Antioxidant Activity *In Vivo* and *In Vitro* of Two Feruloyl Oligosaccharides Preparations Produced from Wheat Bran and Fermented by *Aureobasidium pullulans*

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The antioxidant functions of two feruloyl oligosaccharide (FO1 and FO2) were investigated *in vivo and in vitro*. Effects of FO1 and FO2 on hemolysis of rat red blood cell (RBC) and malondialdehyde (MDA) formation in rat liver homogenate and rat liver mitochondria *in vitro* were studied. Hemolysis of rat RBC and MDA formation in rat liver homogenate and rat liver mitochondria were inhibited in a dosage-dependent manner by FO1 and FO2 in the tested concentration range of 0.5 to 10 mg/mL. The results showed that FO1 and FO2 had antioxidative activity *in vitro*, and the effect of FO2 was better than that of FO1. With increasing dosage, FO1 and FO2 could increase the activity of SOD and GSH-Px in serum of S_{180} tumor-bearing mice, reduce the level of MDA, and thus improve the activity of the antioxidant *in vivo*. When the dosage reached 250 mg/kg/d, FO2 was more likely to improve the capabilities of the antioxidants of tumor-burdened mice than were 5-FU and FO1 *in vivo.* Thus, these oligosaccharides may be used as functional biological materials produced from fermented lignocellulose of WB.

Keywords: Wheat bran; A. pullulans; Feruloyl oligosaccharide; Antioxidant activity; In vivo and in vitro

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INTRODUCTION

Wheat bran (WB) is a good source of dietary fiber and is produced worldwide in enormous quantities as an important by-product of the cereal industry. Many studies have dealt with the isolation of feruloylated oligosaccharides (FOs) from WB (Ishii 1997; Yuan *et al*. 2006; Xie 2010; Yu *et al*. 2013; Yu and Gu 2014a,b). The interest in these oligosaccharides is motivated by their biological activities and their technological applications. A high value is placed on FOs, as they may be used as natural antioxidant materials. The antioxidant activity of FOs is higher than that of ferulic acid（FA) and vitamin C, as they exhibit strong inhibition of the hemolysis of mice red blood cells as well as elimination of Fe^{2+}, H_2O_2, and hydroxyl radicals (Wang *et al.* 2010, 2011). FOs have also been reported to display significant antioxidant capacity in 1-1-diphenyl-2-picrylhydrazyl (DPPH) and lipid peroxidation systems (Wang *et al.* 2008, 2009).

Based on the fact that FOs produced from *A. pullulans*-fermented WB displayed significant antioxidant capacity *in vivo* (Yu and Gu 2014a), there is a motivation to further carry out studies of this topic. However, the manner in which the component purity and degree of polymerization of FOs affect their antioxidant capacity *in vivo* and *in*

vitro is still unknown. In addition, the antioxidant capacity *in vitro* of FOs produced from *A. pullulans*-fermented WB has not been reported.

In our previous work (Yu *et al*. 2013; Yu and Gu 2014a,b), two components (FO1 and FO2) were produced from WB fermented by *A. pullulans via* one- and two-stage pH and temperature controlling processes, respectively. In this study, their antioxidant capacities were investigated *in vivo and in vitro*. The influences of different purities and degrees of polymerization of FOs prepared under the two fermentation conditions on their antioxidant capacity were determined. Because FOs can be produced easily and abundantly from *A. pullulans*-fermented WB, it is feasible to use FOs as potential antioxidant functional foods from WB. Therefore, this study may provide both a theoretical basis and an application prospect to develop FOs as a health-promoting food.

EXPERIMENTAL

Materials

Raw materials and reagents

FO1 and FO2 were prepared by one-stage and two-stage pH and temperature processes for control of WB fermentation by *A. pullulans* 2012, respectively (Yu *et al*. 2013; Yu and Gu 2014a,b). FO1 consisted of feruloyl arabinosyl xylopentose (FAX5, Mw986), feruloyl arabinosyl xylotetraose (FAX4, Mw854), feruloyl arabinosyl xylotriose (FAX3, Mw722), and feruloyl arabinosyl xylobiose (FAX2, Mw590), whereas FO2 contained feruloyl arabinosyl xylohexose (FAX6, Mw 1118), FAX5, and FAX4 (Yu *et al*. 2014).

The fluorouracil injection (5-FU) was from Hengrui Medicine Co. (Lianyungang, Jiangsu, China; batch number: 1111262; specifications: 10 mL, 0.25 g). Fetal bovine serum was purchased from Sigma Chemical Co. (St. Louis, MO, USA). MDA, SOD and GSH-Px kits were purchased from Jiancheng Biotech Co. Ltd. (Nanjing, Jiangsu, China). Vitamin C (Vc) was purchased from Xian Ruilin Biotech Co. Ltd. (Xian, Shanxi, China). Phosphate buffered saline (PBS), H_2O_2 and $FeSO_4$ were purchased from Luke Chemical Product Co., Ltd. (Shouguang, Shandong, China). S_{180} was provided by Jiangsu Provincial Institute of Cancer Research (Nanjing, Jiangsu, China).

The tumor-bearing mice were purchased from the Comparative Medicine Centre of Yangzhou University (Yangzhou, Jiangsu, China; Certificate No. SCXK-2007-0001).

In Vitro Antioxidant Activity of FOs

Effect of FOs on mice erythrocytic osmotic fragility

Male Sprague-Dawley mice (280 to 300 g) were housed for seven days at 18 to 24 °C and a relative humidity of 70% on a 12 h light/12 h dark cycle. Blood (8 mL/rat) from the abdominal aorta was collected into a heparinized tube. Red blood cells (RBCs) were separated from plasma by centrifugation at 10,000 x g for 10 min. The crude RBCs were then washed three times with five volumes of phosphate buffered saline (PBS, pH = 7.4), and suspended in PBS to obtain a 0.5% RBC suspension. In the present study, the method described by Yuan *et al.* (2005) was used to determine the hemolysis of RBC. Five milliliters of RBC suspension was mixed with 2 mL of PBS solution containing varying amounts of FOs, and incubated at 37 °C for 24 h. The resulting solutions were centrifuged at 10,000 x g for 10 min. The absorbance (*A*) of the supernatant at 540 nm was recorded in a spectrophotometer. Percentage inhibition was calculated as the

formula of (%) = (1-*T*/*C*)×100, and hemolysis rate (%) = (*T*/*C*)×100, where *T* and *C* are the averages of the sampling groups and control group (containing no FOs) , respectively.

Effect of FOs on mice erythrocyte hemolysis induced by H_2O_2

The method described by Zhang *et al.* (2002) was used to determine the hemolysis of RBC induced by H_2O_2.

Preparation of sampling group: One milliliter of 0.5% RBC suspension was mixed with 0.2 mL PBS solution containing varying amounts of FOs and 0.1 mL of 100 mM H_2O_2. The incubation mixture was shaken gently in a water bath at 37 °C for 1 h. After incubation, 7.8 mL of PBS solution was added into the reaction mixture, followed by centrifugation at 10,000 x g for 10 min. The absorbance (*A*) of the supernatant at 540 nm was recorded in a spectrophotometer.

Preparation of normal control group: The sampling solution (PBS solution containing varying amounts of FOs) and H_2O_2 were replaced with an equal volume of PBS solution, and the others were kept the same as mentioned above.

Preparation of induced control group: The sampling solution was replaced with an equal volume of PBS solution, and the others were same as mentioned above.

The hemolysis extent of FOs was expressed as the average *A* value of the sampling group relative to the average *A* of the induced control group. The inhibition rate of FOs was calculated as the formula of (%) = (1-*T*/*C*)×100, where *T* and *C* are the average As of the sampling groups and induced control group, respectively.

Effect of FOs on MDA formation in mice liver homogenate

The effect of FOs on MDA formation in mice liver homogenate was determined by the method of Huang *et al.* (2003) with a slight modification. Livers were excised immediately after the animals were sacrificed. The liver was frozen quickly and stored at 4 °C. The thawed tissue samples were homogenized in nine volumes of ice cold PBS and centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant (10% liver homogenate) was used for determination of MDA using commercially available diagnostic kits. The reaction mixture, for the analysis of MDA in mice liver homogenate, containing 0.4 mL of 10% liver homogenate and 0.2 mL of sampling solution, was incubated for 1 h at 37 °C. An equal volume of PBS was added as the control group. The absorbance of the mixture was measured at 532 nm against the blank. The levels of MDA were normalized with protein. The reaction mixture for the analysis of MDA in induced mice liver homogenate was 0.2 mL of 10% liver homogenate mixed with 0.2 mL of sampling solution and 0.2 mL of 1 mM $FeSO_4$ (or H_2O_2), and what followed was similar to the methods for the MDA as mentioned above.

Effect of FOs on MDA formation in mice liver mitochondria

The effect of FOs on MDA formation in mice liver mitochondria was determined by the method of You and Lin (2003). The washed fresh liver was homogenized in nine volumes of PBS and centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was collected and centrifuged repeatedly. The collected precipitation was washed with PBS twice, and then dissolved in PBS to prepare for 0.5 mg/mL mice liver mitochondria suspension. The sampling group reaction mixture, containing 1 mL of the rat mitochondria suspension (0.5 mg/mL), 0.4 mL of sampling solution, 0.4 mL of 0.5 mM $FeSO_4$, and 0.4 mL of 0.5 mM Vc, was incubated for 1 h at 37 °C. Both the sampling solutions, $FeSO_4$ and Vc, were replaced with an equal volume of PBS, which was used

for the normal group. The sampling solution was replaced with an equal volume of PBS, and it was used for the control group. The content of MDA was measured according to the kit method.

In vivo Antioxidant Activity of FOs

In vivo antioxidant activity of FOs was measured according to the method of Yu and Gu (2014a).

Statistical Analysis

Experimental data are expressed as the mean ± SD. All the data are at least triplicate and the results were reproducible within 10% deviation. SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for ANOVA statistical analysis. When $P < 0.05$, the difference was considered statistically significant.

RESULTS AND DISCUSSION

Antioxidant Activity of FOs *in vitro*

Impact of FOs on mice erythrocytic osmotic fragility

The *in vitro* effect of FO1 and FO2 on the mice erythrocytic osmotic fragility is shown in Table 1. Incubation of an erythrocyte suspension with both FO1 and FO2 (0.5, 2.5, 5.0, 7.5, and 10 mg/mL) significantly decreased the hemolysis rate in a dose-dependent manner as compared to the normal control group ($P < 0.05$). This indicated that FOs had inhibitory functions against mice erythrocytic osmotic fragility. The inhibition rate showed an ascendant trend with an increment of FOs concentration. When the dose was 10 mg/mL, FO1 and FO2 inhibited 41.742% and 52.853% of erythrocytic osmotic fragility, respectively. FO2 showed a significantly higher inhibition rate than FO1 did. This indicated that FO2 was more efficient than FO1 in the inhibitory functions against mouse erythrocytic osmotic fragility.

Table 1. Effects of FOs on Mice Erythrocytic Osmotic Fragility

Group	Dose (mg/mL)	A_{540}	Hemolysis rate (%)	Inhibition rate (%)
Normal control	—	0.222±0.009 ^{a}	100	—
FO1	0.5	0.200±0.011 ^{b}	90.090±4.872 ^{a}	9.910±4.872 ^{f}
	2.5	0.169±0.013 ^{c}	76.126±6.049 ^{b}	23.874±6.049 ^{e}
	5	0.149±0.010 ^{d}	67.117±4.601 ^{cd}	32.883±4.601 ^{cd}
	7.5	0.137±0.008 ^{de}	61.712± 3.401^{de}	38.288±3.401 ^{bc}
	10	0.129±0.009 ^{de}	58.108±4.087 ^{de}	41.892±4.087 ^{b}
FO2	0.5	0.190 ±0.008 ^{b}	85.586±3.401 ^{a}	14.414±3.401 ^{f}
	2.5	0.166 ±0.013 ^{c}	74.775±5.644 ^{bc}	25.225±5.644 ^{de}
	5	0.145 ±0.010 ^{de}	65.315±4.375 ^{de}	34.685±4.375 ^{bc}
	7.5	0.127 ±0.008 ^{e}	57.207±3.687 ^{e}	42.793±3.687 ^{b}
	10	0.105 ±0.011 ^{f}	47.297±5.023 ^{f}	52.703±5.023 ^{a}
Different letters in the same column indicate a statistical difference ($P < 0.05$).				

Impact of FOs on mice erythrocyte hemolysis induced by H_2O_2

Table 2 shows the effect of different concentrations of FOs on H_2O_2-induced changes in mice erythrocyte hemolysis. Each dose of FO1 and FO2 showed significant differences from the normal control group and the induced control group ($P < 0.05$). When the erythrocyte suspension was incubated with FOs, a significant dose-dependent decrease in hemolysis rate and increase in inhibition rate of erythrocytes was observed. When the dose of FO1 and FO2 reached 10 mg/mL the inhibition rates against erythrocyte hemolysis were 46.520 ± 5.715% and 62.800 ± 1.361%, respectively. All five doses (0.5, 2.5, 5, 7.5 and 10 mg/mL) of FO2 had significant differences from A_{540}. As a result, FO2 showed more efficient inhibitory functions against erythrocyte hemolysis than FO1 did.

Table 2. Effects of FOs on Mice Erythrocyte Hemolysis Induced by H_2O_2

Group	Dose (mg/mL)	A_{540}	Hemolysis rate (%)	Inhibition rate (%)
Normal control	—	0.222±0.009 ^{h}	—	—
Induced control	—	0.819 ±0.008 ^{a}	100	—
H_2O_2+ FO1	0.5	0.663 ±0.030 ^{b}	80.952±3.602 ^{a}	19.048±3.602 ^{f}
	2.5	0.609 ±0.005 ^{c}	74.359±0.551 ^{b}	25.641±0.551 ^{e}
	5	0.545 ±0.010 ^{d}	66.545±1.186 ^{c}	33.455±1.186 ^{d}
	7.5	0.527± 0.008 ^{d}	64.347± 6.611^{de}	35.653±6.611 ^{d}
	10	0.438 ±0.047 ^{e}	53.480±5.715 ^{f}	46.520±5.715 ^{c}
H_2O_2+ FO2	0.5	0.624 ±0.012 ^{c}	76.190±1.440 ^{ab}	23.810±1.440 ^{e}
	2.5	0.526 ±0.012 ^{d}	64.225±1.480 ^{cd}	35.775±1.480 ^{d}
	5	0.465 ±0.008 ^{e}	56.817±0.979 ^{ef}	43.183±0.979 ^{c}
	7.5	0.350 ±0.017 ^{f}	42.776±2.080 ^{g}	57.224±2.080 ^{b}
	10	0.305 ±0.011 ^{g}	37.200±1.361 ^{g}	62.800±1.361 ^{a}
Different letters in the same column indicate a statistical difference ($P < 0.05$).				

Impact of FOs on MDA formation in mice liver homogenate

The effects of FO1 and FO2 on MDA formation in mice liver homogenate in the absence of inducer, and induced by H_2O_2 and $FeSO_4$, are shown in Table 3. Among the three systems, all five doses (0.5, 2.5, 5, 7.5, and 10 mg/mL) of FO1 and FO2 had significant inhibitory functions against MDA formation in mice liver homogenate from the blank control group, and both FO1 and FO2 improved the inhibitory functions as the dosage increased.

In addition, increasing FOs dose to 5 mg/mL, the inhibitory rate with an inducer was higher than that without an inducer. When the dose was 10 mg/mL in the absence of an inducer and induced by $FeSO_4$, the MDA content of the FO2 controlling group was significantly less than that of the FO1 controlling group. When the dose was 7.5 mg/mL induced by H_2O_2, the MDA content of the FO2 controlling group was significantly less than that of the FO1 controlling group. It was therefore concluded that FO2 had a better effect on the inhibitory functions against MDA formation than FO1 did.

Table 3. Effects of FOs on MDA Formation in Mice Liver Homogenate

Group	Dose (mg/mL)	No inducer		Induced by H_2O_2		Induced by $FeSO_4$	
		MDA (nmol/mg/pr)	Inhibition rate (%)	MDA (nmol/mg/pr)	Inhibition rate (%)	MDA (nmol/mg/pr)	Inhibition rate (%)
Control	0	8.31±0.20 a	—	9.80±0.23 a	—	11.32±0.28 a	—
FO1	0.5	6.45 ±0.30 b	22.42± 3.57 g	8.13 ±0.12 b	17.07± 1.26 e	8.75 ±0.25 b	22.70± 2.18 e
	2.5	6.17±0.09 bcd	25.75± 1.10 efg	7.57 ±0.12 c	22.76± 1.24 d	8.50 ±0.29 bc	24.88± 2.54 de
	5	5.96± 0.11cde	28.24± 1.34 def	6.78 ±0.41 e	30.85± 4.19 c	7.28 ±0.20 d	35.69± 1.74 c
	7.5	5.64 ±0.17efg	32.13± 2.09 bcd	6.39 ±0.18 f	34.83± 1.88 b	6.26 ±0.23 e	44.73± 2.00 b
	10	5.53 ±0.09 fg	33.45± 1.05 bc	6.22 ±0.15 f	36.50± 1.56 b	6.07 ±0.09 e	46.35± 0.76 b
FO2	0.5	6.28 ±0.35 bc	24.43± 4.25 fg	7.96 ±0.15 b	18.78± 1.51 e	8.71 ±0.96 b	23.09± 8.44de
	2.5	6.00 ±0.12 cd	27.76± 1.41 ef	7.23 ±0.16 d	26.26± 1.59 d	7.87± 0.85 cd	30.51± 7.47 cd
	5	5.84±0.17 def	29.76± 1.99 cde	6.41±0.18 ef	34.63± 1.79 b	7.16 ±0.31 d	36.72± 2.70 c
	7.5	5.47±0.12 g	34.14± 1.45 b	5.22 ±0.24 g	46.73± 2.45 a	6.09 ±0.32 e	46.17± 2.83 b
	10	5.06 ±0.04 h	39.15± 0.50 a	5.10± 0.09 g	47.93± 0.88 a	5.13 ±0.13 f	54.71± 1.12 a

Different letters in the same column indicate a statistical difference ($P < 0.05$).

Impact of FOs on MDA formation in mice liver mitochondria

The effects of FOs on MDA formation in mice liver mitochondria are shown in Table 4. MDA formation of mice liver mitochondria was inhibited in a dose-dependent manner by FO1 and FO2 and showed significant differences from blank control group. The inhibitory rates of FO2 were significantly higher ($P < 0.05$) than those of FO1 at the same dosage. When the dosage was 10 mg/mL, the inhibitory rate of FO2 was 47.34%, while that of FO1 was 41.30%. It was therefore concluded that FO2 had a better effect on inhibitory functions against oxidative damage than FO1 did.

Antioxidant Activity of FOs *in vivo*

As shown in Table 5, with increasing the dosage of FO1 (Yu and Gu 2014a) and FO2, the activities of SOD and GSH-Px in S_{180} tumor-bearing mice sera were improved and the content of MDA was reduced, thereby the antioxidant activity of tumor-bearing mice was enhanced. When the dose was 250 mg/kg/d, the enzyme activity of the FOs group hasn't significant difference with that of the 5-FU positive control group, therefore, it indicated that FOs had an excellent *in vivo* antioxidant effect. When the dosage reached 250 mg/kg/d, FO2 improved the antioxidant capability of tumor-burdened mice more than 5-FU and FO1 (Yu and Gu 2014a) *in vivo.*

Table 4. Effects of FOs on MDA Formation in Mice Liver Mitochondria

Group	Dose (mg/mL)	MDA (nmol/mg/pr)	Inhibition rate (%)
Control	0	77.29± 3.14 a	—
FO1	0.5	68.29 ±5.70 b	11.65±7.37 g
	2.5	62.28 ±3.05 c	19.42±3.95 f
	5	55.17 ±2.87 de	28.62± 3.71 de
	7.5	49.79 ±3.35 ef	35.58±4.34 bcd
	10	45.37 ±1.50 fg	41.30±1.94 ab
FO2	0.5	61.95 ±2.29 c	19.84±2.96 f
	2.5	55.95 ±2.64 d	27.61±3.42 e
	5	51.17 ±1.81 def	33.79±2.34 cde
	7.5	46.46 ±2.80 fg	39.89±3.62 bc
	10	40.70 ±2.88 g	47.34±3.73 a
Different letters in the same column indicate a statistical difference ($P < 0.05$).			

Table 5. Effects of FOs on SOD, GSH-Px and MDA of SCR Mice and S180 Tumor-bearing Mice Sera *in vivo*

Group	Dose (mg/kg/d)	MDA (nmol/mL)	SOD (U/mL)	GSH-Px (U/mL)
Normal control	—	2.85 ±0.49 d	237.68 ±6.07 a	577.39 ±8.97 a
Model control	—	5.62 ±0.37 a	210.10 ±6.10 d	415.51± 5.52 f
5-FU	20	4.73 ±0.28 b	223.11 ±3.85 b	524.08 ±9.73 bc
FO1 (Yu and Gu (2014a))	50	4.99 ±0.11 b	213.40 ±3.55 cd	468.00 ±9.88 e
	100	4.12 ±0.11 c	216.49 ±3.98 bcd	496.24±6.74 d
	250	3.94 ±0.13 c	221.71 ±3.02 bc	517.09 ±5.64 c
FO2	50	4.85 ±0.14 b	217.53 ±2.89 bcd	474.67± 4.83 e
	100	3.91 ±0.11 c	225.49±7.16 b	502.91 ±8.42 d
	250	3.14 ±0.12 d	237.61 ±4.18 a	530.42 ±3.48 b
Different letters in the same column indicate a statistical difference ($P < 0.05$).				

The results showed that both FO1 and FO2 presented significant antioxidant capacity *in vivo and in vitro*. Yuan *et al.* (2005) indicated that the FOs could efficiently protect normal rat erythrocytes against hemolysis induced by free radicals under *in vitro* conditions. According to the literature, the membrane of erythrocytes is rich in polyunsaturated fatty acids that are very susceptible to free radical-mediated peroxidation (Yuan *et al.* 2005). It may be deduced that scavenging free radicals may be directly depended on hydrogen atoms and, thus, that the provision of hydrogen atoms enhances the

antioxidant capacity. There is a phenolic hydroxyl group in the parent nucleus for the existence of the FA structure in FOs, therefore, the provision of hydrogen atoms as well as the ability to scavenge free radicals were enhanced (Wang *et al*. 2010). Thereby, the result in our work is similar to the results of other studies (Ohta *et al.* 1994; Katapodis *et al.* 2003; Wang *et al.* 2008, 2011).

It has been reported that the antioxidant activity of oligosaccharides can be affected significantly by the polymerization degree (Chen and Yan 2005). To date, data have revealed that a polymerization degree of six is beneficial to the improvement of antioxidant activity. In the present study, the component purity and polymerization degree of FO2 were higher than those of FO1; therefore, the antioxidant activity *in vivo* of FO2 would be expected to be better. It may be presumed that the antioxidant activity of FOs is closely related to their purity and structure, and this will be further investigated. Thus, the results in our work are similar to the results of other studies (Wang *et al.* 2008, 2011).

CONCLUSIONS

1. FO1 and FO2 inhibited the hemolysis of mice RBCs and the MDA formation of mice liver homogenate and mice liver mitochondria in a dosage-dependent manner in the tested concentration, showing that FOs had an excellent *in vitro* antioxidant effect.
2. As the doses of FO1 and FO2 increased, the activities of SOD and GSH-Px in tumor S_{180}-bearing mice serum were promoted with decreasing MDA content, thus enhancing the *in vivo* antioxidant activity of tumor-bearing mice.
3. FO2 exhibits more efficient antioxidative functions than FO1 does. The structure of FOs affects the antioxidative functions. These oligosaccharides can thus be used as antioxidative agents in the future functional food industry from fermented hemicellulose of WB.

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