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Optimization, Antioxidant Activity and Bile Salts Adsorption Capacity of the Aqueous Enzymatic Extract from Rice Bran

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Abstract

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The optimum extraction conditions of the aqueous enzymatic extract from rice bran (AEERB) were evaluated with respect to γ-oryzanol content and ferric reducing/antioxidant power (FRAP) activity by using response surface methodology. The results showed that the effect of the extraction temperature on γ-oryzanol content and FRAP activity was the most significant. The optimal conditions were determined as follows: Trypsin, 453 U/g at 56°C and pH 7.9 for 2 h at a liquid/solid ratio of 7 : 1. The corresponding γ-oryzanol content and FRAP activity were 0.1749 mg/g and 0.5043 mmol $FeSO_4$/g, respectively. The antioxidant activities of AEERB were comparable with 2,6-di-tert-buty-4-methylphenol (BHT) or ascorbic acid (AA) at the high concentration. Furthermore, the binding capacities of AEERB to sodium taurocholate and sodium deoxycholate were 24.96 and 13.63 μmol/100 mg, respectively. The results indicated that AEERB is a promising method for developing and applying a value-added RB product.

Keywords: antioxidative; aqueous enzymatic extracts; hypolipidemic; rice bran; optimization

Rice bran (RB) is a by-product of the rice milling process. RB is a natural and excellent source of antioxidant compounds (such as polyphenols and flavonoids) and has become a focus for research attention in recent years. The antioxidant ability of phenolics have been well-proven. RB protein is an excellent hypoallergenic cereal protein, and its ability to lower cholesterol is comparable with soy protein (Morita *et al*. 1997). Rice bran oil (RBO) is rich in bioactive compounds such as γ-oryzanol, tocols (tocopherols and tocotrienols) and phytosterols. Numerous studies indicated that the abundant γ-oryzanol and tocols contents in RB have greater effect on lowering serum cholesterol, which can prevent and treat chronic diseases, including coronary heart disease (CHD) and hyperlipidemia (Photchanathip *et al*. 2008; Elisa *et al*. 2009). Especially γ-oryzanol, because of a powerful antioxidant activity, is increasingly focused as an ingredient for drugs and nutraceuticals foods. The above bioactive compounds in RB are mainly divided into two types of water-soluble and fat-soluble, and the difference of polarity is so significant as to be difficultly co-extracted and utilized. Therefore, in recent years, the research on rice bran is mainly focused on the extraction and application of a single functional component, such as RB protein, bioactive peptides, polysaccharides, RB oil and other active ingredients (Aguilar-Garcia *et al*. 2007; Renuka & Arumughan 2007; Adebiyi *et al*. 2009a). However, there are few studies on the composite functional ingredients. Research has shown that there was a synergistic effect between different antioxidant ingredients (Capitani *et al*. 2009; Shi *et al.*2010; Kim *et al.* 2015), and the functional

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characteristics of these composite ingredients may be better than that of a single component, such as polyphenols and β-carotene (And & Gordon 2003), juice and α-tocopherols (Graversen *et al.* 2008), lycopene and vitamin E (Shi *et al.*2010) and so on. Co-extraction might effectively protect the quality of these bioactive compounds, not only making full use of the various nutrients, but also avoiding the waste of raw materials to a certain extent, as well as it provides another path for comprehensive utilization. Therefore, co-extraction of bioactive ingredients from RB is particularly important to improve the functional properties and added value of products.

Enzymatic extraction is a good method for preparing composite ingredients (Alimov 1996; Santa'Anna *et al.* 2003; Teixeira *et al.* 2013). Enzymatic treatment may cause significant changes in the flavour, component and nutrition and improves functional activities (Adebiyi *et al*. 2009b). The functional properties of extractions can be controlled by specific enzyme and reaction conditions (Buchert *et al*. 2009; Kim & Han 2012; Park *et al*. 2012). This method can increase the solubility of proteins and emulsification of solution and promote the dissolution of fat-soluble components. Obviously, it is conducive to co-extraction of antioxidant components from RB. However, there are few reports on the extraction optimization of enzymatic composite functional ingredients from RB.

Natural antioxidants play an important potential role in the inhibition of oxidative stress-related diseases, such as hyperlipidemia and atherosclerosis. Research has showed that hypolipidemic effects are closely related to the antioxidant ability (Cheng *et al*. 2008; Luo *et al*. 2009; Gris *et al*. 2011). Binding of bile acids has been hypothesized as a possible mechanism to lower cholesterol (Kahlon & Smith 2007). Therefore, in the present study, response surface methodology (RSM) was used to optimize the enzymatic extraction conditions for composite functional ingredients from RB–aqueous enzymatic extracts from rice bran (AEERB). Furthermore, the antioxidant capacity and bile salt adsorption capacity assays of AEERB were investigated to assess bioactivities *in vitro*.

MATERIAL AND METHODS

Material. RB was provided by the Great Northern Wilderness Agriculture Co., Ltd (China). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Trypsin (1 : 250) were obtained from Sigma-Aldrich (China). 2,2'-azino-bis (3-ethylbenzo- thiazoline-6-sulphonic acid) (ABTS) and γ-oryzanol were purchased from TCI (Japan). 2,4,6-tripyridyl-*s*-triazine (TPTZ) was obtained from Fluka (Switzerland). Butylated hydroxytoluene (BHT) and ascorbic acid (AA) were purchased from Sinopharm Chemical Reagent Co., Ltd (China). Other analytical grade chemical reagents were obtained from GuangFu Technology Development (China).

Optimization of the aqueous enzymatic extract from RB (AEERB). Briefly, the fresh full-fat RB was passed through a 40-mesh sieve and modified via enzymatic (Trypsin) hydrolysis in water bath (magnetic stirrer) with controlled temperature and pH, using the pH-stat method (NaOH, 0.1 mol/l). Processing of the product followed a different procedure including enzyme inactivation, cooling, centrifugation and filtration. In total, 0.5 ml of extraction solution was dissolved in distilled water to 20 ml. The experiments were established based on the Box-behnken design (BBD) with five factors at three levels, and the levels of independent variables (including pH, temperature and enzyme amount ([E]/[S]), liquid/solid ratio and time) were chosen based on single-factor experiments following our previous report (Wang *et al*. 2015). The γ-oryzanol content and FRAP value of hydrolysate were taken as the responses. The experimental runs are shown in Table 1.

Determination of γ-oryzanol content. The γ-oryzanol content was determined by the method described by Park *et al.* (2013) with a slight modification. Briefly, γ-oryzanol of hydrolysate was extracted using *n*-hexane at 55°C for 0.5 h with a lower rotating speed in dark, followed by centrifugation at 4000 rpm for 20 min (GL-20G-II; Shanghai Anting Scientific Instrument, China). The γ-oryzanol content of supernatant was determined by UV spectrometer (Shimadzu, Japan) at 314 nm.

Antioxidant cctivity. AEERB lyophilized powder was diluted appropriately for the functional activity analysis. In this work, the hydroxyl radical (·OH) scavenging ability and superoxide anion radical (O_2^-·) scavenging ability were used to evaluate the active oxygen free radical scavenging ability. The lipid peroxidation inhibition activity assay was used to evaluate the hydrogen atom transfer ability. The DPPH· scavenging activity, $ABTS^+$ scavenging activity assay and ferric-reducing/antioxidant power (FRAP) belong to the single electron transfer mechanism. The metal ion chelating ability, total antioxidant

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activity and total reducing power were used for the other mechanism assay. The metal ion chelating ability was estimated according to the method of Wang *et al.* (2009). The total reducing power used the SDS-modified ferricyanide assay described by Berker *et al.* (2010). The other antioxidant activity methods were measured according to our previous work (Wang *et al.* 2014, 2015).

Bile salt adsorption capacity. The *in vitro* binding of bile acids (sodium taurocholate and sodium deoxycholate) by AEERB was determined according to the method of Kahlon and Smith (2007) with a slight modification. Briefly, the hydrolysate was evaporated under vacuum (N-1100; Shanghai EYELA Instrument Co., Ltd., China). Fifty mg concentration of AEERB was digested in 1 ml 0.01 mol/l HCl for 1 h in a 37°C shaker bath and the pH of the sample was adjusted to 6.3 with 0.1 mol/l NaOH. Into the each sample was added 4 ml of porcine pancreatin (10 mg/ml, in a 0.1 mol/l phosphate buffer, pH 6.3) for 1 h in a 37°C shaker bath. After incubation, into the mixture was added 4 ml of 0.3 mmol/l cholate solution (sodium taurocholate or sodium deoxycholate, in a 0.1 mol/l phosphate buffer, pH 6.3) for 1 h in a 37°C shaker bath and then centrifuged at 8000 rpm for 10 min at 4°C. Supernatant (2.5 ml) was mixed in 7.5 ml of 60% H_2SO_4 and placed into the water bath for 20 min at the 70°C and then transferred to ice bath for 5 minutes. The absorbance was read at 387 ± 1 nm. The phosphate buffer solution was used as the reagent blank. Values were determined from the standard curves (sodium deoxycholate: $y = 2.9704x + 0.0128$; $R^2 = 0.9986$; sodium taurocholate: $y = 9.6763x + 0.0326$, $R^2 = 0.9993$). Individual substrate blanks were subtracted and the adsorption capacity was calculated as follow:

The adsorption content (μmol/100 mg) = the total content (μmol/100 mg) – the supernatant content (μmol/100 mg)

Statistical analysis. All parameters were expressed as the mean ± SD. The results were statistically analysed using one-way ANOVA followed by Tukey's multiple comparison tests. The criterion for significance was $P < 0.05$. The analysis was performed using the Statistix 8.0 software package (USA).

RESULTS AND DISCUSSION

Optimization of AEERB procedure by RSM. The γ-oryzanol content and FRAP value of hydrolysate under different conditions are listed in Table 1. The quadratic regression equation was obtained as follows:

$$Y_1 = 0.18 - 0.011A + 0.016B - 0.0018C - 0.01D - 0.0044E - 0.012AB - 0.00962AC + 0.017AD + 0.002AE + 0.0062BC + 0.0074BD - 0.001BE - 0.0041CD - 0.0043CE + 0.002DE - 0.041A^2 - 0.043B^2 - 0.0059C^2 - 0.035D^2 - 0.0082E^2 \quad (1)$$

$$Y_2 = 0.5 - 0.029A + 0.044B - 0.01C - 0.028D - 0.018E - 0.038AB - 0.023AC + 0.035AD + 0.0057AE + 0.017BC + 0.016BD + 0.0076BE + 0.023CD - 0.0078CE + 0.02DE - 0.12A^2 - 0.12B^2 - 0.011C^2 - 0.095D^2 - 0.014E^2 \quad (2)$$

where: Y_1 – γ-oryzanol content; Y_2 – FRAP; A – enzyme/substrate ratio (U/g); B – extraction temperature (°C); C – extraction time (h); D – pH; E – solid/liquid ratio (g/ml)

Regression and ANOVA analyses were used to fit the mathematical model and to examine the statistical significances of the model equation. The results of the response surface model in the form of ANOVA are shown in Table 2. The P are mainly used to check the significance of every coefficient and to explain the interaction between the independent variables. The P of the two models were all less than 0.01, which indicated that the mathematical models were highly significant. The lack of fit of P of the γ-oryzanol content and FRAP was non-significant (0.0757 and 0.0911, respectively), which meant that the model equations were adequate for predicting the relevant response. The R^2 and adj-R^2 were used to explain whether the mathematical model can be used to reasonably describe the relationship between the parameters. From the table, the R^2 and adj-R^2 of the γ-oryzanol content and FRAP were all higher than 0.8, showing that the fitting degree of the equations was good, which meant that the models can be used to determine and predict the extraction process. The smaller the CV value is, the better is the reliability of the experimental values (Xu *et al.* 2015). As shown in the table, the low CV % indicated a high degree of precision and a good reliability of the experimental values.

Additionally, in the regression model of the γ-oryzanol content, the items including A, B, D, E, AB, AC, AD, BC, and BD had a highly significant effect on the response values. Whereas CD and CE had a significant effect, and item C had no significant effect. Meanwhile, in the regression model of FRAP, except for AE, BE, and CE, the other items had a highly significant effect on the response values. This result

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Table 1. Box-behnken central composite design for independent variables and their responses

Run	A enzyme amount (U/g)	B temperature (°C)	C time (h)	D pH (–)	E solid/liquid ratio (g/ml)	Y_1 γ-oryzanol (mg/g)	Y_2 FRAP (mmol $FeSO_4$/g)
1	–1 (300)	–1 (50)	0 (2.0)	0 (8.0)	0 (1 : 10)	0.0804	0.2070
2	1 (700)	–1 (50)	0 (2.0)	0 (8.0)	0 (1 : 10)	0.0837	0.2263
3	–1 (300)	1 (60)	0 (2.0)	0 (8.0)	0 (1 : 10)	0.1332	0.3695
4	1 (700)	1 (60)	0 (2.0)	0 (8.0)	0 (1 : 10)	0.0895	0.2379
5	0 (500)	0 (55)	–1 (1.5)	–1 (7.5)	0 (1 : 10)	0.1470	0.4604
6	0 (500)	0 (55)	1 (2.5)	–1 (7.5)	0 (1 : 10)	0.1544	0.3946
7	0 (500)	0 (55)	–1 (1.5)	1 (8.5)	0 (1 : 10)	0.1326	0.3559
8	0 (500)	0 (55)	1 (2.5)	1 (8.5)	0 (1 : 10)	0.1235	0.3820
9	0 (500)	–1 (50)	0 (2.0)	0 (8.0)	–1 (1 : 5)	0.1153	0.3391
10	0 (500)	1 (60)	0 (2.0)	0 (8.0)	–1 (1 : 5)	0.1489	0.4283
11	0 (500)	–1 (50)	0 (2.0)	0 (8.0)	1 (1 : 15)	0.1122	0.2919
12	0 (500)	1 (60)	0 (2.0)	0 (8.0)	1 (1 : 15)	0.1416	0.4113
13	–1 (300)	0 (55)	–1 (1.5)	0 (8.0)	0 (1 : 10)	0.1352	0.3791
14	1 (700)	0 (55)	–1 (1.5)	0 (8.0)	0 (1 : 10)	0.1326	0.3695
15	–1 (300)	0 (55)	1 (2.5)	0 (8.0)	0 (1 : 10)	0.1544	0.4169
16	1 (700)	0 (55)	1 (2.5)	0 (8.0)	0 (1 : 10)	0.1133	0.3143
17	0 (500)	0 (55)	0 (2.0)	–1 (7.5)	–1 (1 : 5)	0.1549	0.4517
18	0 (500)	0 (55)	0 (2.0)	1 (8.5)	–1 (1 : 5)	0.1358	0.3678
19	0 (500)	0 (55)	0 (2.0)	–1 (7.5)	1 (1 : 15)	0.1380	0.3725
20	0 (500)	0 (55)	0 (2.0)	1 (8.5)	1 (1 : 15)	0.1269	0.3690
21	0 (500)	–1 (50)	–1 (1.5)	0 (8.0)	0 (1 : 10)	0.1247	0.3588
22	0 (500)	1 (60)	–1 (1.5)	0 (8.0)	0 (1 : 10)	0.1415	0.4023
23	0 (500)	–1 (50)	1 (2.5)	0 (8.0)	0 (1 : 10)	0.1089	0.2969
24	0 (500)	1 (60)	1 (2.5)	0 (8.0)	0 (1 : 10)	0.1504	0.4075
25	–1 (300)	0 (55)	0 (2.0)	–1 (7.5)	0 (1 : 10)	0.1426	0.3704
26	1 (700)	0 (55)	0 (2.0)	–1 (7.5)	0 (1 : 10)	0.0841	0.2437
27	–1 (300)	0 (55)	0 (2.0)	1 (8.5)	0 (1 : 10)	0.0837	0.2379
28	1 (700)	0 (55)	0 (2.0)	1 (8.5)	0 (1 : 10)	0.0950	0.2524
29	0 (500)	0 (55)	–1 (1.5)	0 (8.0)	–1 (1 : 5)	0.1661	0.4802
30	0 (500)	0 (55)	1 (2.5)	0 (8.0)	–1 (1 : 5)	0.1650	0.4724
31	0 (500)	0 (55)	–1 (1.5)	0 (8.0)	1 (1 : 15)	0.1697	0.4676
32	0 (500)	0 (55)	1 (2.5)	0 (8.0)	1 (1 : 15)	0.1513	0.4286
33	–1 (300)	0 (55)	0 (2.0)	0 (8.0)	–1 (1 : 5)	0.1486	0.4295
34	1 (700)	0 (55)	0 (2.0)	0 (8.0)	–1 (1 : 5)	0.1222	0.3518
35	–1 (300)	0 (55)	0 (2.0)	0 (8.0)	1 (1 : 15)	0.1325	0.3698
36	1 (700)	0 (55)	0 (2.0)	0 (8.0)	1 (1 : 15)	0.1142	0.3148
37	0 (500)	–1 (50)	0 (2.0)	–1 (7.5)	0 (1 : 10)	0.1001	0.2844
38	0 (500)	1 (60)	0 (2.0)	–1 (7.5)	0 (1 : 10)	0.1195	0.3317
39	0 (500)	–1 (50)	0 (2.0)	1 (8.5)	0 (1 : 10)	0.0657	0.1915
40a	0 (500)	1 (60)	0 (2.0)	1 (8.5)	0 (1 : 10)	0.1148	0.3047
41[a]	0 (500)	0 (55)	0 (2.0)	0 (8.0)	0 (1 : 10)	0.1799	0.4952
42[a]	0 (500)	0 (55)	0 (2.0)	0 (8.0)	0 (1 : 10)	0.1808	0.5006
43[a]	0 (500)	0 (55)	0 (2.0)	0 (8.0)	0 (1 : 10)	0.1809	0.4981
44[a]	0 (500)	0 (55)	0 (2.0)	0 (8.0)	0 (1 : 10)	0.1811	0.4865
45[a]	0 (500)	0 (55)	0 (2.0)	0 (8.0)	0 (1 : 10)	0.1759	0.5029
46[a]	0 (500)	0 (55)	0 (2.0)	0 (8.0)	0 (1 : 10)	0.1795	0.4963

[a]central rotation experiments

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Table 2. Analysis of variance of the response model for the γ-oryzanol content and FRAP

Source	DF	γ-Oryzanol content (mg/g)			FRAP ($FeSO_4$ mmol/g)		
		sum of squares	mean squares	*P*	sum of squares	mean squares	*P*
Model	20	4.113E-02	2.056E-03	< 0.0001	3.343E-01	1.672E-02	< 0.0001
A	1	1.931E-03	1.931E-03	< 0.0001	1.376E-02	1.376E-02	< 0.0001
B	1	3.858E-03	3.858E-03	< 0.0001	3.039E-02	3.039E-02	< 0.0001
C	1	4.991E-05	4.991E-05	0.0514	1.611E-03	1.611E-03	0.0003
D	1	1.655E-03	1.655E-03	< 0.0001	1.255E-02	1.255E-02	< 0.0001
E	1	3.115E-04	3.115E-04	< 0.0001	5.448E-03	5.448E-03	< 0.0001
AB	1	5.531E-04	5.531E-04	< 0.0001	5.688E-03	5.688E-03	< 0.0001
AC	1	3.700E-04	3.700E-04	< 0.0001	2.155E-03	2.155E-03	< 0.0001
AD	1	1.217E-03	1.217E-03	< 0.0001	4.979E-03	4.979E-03	< 0.0001
AE	1	1.618E-05	1.618E-05	0.2550	1.292E-04	1.292E-04	0.2521
BC	1	1.531E-04	1.531E-04	0.0014	1.126E-03	1.126E-03	0.0019
BD	1	2.204E-04	2.204E-04	0.0002	1.082E-03	1.082E-03	0.0023
BE	1	4.368E-06	4.368E-06	0.5504	2.291E-04	2.291E-04	0.1310
CD	1	6.750E-05	6.750E-05	0.0253	2.110E-03	2.110E-03	< 0.0001
CE	1	7.445E-05	7.445E-05	0.0194	2.410E-04	2.410E-04	0.1219
DE	1	1.604E-05	1.604E-05	0.2570	1.622E-03	1.622E-03	0.0003
A^2	1	1.494E-02	1.494E-02	< 0.0001	1.230E-01	1.230E-01	< 0.0001
B^2	1	1.585E-02	1.585E-02	< 0.0001	1.232E-01	1.232E-01	< 0.0001
C^2	1	3.068E-04	3.068E-04	< 0.0001	9.934E-04	9.934E-04	0.0033
D^2	1	1.084E-02	1.084E-02	< 0.0001	7.939E-02	7.939E-02	< 0.0001
E^2	1	5.863E-04	5.863E-04	< 0.0001	1.726E-03	1.726E-03	0.0002
Residual	25	2.980E-04	1.192E-05		2.349E-03	9.397E-05	
Lack of Fit	20	2.792E-04	1.396E-05	0.0757	2.187E-03	1.094E-04	0.0911
Pure Error	5	1.883E-05	3.766E-06		1.624E-04	3.247E-05	
Cor Total	45	4.142E-02			3.367E-01		
R^2		0.9928			0.9930		
Adj-R^2		0.9871			0.9874		
CV%		2.59			2.60		
Adeq. Precision		47.575			46.144		

$P < 0.05$ – significant; $P < 0.01$ – highly significant

showed that the relationship of influence factors is not simply linear. Based on the sum of squares, the order of influence factors is as follows:

B > A > D > E > C

The three-dimensional response surface visually reflected the interactions between the two influencing factors as well as the relationships between the response value and experimental levels of each independent variable. From Figure 1A–C, the increase in the enzyme/substrate ratio consequently increased the number of active enzyme centres in the system, resulting in a stronger interaction between the fat components and protein to increase the emulsification degree of the solution (Hamada 1997), which was more conducive to the dissolution of γ-oryzanol. Whereas, when the enzyme/substrate ratio was too high, it would exceed the saturation concentration of the enzyme. The dynamic balance in the system would inhibit the enzymatic reaction, resulting in no significant increase in the ingredient contents, which was consistent with the conclusions reported by Yu *et al.* (2009). The temperature and pH value

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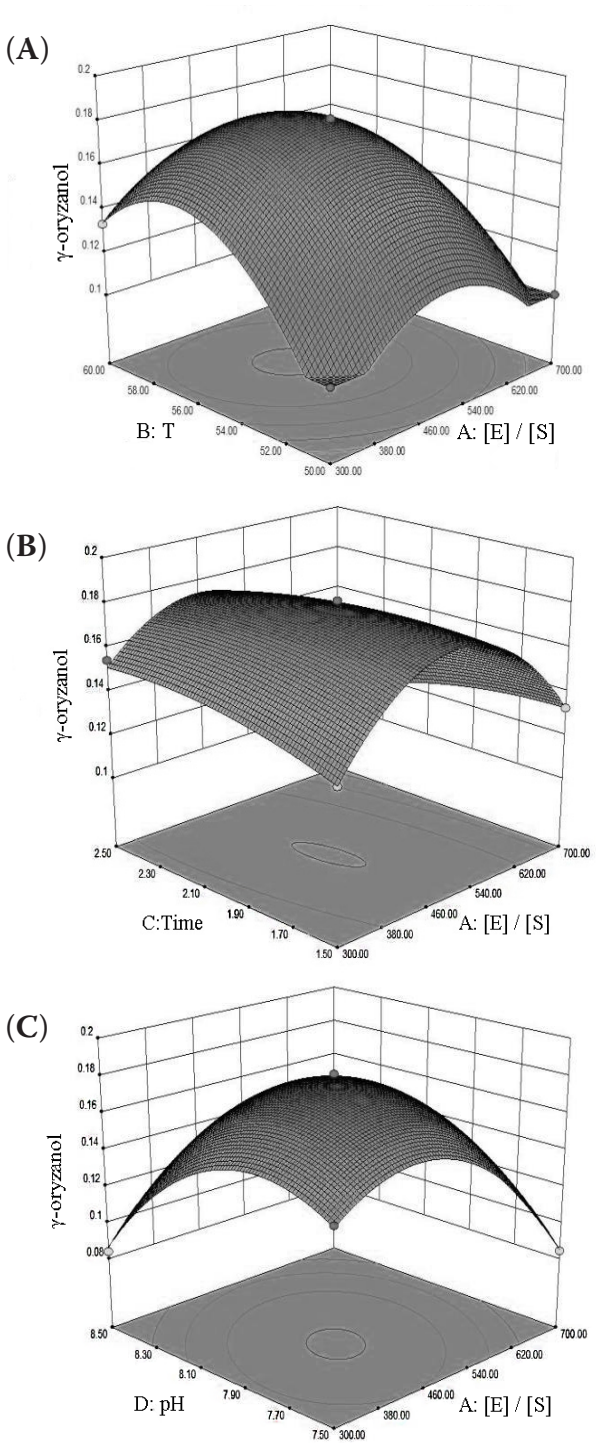

Figure 1. Interaction effects on the γ-oryzanol content. (**A**) the enzyme amount [E]/[S] and temperature; (**B**) [E]/[S] and time; (**C**) [E]/[S] and pH

can play an important role in enzymatic activity. A higher pH value and temperature can accelerate the dissolution of γ-oryzanol, but an excessively high pH value and temperature caused the denaturation of sterol oryzanol in ferulic acid and the irreversible inactivation of trypsin (Figure 1A and C). The effect of hydrolysis conditions on the FRAP value was similar to that of γ-oryzanol (Figure 2). The reason may be that aqueous enzymatic extraction can effectively cleave linkages between polysaccharide, lipid compound and protein to release lipids. It makes minimal change to the raw material composition and minimizes the loss of bioactive components such as γ-oryzanol (Parrado *et al.* 2006). The antioxidant ability of γ-oryzanol is attributed to its' ability to donate phenolic hydrogens (electrons). FRAP is based on a single-electron transfer mechanism (Aguilar-Garcia *et al.* 2007). So the higher the concentration of γ-oryzanol was, the stronger the antioxidant activity was. The correlations of concentration levels of functional ingredients with antioxidant activity were significantly positive (Wang *et al.* 2015).

The optimal conditions were determined as follows: enzyme amount 452.69 U/g, 56.06°C, pH 7.9, time 2.12 h, and liquid/solid ratio 7. The maximum γ-oryzanol content and FRAP value of AEERB predicted by the model were 0.1844 mg/g and 0.5127 mmol $FeSO_4$/g, respectively. To validate the adequacy of the model, the verification experiments were carried out with slight modifications (enzyme amount 453 U/g, 56°C, pH 7.9, time 2 h, and liquid/solid ratio 7). Under these conditions, the γ-oryzanol content of 0.1749 ± 0.009 mg/g and FRAP value of 0.5043 ± 0.007 mmol $FeSO_4$/g were obtained, which closely agreed with the predicted values. The results showed that the response model was not only accurate and reliable but also reflected the expected optimization.

Antioxidant activity. Antioxidant activity is the primary method for evaluating the antioxidant capacity and for exploring the antioxidant mechanism because of its advantages (such as simple, rapid and sensitive). There are many methods for determining antioxidant capacities, but different mechanism assays may give opposite conclusions when used to evaluate the antioxidant activities of a given complex components (Yu *et al.* 2002; Ou *et al.* 2002; Aguilar-Garcia *et al.* 2007). Thus, the antioxidant activities may be better investigated using different assays that depend on different mechanisms. The antioxidant activities are shown in Figure 3. From Figure 3A, the DPPH·scavenging activity of AEERB showed the same tendency with the ascorbic acid (AA) and butylated hydroxytoluene (BHT) (control). An increase in the content gradually increased the DPPH·scavenging activity. At the content greater than 25 μg/ml, the DPPH scavenging activity increased

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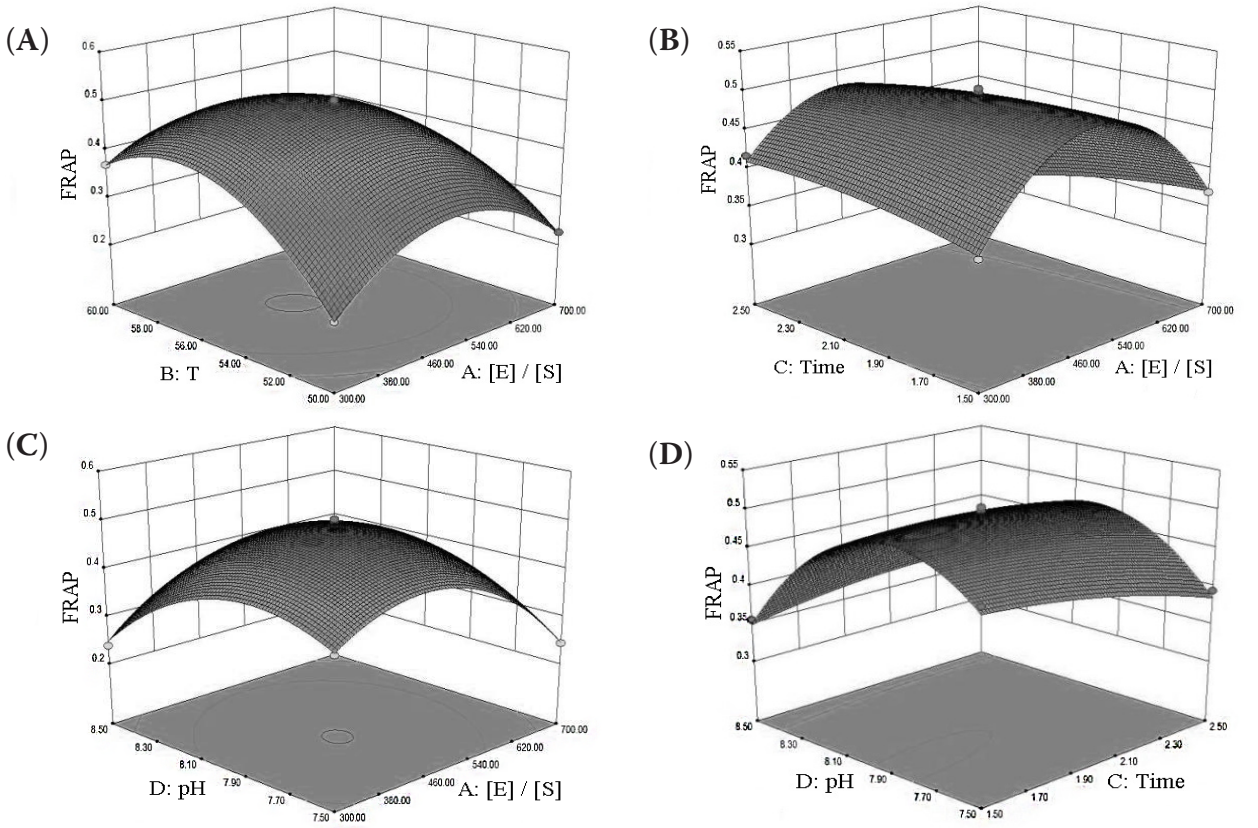

Figure 2. Interaction effects on the FRAP value. (**A**) the enzyme amount [E]/[S] and temperature; (**B**) [E]/[S] and time; (**C**) [E]/[S] and pH; (**D**) time and pH

rapidly. The IC_{50} values of AEERB, AA and BHT were 93.68 ± 3.6 μg/ml, 23.58 ± 5.5 μg/ml and 78.69 ± 5.5 μg/ml, respectively. The results showed that the DPPH· scavenging activity of AEERB was significantly lower than that of BHT and AA($P < 0.05$). When the concentration reached 50 μg/ml, there was an apparent increasing tendency in the ABTS·$^+$ scavenging activity (Figure 3B), which was still much lower than those of AA and BHT. When the concentration was over 150 μg ml, the scavenging activity of AEERB reached 80%, which was close to the lower concentration of AA and BHT. The IC_{50} value of AEERB was 81.51 ± 1.3 μg/ml. The value was clearly lower than that of the DPPH· scavenging activity, perhaps due to a lower selectivity for ABTS·$^+$ than for DPPH· because ABTS·$^+$, but not DPPH·, can react with any hydroxylation aromatic compound (Aguilar-Garcia *et al*. 2007; Wang *et al*. 2015).

·OH is the most reactive and toxic oxygen species. It is able to quickly react with all biological molecules in living cells and rapidly destroy biomolecules (proteins, DNA and nucleic acids) (Wang *et al*. 2012). O_2^-· is the precursor of all oxygen free radicals and its derivatives that damage DNA and cell membrane. Figure 3C and D demonstrate the active oxygen radical scavenging activities increased with an increase in the content of AEERB and AA. The IC_{50} values of ·OH and O_2^-· scavenging activities were 850.90 ± 27.21 g/ml and 138.46 ± 7.08 g/ml, respectively, although the scavenging activities were lower than for AA, which indicated that AEERB effectively reduced the cascade of oxidation reactions and showed a chain-breaking effect.

In Figure 3E, AEERB showed a strong antioxidant ability, which reached that of BHT when the concentration was 300 μg/ml. Compared with the reducing power of the original ferricyanide assay, the SDS-modified ferricyanide assay of total antioxidant capacity can directly determine the absorbance without centrifugation, which is more convenient (Berker *et al*. 2010). The concentration of BHT and AA was positively correlated with reducing power. When the concentration of AEERB was over 100 μg/ml, the reduction capacity rapidly increased with the increase of the concentration, but always lower than AA and BHT (Figure 3F). From Figure 3G,

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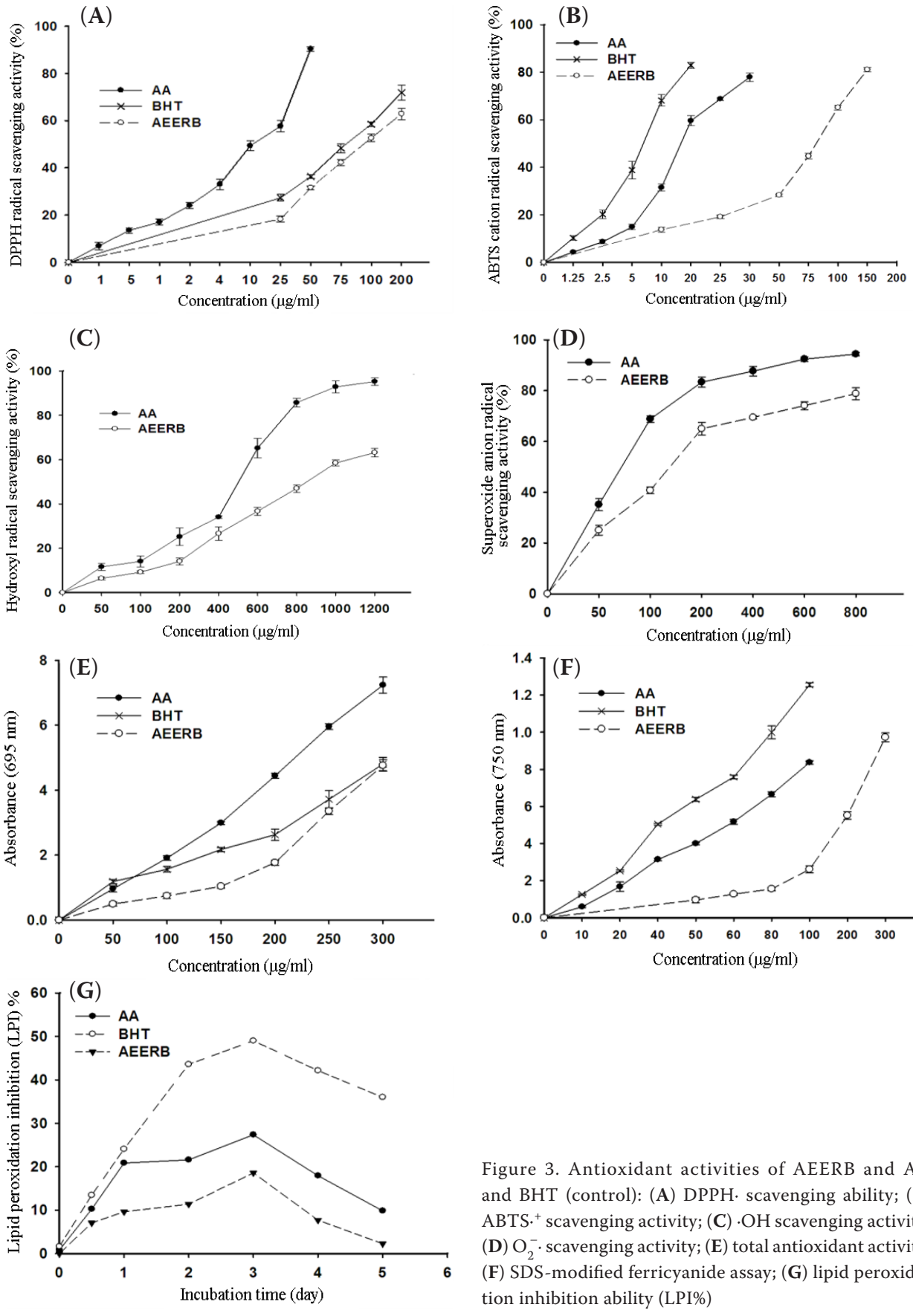

Figure 3. Antioxidant activities of AEERB and AA and BHT (control): (**A**) DPPH· scavenging ability; (**B**) ABTS·$^{+}$ scavenging activity; (**C**) ·OH scavenging activity; (**D**) O_2^{-}· scavenging activity; (**E**) total antioxidant activity; (**F**) SDS-modified ferricyanide assay; (**G**) lipid peroxidation inhibition ability (LPI%)

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Table 3. Bile salt binding capacity by AEERB (μmol/100 mg)

	Sodium taurocholate binding	Sodium deoxycholate binding
AEERB	1.1097 ± 0.001[a]	0.8219 ± 0.0003[b]

AEERB showed better antioxidant ability in the emulsion system, and the lipid peroxidation inhibition activity of AEERB, which was similar to AA (27.11 ± 0.3%), was 22.72 ± 0.7% (at 72 h).

The ferrous ion-chelating ability of AEERB was 18.43 ± 0.80 mg EDTA/g, and its chelation rate was approximately 31.66%. In this system, AA had a rather weak chelating capacity, which was only 8.2% at 20 mg/ml (Wang *et al.* 2009), much lower than that of AEERB. The strong chelating effect of AEERB may be due to the higher concentration of polyphenols and proteins.

Bile salt adsorption capacity. Bile acids are the products of cholesterol decomposition in the liver. The effect of bile acid binding on cholesterol lowering is based on the negative feedback of bile acids in the enterohepatic cycle. Bile acids are commonly present in the form of sodium salt in the body. Thus, we investigated the bile salt-binding capacity using AEERB. As shown in Table 3, the sodium taurocholate binding capacity by AEERB was clearly higher than that of sodium deoxycholate ($P < 0.05$). This may be because the acidity of sulfonic acid group in sodium taurocholate was stronger than that of carboxyl group in sodium deoxycholate, which more easily ionized under neutral conditions (Górecka *et al.* 2002). Additionally, AEERB is rich in polyphenols, compared with sodium deoxycholate. Sodium taurocholate can strongly interact with phenolic hydroxyl groups to form hydrogen bonds. Accordingly, the bile acid binding mechanism of AEERB may be the combination of ionic bonding or hydrogen bonding. The exact binding mechanisms remain for detailed investigation in the future.

CONCLUSIONS

AEERB was prepared via trypsin hydrolysis using RSM. The optimum preparation conditions are as follows: enzyme concentration of 453 U/g, temperature of 56°C, pH 7.9, hydrolysis time of 2 h and the liquid to solid ratio of 7. AEERB is rich in antioxidant compounds. Under the synergetic effect of a variety of functional ingredients, AEERB showed better antioxidant capacity in different systems and response mechanisms. The results revealed that AEERB had good radical scavenging activities, and the radical scavenging activity, antioxidant capacity and reducing power exhibited a good dose-dependent manner. At high concentration, its total antioxidant capacity and lipid peroxidation inhibition are close to BHT or AA. The ferrous ion chelating ability of AEERB is much higher than that of AA. In addition, the sodium taurocholate binding capacity by AEERB is clearly higher than sodium deoxycholate binding capacity. Therefore, AEERB can serve as a natural antioxidative and blood lipid lowering functional food or nutraceutical.

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