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Antioxidant Activity of Solubilized *Tetraselmis suecica* and *Chlorella ellipsoidea* by Enzymatic Digests

Seung-Hong Lee[1], Dong-Uk Chang[2], Bae-Jin Lee[2], and You-Jin Jeon[1†]

[1]*Faculty of Applied Marine Science, Jeju National University, Jeju 690-756, Korea*
[2]*Marine Bioprocess Co. Ltd., Pukyong National University, Busan 608-737, Korea*

Abstract

In this study, we focused on natural water-soluble antioxidants from *Tetraselmis suecica* (*T. suecica*) and *Chlorella ellipsoidea* (*C. ellipsoidea*). They were prepared by enzymatic digestion using five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamex, Alcalase, Flavourzyme, Neutrase, and Kojizyme), and the potential antioxidant activity of each was assessed. Most enzymatic digests from *T. suecica* had a higher radical scavenging activity than those from *C. ellipsoidea*. Among the enzymatic digests, Kojizyme digest from *T. suecica* exhibited the highest effect on DPPH radical scavenging. Viscozyme (30.2%) and Neutrase (34.6%) digests from *T. suecica* exhibited higher hydroxyl radical scavenging activity. Kojizyme digest from *T. suecica* (81.5%) had strong alkyl radical scavenging activity. Neutrase (61.9%) and Kojizyme (61.5%) digest from *T. suecica* possessed the highest effects on hydrogen peroxide scavenging. Among the tested samples, Neutrase (TN) and Kojizyme (TK) digests from *T. suecica* showed the highest antioxidant activity (DPPH, alkyl radical, hydrogen peroxide). Therefore, TN and TK digests were selected for use in the further experiments. Those digests showed enhanced cell viability against H_2O_2-induced oxidative damage, and relatively good hydrogen peroxide scavenging activity in an African green monkey kidney (Vero) cell line. These results suggested that an enzymatic digestion will be an effective way for the production of a potential water-soluble antioxidant from a microalgae, *T. suecica*.

Key words: *Tetraselmis suecica*, *Chlorella ellipsoidea*, enzymatic digest, water-soluble, antioxidant activity

INTRODUCTION

Reactive oxygen species (ROS) such as DPPH, hydroxyl radical and hydrogen peroxide are physiological metabolites formed during aerobic life as a result of the metabolism of oxygen. High ROS levels induce oxidative stress, which can result in a variety of pathological conditions, including cardiovascular disease, cancer, and aging (1,2).

Synthetic antioxidant supplements such as butylated hydroxyl anisole (BHA), butylated hydroxy tolouene (BHT), α-tocopherol and propyl gallate (PG) have been used in order to reduce oxidative damages in human body (3,4). However, it is suspected that those antioxidants are responsible for some side effects such as liver damage and carcinogenesis (5). Antioxidants are involved in the oxidation mechanism by scavenging free radicals, chelating catalytic metals and by acting as oxygen scavengers (6,7).

Natural products produce various antioxidative compounds to protect them from harmful effects of ROS (8). Although many studies regarding antioxidant effects from macroalgae are available, less attention has been paid for microalgae because of difficulties in the isolation and cultivation. Currently, microalgae are being paid more attention as nutraceutical and health food in the market. Microalgae are rich in proteins, carbohydrates, mineral, and diverse functional pigments. The proteins that could be used as health food or animal feed (9,10), carbohydrates that could be used as stabilizers and emulsifiers in food and bioactive materials have potential medicinal values (10-12). In addition, pigments that could be used as natural food dyes (10,13) and mineral (9). So it is thought microalgae would be very useful in functional foods.

Despite a variety of biological functions of microalgae, effective application of microalgae is still limited because of their inefficient cultivation and high production cost. Therefore, in this study, we selected two marine microalgae, *Tetraselmis suecica* and *Chlorella ellipsoidea* that can be massively cultivated and low production cost. Also, it has been reported that *Tetraselmis suecica* and *Chlorella ellipsoidea* exhibits antimicrobial activity and functional biomaterial (14,15).

†Corresponding author. E-mail: youjinj@jejunu.ac.kr
Phone: +82-64-754-3475, Fax: +82-64-756-3493

In our previous study (16), a novel extraction technique using digestive enzymes such as carbohydrases and proteases was employed in order to degrade algae tissues, thereby releasing a variety of bioactive compounds. These enzymes can convert water-insoluble microalgae into water-soluble materials. Enzymatic digests from algae possess water-solubility and safety, as this method does not adapt any organic solvent or other toxic chemicals. Additionally it has a high yield and high radical scavenging activity in comparison with organic extracts.

The objective of this study was to investigate the antioxidant properties of enzymatic digests using five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase) from *Tetraselmis suecica* and *Chlorella ellipsoidea* in order to understand the usefulness of these algae in food and pharmaceutical industry.

MATERIALS AND METHODS

Materials

Marine microalgae used in this study were *Tetraselmis suecica* and *Chlorella ellipsoidea* obtained from Marine bio process Co., Korea. The frozen samples was lyophilized and homogenized with a grinder before extraction. Carbohydrases such as Celluclast 1.5L FG (catalyzing the breakdown of cellulose into glucose, cellobiose and higher glucose polymers) and proteases such as Protamex (hydrolysis of food proteins) were obtained from Novo Co. (Novozyme Nordisk, Bagsvaerd, Danmark). 5,5-Dimethyl-1-pyrrolin N-oxide (DMPO), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), α-(4-pyridyl-1-oxide)-N-t-butylnitrone (4-POBN), DPPH (1,1-diphenyl-2-pricrylhydrazyl), peroxidase, ABTS (2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid), 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) and Hoechst 33342 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest grade available commercially.

Proximate composition

Proximate chemical composition of freeze-dried samples was determined according to the AOAC methods (17). Crude lipid content was determined by Soxhlet method and crude protein content was determined by Kjeldhal method. Ash content was determined by calcinations in furnace at 550°C, the moisture content was determined by keeping in a dry oven at 105°C for 24 hr and Crude carbohydrate content was determined by phenol-sulfuric acid reaction (absorbance at 480 nm, using glucose as the calibration standard)

Preparation enzymatic digests

The preparation of enzymatic digests followed the method previously reported (16). One gram of the ground dried samples powder was homogenized with water (100 mL), and then 100 mg or 100 μL enzyme was added. The pHs of the homogenate were adjusted to its optimal pH value before the digestion. The enzymatic hydrolytic reactions were performed for 24 hr to achieve optimum degree of the hydrolysis. As soon as the enzymatic reaction is completed, the digests were boiled for 10 min at 100°C to inactivate the enzyme. Each sample was clarified by centrifugation (3000 rpm, for 20 min at 4°C) to remove the residue. All the samples were kept in -20°C for further experiments.

Determination of total polyphenolic content

Total polyphenolic compounds in the digests were determined with Folin-Ciocalteu reagent according to the method of Chandler and Dodds (18) using gallic acid as a standard phenolic compound. Sample (1 mL) was mixed with 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 mL of 5% Na_2CO_3 was added. After mixing thoroughly, the mixture was placed in the dark for 1 hr then absorbance was measured at 725 nm.

Radical scavenging assay using an ESR spectrometer

DPPH radical scavenging activity was measured using the method described by Nanjo et al. (19). Methanol solution of 60 μL of each sample (or methanol itself as control) was added to 60 μL of DPPH (60 μmol/L) in methanol. After mixing vigorously for 10 seconds, the solutions were transferred into a 100 μL Teflon capillary tube and fitted into the cavity of the ESR (electron spin resonance) spectrometer (JES-FA machine, JEOL, Tokyo, Japan). The spin adduct was measured on an ESR spectrometer exactly 2 min later. Measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, temperature 298 K.

Hydroxyl radicals were generated by Fenton reaction, and reacted rapidly with nitrone spin trap DMPO; the resultant DMPO-OH adducts was detectable with an ESR spectrometer (20). The ESR spectrum was recorded 2.5 min after mixing in a phosphate buffer solution (PBS; pH 7.4) with 0.3 M DMPO 20 μL, 10 mM $FeSO_4$ 20 μL and 10 mM 20 μL using an ESR spectrometer set at the following conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude

2 G, microwave power 5 mW, gain 6.3×10^5, temperature 298 K.

Alkyl radicals were generated by AAPH. The PBS (pH 7.4) reaction mixtures, containing 40 mmol/L AAPH, 40 mmol/L 4-POBN and indicated concentrations of tested samples, were incubated at 37°C in a water bath for 30 min (21), and then transferred to a 100 μL Teflon capillary tube. The spin adduct was recorded on JES-FA ESR spectrometer. Measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, temperature 298 K.

Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging activity was determined according to the method of Muller (22). A 100 μL of 0.1 M phosphate buffer (pH 5.0) and the sample solution were mixed in a 96-well plate. A 20 μL of hydrogen peroxide was added to the mixture, and then incubated at 37°C for 5 min. After incubation, 30 μL of 1.25 mM ABTS and 30 μL of peroxidase (1 unit/mL) were added to the mixture, and then incubated at 37°C for 10 min. The absorbance was read with an ELISA reader at 405 nm.

Cell culture

Cells of an African green monkey kidney line (Vero) were maintained at 37°C in an incubator, under a humidified atmosphere containing 5% CO_2. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS), streptomysin (100 μg/mL), penicillin (100 unit/mL) and sodium pyruvate (110 mg/L).

Hydrogen peroxide scavenging assay by DCF-DA

For the detection of intracellular H_2O_2, the Vero cells were seeded in 96-well plates at a concentration of 1.0×10^5 cells/mL. After 16 hr, the cells were treated with samples (10 μL) and incubated at 37°C under a humidified atmosphere. After 30 min, H_2O_2 was added at a concentration of 1 mM, and then the cells were incubated for an additional 30 min at 37°C. Finally, 2′,7′-dichlorodi-hydrofluorescein diacetate (DCF-DA; 5 μg/mL) was introduced to the cells, and 2′,7′-dichlorodi-hydrofluorescein fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a Perkin-Elmer LS-5B spectrofluorometer.

Assessment of cell viability

Cell viability was then estimated via an MTT assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is a colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (23). The cells were seeded in 96-well plates at a concentration of 1.0×10^5 cells/mL. After 16 hr, the cells were treated with samples (10 μL). Then, 10 μL of H_2O_2 (1 mM) was added to the cell culture medium, and incubated for 24 hr at 37°C. MTT stock solution (50 μL; 2 mg/mL) was then applied to each of the wells, to a total reaction volume of 200 μL. After 4 hr of incubation, the plates were centrifuged for 5 min at 800× *g*, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μL of dimethylsulfoxide (DMSO), and the absorbance was measured via ELISA at a wavelength of 540 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to present 100% viability. The data are expressed as mean percentage of the viable cells versus the respective control.

RESULTS

Proximate composition

Proximate compositions of freeze dried *Tetraselmis suecica* and *Chlorella ellipsoidea* are shown in Table 1 and extraction yield and total polyphenolic content in the enzymatic digests are depicted in Table 2.

DPPH free radical scavenging activity

The percentage scavenging activity of enzymatic digests against DPPH are shown in Table 3. Significant differences in the activities the different between *T. suecica* and *C. ellipsoidea* and among different digests were observed. The enzymatic digests exhibited less significant effects on DPPH radical scavenging compared to commercial antioxidant such as BHT. However, Neutrase (61.5%) and Kojizyme (71.5%) digest from *T. suecica* exhibited relatively higher effects.

Hydroxyl radical scavenging activity

According to Table 3, most enzymatic digests from *T. suecica* and *C. ellipsoidea* possessed weak hydroxyl radical scavenging effects. Out of them, Viscozyme and

Table 1. Proximate composition of *T. suecica* and *C. ellipsoidea*

Nutrient	*T. suecica*	*C. ellipsoidea*
Moisture (%)	1.99±0.3	5.58±0.3
Carbohydrate (%)	29.89±0.2	18.28±0.4
Protein (%)	57.03±0.1	45.08±0.3
Lipid (%)	3.97±0.2	5.69±0.3
Ash (%)	7.11±0.4	25.37±0.3

Values are means of three replicates±SD.

Table 2. Extraction yield and total polyphenolic content of different enzymatic hydrolysis from *T. suecica* and *C. ellipsoidea*

	Treated enzyme	Yield (g/100 g)	Total polyphenolic (mg/100 g)
T. suecica	Viscozyme	31.0±1.1	181.2±14
	Celluclast	31.6±1.3	195.2±13
	AMG	32.0±1.3	196.5±13
	Termamyl	36.3±1.4	167.3±14
	Ultraflo	29.6±1.6	195.6±12
	Protamex	35.0±1.4	211.2±13
	Alcalase	32.0±1.6	191.7±14
	Flavourzyme	30.3±1.7	191.3±12
	Neutrase	23.0±1.6	280.5±24
	Kojizyme	27.3±1.4	212.3±14
C. ellipsoidea	Viscozyme	24.0±1.3	176.2±24
	Celluclast	26.3±1.4	177.7±12
	AMG	22.6±1.3	186.3±12
	Termamyl	27.0±1.4	181.9±13
	Ultraflo	25.0±1.3	180.8±14
	Protamex	30.3±1.2	177.6±26
	Alcalase	31.3±1.3	177.4±12
	Flavourzyme	30.3±1.3	179.2±12
	Neutrase	32.0±1.3	180.0±29
	Kojizyme	27.0±1.2	179.1±18

Values are means of three replicates±SD.

Neutrase digests from *T. suecica* recorded around 30% of hydroxyl radical scavenging activity.

Alkyl radical scavenging activity

Alkyl radical scavenging effect of enzymatic digests from *T. suecica* and *C. ellipsoidea* is shown in Table 3. As shown in the results, remarkable scavenging effects of all the enzymatic digests were observed in alkyl radical scavenging assay, compared to the other scavenging assays. Several digests yielded approximately 75% scavenging activities. Celluclast, Protamex, and Neutrase digests of *T. suecica* as well as Viscozyme and Flavourzyme digests of *C. ellipsoidea* exhibited relatively higher scavenging activities. Interestingly, Kojizyme digest of *T. suecica* exhibited remarkable alkyl radical scavenging activity (approximately 81%) which was similar to BHT.

Hydrogen peroxide scavenging activity

As depicted in Table 3, significant differences in the activities the different between *T. suecica* and *C. ellipsoidea* and among different digests were observed. All the enzymatic digests from *C. ellipsoidea*, showed weak hydrogen peroxide scavenging effects. However, all the enzymatic digests except Viscozyme, Celluclast, and AMG from *T. suecica* exhibited relatively higher effects. Among them, Neutrase and Kojizyme digests recorded around 61% of hydrogen peroxide scavenging activity. This inhibitory rate was slightly superior to BHT.

Hydrogen peroxide scavenging assay by DCF-DA

As Neutrase (TN) and Kojizyme (TK) digests from *T. suecica* possessed the highest radical scavenging activity, this digests was further evaluated for its H_2O_2 scavenging activity by DCF-DA. The effect of the TN and TK on H_2O_2 scavenging activity by DCFH-DA is exhibited in Fig. 1. Both the samples showed more than 40% H_2O_2 scavenging activity at 100 μg/mL. Comparatively, TN showed better activity than TK. Both the samples showed increased H_2O_2 scavenging activity in a

Table 3. Antioxidative activities of enzymatic hydrolysis from *T. suecica* and *C. ellipsoidea* (tested concentration is 1 mg/mL)

	Treated enzyme	DPPH	Hydroxyl radical	Alkyl radical	Hydrogen peroxide
T. suecica	Viscozyme	9.25±3.2	30.25±2.6	64.25±3.2	41.78±1.2
	Celluclast	23.96±2.6	29.88±2.6	76.45±2.6	46.44±1.6
	AMG	28.15±2.5	27.69±2.7	61.55±2.5	33.15±2.1
	Termamyl	12.76±3.0	29.99±2.6	61.07±3.0	50.15±1.1
	Ultraflo	24.24±2.6	28.72±2.7	64.52±2.6	53.99±2.4
	Protamex	45.30±1.9	26.42±2.7	76.71±1.9	57.62±1.6
	Alcalase	40.82±1.3	20.47±3.0	69.42±2.0	56.30±1.7
	Flavourzyme	23.32±2.0	26.22±2.8	68.41±2.7	57.97±2.4
	Neutrase	61.59±1.3	34.62±2.4	77.64±1.3	61.95±1.7
	Kojizyme	71.55±1.0	14.53±3.2	81.54±1.0	61.59±1.4
C. ellipsoidea	Viscozyme	28.52±1.5	23.30±1.3	76.59±1.5	31.54±1.1
	Celluclast	25.52±1.0	23.31±1.5	72.92±1.4	30.37±0.6
	AMG	27.16±1.7	29.59±1.5	72.55±1.9	28.49±0.4
	Termamyl	28.64±1.2	20.11±1.5	66.05±1.1	28.72±0.1
	Ultraflo	34.92±1.8	19.71±1.8	67.92±1.8	28.54±0.7
	Protamex	35.18±1.7	21.80±1.5	72.35±1.8	36.74±0.4
	Alcalase	25.64±1.7	18.09±1.1	73.85±1.5	38.08±0.7
	Flavourzyme	31.61±1.6	23.18±1.4	75.85±1.5	33.40±0.7
	Neutrase	34.83±1.3	22.77±1.5	71.03±1.3	36.00±0.7
	Kojizyme	27.41±1.8	24.00±1.9	71.96±1.5	31.66±0.7
BHT		83.22±0.5	58.16±0.1	88.45±0.9	55.50±1.8

Values are means of three replicates±SD.

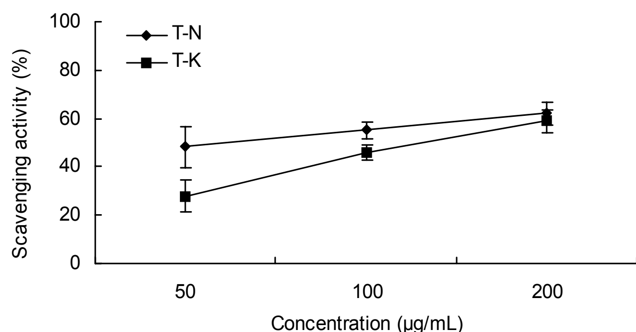

Fig. 1. Effect of the enzymatic digests from *T. suecica* on scavenging intracellular hydrogen peroxide. The intracellular hydrogen peroxide generated was detected by DCF-DA method. T-N; Neutrase digest from *T. suecica*, T-K; Kojizyme digest from *T. suecica*. Experiments were performed in triplicate and the data are expressed as mean±SE.

dose-dependent manner. Especially, TN had the highest H_2O_2 scavenging activity (62%) at 200 μg/mL.

Effect of TN and TK against H_2O_2-induced cell damage

As TN and TK possessed the highest hydrogen peroxide scavenging activity, this digests was further evaluated for its protecting ability against H_2O_2-induced cell damages. The protective effect of the TN and TK on H_2O_2-induced cell damage on Vero cells is exhibited in Fig. 2. In this study, both samples dose-dependently controlled H_2O_2-induced cellular damage. The addition of H_2O_2 to the cell medium without digests rendered only 24.5% cell survival rate, but addition of digests alongwith H_2O_2 to the medium dose-dependently increased cell survival rate. However, the TN and TK, at concentrations of 50~200 μg/mL, evidenced very similar enhancements of activity. In particular, the highest cell viability, 69.5% was recorded with the TN, at a concentration of 200 μg/mL.

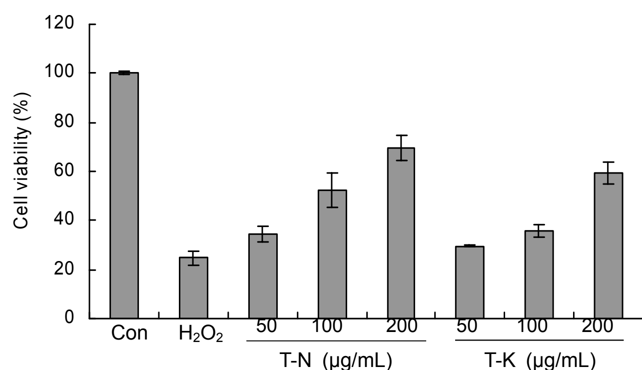

Fig. 2. Protective effect of the enzymatic digests from *T. suecica* on H_2O_2-induced oxidative damage of Vero cells. The viability of Vero cells on H_2O_2 treatment was determined by MTT assay. Abbreviations are the same in Fig. 1. Experiments were performed in triplicate and the data are expressed as mean±SE.

DISCUSSION

Recently many researchers are interested in finding any natural antioxidants having safety and effectiveness, which can be substituted for current and commercial synthetic antioxidants, BHA and BHT. Microalgae have become good candidates for sources of natural antioxidants, as revealed by a number of recent studies (24,25). In the present study, we used the ESR technique. Spin trapping is the most direct method to detect highly reactive free radicals. With this ESR technique, a higher steady-state concentration of free radicals is achieved, which can overcome the sensitivity problem inherent in the detection of endogenous radicals in biological systems (26,27).

Microalgae was successfully digested by five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase) to produce enzymatic digests according to the method of the previous study (28,29). In the present study, we focused on natural water-soluble antioxidants from *T. suecica* and *C. ellipsoidea*, which were prepared by enzymatic digestion and potential antioxidant activities of the resultant enzymatic digests were evaluated using different reactive oxygen species (ROS) scavenging assays, and protective effect against H_2O_2-induced cell damage.

DPPH is stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule, and has often been used as a substrate to evaluate the antioxidative activity of natural compounds (30). In this study, DPPH free radical scavenging activity of proteases digests from *T. suecica* and *C. ellipsoidea* was relatively higher than the digests by treated with carbohydrases. Especially, Kojizyme digests of *T. suecica* indicated the highest scavenging activity (above 70%). Many researchers have reported a positive correlation between free radical scavenging activity and total phenolic compounds. The two studies conducted by Lu and Foo (31) and Siriwardhana et al. (32) reported a high correlation between DPPH radical scavenging activities and total polyphenolics. In this study, Neutrase digests of *T. suecica* showed a lower DPPH free radical scavenging activity than Kojizyme digest, although that contained as much phenolic compounds as the Kojizyme digests. It is thought that other bioactive components, such as proteins, polysaccharides and different kinds of pigments. For example, it was found that oligosaccharides, sulfate and glycoprotein components in red microalga *Porphyridium sp*. exhibited antioxidant activities (33). Thus, it can be concluded that all those factors

influenced the antioxidant activity.

Hydroxyl radicals generated in the Fenton system (Fe^{2+}/H_2O_2) were trapped by DMPO, forming a spin adduct as detected by the ESR spectrometer. All the tested enzymatic digests scavenged less than 35%. Moreover, proteases-treated digests and carbohydrases-treated digests showed very similar hydroxyl radical scavenging of activity. In particular, the highest hydroxyl radical scavenging activity, 34.6% was recorded with the Neutrase digest of *T. suecica*. Among the ROS, the hydroxyl radical is the most reactive and it induces severe damage to the adjacent biomolecules.

The alkyl radical has been found to be a primary intermediate in many hydrocarbon reactions. These radicals were easily detected with ESR, a technique which has been found to be very useful in the characterization of solid surfaces and in the elucidation of active surface sites as well as surface reactions (34). The alkyl radical spin adduct was observed when AAPH was incubated with the spin trap 4-POBN at 37°C for 30 min. All the tested enzymatic digests exhibited activities more than 60%. Especially, Kojizyme digest of *T. suecica* showed around 80% of scavenging activity. This result suggests that enzymatic digests from *T. suecica* and *C. ellipsoidea* have remarkable scavenging abilities on the alkyl radicals.

Although H_2O_2 itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl radicals. Therefore, it is very important to remove H_2O_2 for the protection of living systems. Addition of H_2O_2 to cells in culture can lead to transition metal ion-dependent $HO^\cdot$ mediated oxidative DNA damage (35). According to the H_2O_2 scavenging activity results, enzymatic digests from *T. suecica* was relatively higher than the digests from *C. ellipsoidea*. All the enzymatic digests of *T. suecica* except AMG digest exhibited more than 40% H_2O_2 scavenging activity, and proteases-treated digests exhibited greater effects than carbohydrases-treated digests, suggesting that proteases have more capability to liberate H_2O_2 scavenging compounds. Specifically, the Neutrase and Kojizyme digests evidenced more profound activities (~61.9 and 61.5%, respectively) than was observed in their counterparts.

Therefore, the Neutrase (TN) and Kojizyme (TK) digests of *T. suecica* was selected for use in further experiments. H_2O_2 has been extensively used as an inducer of oxidative stress *in vitro* model. The exposure of cultured cells to H_2O_2 results in an imbalance in energy metabolism and the deleterious effects of hydroxyl and peroxyl radicals on membrane lipids and proteins. Therefore, in this study, we investigated scavenging activity of the TN and TK after the administration of H_2O_2 treatment in Vero cell lines. The DCF-DA method was used to detect the intracellular hydrogen peroxide level (36). DCF-DA diffuses into cells, where it is hydrolyzed by intracellular esterase to polar 2′,7′-dichlorodihydrofluorescein. This non-fluorescent fluorescein analog gets trapped inside the cells and is oxidized by intracellular oxidants to a highly fluorescent, 2′,7′-dichlorodi-hydrofluorescein. As shown in Fig. 1, the intracellular H_2O_2 scavenging activity of TN and TK was expressed as 62.06 and 59.03% at the concentration of 200 μg/mL, and the scavenging activities increased when increasing the sample concentration. As the TN and TK generated in this study evidenced such good H_2O_2 radical scavenging activity, this samples was evaluated further with regard to its protective effects against H_2O_2-induced cellular damage. The protective effects of TN and TK on cell viability in H_2O_2-induced Vero cells were measured via an MTT assay. H_2O_2-induced cells recorded 24.58% cell survival rate, whereas treatment of the TN and TK increased the cell viability. Especially, TN exhibited significant cell survival rates even at 200 μg/mL (69.55%). TN contain high level of polyphenol compounds and it could be assumed that polyphenol compounds are responsible for the increased scavenging intracellular hydrogen peroxide and protective effect on H_2O_2-induced cell damage.

In this study, enzymes (carbohydrases and proteases) were used for extraction of antioxidant compounds. Materials of the microalgae wall may interfere with the proper extraction of bioactive compounds. In order to overcome such barriers, enzymes can be applied to efficiently extract the desired compounds with higher yields. Enzymatic digests from algae have reported significant yields of desired compounds in the previous studies (16,37). Additionally, enzymatic digests possesses innovative advantages and characteristics over conventional extraction procedures such as water solubility, higher extraction efficacy, greater variation of constituents and comparative inexpensiveness (16,38,39).

The study has shown that *Tetraselmis suecica* and *Chlorella ellipsoidea* have different antioxidative activities. Enzymatic digests exhibited appreciable antioxidative activities. In conclusion, *Tetraselmis suecica* and *Chlorella ellipsoidea* exhibited antioxidant potential against ROS and it would be a potential candidate as a natural water-soluble antioxidative source, which can be applied in food or food related industries.

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