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THE COMPARISON OF ANTIOXIDATIVE PROPERTIES OF SEAWEED OLIGOSACCHARIDES FERMENTED BY TWO LACTIC ACID BACTERIA

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THE COMPARISON OF ANTIOXIDATIVE PROPERTIES OF SEAWEED OLIGOSACCHARIDES FERMENTED BY TWO LACTIC ACID BACTERIA

Shao-Chi Wu*, Fu-Jin Wang**, and Chorng-Liang Pan***

Key words: agarases, seaweed oligosaccharides, lactic acid bacteria, antioxidative properties.

ABSTRACT

The aim of this study was to investigate if the antioxidant properties of lactic acid bacteria (LAB) fermentation products have the potential to be used in future food materials. Twenty four LAB fermentation products, derived from two LAB strains, *Enterococcus* (*Ent.*; formerly *Streptococcus*) *faecalis* BCRC13076 and/or *Lactobacillus* (*Lb.*) *rhamnosus* BCRC14068 fermented through four seaweed polysaccharide extracts (SwPSExts) from *Gelidium* sp., *Gracilaria* sp., *Monostroma* (*Mon.*) *nitidum*, and *Porphyra* (*Por.*) *dentate* or their seaweed-oligosaccharide-lysates (SwOSLys) source digested by agarases, had their antioxidative properties evaluated by six *in vitro* methods. Seaweed oligosaccharide LAB fermentation product (SwOS-LAFP) showed a significantly greater reducing power, an increased chelating effect upon ferrous ions, an increased inhibition effect upon the hemoglobin-catalyzed peroxidation of linoleic acid, and an increased scavenging capacity upon hydrogen peroxide. However, it showed a reduced activity upon DPPH radicals compared to the seaweed polysaccharide LAB fermentation product (SwPS-LAFP). In 24 LAB fermentation products only two exhibited a scavenging effect upon hydroxyl radicals. These findings demonstrate that SwPS-LAFP and SwOS-LAFP possess significant antioxidant activity and suggest that AgPS-LAFPs and AgOS-LAFSs may be considered among the more promising food components for preventing oxidative damage.

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I. INTRODUCTION

Oxidative stress has been reported to be the result of an imbalance that occurs when survival mechanisms are unable to deal adequately with reactive oxygen species (ROS) in the cells [10, 14, 48]. The interest in ROS in biology and medicine is obvious because of their strong relationship with phenomena such as Alzheimer's disease [16, 52], arthritis [30], cancer [35, 54], cardiovascular diseases [6, 31], diabetes [2, 8], tissue damage caused by ischemia-reperfusion [47], and Parkinson's disease [11, 61].

It has been observed that the production of reactive oxygen species (ROS) in algae is stimulated by various environmental stresses, such as high light levels, heavy metals, high salt concentrations, UV irradiation etc. Algae generally have higher antioxidative activity due to a higher content of various nonenzymatic antioxidant components, such as reduced glutathione (GSH), ascorbic acid, α-tocopherol, β-carotenoids, flavonoids, hydroquinones, phycocyanin, proline, mannitol, myoinositol, phenolics, and/or polyamines [15, 41, 51]. As a result, according to many studies in the past few years, algae have become good candidates as a source of natural antioxidants [17, 19, 23, 40, 63].

The ability of lactic acid bacteria to create the low oxidation-reduction potential needed for their optimum growth is probably related to some of these systems [25, 34]. Starter cultures with free radical scavenger properties would be useful in the food manufacturing industry. They could benefit the consumer by providing another dietary source of antioxidants, or by providing probiotic bacteria with the potential of producing antioxidants during growth in the intestinal tract [38, 44]. Some species of *Lactobacillus* (*Lb.*) and *Streptococcus* (*Strep.*) have been reported to produce antioxidative activity [12, 37]. The intensity of antioxidative activity varied among the different cultures in each study. Most of these studies based the results on their evaluation of cell-free extracts of the lactic acid bacteria.

Our previous studies tested various seaweed oligosaccharides that were produced by agarases digested seaweed polysaccharide extract which were then fermented by two LAB

strains, *Ent*. (formerly *Strep*.) *faecalis* BCRC13076 and *Lb*. *rhamnosus* BCRC14068 [56, 57]. Based on the fact that both algae and lactic acid bacteria have antioxidative capacities, the purpose of this study was to examine the effects of the antioxidative properties of the LAB fermentation product from seaweed oligosaccharides or polysaccharides by various antioxidative assays.

II. MATERIALS AND METHODS

1. Crude Enzyme Preparation

The preparation of two crude enzyme solutions of agarases from the strains *Aeromonas salmonicida* MAEF108 and *Pseudomonas vesicularis* MA103 were obtained according to the method used by Wu and Pan [55], and the assay of agarase activity was carried out according to the same reference.

2. Lactic Acid Bacteria (LAB) Starter

Two LAB strains, *Ent. faecalis* BCRC13076 and *Lb. rhamnosus* BCRC14068 were used as fermentation starter. They were obtained from the Bioresources Collection and Research Center (BCRC), Hsinchu, Taiwan. In the article of Wu *et al*. [56, 57], three LAB groups were carried out. These three LAB groups are: (1) *Ent. faecali*s BCRC13076, (2) *Lb. rhamnosus* BCRC14068, and (3) *Ent. faecalis* BCRC13076 and *Lb. rhamnosus* BCRC14068. Their abbreviations are: (1) LAB-BCRC13076, (2) LAB-BCRC14068, and (3) LAB-Comb, respectively.

3. Types of Seaweed Polysaccharide Extract (SwPSExt)

The seaweed polysaccharide extract (SwPSExt) of four seaweeds, *Gelidium* sp., *Gracilaria* sp., *Monostroma* (*Mon*.) *nitidum*, and *Porphyra* (*Por*.) *dentate* were prepared according to the protocol described in Wu and Pan [55]. The four SwPSExts from *Gelidium* sp., *Gracilaria* sp., *Mon. nitidum*, and *Por. dentate* are abbreviated as Gel, Gra, Mon, and Por, respectively. The seaweed extracts were composed of ash (4.4%-11.33%), protein (0.31%-4.13%), and the rest was carbohydrate (28.72%-49.25%) in the form of polysaccharide (data not shown).

4. Preparation of Seaweed Polysaccharide LAB Fermentation Solution (SwPS-LAFS)

According to the method used by Wu *et al*. [56, 57], 100 mL of 0.5% four SwPSExt (Gel, Gra, Mon, and Por) were placed in a 125 mL glass bottle with a screw cap, and 5 mL of a nitrogen source solution was added which contained 1.0 g of beef extract (Sigma, Chemical Co., St. Louis, MO, U.S.A.) and 0.5 g of yeast extract (Sigma). Then, LAB starter of three LAB groups as mentioned above were used for the seaweed polysaccharide fermentation by lactic acid bacteria until the pH value reached 4.4 to 4.6, which was taken as the terminal point of the fermentation. The seaweed polysaccharide solutions which were obtained from the 12 LAB fermentation samples are referred to as seaweed polysaccharide LAB fermentation solution (SwPS-LAFS). The fermentation solutions derived from Gel fermented with LAB-BCRC13076 is referred to as 13076-Gel-SwPS-LAFS in this article; all other 11 SwPS-LAFS are named by the same rule.

5. Preparation of Seaweed Oligosaccharides LAB Fermentation Solution (SwOS-LAFS)

According to the method used by Wu *et al*. [58], 105 mL of seaweed oligosaccharides solution (SwOSS) was obtained from four different seaweed types (Gel, Gra, Mon, and Por, respectively), and contained 5 mL nitrogen source solution composed of 1.0 g of beef extract and 0.5 g of yeast extract. This was placed in a 125 mL glass bottle with a screw cap. Then, LAB starter of three LAB groups as mentioned above were used for the fermentation of the SwOSS by lactic acid bacteria until the pH value reached 4.4 to 4.6, which was taken as the terminal point of the fermentation. The 12 LAB fermentation samples obtained from the LAB fermented SwOSS are referred to as the seaweed oligosaccharide LAB fermentation solution (SwOS-LAFS). The fermentation solution derived from Gel that fermented with LAB-BCRC13076 is called 13076-Gel-SwOS-LAFS in this article; all other 11 SwOS-LAFS are named using the same method.

6. Preparation of LAB Fermentation Product for Testing

First, the LAB fermentation solution of (A) SwPS-LAFS or (B) SwOS-LAFS were centrifuged at 10,000 × g at 4°C for 30 min using a high-speed refrigerated centrifuge (Himac CR 21, Hitachi, Tokyo, Japan). Then, the upper layer was placed into 50 mL centrifuge tubes (Hitachi, Tokyo, Japan) using 0.2 μm filter membrane (47 mm; Millex-GS, Millipore Corp., U.S.A.) to filter the LAB fermentation solution and obtain the sterile samples. Finally, the sterile LAB fermentation samples were named (a) seaweed polysaccharide LAB fermentation product (SwPS-LAFP) or (b) seaweed oligosaccharide LAB fermentation product (SwOS-LAFP). Due to the fact that the reducing sugar concentrations of the LAB fermentation product ranged from 5.2-19.2 mg/mL D-galactose (data not shown), the reducing sugar concentration of the LAB fermentation products were adjusted to 5 mg/mL. Finally, the sterilized LAB fermentation products were stored in a refrigerator (GR-B500A, LG, Seoul, Korea) at 4°C for determining their antioxidative effects.

7. Methods to Determine Anti-oxidative Effects

1) Reducing Power

The reducing power of the LAB fermentation products were measured by a method derived from a previous method [55, 58]. Each LAB fermentation product was mixed with 200 mM sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide (Sigma). This mixture was then incubated at 50°C for 20 min. Then, 10% trichloroacetic acid (w/v) (Panreac, Spain) was added, and the mixture was centrifuged. The

upper layer was mixed with de-ionized water and 0.1% ferric chloride (Merck, Germany), and the absorbance was determined at 700 nm. The higher the absorbance, the higher the reducing power; reducing power is expressed as: x μg/mL of ascorbic acid (AA).

2) α-Diphenyl-α-Picrylhydrazyl (DPPH) Assay

The DPPH free radical scavenging capacities of the LAB fermentation products were measured using the method reported previously [55, 58]. LAB fermentation products were mixed with 0.1 mM DPPH (Sigma) ethanol solution and 50 mM Tris-HCl buffer (pH 7.4) solution. Methanol (Panreac) was used as a control. After 30 min, the reduction of the DPPH free radicals was measured at 517 nm absorbance. As reported in Ahn *et al*. [1], the concentration of L-ascorbic acid (Sigma) at 1 mg/mL was used as a positive control. The inhibition ratio was calculated from Equation (1) as follows.

$$\% \text{ inhibition} = [(\text{absorbance of control} - \text{absorbance of test sample})/\text{absorbance of control}] \times 100. \quad (1)$$

3) Inhibition of the Hemoglobin-Catalyzed Peroxidation of Linoleic Acid

Inhibition of the hemoglobin-catalyzed peroxidation of linoleic acid was determined after the method of Kuo *et al*. [26]. The LAB fermentation products (20 μL) were mixed with 0.3 mL of 0.05 M phosphate buffer (pH 7.0) containing 0.04% Tween 20 (Sigma) and 20 μL of 100 mM linoleic acid (Sigma). They were then equilibrated at 37°C for 3 min. The peroxidation of linoleic acid in the above reaction mixture was initiated by adding 50 μL of 0.003% hemoglobin (Sigma) in water, followed by incubation at 37°C for 10 min, and then stopped by adding 5 mL of 0.6% HCl (in ethanol). The thiocyanate (FTC) method was then used to quantify the peroxidation of linoleic acid, in the order of 0.1 mL of $FeCl_2$ solution (0.02 M) and 0.1 mL of ammonium thiocyanate (Panreac) solution (30%) were added to 1.6 mL of the sample solution at 37°C for 5 min. The absorbance of the mixture was determined at 480 nm. As reported in Rupérez *et al*. [43] the concentration at 1 mg/mL of Trolox (Acros Organics N. V., Geel, Belgium) was used as a positive control. The inhibition effect (percent) was calculated according to Equation (1).

4) Chelating Effect on Ferrous Ions

The chelating effect on ferrous ions was determined according to a previous method [55, 58]. The LAB fermentation products were mixed with methanol and 400 μM $FeCl_2$ (Merck), followed by the addition of 2 mM ferrozine (Sigma). After 10 min the absorbance of the mixture was determined at 562 nm. Trolox was used as a positive control [43]. The chelating effect (%) was calculated by Equation (1).

5) Scavenging Capacity of Hydrogen Peroxide (H_2O_2)

The scavenging capacity of H_2O_2 was measured using a previous method [55, 58]. The LAB fermentation products were added to 5 mM H_2O_2 (Showa, Tokyo, Japan). After preincubation for 20 min, HRPase-phenol red in a 0.1 M phosphate buffer was added. After 10 min the absorbance at 610 nm was measured. The method reported by Yen *et al*. [60] was followed, using 1 mg/mL L-ascorbic acid (Sigma) as the positive control. The scavenging capacity (%) was calculated in a manner similar to Equation (1).

6) Hydroxyl Radicals Scavenging Effect

The scavenging effect of hydroxyl radicals was measured using the method of Halliwell *et al*. [21], and the results depended on the effect of deoxyribose degradation catalyzed by Fe(III)-EDTA. Then 0.1 mL of 28 mM deoxyribose (Sigma), 0.4 mL of 40 mM phosphate buffer (pH 7.4), 0.1 mL of 1 mM $FeCl_3 \cdot 6H_2O$ (Merck), 0.1 mL of 1.04 mM EDTA (Panreac), 0.1 mL of 10 mM H_2O_2 (Showa), 0.1 mL of 1 mM L-ascorbic acid (Sigma), and 0.1 mL of the LAB fermentation products were added together in test tubes, and mixed well. The reaction was carried out at 37°C in a water bath for 1 h. Next, 0.5 mL of 1% 2-Thiobarbituric acid (Sigma) and 0.5 mL of 2.8% Trichloroacetic acid (Panreac) were added to each of these test tubes. The mixture was placed at 100°C in a hot water bath for 10 min, and then cooled to room temperature. The mixture was centrifuged at 1,630 × g for 10 min, using a low-speed refrigerated centrifuge (CF 7D2, Hitachi). The upper layer was taken to measure the absorbance at 532 nm. D-mannitol (Sigma) was used as a positive control [43]. The scavenging effect (%) was calculated by Equation (1).

8. Statistical Analysis

All antioxidative results from the LAB fermentation products are expressed as mean ± SD (n = 3). Data were analyzed by one-way analysis of variance (ANOVA). When the ANOVA identified differences among groups, multiple comparisons among means were made using Duncan's new multiple range test. Statistical significance was determined by setting the aggregate type I error at 5% ($p < 0.05$) for each set of comparisons, using the Statistical Analysis System software package [46].

III. RESULTS AND DISCUSSION

1. Reducing Power

Some studies reported that the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [9, 22, 29]. The results of the reducing power of (A) 12 SwPS-LAFP which is derived from SwPS-LAFS or (B) 12 SwOS-LAFP which is derived from SwOS-LAFS fermented by LAB BCRC13076 and/or BCRC14068 are shown in Table 1. The reducing power of the 24 LAB fermentation products was in the range of 10.1 ± 0.4 (14068-Por-SwPS-LAFP) to 27.0 ± 1.3 (13076-Gel-SwOS-LAFP) μg/mL of ascorbic acid (AA), and SwOS-LAFPs were better than SwPS-LAFPs for the same seaweed type. In addition, the

Table 1. Reducing power of (A) 12 SwPS-LAFP which is derived from SwPS-LAFS or (B) 12 SwOS-LAFP derived from SwOS-LAFS fermented with LAB BCRC13076 and/or BCRC14068.

Strain	Fermentation product	Equal to x μg/mL ascorbic acid			
		Gel***	Gra	Mon	Por
13076	SwPS-LAFP**	$16.1 \pm 0.4^{c\,[B]}$	$15.1 \pm 0.7^{c\,[C]}$	$17.4 \pm 0.4^{c\,[A]}$	$14.6 \pm 0.4^{c\,[C]}$
	SwOS-LAFP	$27.0 \pm 1.3^{a\,[A]}$	$18.2 \pm 0.7^{b\,[C]}$	$24.4 \pm 0.5^{a\,[B]}$	$26.9 \pm 0.5^{a\,[A]}$
14068	SwPS-LAFP	$14.3 \pm 0.9^{d\,[A]}$	$14.3 \pm 0.9^{c\,[A]}$	$14.7 \pm 0.6^{d\,[A]}$	$10.1 \pm 0.4^{e\,[B]}$
	SwOS-LAFP	$25.9 \pm 0.4^{ab\,[A]}$	$25.4 \pm 2.1^{a\,[A]}$	$21.1 \pm 0.5^{b\,[B]}$	$25.2 \pm 0.5^{b\,[A]}$
Comb	SwPS-LAFP	$16.6 \pm 0.3^{c\,[A]}$	$14.8 \pm 0.1^{c\,[B]}$	$17.1 \pm 0.4^{c\,[A]}$	$12.6 \pm 0.2^{d\,[C]}$
	SwOS-LAFP	$25.0 \pm 0.6^{b\,[B]}$	$24.5 \pm 0.7^{a\,[B]}$	$24.1 \pm 0.4^{a\,[B]}$	$26.4 \pm 0.5^{a\,[A]}$

* Data are mean values of triplicate determinations ± standard deviation. Different superscript small letters in the same column (vertical comparison) or capital letters in the same row (horizontal comparison) of brackets indicate significantly different values ($p < 0.05$).
** SwPS-LAFP, seaweed polysaccharide LAB fermentation product; SwOS-LAFP, seaweed oligosaccharide LAB fermentation product.
*** Gel, Gra, Mon, and Por represent SwPSExt are derived from *Gelidium* sp., *Gracilaria* sp., *Mon. nitidum*, and *Por. dentate*, respectively.

Table 2. DPPH radical scavenging effect (%) of (A) 12 SwPS-LAFP which is derived from SwPS-LAFS or (B) 12 SwOS-LAFP derived from SwOS-LAFS fermented with LAB BCRC13076 and/or BCRC14068.

Strain	Fermentation product	Scavenging effect (%)			
		Gel***	Gra	Mon	Por
13076	SwPS-LAFP**	$57.1 \pm 1.2^{d\,[B]}$	$61.5 \pm 1.8^{c\,[A]}$	$57.0 \pm 2.0^{c\,[B]}$	$63.6 \pm 0.2^{d\,[A]}$
	SwOS-LAFP	$36.2 \pm 2.2^{e\,[C]}$	$42.9 \pm 0.5^{e\,[B]}$	$44.2 \pm 1.9^{d\,[B]}$	$55.3 \pm 1.9^{e\,[A]}$
14068	SwPS-LAFP	$65.9 \pm 0.7^{b\,[D]}$	$71.5 \pm 1.2^{a\,[C]}$	$73.8 \pm 0.3^{a\,[B]}$	$79.6 \pm 1.0^{a\,[A]}$
	SwOS-LAFP	$63.7 \pm 0.6^{bc\,[B]}$	$55.9 \pm 1.6^{d\,[C]}$	$72.5 \pm 1.5^{a\,[A]}$	$75.0 \pm 1.8^{b\,[A]}$
Comb	SwPS-LAFP	$69.6 \pm 1.4^{a\,[B]}$	$68.7 \pm 0.5^{b\,[B]}$	$64.5 \pm 0.9^{b\,[C]}$	$72.5 \pm 2.1^{bc\,[A]}$
	SwOS-LAFP	$62.2 \pm 1.0^{c\,[B]}$	$54.7 \pm 0.7^{d\,[C]}$	$55.9 \pm 0.7^{c\,[C]}$	$70.6 \pm 1.8^{c\,[A]}$

* Data are mean values of triplicate determinations ± standard deviation. Different superscript small letters in the same column (vertical comparison) or capital letters in the same row (horizontal comparison) of brackets indicate significantly different values ($p < 0.05$).
** SwPS-LAFP, seaweed polysaccharide LAB fermentation product; SwOS-LAFP, seaweed oligosaccharide LAB fermentation product.
*** Gel, Gra, Mon, and Por represent SwPSExt are derived from *Gelidium* sp., *Gracilaria* sp., *Mon. nitidum*, and *Por. dentate*, respectively.

reducing power of SwPS-LAFP and SwOS-LAFP which were fermented by LAB-BCRC13076, LAB-BCRC14068, or LAB-Comb, were as follows: (i) 13076-Por-SwOS-LAFP was 1.8-fold higher than 13076-Por-SwPS-LAFP; (ii) 14068-Por-SwOS-LAFP was 2.5-fold higher than 14068-Por-SwPS-LAFP; and (iii) com-Por-SwOS-LAFP was 2.1-fold higher than Com-Por-SwPS-LAFP, respectively.

Thus, it appears necessary to determine the reducing power of SwPS-LAFP or SwOS-LAFP to elucidate the relationship between their antioxidant effect and their reducing power. In our previous study, the SwPSExts or SwOSLys which was the raw material for SwPS-LAFP or SwOS-LAFP also showed antioxidative properties and reducing power. The reducing power of SwPS-LAFPs was greater than that of SwPSExts, except for SwPSExt from *Por. dentate*. However, it appears that an increase of either SwOS-LAFP or SwOSLys had no relationship with the reducing power. Several workers have found that the reducing power of fermented milk was significantly greater than milk and suggested that certain metabolites, such as glutathione, that demonstrate superior reducing power might be produced during kefir fermentation and that they could possibly react with free radicals to stabilize and terminate radical chain reactions [20, 28, 39].

2. α-Diphenyl-α-Picrylhydrazyl (DPPH) Assay

Proton radical-scavenging has been reported to be an important mechanism for antioxidation. The decrease in absorbance of DPPH radicals is caused by antioxidants through the reaction between antioxidant molecules and radicals, resulting in the scavenging of the radicals by hydrogen donation. This is visually noticeable as a discoloration from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the activity of antioxidants [4]. The reduction in the concentration of the DPPH allows monitoring the decrease in absorbance at a characteristic wavelength when proton-radical scavengers are encountered [59]. The scavenging effects (%) on DPPH radicals of (A) 12 SwPS-LAFP which are derived from SwPS-LAFS or (B) 12 SwOS-LAFP which are derived from SwOS-LAFS fermented by LAB BCRC13076 and/or BCRC14068 are shown in Table 2. The scavenging effects

Table 3. Ferrous ion chelating effect (%) of (A) 12 SwPS-LAFP which is derived from SwPS-LAFS or (B) 12 SwOS-LAFP derived from SwOS-LAFS fermented with LAB BCRC13076 and/or BCRC14068.

Strain	Fermentation product	Chelating effect (%)			
		Gel***	Gra	Mon	Por
13076	SwPS-LAFP**	$40.4 \pm 2.9^{d\,[B]}$	$41.0 \pm 2.0^{d\,[B]}$	$75.2 \pm 2.2^{b\,[A]}$	$36.1 \pm 1.4^{d\,[C]}$
	SwOS-LAFP	$74.9 \pm 1.9^{b\,[B]}$	$73.2 \pm 1.5^{b\,[B]}$	$82.1 \pm 1.2^{a\,[A]}$	$66.4 \pm 0.8^{c\,[C]}$
14068	SwPS-LAFP	$20.6 \pm 1.4^{e\,[D]}$	$40.0 \pm 0.5^{d\,[A]}$	$30.6 \pm 1.9^{d\,[C]}$	$36.8 \pm 1.1^{d\,[B]}$
	SwOS-LAFP	$56.2 \pm 1.1^{c\,[C]}$	$56.1 \pm 0.9^{c\,[C]}$	$82.1 \pm 0.2^{a\,[A]}$	$78.3 \pm 0.7^{b\,[B]}$
Comb	SwPS-LAFP	$41.2 \pm 1.3^{d\,[AB]}$	$24.0 \pm 1.9^{e\,[C]}$	$43.1 \pm 2.9^{c\,[A]}$	$37.7 \pm 0.4^{d\,[B]}$
	SwOS-LAFP	$78.5 \pm 0.2^{a\,[C]}$	$78.3 \pm 0.3^{a\,[C]}$	$84.4 \pm 0.3^{a\,[A]}$	$83.0 \pm 0.6^{a\,[B]}$

* Data are mean values of triplicate determinations ± standard deviation. Different superscript small letters in the same column (vertical comparison) or capital letters in the same row (horizontal comparison) of brackets indicate significantly different values ($p < 0.05$).

** SwPS-LAFP, seaweed polysaccharide LAB fermentation product; SwOS-LAFP, seaweed oligosaccharide LAB fermentation product.

*** Gel, Gra, Mon, and Por represent SwPSExt are derived from *Gelidium* sp., *Gracilaria* sp., *Mon. nitidum*, and *Por. dentate*, respectively.

(%) on DPPH radicals of the 24 LAB fermentation products were in the range of 36.2 ± 2.2 (13076-Gel-SwOS-LAFP) to 79.6 ± 1.0 (14068-Por-SwPS-LAFP), and their scavenging effects (%) on DPPH radicals of SwOS-LAFPs were smaller than SwPS-LAFPs for the same seaweed type. In addition, when comparing SwPS-LAFP and SwOS-LAFP, which were fermented by LAB-BCRC13076, LAB-BCRC14068, or LAB-Comb, the results were as follows (i) 13076-Gra-SwOS-LAFP or 13076-Por-SwOS-LAFP were 0.6-fold lower than 13076-Gra-SwPS-LAFP or 13076-Por-SwPS-LAFP; (ii) 14068-Gel-SwOS-LAFP or 14068-Mon-SwOS-LAFP were 1.0-fold lower than 14068-Gel-SwPS-LAFP or 14068-Mon-SwPS-LAFP; and (iii) com-Por-SwOS-LAFP was 1.0-fold lower than Com-Por-SwPS-LAFP, respectively.

In the present study, the antioxidative capability of 12 SwOS-LAFP on DPPH radical-scavenging activity was smaller than that of SwPS-LAFP. Many investigators explain decreasing of DPPH radical-scavenging activity, such as McCuea and Shetty [32, 33] believed that the degradation of polymeric phenolic structures after being fermented by microorganisms may explain the increase in soluble phenolic content thereby decreasing antioxidant activity. Rohn *et al*. [42] prove the covalent attachment of quercetin to bovine serum albumin decreases the total antioxidant activity in comparison to an equivalent amount of free quercetin depending on the degree of derivatization. Kevers *et al*. [24] explain antioxidant capacity (DPPH) decrease drastically during storage that reserve at low temperature, cause by phenol content reduce.

3. Chelating Effect on Ferrous Ions

Iron, the most abundant transition-metal ion in the human body, may work as a catalyst for the generation of reactive oxygen species under pathological conditions [50]. In the presence of low concentrations of transition-metal ions, hydroxyl radicals are formed from hydrogen peroxide via the Fenton reaction [3]. Table 3 shows the test results of (A) 12 SwPS-LAFP which are derived from SwPS-LAFS or (B) 12 SwOS-LAFP which are derived from SwOS-LAFS fermented by LAB BCRC13076 and/or BCRC14068, on the chelating effect on ferrous ions (%). The results of the chelating capacity on ferrous ions on the 24 LAB fermentation products was in the range of 24.0 ± 1.9 (Comb-Gra-SwPS-LAFP) to 84.4 ± 0.3 (Comb-Mon-SwOS-LAFP), and their chelating capacity on ferrous ions of SwOS-LAFPs were better than SwPS-LAFPs for the same seaweed type. In addition, when comparing SwPS-LAFP and SwOS-LAFP, which were fermented by LAB-BCRC13076, LAB-BCRC14068, or LAB-Comb, the results of (i) 13076-Gel-SwOS-LAFP was 1.9-fold higher than 13076-Gel-SwPS-LAFP; (ii) 14068-Gel-SwOS-LAFP or 14068-Mon-SwOS-LAFP were 2.7-fold higher than 14068-Gel-SwPS-LAFP or 14068-Mon-SwPS-LAFP; and (iii) Comb-Gra-SwOS-LAFP was 3.6 fold higher than Comb-Gra-SwPS-LAFP.

Because SwPS-LAFPs or SwOS-LAFPs that used beef extract and yeast extract as a nitrogen source were fermented by LAB, the protein contents in the beef extract and yeast extract could bind ferric or ferrous ions. Liu *et al*. [28] also pointed out the ability of milk proteins to bind ferric or ferrous ions, e.g., lactoferrin, serum albumin, casein, and a high molecular-weight fraction of whey, all of which have been reported to demonstrate some iron-chelating activity.

4. Inhibition of the Hemoglobin-Catalyzed Peroxidation of Linoleic Acid

Lipid peroxidation is a process by which polyunsaturated fatty acids present in cell membranes are broken down into various products such as hydrocarbon gases, cytotoxic aldehydes and free oxygen radicals [18]. Therefore, the inhibition of lipid peroxidation is of great importance to the prevention of certain human disease processes involving free radicals. Table 4 shows the inhibition effects (%) on the hemoglobin-catalyzed peroxidation of linoleic acid of (A) 12 SwPS-LAFP which is derived from SwPS-LAFS or (B) 12 SwOS-LAFP derived from SwOS-LAFS fermented by LAB BCRC13076 and/or BCRC14068. The hemoglobin catalyzed peroxidation of linoleic acid inhibition of the 24 LAB fermentation products was in the range of 7.9 ± 5.5 (14068-Gra-SwPS-LAFP)

Table 4. Inhibition (%) of the hemoglobin-catalyzed peroxidation of linoleic acid of (A) 12 SwPS-LAFP which is derived from SwPS-LAFS or (B) 12 SwOS-LAFP derived from SwOS-LAFS fermented with LAB BCRC13076 and/or BCRC14068.

Strain	Fermentation product	Inhibition (%)			
		Gel***	Gra	Mon	Por
13076	SwPS-LAFP**	$18.9 \pm 4.0^{c\,[B]}$	$36.6 \pm 0.0^{c\,[A]}$	$12.6 \pm 4.5^{d\,[C]}$	$18.9 \pm 1.0^{e\,[B]}$
	SwOS-LAFP	$87.1 \pm 3.1^{a\,[A]}$	$53.1 \pm 8.0^{b\,[C]}$	$64.7 \pm 4.1^{a\,[B]}$	$73.2 \pm 4.8^{b\,[B]}$
14068	SwPS-LAFP	$19.0 \pm 4.8^{c\,[A]}$	$7.9 \pm 5.5^{d\,[B]}$	$17.5 \pm 2.7^{cd\,[A]}$	$22.2 \pm 2.7^{e\,[A]}$
	SwOS-LAFP	$24.2 \pm 6.9^{bc\,[C]}$	$33.3 \pm 2.0^{c\,[C]}$	$45.8 \pm 7.9^{b\,[B]}$	$63.4 \pm 2.3^{c\,[A]}$
Comb	SwPS-LAFP	$32.9 \pm 9.7^{b\,[AB]}$	$35.4 \pm 5.7^{c\,[AB]}$	$24.8 \pm 2.8^{c\,[B]}$	$40.4 \pm 4.9^{d\,[A]}$
	SwOS-LAFP	$77.5 \pm 1.3^{a\,[BC]}$	$84.8 \pm 3.8^{a\,[B]}$	$73.2 \pm 7.6^{a\,[C]}$	$93.5 \pm 2.2^{a\,[A]}$

* Data are mean values of triplicate determinations ± standard deviation. Different superscript small letters in the same column (vertical comparison) or capital letters in the same row (horizontal comparison) of brackets indicate significantly different values ($p < 0.05$).

** SwPS-LAFP, seaweed polysaccharide LAB fermentation product; SwOS-LAFP, seaweed oligosaccharide LAB fermentation product.

*** Gel, Gra, Mon, and Por represent SwPSExt are derived from *Gelidium* sp., *Gracilaria* sp., *Mon. nitidum*, and *Por. dentate*, respectively.

to 93.5 ± 2.2 (Comb-Por-SwOS-LAFP), and their inhibition effects on the hemoglobin-catalyzed peroxidation of linoleic acid of SwOS-LAFPs were better than SwPS-LAFPs for the same seaweed type. In addition, when comparing SwPS-LAFP and SwOS-LAFP which were fermented by LAB-BCRC13076, LAB-BCRC14068, or LAB-Comb, the results of (i) 13076-Gel-SwOS-LAFP was 4.6-fold higher than 13076-Gel-SwPS-LAFP; (ii) 14068-Gra-SwOS-LAFP was 4.2-fold higher than 14068-Gra-SwPS-LAFP; and (iii) Comb-Mon-SwOS-LAFP was 2.9-fold higher than Comb-Mon-SwPS-LAFP, respectively.

Terahara *et al*. [49] continued research also showed that ether extracts from two yogurt starters, *Strep. thermophilus* 1131 and *Lb. delbrueckii* subsp. *bulgaricus* 2308, could inhibit the oxidation of erythrocyte membranes *in vitro* and low-density lipoproteins *in vivo*. Zommara *et al*. [62] investigated the effect of whey from bovine skim milk fermented with bifidobacteria and found that lactic acid bacteria had a specific effect on oxidative stress. Liu *et al*. [28] found that the inhibitory effect upon linoleic acid peroxidation of milk-kefir and soymilk-kefir were significantly greater than those of milk and soymilk after being incubated for 32 h. Pena-Ramos and Xiong [36], and Wong and Kitts [53] reported that proteins derived from dairy products, or peptides derived from milk protein hydrolysates inhibited lipid oxidation. This suggested that the specific amino acid residue side-chain groups or the specific peptide structure of the antioxidative peptides may be attributable to the chelation of prooxidative metal ions and the termination of the radical chain reactions. Sakanaka *et al*. [45] reported that peptides hydrolysates from casein calcium have a strong effect on the discoloration of β-carotene when analyzed for their antioxidant activity by the β-carotene bleaching assay. In our data (not shown) the soluble protein content of SwPS-LAFPs or SwOS-LAFPs was greater than that of their SwPSExts or SwOSSs. Thus, we suggest that the peptides derived from beef extract and yeast extract proteins might contribute to the inhibitory effects of SwPS-LAFPs or SwOS-LAFPs upon lipid peroxidation.

5. Hydrogen Peroxide (H_2O_2) Scavenging Capacity and Hydroxyl Radicals Scavenging Effect

It is well known that the O_2 molecule has low reactivity and that its toxicity stems mainly from its excited state (singlet oxygen) or from its semi-reduced radical forms that can cause deleterious or lethal oxidative damage to the cells [13]. The four electron reduction of O_2 to H_2O gives rise successively to the formation of the reactive oxygen intermediates superoxide radical anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$). Several models have been proposed to explain the mechanism of O_2 sensitivity in microorganisms. $O_2^{\cdot-}$ and H_2O_2 are moderately reactive in aqueous solutions, but they both contribute to the formation of the highly reactive oxidant $HO^{\bullet}$ via the Fenton and Haber-Weiss reactions. In bacteria, generation of the $HO^{\bullet}$ radical is facilitated by the presence of $O_2^{\cdot-}$, H_2O_2 and free iron. Cellular components such as hemoproteins, lipids and DNA are targets for $HO^{\bullet}$ forming a site-specific Fenton-driven mechanism. Therefore, its removal is required to ensure cellular homeostasis [5].

The H_2O_2 scavenging capacity (%) of (A) 12 SwPS-LAFP which is derived from SwPS-LAFS or (B) 12 SwOS-LAFP which is derived from SwOS-LAFS fermented by LAB BCRC13076 and/or BCRC14068 are shown in Table 5. The H_2O_2 scavenging capacity of the 24 LAB fermentation products was found to be in the range of 37.3 ± 0.9 (Comb-Gel-SwPS-LAFP) to 76.0 ± 2.0 (14068-Mon-SwOS-LAFP), and their chelating capacity on ferrous ions of SwOS-LAFPs were better than SwPS-LAFPs for the same seaweed type. In addition, when comparing SwPS-LAFP and SwOS-LAFP which were fermented by LAB-BCRC13076, LAB-BCRC14068, or LAB-Comb, the results were as follows. (i) 13076-Por-SwOS-LAFP was 1.1-fold higher than 13076-Por-SwPS-LAFP; (ii) 14068-Mon-SwOS-LAFP was 1.4-fold higher than 14068-Mon-SwPS-LAFP; and (iii) Comb-Gel-SwOS-LAFP was 1.6 fold higher than Comb-Gel-SwPS-LAFP, respectively. The hydroxyl radicals scavenging capacity (%) of (A) 12 SwPS-LAFP which is derived from SwPS-LAFS or (B) 12 SwOS-

Table 5. Hydrogen peroxide scavenging effect (%) of (A) 12 SwPS-LAFP which is derived from SwPS-LAFS or (B) 12 SwOS-LAFP which is derived from SwOS-LAFS fermented with LAB BCRC13076 and/or BCRC14068.

Strain	Fermentation product	Scavenging effect (%)			
		Gel***	Gra	Mon	Por
13076	SwPS-LAFP**	$62.3 \pm 0.2^{c\,[B]}$	$56.2 \pm 1.0^{d\,[D]}$	$66.4 \pm 0.4^{c\,[A]}$	$57.9 \pm 0.1^{d\,[C]}$
	SwOS-LAFP	$68.5 \pm 0.2^{a\,[B]}$	$63.8 \pm 0.6^{a\,[D]}$	$69.6 \pm 0.1^{b\,[A]}$	$65.8 \pm 0.6^{c\,[C]}$
14068	SwPS-LAFP	$57.2 \pm 0.9^{e\,[B]}$	$57.1 \pm 0.1^{cd\,[B]}$	$55.7 \pm 0.4^{d\,[C]}$	$67.5 \pm 0.5^{b\,[A]}$
	SwOS-LAFP	$64.9 \pm 0.2^{b\,[C]}$	$60.7 \pm 1.3^{b\,[D]}$	$76.0 \pm 2.0^{a\,[A]}$	$72.3 \pm 0.6^{a\,[B]}$
Comb	SwPS-LAFP	$37.3 \pm 0.9^{f\,[D]}$	$46.7 \pm 0.7^{e\,[B]}$	$50.9 \pm 0.3^{e\,[A]}$	$45.0 \pm 1.0^{e\,[C]}$
	SwOS-LAFP	$60.5 \pm 0.3^{d\,[B]}$	$57.8 \pm 0.3^{c\,[C]}$	$68.7 \pm 0.5^{b\,[A]}$	$57.9 \pm 0.4^{d\,[C]}$

* Data are mean values of triplicate determinations ± standard deviation. Different superscript small letters in the same column (vertical comparison) or capital letters in the same row (horizontal comparison) of brackets indicate significantly different values ($p < 0.05$).
** SwPS-LAFP, seaweed polysaccharide LAB fermentation product; SwOS-LAFP, seaweed oligosaccharide LAB fermentation product.
*** Gel, Gra, Mon, and Por represent SwPSExt are derived from *Gelidium* sp., *Gracilaria* sp., *Mon. nitidum*, and *Por. dentate*, respectively.

Table 6. Hydroxyl radicals scavenging effect (%) of (A) 12 SwPS-LAFP which is derived from SwPS-LAFS or (B) 12 SwOS-LAFP derived from SwOS-LAFS fermented with LAB BCRC13076 and/or BCRC14068.

Strain	Fermentation product	Scavenging effect (%)			
		Gel***	Gra	Mon	Por
13076	SwPS-LAFP**	-	-	-	-
	SwOS-LAFP	-	-	-	25.6 ± 4.4
14068	SwPS-LAFP	-	-	-	-
	SwOS-LAFP	-	-	-	-
Comb	SwPS-LAFP	-	-	-	-
	SwOS-LAFP	-	-	18.2 ± 12.0	-

* Data are mean values of triplicate determinations ± standard deviation. Different superscript small letters in the same column (vertical comparison) or capital letters in the same row (horizontal comparison) of brackets indicate significantly different values ($p < 0.05$).
** SwPS-LAFP, seaweed polysaccharide LAB fermentation product; SwOS-LAFP, seaweed oligosaccharide LAB fermentation product.
*** Gel, Gra, Mon, and Por represent SwPSExt are derived from *Gelidium* sp., *Gracilaria* sp., *Mon. nitidum*, and *Por. dentate*, respectively.

LAFP which is derived from SwOS-LAFS fermented by LAB BCRC13076 and/or BCRC14068 are shown in Table 6. The hydroxyl radicals scavenging capacity, except for 13076-Por-SwOS-LAFP and Comb-Mon-SwOS-LAFP was 25.6 ± 4.4 and 18.2 ± 12.0, the other 22 LAB fermentation products had no hydroxyl radica.

Lin and Yen [27] demonstrated that *Lb. delbrueckii* ssp. *bulgaricus* Lb possesses the highest hydroxyl radical scavenging ability at 234 mM, and *Strep. thermophilus* MC and 821 and *Lb. delbrueckii* ssp. *bulgaricus* 448 and 449 scavenged the most hydrogen peroxide at approximately 50 mM in 11 tested LAB strains. Sakanaka *et al.* [45] reported that peptides hydrolysates from casein calcium showed hydroxyl radical-scavenging activity, and that this activity increased with the increase in concentration of casein calcium peptides. A 0.5% concentration of casein calcium peptides exhibited 78.4% scavenging activity. Hence, the peptides derived from beef extract and yeast extract proteins might also contribute to the scavenging capacity of SwPS-LAFPs or SwOS-LAFPs upon hydrogen peroxide.

In our previously study [57], we described that seaweed oligosaccharide solution could be fermented by LAB, and that probably two different strategies could be used to metabolize lactose or galactose depending on their mode of transport, either via the phosphenolpyruvate-dependent phosphotransferase system (PEP-PTS) or via a permease system. LAB has a complex proteolytic system that together with other proteolytic enzymes convert proteins into peptides, and increasing with LAB fermentation time [7]. Beef extract and yeast extract as the nitrogen source also promote lactic acid fermentation. These fermentation conditions could produce many metabolism products such as peptide hydrolysates that contribute antioxidative properties of SwPS-LAFPs or SwOS-LAFPs. Therefore, proteins in SwPS-LAFPs or SwOS-LAFPs provide antioxidative abilities and have a chelating effect on ferrous ions. Moreover, peptide hydrolysates in SwPS-LAFPs or SwOS-LAFPs supply antioxidative abilities of reducing power, antioxidative abilities of inhibition to the hemoglobin-catalyzed peroxidation of linoleic acid, and H_2O_2 scavenging capacity. Therefore peptides and proteins contribute to the

mainly antioxidative capability of lactic acid fermentation products.

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