHg) by Fourier transform infrared spectroscopy (FT-IR), fluorescence spectroscopy, and scanning electron microscopy (SEM). Furthermore, the in vitro antioxidant activities of the MPH and its glycosylated derivatives were determined using different model systems. The results of the present study may provide experimental evidence in support of the utilization of MPH and its glycosylated derivatives as highly effective natural antioxidants in health products and functional foods.

2. Materials and methods

2.1. Strain and liquid fermentation

Morchella strain ACCC 50537 was provided by the Agricultural Culture Collection of China. The strain was cultured on potato dextrose agar slants at 25°C for 7 days, and then stored at 4°C. It was subcultured every 2 months. The fungal culture (0.5 cm²) was inoculated in a 250-mL culture flask with 100 mL culture media containing 100 g/L potato, 30 g/L dextrose, 5 g/L yeast extract, 1 g/L peptones, 1 g/L MgSO₄·7H₂O, and 1 g/L KH₂PO₄. The culture was incubated at 25°C for 3 days with shaking at 150 rpm. The fermented broth was filtered with a filter cloth, washed with distilled water, and freeze-dried to obtain the Morchella mycelia.

2.2. Morchella protein extraction and MPH preparation

The Morchella mycelia were homogenized, resuspended in NaOH solution (pH 12.0) at a ratio of 1:40 (w/v), and the proteins were extracted for 1 h in a 45°C water bath. The solution was centrifuged at 4000 g for 20 min, and the supernatant was adjusted to pH 4.1 with 2 mol/L HCl, followed by a further centrifugation at 4000 g for 20 min. After removal of the supernatant, the pellet was dissolved in a small amount of distilled water and freeze-dried to obtain the Morchella protein isolate (MPI). The protein content of the MPI was 63.03 ± 2.08% (w/w), as determined by the Bradford method (Bradford, 1976).

The MPI solution with a mass fraction of 5% was denatured at 100°C for 30 min and then cooled. Papain was added to the solution at an enzyme/protein (E/S) ratio of 2%, and the mixture was hydrolyzed in a shaking water bath at 45°C for 3 h at pH 6.0 using the pH-stat technique. At the end of hydrolysis, the enzyme was inactivated for 10 min in a boiling water bath, and the mixture was then centrifuged at 4000 g for 15 min. The supernatant was freeze-dried to obtain MPH.

2.3. MPH glycosylation

MPH (10 mL) with a mass fraction of 0.5% was placed in a screw-capped plastic centrifuge tube. The glycosylation reaction was conducted in a boiling water bath under a range of experimental conditions (saccharide type, pH, saccharide/MPH mass ratio, and reaction time), and then stopped by cooling in an ice bath to obtain MPHg for subsequent analysis.

2.3.1. Single factor experiment

Based on the glycosylation process, the effect of a single factor, such as saccharide type (xylose, glucose, lactose, and dextran-20), pH (9.0–13.0), saccharide/MPH mass ratio (1:2–4:1), and reaction time (0–100 min), on the glycosylation reaction was examined using total antioxidant activity (TAA) as an indicator.

2.3.2. Response surface experiment

Based on the previous single factor experiments, MPH glycosylation was optimized using Design-Expert software (version 8.06) based on the three-factor, three-level Box–Behken design, where pH, saccharide/MPH mass ratio, and reaction time were the observed variables, and TAA was the response value. The experimental factors and design levels are shown in Table 1.

2.4. Fourier transform infrared spectroscopy

Samples of native MPH (MPHn), heat-treated MPH (MPHd), MPH–xylose mixture (MPH–Xylm), MPHg, and native xylose (XYLn) were each mixed with potassium bromide powder at a concentration of 1:100, and then ground into powders in an agate mortar. The specimens were scanned at 400–4000 cm⁻¹ with a spectral resolution of 4 cm⁻¹ under transmission mode of a Nicolet 380 infrared spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) following compression molding.
A μ/C0 is the absorbance of the H sample solution; and C0/C1 represents the absorbance without and with the sample present, respectively.

Box–Behnken experimental design and results for glycation of Morchella protein hydrolysate (MPH).

| Standard order | X1 (pH) | X2 (Xylitol/MPH mass ratio) | X3 (reaction time, min) | Total antioxidant activity (695 nm) |
|----------------|---------|-----------------------------|-------------------------|-----------------------------------|
| 1              | −1 (11) | −1 (2:1)                    | 0 (60)                  | 0.87                              |
| 2              | 1 (13)  | −1 (2:1)                    | 0 (60)                  | 0.32                              |
| 3              | −1 (11) | 1 (4:1)                     | 0 (60)                  | 1.18                              |
| 4              | 1 (13)  | 1 (4:1)                     | 0 (60)                  | 1.02                              |
| 5              | −1 (11) | 0 (3:1)                     | −1 (40)                 | 0.98                              |
| 6              | 1 (13)  | 0 (3:1)                     | −1 (40)                 | 0.73                              |
| 7              | −1 (11) | 0 (3:1)                     | 1 (80)                  | 1.18                              |
| 8              | 1 (13)  | 0 (3:1)                     | 1 (80)                  | 0.77                              |
| 9              | 0 (12)  | −1 (2:1)                    | −1 (40)                 | 0.68                              |
| 10             | 0 (12)  | 1 (4:1)                     | 1 (80)                  | 1.26                              |
| 11             | 0 (12)  | −1 (2:1)                    | 1 (80)                  | 0.96                              |
| 12             | 0 (12)  | 1 (4:1)                     | 1 (80)                  | 1.22                              |
| 13             | 0 (12)  | 0 (3:1)                     | 0 (60)                  | 1.21                              |
| 14             | 0 (12)  | 0 (3:1)                     | 0 (60)                  | 1.23                              |
| 15             | 0 (12)  | 0 (3:1)                     | 0 (60)                  | 1.22                              |
| 16             | 0 (12)  | 0 (3:1)                     | 0 (60)                  | 1.32                              |
| 17             | 0 (12)  | 0 (3:1)                     | 0 (60)                  | 1.32                              |

Actual parameters are represented in brackets. Experimental values represent the mean of triplicate measurements. Los valores experimentales representan la media de mediciones en triplicado.

2.5. Fluorescence spectroscopy

Solutions (0.05 mg/mL) of MPHNa, MPHNa, MPH-X, and MPH were prepared by mixing with 20 mM (pH 7.4) phosphate buffer. The fluorescence emission spectrum of each sample within the wavelength range of 300–400 nm was measured under a 291 nm excitation wavelength (5 nm slit) using a fluorescence spectrophotometer (F-4600, Hitachi, Japan).

2.6. Scanning electron microscopy

Freeze-dried samples were fixed onto a round sampling table with double-sided adhesive tape and scanned by a scanning electron microscope (ZEISS-EVO18, Germany) at an accelerating voltage of 20 kV under vacuum conditions. Images were recorded at appropriate magnification for structural analysis.

2.7. Antioxidant activity

2.7.1. Total antioxidant activity

TAA was determined in accordance with a previously published method (Salla, Sunkara, Ogutu, Walker, & Verghese, 2016). Briefly, 0.3 mL of a 1:10 diluted sample was added to a 3-mL ammonium molybdate reaction system containing 28 mM sodium phosphate, 0.6 M sulfuric acid, and 4 mM ammonium molybdate. The mixture was mixed well, sealed, and incubated at 95°C in a water bath for 90 min. After the sample was cooled, its absorbance was measured at 695 nm in a spectrophotometer (Model V-5000, Shanghai Yuanxi Instrument Co., Ltd., Shanghai, China). An equivalent volume of sample preparation mixture without MPH was used as the blank, and ascorbic acid (VC), at the same concentration as the samples, was used as a positive control.

2.7.2. Reducing power

The reducing power of the samples was determined using the method of Wang et al. (2016), with minor modifications. Briefly, 1 mL of sample solution was mixed with 1.5 mL of phosphate buffer solution (PBS) (0.2 M, pH 6.6), followed by the addition of 2.5 mL of 1% potassium ferricyanide. After 20 min incubation at 50°C in a water bath, 2.5 mL of 10% trichloroacetic acid was added, and the mixture was then centrifuged at 2000 g for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL distilled water and 0.5 mL of 0.1% FeCl3, and the absorbance was read at 700 nm. As before, VC served as a positive control.

2.7.3. H2O2 scavenging activity

H2O2 scavenging activity was measured using the method of Al-Amiery, Al-Majedy, Kadhum, and Mohammad (2015), with slight modifications. Briefly, 0.1 mL sample was mixed with 1 mL 40 mM H2O2 (prepared in 0.2 M pH 7.4 PBS), and PBS was added to bring the final volume up to 4 mL. The reaction was carried out at room temperature for 10 min in the dark, and sample absorbance was then measured at 230 nm. As before, VC served as a positive control. The following formula was used to calculate the H2O2 scavenging activity:

\[ \text{H}_2\text{O}_2\text{scavenging activity(\%) } = \frac{A_1 - (A_1 - A_2)}{A_3} \times 100 \]  

where A1 is the absorbance of the H2O2 and sample solution; A2 is the absorbance of the sample solution; and A3 is the absorbance of the H2O2 solution.

2.7.4. Nitrite scavenging activity

Nitrite scavenging activity was determined based on a previously reported method (Fu, Zhang, Guo, & Chen, 2014), with slight modifications. Briefly, 1.0 mL sample was mixed with 1.0 mL NaNO2 (5 μg/mL), and distilled water was used to bring the total volume up to 3 mL. The mixture was incubated at 37°C for 30 min and then 2 mL of 0.4% sulfanilic acid (prepared in 20% HCl) was added. The mixture was placed at room temperature for 5 min, followed by the addition of 1 mL 0.2% naphthyl ethylenediamine dihydrochloride. The mixture was placed at room temperature for another 15 min, and the sample absorbance was then read at 538 nm using distilled water as a blank. As before, VC served as a positive control. Nitrite scavenging activity was calculated according to the following formula:

\[ \text{Nitrite scavenging activity(\%) } = \left(\frac{A_0 - A_1}{A_0}\right) \times 100 \]  

where A0 and A1 represent the absorbance without and with sample present, respectively.
2.7.5. Free radical scavenging activity

The DPPH and ABTS free radical scavenging activity of the samples was measured using DPPH and ABTS as the free radical models, respectively (Zhuang, Tang, & Yuan, 2013). The superoxide anion free radical scavenging activity of the samples was determined by the pyrogallol autoxidation method (Liu et al., 2013). The free radical scavenging activities of the experimental samples were compared with that of positive control VC.

2.8. Statistical analysis

Data from the three experiments were expressed as mean ± standard deviation. Data from the response surface experiment were analyzed by the Design-Expert 8.06 software, whereas differences between groups in the other experiments were determined by the SPSS 19.0 software using an ANOVA-Tukey test. Statistical significance was determined at p < 0.01.

3. Results and discussion

3.1. Effect of different experimental factors on MPH glycosylation

The MR is a complex chemical reaction, and the extent of the reaction is influenced by the type and the nature of the chemical substrates, as well as the reaction parameters such as substrate concentration, pH, and reaction time (Jiang, Wang, Che, & Tian, 2014). Examination of the effects of sugar type and reaction time on MPH glycosylation at pH 11.0 and a saccharide/MPH mass ratio of 1:1 indicated that glycosylation could significantly enhance the TAA of MPH (Figure 1(a)). Xylose showed the best glycosylation effect, followed by glucose, lactose, and dextran-20. This could be due to the shorter carbon chain of xylose, compared to hexose, disaccharides, and polysaccharides, which results in less steric hindrance and better reactivity (Huang et al., 2012). During the reaction, the overall TAA of different MRPs initially increased and then decreased as the reaction time increased, and TAA reached a maximum at 60 min when MPH was modified by xylose. This result is consistent with the findings of Yilmaz and Toledo (2005), and demonstrates that the antioxidant activity of MRPs is not necessarily proportional to the reaction time.

Examination of the effects of pH and xylose/MPH mass ratio on MPH glycosylation, using xylose as the substrate and a reaction time of 60 min, showed that the TAA of MRPs generally increased and then decreased as pH and mass ratio increased (Figure 1(b)). The TAA of the MRPs was the highest at pH 12.0, and a 3:1 xylose/MPH mass ratio. The MR is essentially a base-catalyzed reaction that is facilitated by alkaline conditions (Vhangani & Van Wyk, 2016). However, excessively high pH can have a negative effect on the reaction (Dworschák & Carpenter, 1980). Research by Luo et al. (2013) has shown that although higher proportions of xylose could improve the MR rate and the antioxidant activity of the reaction products, excessive xylose could reduce the antioxidant activity of MRPs. The results from the present study are consistent with these studies.

3.2. Optimization of MPH glycosylation

3.2.1. Model prediction and statistical analysis

The matrix design of the RSM and the corresponding results are shown in Table 1. The quadratic polynomial equation of TAA in terms of pH (X₁), xylose/MPH mass ratio (X₂), and reaction time (X₃) was obtained by quadratic multinomial regression fitting, using the data in Table 1, and was

\[ Y = 1.26 - 0.17X_1 + 0.23X_2 + 0.06X_3 + 0.10X_1X_2 - 0.04X_1X_3 - 0.08X_2X_3 - 0.26X_2^2 - 0.15X_3^2 - 0.08X_3^2 \]

The results of variance analysis (Table 2) showed that the regression model was highly significant (p < 0.0001), with no significant lack of fit (p = 0.39), and that the actual values and the predicted values were highly correlated (determination coefficient (R²) = 0.987, adjusted determination coefficient (Adj R²) = 0.970). The adequate precision was far greater than 4 (25.39) and the coefficient of variation was relatively small (CV was 4.67%), indicating that the model fitted well, had high reliability and accurately reflected the variation in the response values to each factor. Furthermore, the magnitude of the effects of the three independent factors on TAA was examined using an ANOVA-Tukey test. Statistical significance was determined at p < 0.01.
Table 2. Results of variance analysis for the response surface quadratic polynomial model.

| Source | Sum of squares | DF | Mean square | F-value | P-value |
|--------|----------------|----|-------------|---------|---------|
| Model  | 1207           | 9  | 0.134       | 58.56   | <0.0001 |
| $X_1$  | 0.237          | 1  | 0.237       | 103.6   | <0.0001 |
| $X_2$  | 0.422          | 1  | 0.422       | 184.25  | <0.0001 |
| $X_3$  | 0.029          | 1  | 0.029       | 12.5    | 0.0095  |
| $X_1X_2$ | 0.037      | 1  | 0.037       | 16.27   | 0.0050  |
| $X_1X_3$ | 0.006      | 1  | 0.006       | 2.64    | 0.1482  |
| $X_2X_3$ | 0.026      | 1  | 0.026       | 11.32   | 0.0120  |
| $X_1^2$ | 0.295         | 1  | 0.295       | 128.68  | <0.0001 |
| $X_2^2$ | 0.091          | 1  | 0.091       | 39.68   | 0.0004  |
| $X_3^2$ | 0.028          | 1  | 0.028       | 12.14   | 0.0102  |
| Residual | 0.016       | 7  | 0.002       | 0.39    | 0.7695  |
| Lack of fit | 0.004     | 3  | 0.001       | 0.39    | 0.7695  |
| Pure error | 0.012     | 4  | 0.003       |         |         |
| Total | 1223          | 16 |             |         |         |

$R^2 = 0.987$, Adj $R^2 = 0.970$, Adeq Precision = 25.39, CV = 4.67% 

DF: degrees of freedom; $R^2$: determination coefficient; Adj $R^2$: adjusted determination coefficient; Adeq Precision: adequate precision; CV: coefficient of variation. 

Variables on the response values was determined to be in the following order: xylose/MPH mass ratio ($X_2$) > pH ($X_1$) > reaction time ($X_3$) (Table 2). It was also found that the model’s linear terms ($X_1$, $X_2$, $X_3$), quadratic terms ($X_1^2$ and $X_2^2$), and interaction terms ($X_1X_2$, $X_1X_3$, $X_2X_3$) all had a significant effect on the response values ($p < 0.01$), suggesting that the effects of the different factors on MPH glycosylation were not a simple linear relationship.

3.2.2. Response surface analysis and optimization

The effects of various factors and their interactions on the response values are shown by three-dimensional response surface and two-dimensional contour plots in Figure 2. The presence of a maximum value on every curved surface indicates that the densities and shapes of the contour lines (Figure 2(b)) demonstrate that xylose/MPH mass ratio and pH were the major influencing factors on MPH glycosylation among the three variables, while the effect of reaction time was relatively small. This finding was consistent with the results from the variance analysis shown in Table 2. Within the range of the selected experimental factors, regression model analysis by Design-Expert software showed that the optimal conditions for MPH glycosylation were as follows: pH 11.80, xylose/MPH mass ratio 3.69:1, and reaction time 61.46 min. Under these conditions, the TAA of the products could be up to 1.36. For operational convenience, the adjusted optimal reaction conditions were as follows: pH 11.80, xylose/MPH mass ratio 3.7:1, and 60 min reaction time. Under these optimal conditions, the TAA of the glycosylated MPH was determined to be 1.33 from three parallel experiments, which was consistent with the theoretical prediction, indicating that the model could be used to accurately predict the conditions of MPH glycosylation.

3.3. FT-IR analysis

FT-IR is a commonly used method for analyzing protein and polypeptide secondary structures and food composition, and a powerful tool for identifying protein–saccharide polymers (Van et al., 2002; Wang, Bao, & Chen, 2013). The FT-IR spectra of MPH, MPH-X, MPH-X_M, MPH-X_G, and XYL are shown in Figure 3. The absorption peak near 1654cm$^{-1}$ is attributed to the amide I band (C=O stretching vibration). MPH molecules in this region were clearly modified by the MR because the intensity of the absorption peak was significantly lower than that of MPH, MPH-X, and MPH-X_M, which is consistent with the findings of Hong, Meng, and Lu (2015). The absorption peak at wavenumber 1180–953 cm$^{-1}$ is the characteristic absorption peak of carbohydrate, belonging to C–C, C–O stretching vibrations and C–H bending vibrations. Most of the protein molecules showed very weak absorptions in this region (Gu et al., 2010). The absorption peak of MPH was markedly greater in this region than those of MPH-X and MPH-X_M.
Morchella protein hydrolysate (MPH), but was significantly lower than those of MPH-X (and de Morchella nativa (MPH was not a simple mixture of are shown in Figure 5. Fourier transform infrared spectra of native Morchella protein hydrolysate (MPH and XYL Intrinsic emission fluorescence spectra of native Morchella protein 2016. The redshift of the fluorescence peaks indicates that the fluorescent emission groups are more exposed to the solvent, and results in an increase in the polarity of the microenvironment of these groups (Pallares, Vendrell, Aviles, & Ventura, 2004). The dramatic decrease in fluorescence intensity of the MPHs may be because of steric hindrance caused by the addition of carbohydrate, which could largely shield the signals from the fluorescence emission groups, and thereby reduce the fluorescence of the copolymers (Huang et al., 2012).

3.5. SEM analysis

SEM has been widely used to detect the effects of glycosylation on the microstructure of samples (Liu, Xu, Zhang, Zhao, & Ding, 2016; Moeckel, Duerasch, Weiz, Ruck, & Henle, 2016). Observations of MPHs, MPH-X, MPH-xyllose mixture, and MPH by SEM are shown in Figure 5. The structure of MPH (Figure 5(a)) was loose and irregular in shape, while the structure of MPHs (Figure 5(b)) was mainly comprised of compact spherical micelles, indicating that heating had a particular effect on the conformation of MPH. The molecular morphology of MPH-X (Figure 5(c)) was significantly larger than that of MPH, and was flat and compact in structure with a small amount of debris on the surface. The morphology of MPH (Figure 5(d)) was also flat and compact, similar to the structure of MPH-X, and the volume was likewise significantly larger compared to MPH. However, its surface was smooth and clean with obvious protrusions, indicating that MPH was not a simple mixture of
MPH and xylose, and that polymerization reactions were likely to have occurred between the two, which changed the microstructure of MPH. The mechanisms of MPH-xylose graft copolymer formation could be due to the breaking of intramolecular non-covalent bonds in MPH during MR (Niu, Jiang, Pan, & Zhai, 2011), paralleled by the covalent bonding of xylose molecules to MPH, which surrounded the MPH and extended the size of the molecule. As a result, the volume was increased and the structure became more compact, thereby leading to the formation of copolymers along with a unique microstructure.

3.6. In vitro antioxidant activity analysis

A variety of experimental models were used to analyze the in vitro antioxidant activities of MPH\(_N\), MPH\(_H\), MPH-X\(_M\), and MPH\(_G\) (Figure 6). Among the four molecules, MPH\(_G\) exhibited the highest antioxidant activity, although this was less than that of the positive control (VC), at the same concentration. Its TAA and reducing power were 7.77 and 17.03 times those of MPH\(_N\), respectively. Moreover, its nitrite and H\(_2\)O\(_2\) scavenging activities were 11.70 and 2.09 times those of MPH\(_N\), respectively, and its DPPH, ABTS, and superoxide anion radical scavenging activities were 426.29%, 37.33%, and 320.14% higher than those of MPH\(_N\), respectively. These findings indicate that MPH glycosylation by xylose through MR significantly increased the in vitro antioxidant activities of MPH, which is consistent with results of previous research (Wang et al., 2013). Although the reducing powers of MPH\(_H\) and MPH-X\(_M\), and the nitrite and superoxide anion free radical scavenging activities of MPH\(_H\), were significantly enhanced compared to those of MPH\(_H\), these enhancements were significantly lower than those of MPH\(_G\) (\(p < 0.01\)). According to a previously published study, reductone (an intermediate stage product), melanoidin, and heterocyclic compounds (advanced stage products) from the MR play important roles in antioxidation (Yin, Yang, Zhao, & Li, 2014), by breaking the free radical chain, chelating metal ions, providing hydrogen and electrons, and scavenging active oxygen (Kim & Lee, 2009; Nooshkam & Madadlou, 2016). The glycosylation of MPH conducted via MR in this

Figure 6. Antioxidant activity of native Morchella protein hydrolysate (MPH\(_N\)), heat-treated MPH (MPH\(_H\)), MPH-xylose mixture (MPH-X\(_M\)), glycosylated MPH (MPH\(_G\)), and positive control ascorbic acid (VC). (a) Total antioxidant activity and scavenging capacities toward ABTS and DPPH radicals, and H\(_2\)O\(_2\); (b) Reducing power and scavenging capacities toward nitrite and superoxide radicals. Values are presented as mean ± standard deviation (\(n = 3\)). Different letters (a–e) indicate significant differences (\(p < 0.01\)) among samples at the same concentration.

Figure 6. Actividad antioxidante de proteína hidrolizada de Morchella nativa (MPH\(_N\)), de MPH tratada térmicamente (MPH\(_H\)), de una mezcla MPH – xilosa (MPH-X\(_M\)), de MPH glicosilada (MPH\(_G\)) y del control positivo de ácido ascórbico (VC). (a) Actividad antioxidante total y capacidad de eliminación de los radicales ABTS y DPPH y H\(_2\)O\(_2\); (b) Poder reductor y capacidad de eliminación de los radicales de nitrito y superóxido. Los valores figuran como la media ± la desviación estándar (\(n = 3\)). Las distintas letras (a–e) indican diferencias significativas (\(p < 0.01\)) entre las muestras sometidas a prueba con la misma concentración.
study is a “natural” process, whereas other existing modification methods are artificial. Therefore, this study has revealed a promising means for improving the antioxidant activity of MPH, and provides experimental support for the development and application of MPH and its glycosylated derivatives as natural antioxidants in health products and functional foods.

4. Conclusions

In this study, MPH glycosylation was successfully optimized by the response surface experiment, and the presence of structural modification of the MPH was confirmed by infrared spectroscopy, fluorescence spectroscopy, and SEM. In addition, changes in the antioxidant activities of MPH before and after glycosylation were examined using in vitro experimental models. The results of the response surface experiment showed that the optimal MPH glycosylation conditions were as follows: pH 11.80, xylose/MPH mass ratio of 3.7:1, and reaction time of 60 min. The in vitro antioxidation experiments demonstrated that the antioxidant activities of MPH were significantly enhanced compared to those of MPH0, MPH1, and MPH-XM. These results demonstrate that the glycosylation of MPH through the MR is an effective way to improve its antioxidant activity. However, the present study has not considered whether the harmful advanced glycation end products are produced during the glycosylation of MPH, which remains to be done for further study.

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Disclosure statement

No potential conflict of interest was reported by the authors

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