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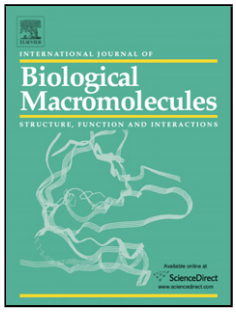

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Author: Bing-Jie Chen Mei-Jia Shi Shuai Cui Shu-Xian Hao Robert C Hider Tao Zhou

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Improved antioxidant and anti-tyrosinase activity of polysaccharide from *Sargassum fusiforme* by degradation

Bing-Jie Chen[a], Mei-Jia Shi[a], Shuai Cui[a], Shu-Xian Hao[b], Robert C Hider[c], Tao Zhou[a]*

[a] *School of Food Science and Biotechnology, Zhejiang Gongshang University, Xiasha, Hangzhou, Zhejiang, 310018, P. R. China.*

[b] *Department of Food processing and Safety Control, South China Sea Fishery Research Institute, Guangzhou, P. R. China.*

[c] *Institute of Pharmaceutical Science, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London, SE1 9NH, UK.*

*****Corresponding Author: T. Zhou

School of Food Science and Biotechnology, Zhejiang Gongshang University

E-mail: taozhou@zjgsu.edu.cn; tel: +86 571 28008976; fax: +86 571 88905733

Highlights

- The degradation conditions of polysaccharide from *S. fusiforme* has been optimized.
- The antioxidant activity of the degraded polysaccharides was greatly improved.
- The degraded polysaccharides possess superior anti-tyrosinase activity.

Abstract: An efficient method for the degradation of polysaccharides isolated from *Sargassum fusiforme* (PSF) was developed by using ascorbic acid in combination with H_2O_2. The degradation conditions were optimized using a Box-Behnken response surface design (BBRS). The optimum conditions were established as: concentration of ascorbic acid (V_C) and H_2O_2 17.26 mM, degradation temperature 51°C and degradation time 1.6 h. The DPPH radical scavenging rate of the degraded polysaccharides from *S. fusiforme* (DPSF) obtained under the optimal conditions was determined to be 75.22±0.02%, which was well matched with the value (75.21%) predicted by the BBRS model. *In vitro* antioxidant activity of the polysaccharides was evaluated by determining their radical (hydroxyl radical, superoxide anion radical and DPPH radical) scavenging abilities, and ferric iron reducing power. The inhibitory activity on tyrosinase of DPSF was also evaluated. The results indicate that the degraded polysaccharide has superior antioxidant activity and anti-tyrosinase effect to those of the original polysaccharide.

Keywords: polysaccharide from *Sargassum fusiforme*; antioxidant activity; anti-tyrosinase activity

1. Introduction

Natural polysaccharides are widely distributed in animals, plants and microorganisms. Many of them possess marked immunological properties by nonspecific stimulation of host immune system, resulting in a range of biological activities, such as anti-tumor, anti-viral, anti-infection, antioxidant, anti-mutagenic and hematopoietic activity. *Sargassum fusiforme*, a type of brown algae widely distributed in China, Korea, and Japan, has been applied as a therapeutical agent for thousands of years [1]. The polysaccharides extracted from *S. fusiforme* have been demonstrated to have multiple functions, such as antitumor [2], vibriosis resistance, enhancing immunity [3], and anti-hyperlipidemia [4]. However, the activities of the polysaccharides are insufficiently potent, which limits their pharmaceutical application. Many studies have demonstrated that molecular weight distributions of polysaccharides have a considerable influence on their biological activities [5]. Therefore, it is necessary to prepare polysaccharides from *S. fusiforme* with relatively low molecular weight to improve the activity. The degradation of polysaccharides can be achieved by either enzymatic methods [6], chemical methods [7], or physical methods [8]. Free radicals, for example, the hydroxyl radical (HO·), which degrade polysaccharides by attacking and breaking the glycosidic linkages is a short-lived but powerful oxidant that can be generated by H_2O_2 reaction with reduced transition metal ions. Ascorbic acid (V_C) can also reduce H_2O_2 to yield hydroxyl radicals in the presence of trace metals. Therefore, H_2O_2 in combination with V_C has attracted interest for polysaccharide degradation [9,10]. At present, little information is

available regarding the optimal conditions for degradation of polysaccharides from *S. fusiforme*. In this study, a method involving the use of H_2O_2/Vc was employed in order to degrade the polysaccharide. The degradation conditions were optimized by a response surface methodology (RSM).

Tyrosinase (EC 1.14.18.1) is widely distributed in nature and plays an important role in melanin production. It is well known that tyrosinase is responsible for the pigmentation of skin [11], the browning of fruits and vegetables [12]. Alterations in melanogenesis may be responsible for part of the clinical and histopathological features unique to malignant melanoma, a cancer with a rapidly increasing incidence [13]. Thus, tyrosinase inhibitors may be clinically useful for the treatment of skin cancer and some dermatological disorders associated with melanin hyperpigmentation. Recently, it was reported that some polysaccharides inhibit tyrosinase [14].

Herein, the antioxidant and anti-tyrosinase activities of the degraded polysaccharide from *S. fusiforme* (DPSF) are compared with the native polysaccharide preparations (PSF).

2. Materials and methods

2.1 Materials and chemicals

Sargassum fusiforme was collected in Dongtou, Zhejiang province (China). papain, hydrogen peroxide (H_2O_2, 30%, V/V), 1,1-diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), *L*-tyrosine and dihydroxyphenylalanine (*L*-DOPA) were purchased from Aladdin (Shanghai, China). Tyrosinase, dextran standards were

purchased from the Sigma-Aldrich Chemical Co. (USA). All other chemicals and reagents used were of analytically pure agent grade.

2.2 Preparation of crude polysaccharide

The crude polysaccharide from *S. fusiforme* (PSF) was prepared according to Chen's method with slight modifications [15]. Briefly, *S. fusiforme* was dried at 55°C and crushed into powder (100 mesh) by a disintegrator. The powder (10.0 g) was soaked with 95% ethanol for 20 h and extracted with 95% ethanol (1:5, W/V) refluxing at 90°C for 2 h（two times）to remove the pigments and small lipophilic molecules. The residue was then extracted with a 20-fold volume of distilled water at 95°C for 2h for three cycles. The combined aqueous extracts were filtered, concentrated, to about 100 mL, and then 95% ethanol (1:3, V/V) was added. The solution was allowed to stand at 4°C overnight. The resulting precipitate was collected by centrifugation at 8000 rpm for 10 min, redissolved in distilled water and deproteinated with papain (0.1%, W/V) and Sevag reagent (n-butanol:chloroform=1:4, V/V). The resulting aqueous solution was dialyzed (Mw cut-off 3500 Da) against ultrapure water for 3 days. Finally the solution was concentrated and lyophilized, yielding the crude polysaccharide

2.3 Degradation of PSF

PSF was degraded using a H_2O_2/V_C method with some modifications [16]. In brief, PSF (1.0 g) was dispersed in 200 mL distilled water, and different concentrations of H_2O_2 and V_C (molar ratio 1:1) were introduced directly into the

solution. Hydrolysis of PSF was performed for the designated reaction times (0.5, 1, 1.5, 2 and 2.5 h) and temperatures (30, 40, 50, 60 and 70 ºC). The reaction mixture was concentrated, and precipitated with 95% ethanol (1:3, V/V) at 4ºC overnight. After centrifugation at 8000 rpm for 10 min, the sediment was collected, dissolved in distilled water. The resulting solution was dialyzed, concentrated and lyophilized (as described in 2.2) to yield degraded polysaccharides (DPSF).

2.4 Characterization of polysaccharide

The total sugar content of polysaccharide samples was estimated by a phenol–sulfuric acid colorimetric method using glucose as a standard [17]. The sulfate content was determined by a barium chloride method [18]. The protein content was determined by the method of Bradford, using bovine serum albumin as a standard [19]. The content of uronic acid was determined according to the method of Blumenkrantz and Asboe-Hansen using D-glucuronic acid as a standard [20]. The moisture content was measured by employing vacuum drying method according to the Chinese National Standard (GB 5009.3-2010).

2.5 Molecular weight analysis

The molecular weight of polysaccharide was measured using high-performance gel permeation chromatography (HPGPC) on a Waters 1000 HPLC system, using a Waters 2414 Refractive Index Detector. Samples (10.0 mg) were dissolved in distilled water (10.0 mL), passed through a 0.45 μm filter and applied to a gel-filtration

chromatographic column of Ultrahydrogel™ Linear (300 mm × 7.8 mm, Waters, USA). Deionized water was used as a mobile phase at a flow rate of 0.5 mL/min. The temperature of the column was maintained at 30°C and the injection volume was 10 μL. Preliminary calibration of the column was carried out using dextran standards with different molecular weights.

2.6 Optimization of hydrolysis conditions for the preparation of DPSF

Based on the results of single-factor experiments, response surface methodology (RSM) was employed to further optimize the degradation conditions, three key independent variables, including concentration of Vc/H_2O_2, degradation temperature, and degradation time. Box–Behnken design (BBD) was used to survey the effects of independent variables at three levels on the response dependent variable, namely DPPH free radical scavenging ability. Each coded parameter was set at three different levels. The entire design consisted of 17 experimental runs carried out in random order (Table1). Each experiment was carried out in triplicate. Degraded polysaccharide obtained under the optimum conditions was used for more detailed antioxidant activity and anti-tyrosinase activity investigations.

Insert Table 1

2.7 Antioxidant activity assays for polysaccharides

2.7.1 Ferric iron reducing power

The ferric iron reducing power assay was carried out in reference to a previously reported method with some modifications [21]. Polysaccharide samples (2mL) with

different concentrations were mixed with phosphate buffer (2 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2 mL, 1%, W/V), then incubated at 50°C for 20 min. Trichloroacetic acid (2mL, 10%, W/V) was added to the mixture, centrifuged at 3000 rpm for 10 min. The supernatant (2 mL) was mixed with distilled water (2 mL) and $FeCl_3$ (0.4 mL, 0.1%, W/V). The absorbance of the resulting solution was measured at 700 nm after 10 min. V_C served as a positive control.

2.7.2 Scavenging activity of DPPH radicals

The DPPH-scavenging activity was determined according to the reported method with some modification [22]. In brief, samples (2.0 mL) were mixed with DPPH solution (2.0 mL, 0.1mmol/L in ethanol), followed by vigorous blending. After 30 min incubation in the dark, the absorbance at 517 nm was measured. The DPPH-scavenging activity was calculated as:

DPPH-scavenging activity (%) = $[1 - (A_{sample}-A_{background})/A_{blank}]\times 100$

$A_{background}$ is the absorbance of the sample under identical conditions as A_{sample} with ethanol instead of DPPH solution, and A_{blank} is the absorbance of the control solution (distilled water instead of the sample).

2.7.3 Scavenging activity of hydroxyl radical (HO·)

The HO· scavenging ability of polysaccharide samples was determined by an improved Fenton-type reaction as previously described [23]. sample solutions (1.0 mL) mixed with $FeSO_4$ (1.0 mL, 9 mM), salicylic acid-ethanol (1.0 mL, 9 mM) and H_2O_2 (1.0 mL, 9 mM) were incubated at 37°C for 0.5 h, and absorbance was measured at 510 nm. The scavenging activity was calculated according to the following formula:

HO· scavenging activity (%) = [1 − (A_{sample}− $A_{background}$)/A_{blank}]×100

2.7.4 Superoxide anion radical ($O_2^{\bullet-}$)-scavenging activity

Superoxide anion radical-scavenging activity of polysaccharide was conducted according to Marklund's method [24]. Sample solution (0.1 mL) was mixed with 3mL Tris-HCl buffer (pH 8.2, 50 mM). After incubation at 25°C for 20 min, pyrogallol (3 mL, 7 mM) preheated at 25°C was added and mixed thoroughly. Absorbance of the resulting mixture was determined at 420 nm after 5 min. Radical scavenging ability was calculated using the equation below:

$O_2^{\bullet-}$-scavenging activity (%) = (A_{sample}−$A_{background}$)/A_{blank}]×100

2.8 Assay of tyrosinase activity

The inhibitory activity of polysaccharides on mushroom tyrosinase was determined spectrophotometrically according to a previously described method with minor modification [25]. Briefly, a solution of *L*-tyrosine or *L*-DOPA (0.5 mL, 2 mM) was mixed with phosphate buffer (0.4 mL, 25 mM, pH 6.8), followed by the addition of polysaccharide (0.05 mL) at different concentrations (0, 5, 10, 15 and 20 mg/mL). After 10 min of incubation, mushroom tyrosinase (0.05 mL, 500 U/mL) was added to the solution. The optical density (OD) was recorded after 30 min. The increase in the absorbance value at 475 nm due to the formation of dopachrome was monitored using a microplate reader. The inhibition rate of tyrosinase was calculated according to the following formula:

Inhibition rate (%) = [1− (C−D)/(A−B)]×100

where A is the OD without testing polysaccharide, B is the OD without testing

polysaccharide and substance, C is the OD of testing group, D is the OD with testing substance but without tyrosinase.

The inhibitory kinetics of polysaccharide for tyrosinase was analyzed using the Lineweaver-Burk method based on the results of inhibitory effect on the diphenolase activity of mushroom tyrosinase [26].

2.9 Statistical analysis

All the data are expressed as mean ± standard deviation (SD) of at least three replicates. Data was subjected to analysis of variance (ANOVA) followed by Duncans multiple-range tests, $P<0.05$ was considered as a statistically significant difference. Design-Expert (Version 8.0.5, State-Ease Inc., Minneapolis, MN, USA) was used for the experimental design and the regression analysis of experimental data.

3. Results and discussion

3.1 Optimization of the degradation parameters of polysaccharide

3.1.1 Statistical analysis and the model fitting

The designed experiments were carried out for different combinations of the physical parameters, and the empirical relationship between response variable and the test variables by a second-order polynomial equation was obtained as follows:

DPPH scavenging rate (%) = $74.48+2.95A+0.63B+0.45C-0.048AB+0.1AC-0.027BC-3.26A^2-2.60\ B^2-2.35C^2$.

The *F*-value and *P*-value are two important indicators for ANOVA. A high

F-value and a low *P*-value indicate the quadratic polynomial model is significant. The *F*-value of this quadratic polynomial model was 459.46 (Table 2), while the corresponding *P*-value was less than 0.0001, which implied the model was extremely significant. Non-significant lack-of-fit is desirable. The *F*-value and *P*-value of lack-of-fit in the regression model were respectively 1.30 and 0.3898, which implied the lack-of-fit was not significant relative to the pure error and confirmed the validity of the model. The value of the determination coefficient (R^2= 0.9983) of the quadratic regression model indicated that only 0.17% of the total variations could not be explained by the model. The adjusted determination coefficient (R^2_{adj}= 0.9961) was also high, showing the high degree of correlation between the experimental and predicted values. In addition, the R^2_{adj} was close to the R^2 value, which exhibited the large enough sample scale [27]. At the same time, a low value 0.30% of the coefficient of the variation (C.V.) clearly indicated a high degree of precision and good reliability of the experimental values. Besides, Table 2 showed that the linear coefficients (A, B and C), quadratic term coefficient (A^2, B^2 and C^2,) were significant, the other term coefficients were not significant (P>0.05).

Insert Table 2

3.1.2 Response surface plot and contour plot analyses

The graphical representations of three-dimensional (3D) response surface and two-dimensional (2D) contour plots were employed to express the regression equation visually, which could accomplish a better understanding of the relationship between the responses and each tested variable, and the interactions between any two variables.

As presented in Fig. 1A-I and A-II, the effects of concentration of Vc/H_2O_2, degradation temperature, and their interactions on DPPH-scavenging activity of polysaccharide were illustrated. When the degradation temperature was higher than 50 ^oC, the concentration of Vc/H_2O_2 produced an outstanding response value from 5 to 17 mM. Therefore, the concentration of Vc/H_2O_2 had the main impact on the response value, and the result was in agreement with the preceding ANOVA analysis. The DPPH-scavenging activity of the resulting degraded polysaccharide increased markedly with the increase of the concentration of Vc/H_2O_2 until 17 mM, after which it increased slightly. The effects of concentration of Vc/H_2O_2 and degradation time on DPPH-scavenging activity of polysaccharide and their mutual interactions are presented in Fig. 1B-I and B-II, the DPPH-scavenging activity of DPSF increased with increasing degradation time until about 1.6h, and then slightly decreased with increasing degradation time. However, this analysis demonstrated that there was a small reciprocal interaction on the DPPH-scavenging activity of polysaccharide between the concentration of Vc/H_2O_2 and degradation time, which was consistent with the analytical results of coefficients estimate (0.1) of the regression equation. Fig. 1C-I and C-II display the effects of degradation temperature and degradation time on DPPH-scavenging activity of polysaccharide. The DPPH-scavenging activity of polysaccharide increased with increasing degradation temperature from 40 until 51^oC, thereafter it declined slightly. This is probably due to the fact that higher temperatures cause decomposition of H_2O_2 and V_C.

Insert Fig. 1

3.1.3 Verification of predictive model

Based on Fig. 1, it was concluded that the optimized parameters for the degradation of polysaccharide from *S. fusiforme* are as follows: concentrations of Vc/H_2O_2 17.26 mM, degradation temperature 51°C and degradation time 1.6 h. Under these conditions, the model predicted a maximum response of 75.21%. For the validation of the suitability of the model equations, triplicate confirmatory experiments were performed under the optimal conditions, and the DPPH-scavenging activity of polysaccharide was found to be 75.22±0.02%, which indicated that the model was appropriate for the degradation process.

3.2 Characteristics of the sample

The chemical composition of PSF and DPSF are presented in Table 3. As shown in Table 3, DPSF contained a higher content of total sugar and sulfate than PSF, but less uronic acid.

Insert Table 3

3.3. Antioxidant activities of polysaccharide

3.3.1 Assay of ferric iron reducing power

Total antioxidant ability can be evaluated by measuring the ferric iron reducing power. $[Fe(CN)_6]^{3-}$ is reduced to $[Fe(CN)_6]^{4-}$ in the presence of polysaccharides. The latter reacts with ferric ion to form $Fe_4[Fe(CN)_6]_3$ (Prussian blue). Thus, the formation of Fe(II) can be monitored by measuring the absorbance at 700 nm which is positively correlated to the concentration of Prussian blue. Higher absorbance values correspond

to a stronger ferric iron reducing power. As shown in Fig. 2A, the ferric reducing power of both PSF and DPSF behaved in a concentration-dependent manner over the tested concentration ranges. At a concentration of 2.5 mg/mL, the absorbance values of the solution obtained with PSF and DPSF were determined to be 0.266 and 0.669, respectively, indicating that the ferric iron reducing power of DPSF was significantly improved in comparison with PSF (P<0.05). This finding is in accordance with the results of previous studies that demonstrated that the polysaccharides with lower molecular weight possessed higher reducing power [28]. However, the reducing power of DPSF was weaker than V_C. Reducing properties were generally associated with the presence of reductones, which exhibited antioxidant activity by donating hydrogen atoms and electron donors. Thus, hydrolysis of PSF might produce reductone, which could react with free radicals to convert them into more stable products, thereby terminating radical chain reactions [29].

Insert Fig. 2

3.3.2 DPPH free-radical scavenging activity

DPPH is a stable free-radical compound which shows maximum absorbance at 517 nm. It was widely used in evaluating the ability of antioxidants to scavenge radicals. As presented in Fig. 2B, the scavenging effect of tested samples increased with increasing concentration. The DPPH scavenging effect of DPSF was higher than that of PSF, however both showed lower scavenging activity than V_C. The concentration of antioxidant needed to decrease the initial radical concentration by 50% (IC_{50}) is widely used to assess the antioxidant activity. The IC_{50} values of PSF,

DPSF were 0.611 and 0.270 mg/mL, respectively. The results implied that degradation of polysaccharides may expose more active moieties, which could serve as hydrogen donors to scavenge DPPH radicals.

3.3.3 Hydroxyl radical scavenging activity

Hydroxyl radical is one of the most reactive and dangerous free radicals amongst reactive oxygen species and is mainly responsible for the oxidative injury of biomolecules functioning in living cells. In this study, hydroxyl radicals were generated by a Fenton-type reaction, namely the reaction of Fe(II) complex with H_2O_2 in the presence of salicylic acid. The scavenging ability of DPSF was found to be improved as compared to that of PSF ($P<0.05$) (Fig. 2C). The results suggest that the molecular weights of these polysaccharides play an important role on their bioactivity, which is similar to Zhao's report that higher antioxidant activities were found when the molecular weight decreased [5]. In addition, the polysaccharides were found to have a lower antioxidant activity than V_C. At a concentration of 1.0 mg/mL, the scavenging effects of PSF and DPSF were determined to be 18.33% and 32.98%, respectively, while the scavenging rate of V_C was 30.76% even at 0.1 mg/mL.

3.3.4 Superoxide anion radical scavenging activity

The superoxide anion radical is generated by numerous biological and photochemical reactions. In addition to directly attacking important biological molecules, the superoxide anion radical may also decompose to form singlet oxygen and hydroxyl radicals that may increase local oxidative stress and initiate cellular damage or lipid peroxidation and pathological incidents [30]. Thus, removing the

hydroxyl radical is important for antioxidant defense in cell or food systems. The decrease of absorbance at 420 nm after addition of polysaccharides can represent the decrease in content of superoxide radicals. Thus, a lower absorbance at 420 nm indicates a higher antioxidant activity of the sample. As shown in Fig. 2D, the scavenging effects of PSF, DPSF and V_C on superoxide anion radical were dose-dependent. The scavenging abilities on superoxide radical could be ranked as the following order: PSF<DPSF<V_C. The IC_{50} values of PSF and DPSF were 0.577 and 0.388 mg/mL, respectively. The superoxide anion radical-scavenging rate was 43.38 % for PSF, 53.56 % for DPSF and 99.58% for V_C at 0.5 mg/mL. Sulphate content is an important factor for superoxide anion scavenging ability in fucoidan [31], and the superior activity of DPSF to PSF is assumed to be attributed to its higher sulphate content .

3.4 Inhibition of tyrosinase activity

As shown in Fig. 3, the inhibitory effects of polysaccharide on both monophenolase and diphenolase activities of mushroom tyrosinase exhibited a dose-dependent manner. DPSF possesses a superior anti-tyrosinase activity to that of PSF. At 1.0 mg/mL, the inhibitory rates of PSF and DPSF against monophenolase activity of tyrosinase were determined to be 14.3% and 37.1%, respectively (Fig. 3A), while for diphenolase activity, the inhibitory rates were 19.9% and 49.7%, respectively (Fig. 3B). Fig. 3C shows the relationship between the catalytic activity of tyrosinase and the enzyme dosage at different concentrations of DPSF. Plots of the enzyme activity versus the enzyme dosage used in the presence of different

concentrations of polysaccharide provided a family of straight lines, all of which passed through the origin. The increase of polysaccharide concentration resulted in a decrease in the line slope, indicating that the inhibition of tyrosinase by polysaccharide was reversible. The kinetic data of the inhibition of L-DOPA oxidation by polysaccharide were analysed using Lineweaver–Burk double-reciprocal plots (Fig. 3D). The plots give a family of straight lines with different slopes intersecting in the second quadrant, indicating that DPSF can bind, not only with free enzyme, but also with the enzyme–substrate complex, namely, DPSF was a competitive-uncompetitive mixed type inhibitor. This result was consistent with previously published work.[32]

It is well known that tyrosinase can catalyze hydroxylation of *L*-tyrosine to *L*-DOPA (monophenolase activity) and also the subsequent oxidation of DOPA to dopaquinone (diphenolase activity). Dopaquinone is highly reactive and can polymerize spontaneously to form melanin in a series of reaction pathways. Reduction of dopaquinone formation results in the inhibition of melanin formation. Thus, it can be reasonably assumed that the anti-tyrosinase activity of polysaccharide is attributed to its antioxidant property [33]. Hydroxyl groups of polysaccharide could form hydrogen bonds to a site on the enzyme, leading to steric hindrance or conformational change [34]. Indeed, it has been demonstrated that binding of polysaccharide from *S. fusiforme* with tyrosinase results in an obvious change in the tertiary structure of tyrosinase [32]. Therefore, superior anti-tyrosinase activity of DPSF to that of PDF can be partially attributed to its superior anti-oxidant activity, while the relatively low

molecular weight of DPSF possibly means its ability to bind tyrosinase more tightly.

Insert Fig. 3

4. Conclusions

The technology for the degradation of polysaccharides from *Sargassum fusiforme* has been developed in order to increase the antioxidant and anti-tyrosinase activities of polysaccharides. The degradation conditions optimized by RSM are as follows: concentrations of Vc/H_2O_2 17.26 mM, degradation temperature 51^oC and degradation time 1.6 h. The degraded polysaccharide obtained under optimized conditions possesses superior antioxidant activities and anti-tyrosinase activity than undegraded polysaccharide. Furthermore, the water solubility of degraded polysaccharide was also greatly improved in comparison with PSF. It is proposed that the newly generated functional groups in the degraded polysaccharides have greater chance to interact with radicals because of their low molecular weight, improved water solubility and greater surface area. DPSF developed in this investigation could have potential applications in the fields of medicine, cosmetics and food production.

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References

[1] W. Zhu, V.E.C. Ooi, P.K.S. Chan, P.O. Ang, Jr, Isolation and characterization of a sulfated

polysaccharide from the brown alga *Sargassum patens* and determination of its anti-herpes activity, Biochem. Cell Biol. 81 (2003) 25-33.

[2] X.M. Chen, W.J. Nie, G.Q. Yu, Y.L. Li, Y.S. Hu, J.X. Lu, L.Q. Jin, Antitumor and immunomodulatory activity of polysaccharides from Sargassum fusiforme, Food Chem. Toxicol. 50 (2012) 695-670.

[3] X.X. Huang, H.Q. Zhou, H. Zhang, The effect of *Sargassum fusiforme* polysaccharide extracts on vibriosis resistance and immune activity of the shrimp *Fenneropenaeus chinensis*, Fish Shellfish Immunol. 20 (2006) 750-757.

[4] W.J. Mao, B.F. Li, Q.Q. Gu, Y.C. Fang, H.T. Xing, Preliminary studies on the chemical characterization and antihyperlipidemic activity of polysaccharide from the brown alga *Sargassum fusiforme*, Hydrobiologia. 512 (2004) 263-266.

[5] X. Zhao, B.F. Li, C.H. Xue, L.P. Sun, Effect of molecular weight on the antioxidant property of low molecular weight alginate from *Laminaria japonica*, J. Appl. Phycol. 24 (2012) 295-300.

[6] H.U. Rehman, A. Aman, A. Silipo, S.A.U. Qader, A. Molinaro, A. Ansari, Degradation of complex carbohydrate: Immobilization of pectinase from Bacillus licheniformis KIBGE-IB21 using calcium alginate as a support, Food Chem. 139 (2013) 1081-1086.

[7] X.T. Meng, R.E. Xing, S. Liu, H.H. Yu, K.C. Li, Y.K. Qin, P.C. Li, Molecular weight and pH effects of aminoethyl modified chitosan on antibacterial activity in vitro, Int. J. Biol. Macromol. 50 (2012) 918-924.

[8] J.I. Choi, J.K. Kim, J.H. Kim, D.K. Kweon, J.W. Lee, Degradation of hyaluronic acid powder by electron beam irradiation, gamma ray irradiation, microwave irradiation and

thermal treatment: A comparative study, Carbohydr. Polym. 79 (2010) 1080-1085.

[9] Z.S. Zhang, X.M. Wang, M.X. Zhao, H.M. Qi, Free-radical degradation by Fe^{2+}/Vc/H_2O_2 and antioxidant activity of polysaccharide from *Tremella fuciformis*, Carbohydr. Polym. 112 (2014) 578-582.

[10] Z.S. Zhang, X.M. Wang, X.F. Mo, H.M. Qi, Degradation and the antioxidant activity of polysaccharide from *Enteromorpha linza*, Carbohydr. Polym. 92 (2013) 2084-2087.

[11] C. Olivares, F. Solano, New insights into the active site structure and catalytic mechanism of tyrosinase and its related proteins, Pigment Cell & Melanoma Res. 22 (2009) 750-760.

[12] Y.-J. Kim, H. Uyama, Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future, Cell. Mol. Life Sci. 62 (2005) 1707-1723.

[13] N. Baurin, E. Arnoult, T. Scior, Q.T. Do, P. Bernard, Preliminary screening of some tropical plants for anti-tyrosinase activity, J. Ethnopharmacol. 82 (2002) 155-158.

[14] S. Rout, R. Banerjee, Free radical scavenging, anti-glycation and tyrosinase inhibition properties of a polysaccharide fraction isolated from the rind from *Punica granatum*, Bioresour. Technol. 98 (2007) 3159-3163.

[15] X.M. Chen, W.J. Nie, S.R. Fan, J.F. Zhang, Y.X. Wang, J.X. Lu, L.Q. Qin, A polysaccharide from *Sargassum fusiforme* protects against immunosuppression in cyclophosphamide-treated mice, Carbohydr. Polym. 90 (2012) 1114-1119.

[16] T.T. Zhao, Q.B. Zhang, H.M. Qi, H. Zhang, X.Z. Niu, Z.H. Xu, Z.E. Li, Degradation of porphyran from *Porphyra haitanensis* and the antioxidant activities of the degraded porphyrans with different molecular weight, Int. J. Biol. Macromol. 38 (2006) 45-50.

[17] Y. Chen, M.Y. Xie, S.P. Nie, C. Li, Y.X. Wang, Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of Ganoderma atrum, Food Chem. 107 (2008) 231-241.

[18] Y. Kawai, N. Seno, K. Anno, A modified method for chondrosulfatase assay, Anal. Biochem. 32 (1969) 314-321.

[19] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

[20] N. Blumenkrantz, G. Asboe-Hansen, New method for quantitative determination of uronic acids, Anal. Biochem. 54 (1973) 484-489.

[21] G.C. Yen, H.Y. Chen, Antioxidant activity of various tea extracts in relation to their antimutagenicity, J. Agric. Food. Chem. 43 (1995) 27-32.

[22] J. Xu, L.L. Xu, Q.W. Zhou, S.X. Hao, T. Zhou, H.J. Xie, Isolation, purification, and antioxidant activities of degraded polysaccharides from *Enteromorpha prolifera*, Int. J. Biol. Macromol. 81 (2015) 1026-1030.

[23] Y. Liu, Y.Q. Du, J.H. Wang, X.Q. Zha, J.B. Zhang, Structural analysis and antioxidant activities of polysaccharide isolated from Jinqian mushroom, Int. J. Biol. Macromol. 64 (2014) 63-68.

[24] S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur. J. Biochem, 47 (1974), 469-474.

[25] P. Maisuthisakul, M.H. Gordon, Antioxidant and tyrosinase inhibitory activity of mango seed kernel by product, Food Chem. 117 (2009) 332-341.

[26] D.F. Li, P.P. Hu, M.S. Liu, X.L. Kong, J.C. Zhang, R.C. Hider, T. Zhou, Design and synthesis of hydroxypyridinone-L-phenylalanine conjugates as potential tyrosinase inhibitors, J. Agri. Food Chem. 61 (2013) 6597-6603.

[27] J.P. Maran, V. Mekala, S. Manikandan, Modeling and optimization of ultrasound-assisted extraction of polysaccharide from *Cucurbita moschata*, Carbohydr. Polym. 92 (2013) 2018-2026.

[28] C. Zhou, Y. Wang, H. Ma, R. He, Effect of ultrasonic degradation on in vitro antioxidant activity of polysaccharides from Porphyra yezoensis (Rhodophyta), Food Sci. Technol. Int.14 (2008) 479-486.

[29] Y.F. Wang, Z.W. Yang, X.L. Wei, Antioxidant activities potential of tea polysaccharide fractions obtained by ultra filtration, Int. J. Biol. Macromol. 50 (2012) 558-564.

[30] W. Liu, H.Y. Wang, X.B. Pang, W.B. Yao, X.D. Gao, Characterization and antioxidant activity of two low-molecular-weight polysaccharides purified from the fruiting bodies of *Ganoderma lucidum*, Int. J. Biol. Macromol. 46 (2010) 451-457.

[31] M.C.R. de Souza, C.T. Marques, C.M.G. Dore, F.R.F. da Silva, H.A.O. Rocha, E. L. Leite, Antioxidant activities of sulfated polysaccharides from brown and red seaweeds, J. Appl. Phycol. 19 (2007) 153-160.

[32] J. Zhang，Z.H. Chen, S. Ye, Y.X. Si，C.Y. Li, Study on inhibition effect of *Sargassum fusiforme* polysaccharide extracts on activity of tyrosinase, Sci. Technol. Food Industry 33 (2012) 116-120.

[33] I. Kubo, Q.X. Chen, K.I. Nihei, Molecular design of antibrowning agents: Antioxidative tyrosinase inhibitors, Food Chem. 81 (2003) 241-247.

[34] B. Yang, M.M. Zhao, Y.M. Jiang, Optimization of tyrosinase inhibition activity of ultrasonic-extracted polysaccharides from longan fruit pericarp, Food Chem. 110 (2008) 294-300.

Figure Captions

Fig. 1. Contour plots (A-I–C-I) and response surface (A-II–C-II) demonstrating the effects of concentration of Vc/H_2O_2, degradation temperature, and degradation time on DPPH-scavenging activity of polysaccharide.

Fig. 2. The antioxidant activities of *Sargassum fusiforme* polysaccharide. (A) ferric iron reducing power, (B) DPPH scavenging activity, (C) •OH scavenging activity, (D) $O_2^{\bullet-}$ scavenging activity. Values were expressed as the mean ± SD (n = 3).

Fig. 3. Inhibitory effect of the PSF and DPSF on tyrosinase activity. (A) monophenolase activity; (B) diphenolase activity. (C) Relationship between the catalytic activity of tyrosinase and the enzyme dosage at different concentrations of DPSF. The *L*-DOPA concentration is 2 mmol/L. (D) Lineweaver-Burk plots of mushroom tyrosinase with *L*-DOPA as a substrate in the presence of different concentration of DPSF. The enzyme dosage is 20 U.

Fig. 1

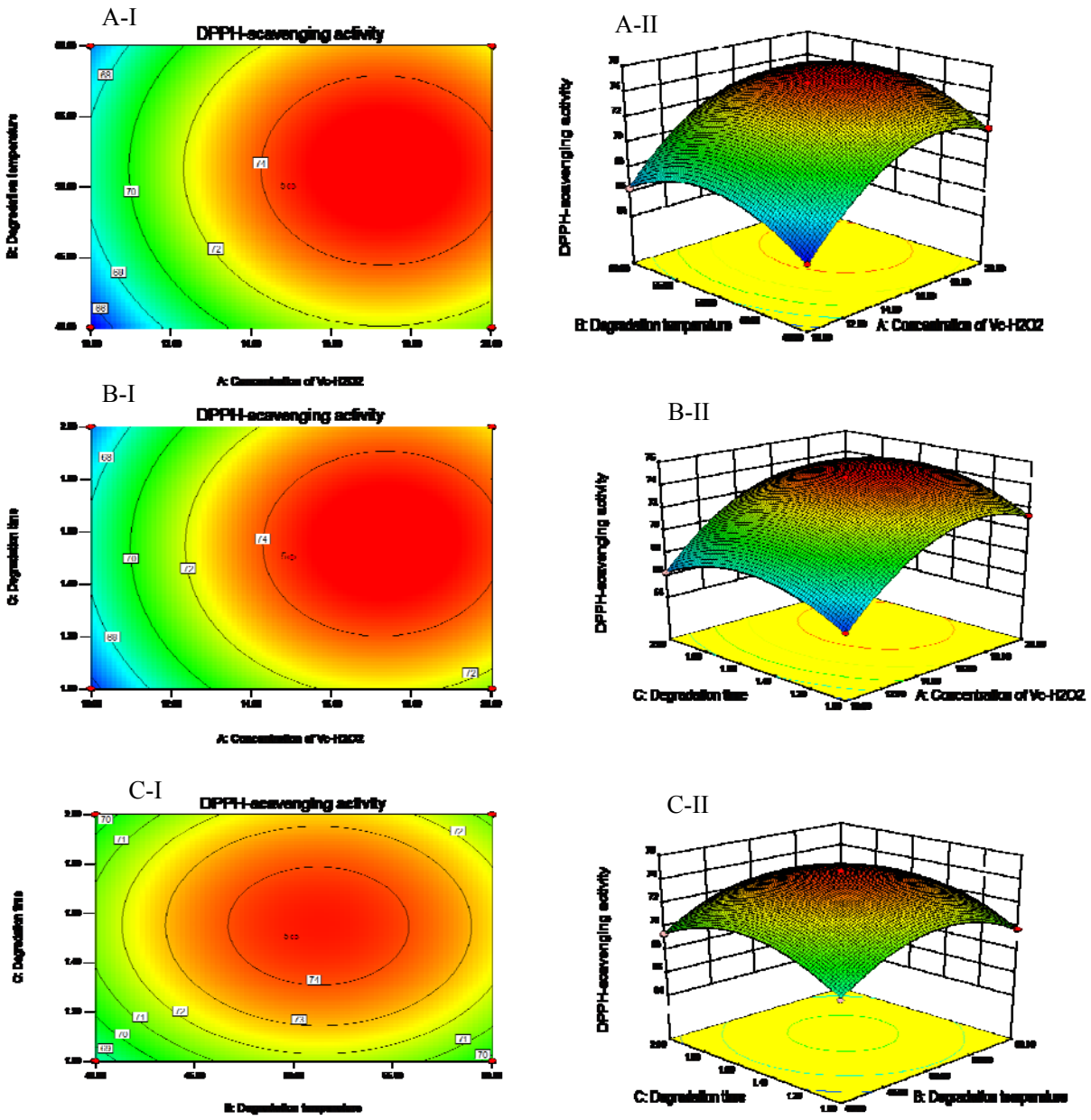

Fig. 2

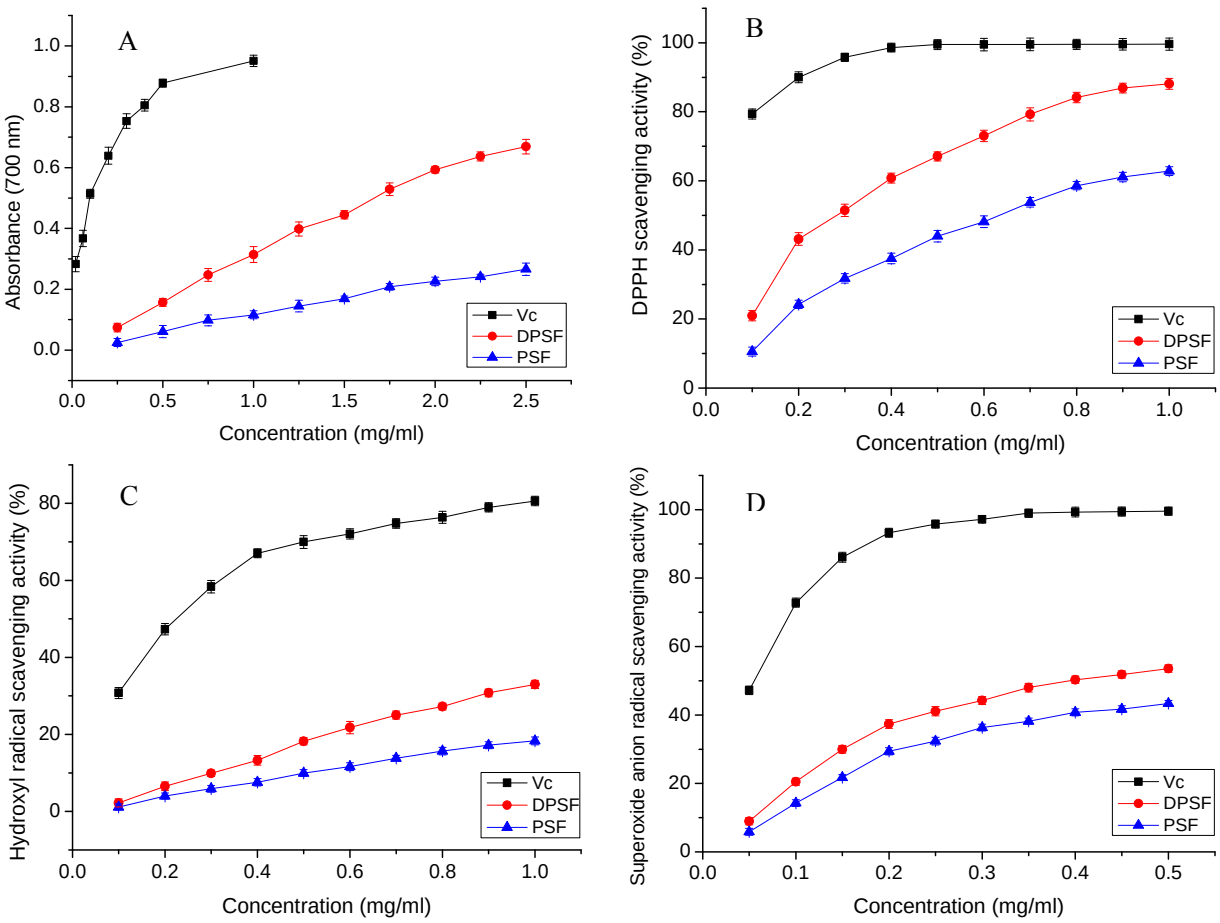

Fig. 3

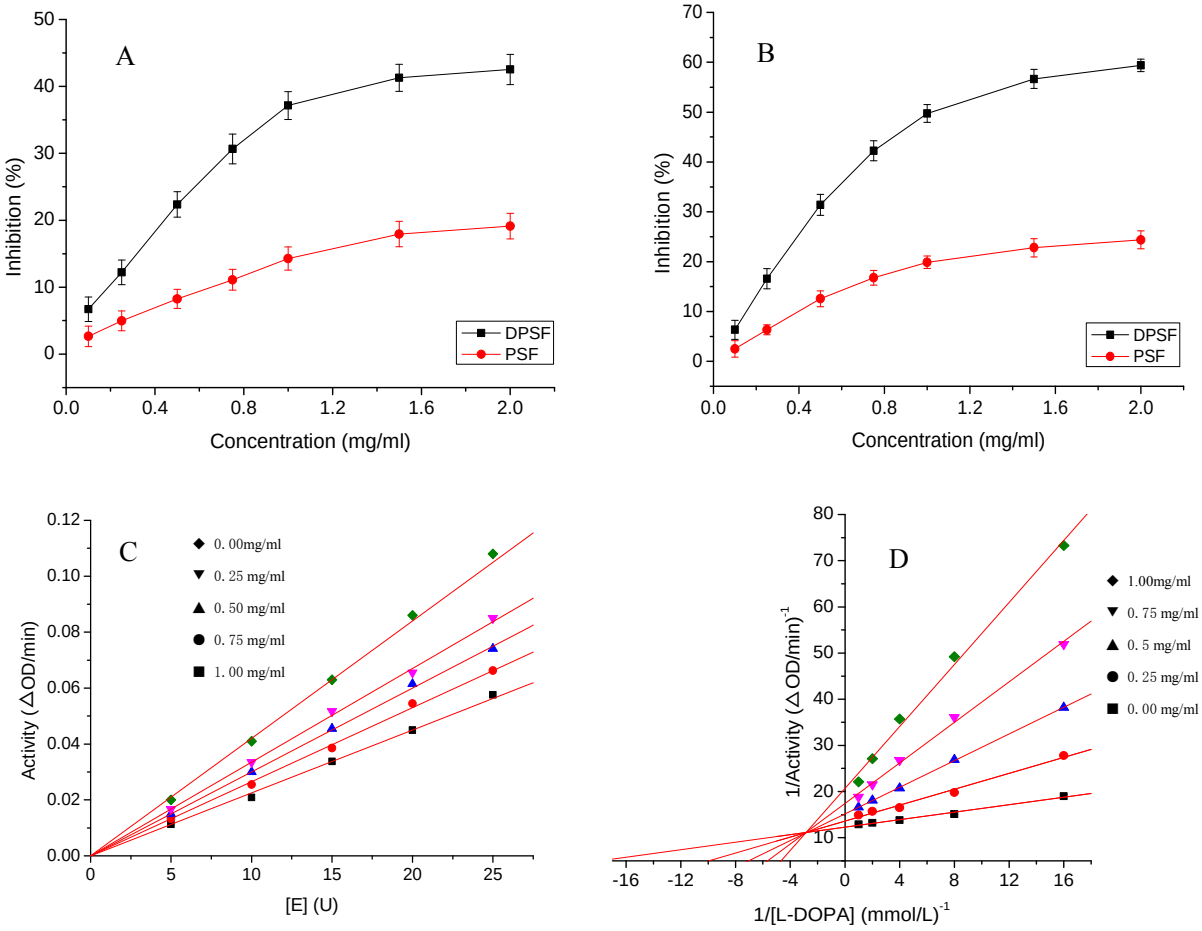

Table 1. Experimental design and results of the Box-Behnken design for optimization of the DPPH-scavenging activity of polysaccharide

No.	A (mM)	B (°C)	C (h)	DPPH-scavenging activity (%)
1	15	40	1	68.23
2	10	50	1	65.66
3	15	60	1	69.77
4	20	50	1	71.33
5	10	40	1.5	65.1
6	20	40	1.5	71.11
7	10	60	1.5	66.23
8	20	60	1.5	72.05
9	10	50	2	66.21
10	20	50	2	72.28
11	15	40	2	69.35
12	15	60	2	70.78
13	15	50	1	74.25
14	15	50	1	74.65
15	15	50	1	74.4
16	15	50	1	74.38
17	15	50	1	74.72

Note: A, B and C represent concentration of Vc-H_2O_2, reaction temperature and reaction time, respectively. DPPH-scavenging activity was measured when concentration of DPSF was 0.65 mg/mL.

Table 2. Analysis of variance for regression model of DPPH of polysaccharide

Source	SS	DF	MS	*F*-value	*P*-value
Model	181.76	9	20.20	459.46	<0.0001
A	69.44	1	69.44	1579.92	<0.0001
B	3.18	1	3.18	72.24	<0.0001
C	1.65	1	1.65	37.47	0.0005
AB	9.025E-003	1	9.025E-003	0.21	0.6642
AC	0.040	1	0.04	0.91	0.3719
BC	3.025E-003	1	3.025E-003	0.069	0.8006
A^2	44.75	1	44.75	1018.07	<0.0001
B^2	28.41	1	28.41	646.33	<0.0001
C^2	23.25	1	23.25	529.03	<0.0001
Residual	0.31	7	0.044		
Lack of fit	0.15	3	0.051	1.30	0.3898
Pure error	0.16	4	0.039		
Cor total	182.06	16			
R^2=0.9983; Adj R^2=0.9961; Pred R^2=0.9853; Adeq Precision=58.965; C.V.%=0.30					

Note: DF, degree of freedom; SS, sum of squares; MS, mean squares

Table 3. The chemical characteristics of polysaccharides.

Sample	Yield (%)	Total sugar (%)	Sulfate (%)	Protein (%)	Uronic acid (%)	Moisture (%)	Mw (kDa)
PSF	11.10	41.90	14.72	2.40	27.23	16.20	987
DPSF	90.10	54.88	16.38	1.60	20.15	17.86	407