A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Nutritional and Functional properties from high temperature and disease –resistant strains of *Pyropia yezoensis*

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GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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CONTENTS

국문초록

이 연구는 국내에서 생산되는 영양 및 기능성 면에서 우수한 수산 양식 종자를 개발하여 수출하기 위한 과제로서 2013년도부터 시행되어온 Golden Seeds Project(GSP)의 한 부분으로 진행된 연구이다. 현재 우리나라 수산 양식 생산량은 계속 증가를 하고 있으며, 그 중 홍조류인 방사무늬 김 양식 생산량은 넙치 양식에 이어 두 번째로 가장 많은 양식 생산이 이루어 지고 있다. 이와 더불어 김의 수출량 또한 지속적으로 증가를 하고 있어 이전에는 주로 식품으로서만 이용하였던 방사무늬 김을 더욱 더 고부가가치 산업으로 이용을 하기 위해 연구를 하고 있다. 그러나, 방사무늬 김의 유리사상체 시기는 주로 고수온기인 여름철이며, 전 지구적으로 수온이 증가하는 추세에 따라 고수온기에 여러 질병에 감염되며, 또한 저수온기에 주로 생산되는 방사무늬 김 양식 생산에 있어서 큰 타격을 준다. 이에 많은 연구자들은 붉은 갯병과 같은 질병에 내성을 가지며, 고수온기에 내성을 가진 양식 생산이 가능한 품종을 개발하는 연구 중에 있다. 대표적으로 고수온기인 여름철 김 양식장에서 발생하는 질병을 예방하기 위해 유전적 조작을 이용한 내병성 및 고온 내성 김 품종을 개발하는 것이다. 그러나 유전적 조작으로 개발이 된 것에 대해 반감을 가지기 때문에 유전적 조작으로 개발된 품종인 내병성 및 고온내성 김 품종과 일반 양식산 방사무늬 김의 영양 및 기능성 성분의 함량을 비교 분석 하였을 때, 영양 및 기능성 성분에서 차이가 나는지 확인 하였다. 그 결과, 단백질 및 아미노산은 내병성 김(DP)에서 더 많았고, 탄수화물 및 단당의 함량에서는 고온 내성 김(HP)에서 더 많았으며, 지질과 회분은 매우 적은 함량을 보였다. 김 유래 기능성 성분으로 알려진 Phycoerythrin 과 sterol을 분석한 결과, 일반 양식산 방사무늬 김에서

개발된 내병성 및 고온내성 품종보다 더 높은 함량을 보였으나, 내병성 및 고온내성 품종만으로 비교하였을 때, 고온내성 품종에서 내병성 품종보다 더 높은 함량을 보였다. 이는 김 유래 기능성 성분이 환경에 영향을 많이 받기 때문에 일반 양식산 방사무늬 김에서 높게 나타난 것으로 사료된다.

그리고 내병성 및 고온내성 방사무늬 김을 실제로 먹었을 때 장내 소화 효소에 의해서 분해가 되어 나타나는 생리활성을 내병성 및 고온내성 품종과 일반 양식산 김의 차이를 비교하였다. 그 중 당뇨, 비만 등의 대사성 질병을 유발하는 활성산소(reactive oxygen species, ROS)와 염증을 소거 및 완화 시킬 수 있는 기능성에 대하여 평가하였다. 그 결과, 내병성 김 품종보다 고온내성 김 품종에서 일반 양식산 김보다 라디칼 소거능이 뛰어났으며, 세포독성을 보이지 않으며, 뛰어난 세포 내 ROS 소거능을 확인하였다. 특히, trypsin 과 α-chymotrypsin 효소 가수분해물에서 뛰어난 항산화 활성을 가짐을 확인 하였다. 그리고 대식세포(RAW 264.7) 내의 NO 소거능을 통한 항염증 효능을 확인한 결과, 내병성 및 고온내성 김을 비교 했을 때, 고온내성 김 품종에서 NO 소거능이 뛰어났지만, 일반 양식산 김과 비교 했을 때 유의적 차이를 보이지 않았다. 종합적으로 살펴 보았을 때, 내병성 김 품종에 비해 고온내성 김 품종에서 탄수화물의 함량이 제일 높았으며, 뛰어난 항산화 및 항염증 활성을 보였다. 이를 통해서, 고온내성 김 품종의 종자를 이용하면 보다 넓은 범위의 수온 환경에서 양식생산이 가능하며, 그 영양 및 기능성 성분 면에서 일반 양식산 김과 비교했을 때 더 적은 함량을 보이지만 기능성 면에서 뛰어난 항산화 및 항염 활성을 나타내는 것으로 보아 고온 내성 김 품종이 종자 산업에 있어서 경쟁력 있는 우수한 종자가 될 것으로 사료된다.

LIST OF FIGURE

LIST OF TABLE

INTRODUCTION

Developing excellent seeds is critical for agriculture and aquaculture industries but most seeds are very sensitive to their environment (Radhika et al, 2017). Among of them, *Pyropia yezoensis* (*P. yezoensis*) which is red seaweed (Rhodophyta) is a very important resource for food and oriental medicine industry (Lee et al, 2015; Sandra and Suzzanne, 2003). Yearly total aquaculture production and export volume of *P. yezoensis* has dramatically increased (Lee et al, 2012). It has attracted in functional foods and medical industry (Liu et al, 2010). *Pyropia yezoensis* has various nutritional and functional components such as polysaccharides, proteins, mycosporine-like amino acids (MAAs) and sterols (Jung et al, 2016; Mok et al, 2011). These components have a variety of biological effects such as antioxidant, anti-inflammation and anticancer activity (Shin et al, 2011; Lee et al, 2016). And functional components are;

- Phycoerythrin is a red protein complex with antioxidant and anti-inflammation activity from the light harvesting phycobiliprotein family of red algae (Jung et al, 2016; Wu et al, 2017).
- Plant sterols or phytosterols vary from other sterols with the inclusion of carbon side chains and have known to have biological activities (Eman et al, 2016; Katarzyna et al, 2013).
- β-carotene is strongly red-orange pigment abundant in plants and fruits. It is a member of the carotenes which are terpenoids synthesized biochemically from eight isoprene units and hence have 40 carbons (Dinesh and Yashika, 2014; Berti et al, 2014).
- Mycosporine-like amino acids (MAAs) are low molecular weight functional components that provide protection against UV radiation (310–365 nm) (Suh et al,

2014; Sinha et al, 2007). Among these MAAs, Porphyra-334 is the main component from *P. yezoensis* (Suh et al, 2014; Oren & Cimerman, 2007).

All these components can be affected by environmental factors (Shin et al, 2011; Lee et al, 2015). It faces significant risk of quality and aquaculture production impact by increasing the water temperature (Kim et al, 2014). Aquaculture production of *P. yezoensis* decreases during high water temperature seasons (Yan et al, 2013). Therefore, many studies have investigated strains that have tolerance against disease and high temperature (Camilo et al, 2015; Yan et al, 2013).

Many organisms have been exposed to various oxidative stresses by the environment (Kang et al., 2012; Heo et al., 2006). Reactive oxygen species (ROS) which commonly called free radicals are produced in the body under oxidative stress such as hydrogen peroxide (H_2O_2), hydroxyl radicals (HO˙) and peroxyl radicals (ROO-) (Kang et al, 2012; Hong et al., 2011). When human defense mechanisms do not work properly, ROS damage normal cells (Alfadda et al., 2012; Baek et al, 2015). A number of synthetic antioxidants such as BHA (butylated hydroxyanisol) and BHT (butylated hydroxytoluene) also have side effects (Branen, 1975; Song et al., 2000). Many studies have investigated natural antioxidants (Kim et al., 2005; Choi and Lee, 2014).

Inflammation which plays a crucial role in metabolic disease such as cancer and cardiovascular disease in human is a part of non-specific protective response to harmful stimuli that include damage to tissues, pathogens and specific disease conditions (Fernando et al, 2016). Macrophages are important mediators of the inflammatory responses and they produce pro-inflammatory cytokines such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and various pro-inflammatory cytokines which serve as mediators of the inflammatory response (Fernando et al, 2016; Lee et al, 2015). However, excessive and uncontrolled production of these inflammatory mediators and cytokines are

associated with autoimmune disorders, neuropathological diseases and rheumatoid arthritis. Suppression of these inflammatory mediators may be an effective therapeutic strategy to prevent diseases caused by inflammatory disorders (Fernando et al, 2016; Lee et al, 2015). Many researchers have investigated natural products with biological effects such as antioxidant and anti-inflammatory activity (Fernando et al, 2016; Lee et al, 2015)

Enzymatic extracts from marine organisms have been reported that they have higher yield and biological effects (Kim et al, 2016; Lee et al, 2016). Lee et al (2016) reported that AMG-assisted extracts from *P. yezoensis* were shown as higher yield and carbohydrate contents than other enzymes. Kim et al (2016) reported that pepsin- assisted extracts from *Hippocampus abdominalis* were shown as higher yield, protein contents and biological effects.

In this study, we investigate the nutritional and functional properties of disease and high temperature resistant strains of *P. yezoensis* (DHP) which was developed by Kongju National University and cultivated in Seocheon for one month after seed collection (Tatyana et al, 2016; Wang et al, 2017). And we evaluated that DHP has been to good potential seeds. Part I analyzes nutritional and functional components from DHP strains. Part II screens biological effects such as antioxidant and anti-inflammation activity of enzymatic hydrolysates from DHP strains.

Part I.

Nutritional and functional components for disease and high temperature resistant *Pyropia yezoensis* strains

1. Abstract

Pyropia yezoensis (*P. yezoensis*) has been used for food and oriental medicine in Southeast Asia due to its many nutritional and functional components including carbohydrates, proteins, phycobiliproteins and sterols. Previous studies have investigated these components can be changed by environmental factors including water temperature and amount of sunshine. And they have been considered as indicator of good quality which has amino acids related to flavor. Disease and high temperature resistant strains of *P. yezoensis* (DHP) were developed using genomic methods at Kongju National University and cultivated in Seocheon. Disease resistant strains (DP) had the highest protein and amino acids contents whereas high temperature resistant strains (HP) had the lowest. However, HP strains had the highest carbohydrate and monosaccharide contents, Ash and lipid content from both HP and DP strains were lower than marine cultivated *yezoensis*. HP and DP strains were shown as higher magnesium and calcium contents. But HP and DP strains were shown as lower functional components such as sterol, mycosporine-like amino acids and phycoerythrin. In conclusion, DP strains were shown as higher protein and amino acid contents than HP and CP strains but HP strains were shown as higher carbohydrate and monosaccharide contents than DP and CP strains.

2. MATERIALS AND METHODS

2.1. Preparation of DHP strains

Disease and high temperature- resistant strains from Kongju National University were cultivated in Seocheon from November (2016) to January (2017). Samples were desalted by washing and then dried using a far infrared dryer (KEC-60, Korea) at 50℃ for 24 h. The dried samples were then ground to under the size of 50 meshes.

2.2. Proximate composition

The proximate composition of DHP strains was measured following the appropriate AOAC international methods: protein contents were measured by Kjeldahl, lipid contents by soxhlet, ash contents by electric muffle furnace (JSR, 600℃, Korea) and moisture contents by HB43-S (Mettler Toledo, Canada). Carbohydrate content was calculated as 100% - (moisture + ash + lipid + protein).

2.3. Monosaccharide composition

Monosaccharides were determined following Furneaux et al. (1990) and Shin et al. (2013). Dried samples were hydrolyzed with 2.5% sulfuric acid (H_2SO_4) at 110–150℃ and the acidic smell removed using a speed-back instrument. Samples were prepared at 1 mg/ml concentration and analyzed by liquid chromatograph (Shimadzu LC, column: Shodex Asahipack NH2P-50 4E, 4.6×250 mm, detector: RI, Japan). We used mobile phase that equipped with isocratic (250 mM H_3PO_4: Acetonitrile = 20: 80) to measure fructose and glucose content including galactose and mannose which are the main monosaccharides in porphyran which are the functional polysaccharide from *P. yezoensis*.

2.4. Amino acid composition

We measured 16 amino acid contents which are flavor amino acid in *P. yezoensis* by the methods which were modified by Jung et al. (2016). Samples were hydrolyzed with 6N HCl at 110℃ for 22 h. Then evaporated at 70–80℃ and diluted by 0.02N HCl. Pretreated samples were analyzed by amino acid analyzer (50℃, 4.6 mm I.D. ×60 mm column packed with ion exchange resin, detector: 570 and 440 nm, flow rate: 0.4 ml/min). We measured 16 amino acid levels including glutamic acid and alanine which are flavor amino acids in *P. yezoensis*.

2.5. Mineral contents

We measured macro mineral contents (including calcium, potassium, magnesium and sodium) by the methods which were modified by Jung et al. (2016). Samples were hydrolyzed with HNO_3 at 50℃ and dried to remove acid smell and analyzed by ICP-OES (Perkin-Elmer ICP-OES 2100DV, USA). We measured macro mineral contents including calcium, potassium, magnesium and sodium.

2.6. Fatty acid contents

We measured total fatty acid, saturated fatty acid and unsaturated fatty acid contents by the method which modified Lepage and Roy (1986) and Shin et al. (2013). Dried samples were treated with methyl ester and analyzed by gas chromatograph (Agilent GC/Pegasus 4D, USA).

2.7. Functional components

2.7.1. Phycoerythrin extraction and quantification

Phycoerythrin was extracted following a modified freeze-thawing method introduced by Niu et al. (2007). DHP powder (1 g) was extracted in 25 ml distilled water. And the extract placed in conical tubes (FALCON, USA) and frozen to -80℃ then thawed to 25℃. The freeze-thaw process was repeated 4 times and then extracts were centrifuged (12000 rpm, 10 min, 4℃). After centrifugation, supernatants were collected in other tube and this process repeated 4 times. Finally, we obtained crude phycobiliprotein extracts and specific extracts were calculated as following the formular;

$$PC = \{A_{615} - (0.474x\ A_{652})\}/5.34,$$

$$APC = \{A_{652} - (0.208\ x\ A_{615})\}/5.09,$$

$$PE = \{A_{562} - (2.41\ x\ PC) - (0.849\ x\ APC)\}/9.62,$$

Where PC, APC and PE are named phycocyanin, allophycocyanin, and phycoerythrin content (%); A_{615} is measured at absorbance 615nm; A_{652} is measured at 652 nm; A_{562} is measured at 562nm and the various constants were determined by ELISA instrument.

2.7.2. Sterol extraction and quantification

Phytosterol extraction followed the modified method proposed by Machado et al. (2004) and included saponification or non-saponification. For saponification, 1 g of DHP powder was extracted in 27 ml KOH solution (8:1, v/v) and the extract heated at 80℃ for 30 min. The samples were then cooled to room temperature and filtered. Extracts were evaporated at 40℃ and then mixed with n-hexane (20 ml) and distilled water (20 ml), transferred to a conical tube and centrifuged (2000 rpm, 10 min). Finally, the supernatant was analyzed by GC/MS (GCMS-TQ8040, Japan). For non-saponification, 1 g DHP powder was extracted by Hexane (20 ml) and distilled water (20 ml) at room temperature for 30 min and then filtered. And the supernatant analyzed by GC/MS (GCMS-TQ8040).

2.7.3. Mycosporine–like amino acid extraction and quantification

Mycosporine-like amino acid extraction followed the modified method proposed by Suh et al. (2014). DHP powder (0.25 g) was dissolved in 3 ml distilled water and incubated overnight at 4℃. Then 14 ml MeOH was added and the solution was extracted by sonication for 5 min in an ice-bath and centrifuged (4℃, 15 min, 3000 rpm) and supernatant collected. Residue was extracted two further times with 17 ml MeOH and collected the supernatant. The extract was concentrated at 40℃ and freeze dried. Finally, extracts were dissolved in 400 ml distilled water and sonicated for 5 min and analyzed by HPLC (column: Sun fire ODS, flow rate: 1.0 ml/min, UV wavelength: 330 nm) as shown in **Table 1-1**.

2.7.4. β-carotene extraction and quantification

β- carotene extraction followed the modified method of Rudolf et al. (1980). DHP powder (1 g) was extracted by n-hexane (20 ml) at room temperature for 24 h and supernatants collected in another flask. Residues were extracted by ethyl acetate (20 ml) at room temperature for 24 h and supernatants collected and residues were extracted by methanol (20 ml) at room temperature. All extracts were filtered and evaporated under dark conditions at 37℃ and analyzed by HPLC using β-carotene standards from Sigma Aldrich (855553, β-carotene (95%)) as shown in **Table 1-2**.

2.8. Statistical analysis

Figure 1.1 shows the extraction standardization regime applied. Marine cultivated (CP) *yezoensis* strains are shown as seasonal and regional total average (maximum, average maximum, minimum, and average minimum) and DP and HP stains are expressed as mean ± standard deviation.

Table 1-1. HPLC conditions of mycosporine-like amino acid content (UV = 330 nm)

Time	**Flow**	**%A**	**%B**
0.0	1.00	0.0	100.0
7.0	1.00	0.0	100.0
15.0	1.00	70.0	30.0
20.0	1.00	80.0	20.0
22.0	1.00	100.0	0.0
32.0	1.00	100.0	0.0
Columen: Sunfire ODS (4.6x250mm)			

Table 1-2. HPLC analysis conditions of β-carotene from DHP

Time	Flow	%A	%B
0	1.0	90	10
10	1.0	100	0
20	1.0	100	0
Column: Sun fire ODS column(4.6x250mm)			

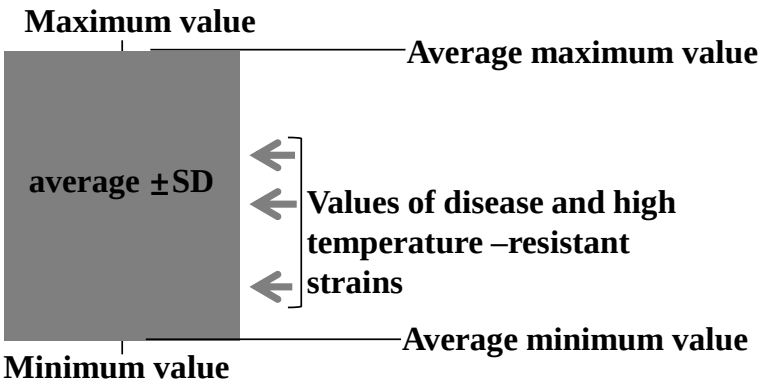

Figure 1-1. Standardization of nutritional and functional components.

All data are expressed here as mean ± standard deviation (SD) of triplicate experiments.

3. RESULTS AND DISCUSSION

3.1. Proximate composition

Figure 1-2 shows that protein content from marine cultivated species = 42.56 ± 1.17 % whereas DP and HP had higher and lower protein content. Jung et al. (2016) reported that protein content decreased with increasing water temperature. That result is consistent with the current research. However, HP strains were shown as lower contents than DP and CP strains. **Figure 1-3** shows that carbohydrate content from marine cultivated species = 39.97 % whereas HP strains were higher carbohydrate content than DP and CP strains. Shin et al. (2013) reported an inverse correlation between carbohydrate and protein contents. And Carbohydrate contents were increased by increasing water temperature. This result is consistent with the current research. Although HP strains have a high temperature resistant gene, HP strains have lower protein content and higher carbohydrate content than DP and CP strains. Thus, the HP strains do not show significant influence from the high temperature resistant gene. **Figure 1-4** shows that lipid content from marine cultivated species = 0.40 %. HP strains are similar to that whereas DP strains had lower lipid content. Jung et al. (2016) reported that *P. yezoensis* had small lipid content with no difference due to environment. This is consistent with the current results. **Figure 1-5** shows that crude ash content = 12.50 %. Both HP and DP strains have lower content. Jung et al. (2016) were reported that crude ash contents from each region (Korea, China, and Japan) were similar to that and Shin et al. (2013) reported that crude ash contents decreased by increasing water temperature from November to February. These results suggest that DP and HP strains were cultivated for shorter time than CP strains.

3.2. Monosaccharide contents

Porphyran is the functional carbohydrate from *P. yezoensis* (Lee et al, 2016, Zhou et al, 2012; Zhang et al, 2010) which are composed of galactose and mannose (Lee et al, 2016, Zhang et al, 2010). Shin et al. (2013) reported that galactose content from *P. yezoensis* was 70–79%. And it was sensitive to environmental factors such as water temperature and salinity. The current results were similar to that. CP and DP and HP strains were shown as **figure 1-6**. HP strains were shown as higher galactose contents than CP strains shown as 13.33%. However, DP and HP strains have no detected glucose and fucose. This suggests that DP and HP strains have galactose as the major monosaccharide. HP strains have the highest galactose content. Mannose content from marine cultivated species = 3.61%. But, DP and HP strains have lower contents than CP strains.

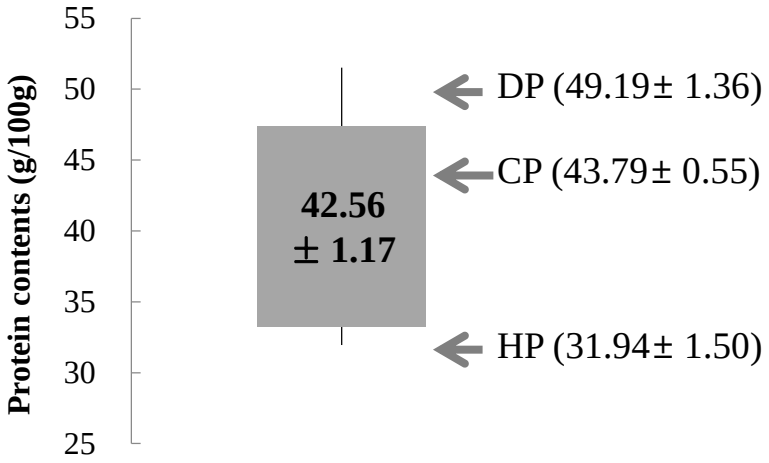

Figure 1-2. Protein content from control strains (CP), disease resistant strains (DP) and high temperature resistant strains (HP) of *P. yezoensis*.

Data are mean ± standard deviation of triplicate experiments.

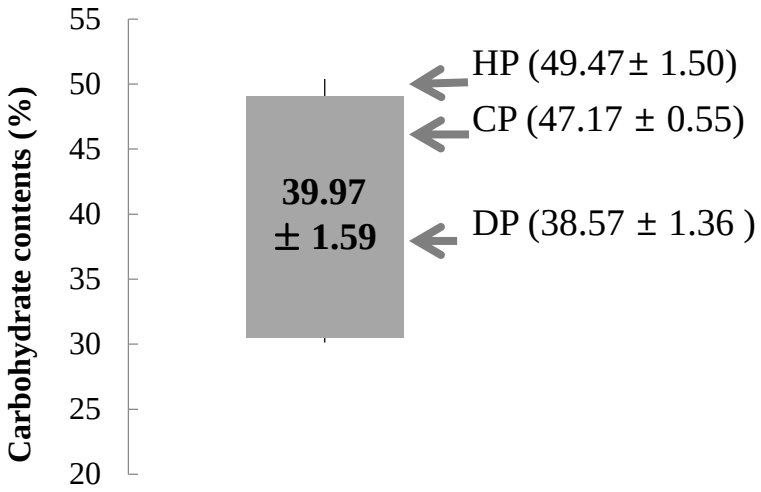

Figure 1-3. Carbohydrate content from control strains (CP), disease resistant strains (DP) and high temperature resistant strains (HP) of *Pyropia yezoensis* .

Data are mean ± standard deviation of triplicate experiments**.**

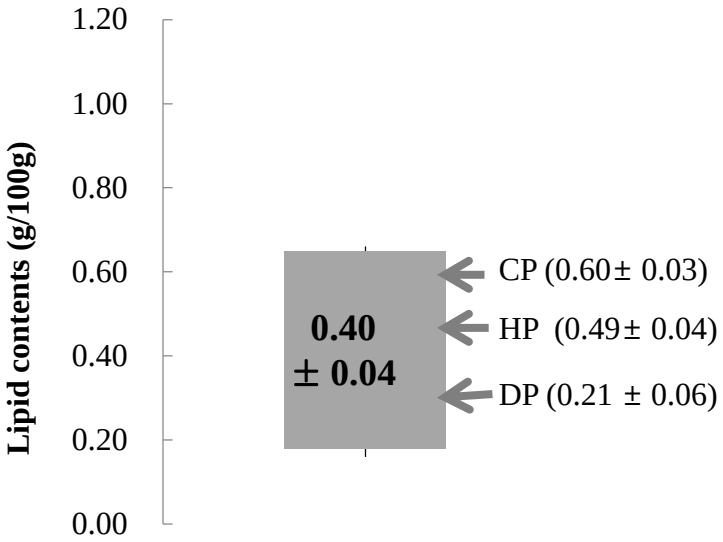

Figure 1-4. Crude lipid content from control strains (CP), disease resistant strains (DP) and high temperature resistant strains (HP) of *Pyropia yezoensis* .

Data are mean ± standard deviation of triplicate experiments**.**

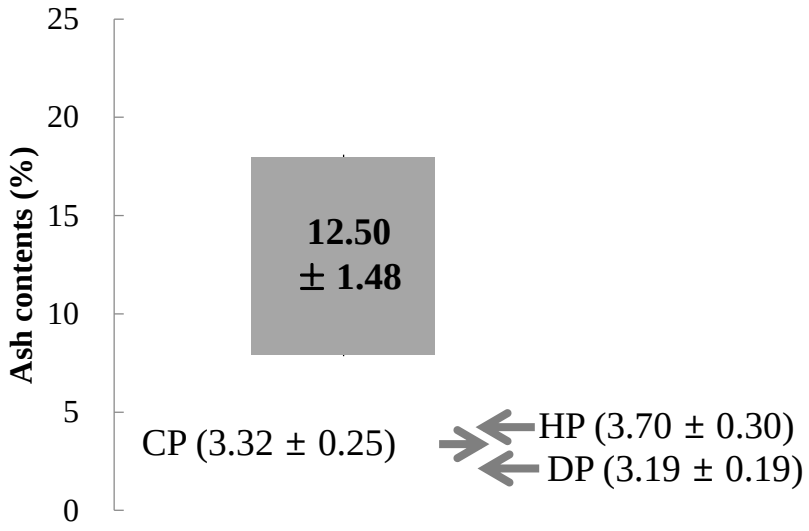

Figure 1-5. Crude ash content from control strains (CP), disease resistant strains (DP) and high temperature resistant strains (HP) of *Pyropia yezoensis*.

Data are mean ± standard deviation of triplicate experiments.

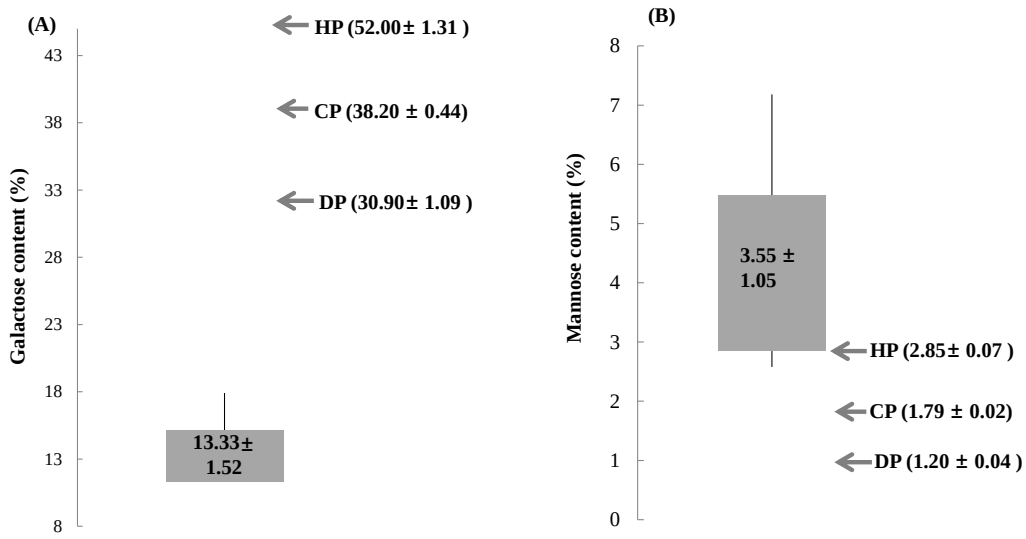

Figure 1-6. Monosaccharide content from control strains (CP), disease resistant strains (DP) and high temperature resistant strains (HP) of *Pyropia yezoensis* :

(A) galactose, and (B) mannose.

Data are mean ± standard deviation**.**

3.3. Amino acid contents

Protein accounts for 20% of human body mass which is responsible for many important functions. Proteins are themselves composed of many small molecules or amino acids (Cian et al, 2015; Aluko et al, 2015). And amino acids are also important components of cells, tissues and muscles as well as being used to store and transfer nutrients (Cian et al, 2015; Aluko et al, 2015). Amine and carboxylic groups have a positive impact on many metabolic diseases including inflammation, obesity, diabetes and cardiovascular disease (Shankar and Rhim, 2015; Cian et al, 2015). Essential amino acids are important for the metabolic pathway and be obtained in outside and belong to phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine and histidine (Harnedy and Richard, 2011; Shankar and Rhim, 2015). **Figure 1-7** shows that amino acid content for the CP, DP and HP strains. Jung et al. (2016) reported that aspartic acid, alanine and glutamic acid content were higher than other amino acids at all regions. In the current study, CP strains have higher total amino acid content than both DP and HP strains (**Figure 1-7 (A)**). But DP strains have higher essential amino acid content than CP strains.

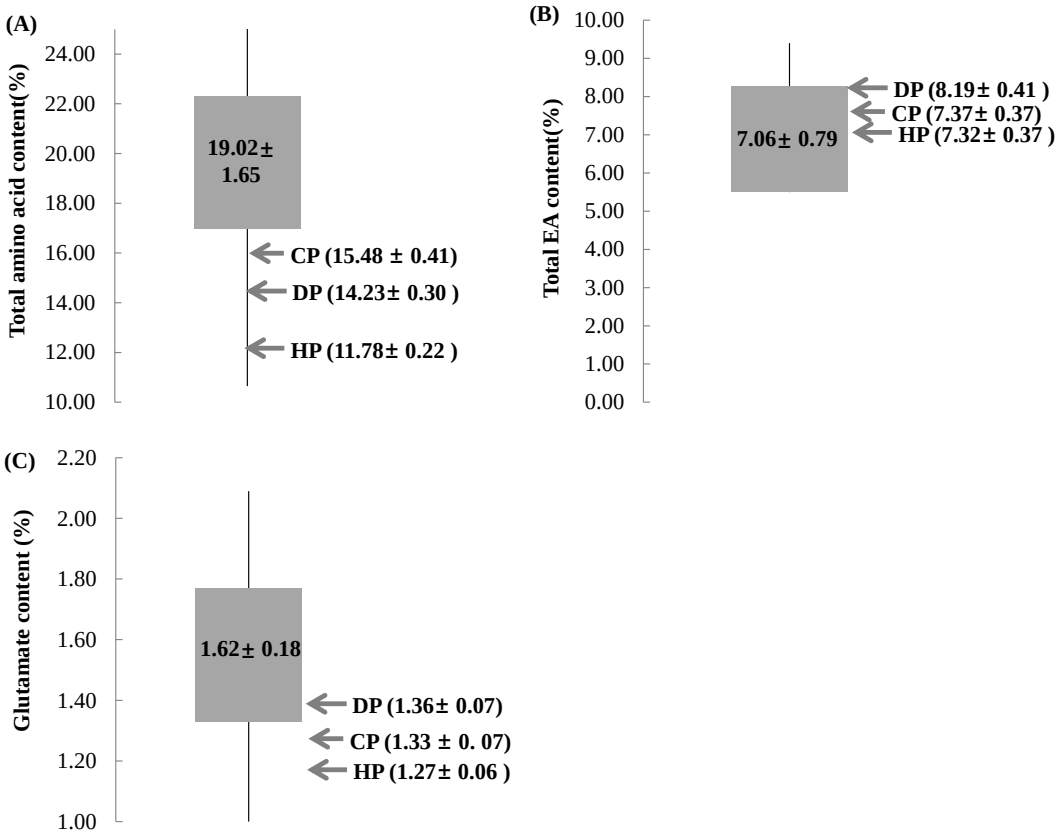

Figure 1-7. Amino acid content from control strains (CP), disease resistant strains (DP) and high temperature resistant strains (HP) of *Pyropia yezoensis* :

(A) Total amino acids, (B) essential amino acids and (C) glutamic acid.

Data are mean ± standard deviation

3.4. Fatty acid contents

Figure 1-8 shows that fatty acid content for CP, DP and HP strains. **Figure 1-8 (A)** shows that total fatty acid content from marine cultivated species = 0.51%. But DP and HP strains have lower content. Although HP strains were appeared to have slightly higher content than the DP strain (**Figure 1-8(A)**). **Figure 1-8 (B)** shows that total saturated fatty acid content from marine cultivated species= 0.16% and HP strains have higher content than CP strains whereas DP strains are similar CP. **Figure 1-8 (C)** shows that total unsaturated fatty acid content from marine cultivated species = 0.36% whereas DP and HP strains are lower contents than marine cultivated species. Shin et al (2013) reported that unsaturated fatty acid is higher contents than saturated fatty acid in *P. yezoensis*. However, DHP strains have a higher saturated fatty acid contents than unsaturated fatty acid contents.

3.5. Mineral contents

Figure 1-9 shows mineral contents from CP, DP and HP strains. Compared to other components, mineral contents are small amounts. However, mineral contents such as Zinc and magnesium strongly influenced quality of *Pyropia yezoensis* (Lee et al, 1974; Ye et al, 2014). The current study investigated macro minerals such as sodium, potassium, calcium and magnesium contents. **Figure 1-9(A)** shows that sodium content from marine cultivated species= 1.11% whereas DHP strains have lower contents. As shown as **Figure 1-9 (B),** potassium content from marine cultivated species = 6.65%. In contrast, DHP strains have higher magnesium content than CP strains. As shown in **Figure 1-9 (C),** calcium content from marine cultivated species = 0.35% and HP strains have higher (0.47%) contents than DP strains (0.38%). Thus, CP strains have more sodium and potassium content but HP strains have higher magnesium and calcium contents than CP strains.

3.6. Functional components

Phycoerythrin is a red protein complex with antioxidant and anti-inflammation activity from the light harvesting phycobiliprotein family of red algae (Jung et al, 2016; Wu et al, 2017). **Figures 1-10** shows that phycoerythrin content from marine cultivated species= 1.00 ± 0.20% and DP strains have higher and HP strains lower content. Plant sterols or phytosterols vary from plant or marine algae with the inclusion of carbon side chains and have known biological activities (Eman et al, 2016; Katarzyna et al, 2013). **Figure 1-11** shows that total sterol content from marine cultivated species = 0.25 ± 0.06% and DP and CP strains have lower contents. β- carotene is a member of the carotenes which are terpenoids synthesized biochemically from eight isoprene units (Dinesh and Yashika, 2014; Berti et al, 2014). **Figure 1-12** shows that β-carotene content from marine cultivated species = 0.15% and DHP strains have larger contents. Mycosporine-like amino acids (MAAs) are functional components with low molecular weight that have protection activity against UV radiation (310–365 nm) (Suh et al, 2014; Sinha et al, 2007). Among these MAAs, Porphyra-334 is the main MAAs components from *P. yezoensis* and has a specific absorption at 330 nm (Suh et al, 2014; Oren & Cimerman, 2007). **Figure 1-13** shows that MAAs component (Palythine, Shinorine, Asterine-330, Porphyra-334, and Usujirene) contents for WT, CP, DP and HP strains. MAAs from DHP strains compared with WT were little amount. So, MAAs contents of disease and high temperature – resistant strains (DHP) were shown as **figure 1- 13**. As shown **figure 1-13**, disease resistant strains (DP) have higher MAAs contents than high temperature resistant strains (HP) and control strains (CP). Porphyra-334 is the largest MAAs component for DP strains. Thus, DP strains have higher MAAs content than HP.

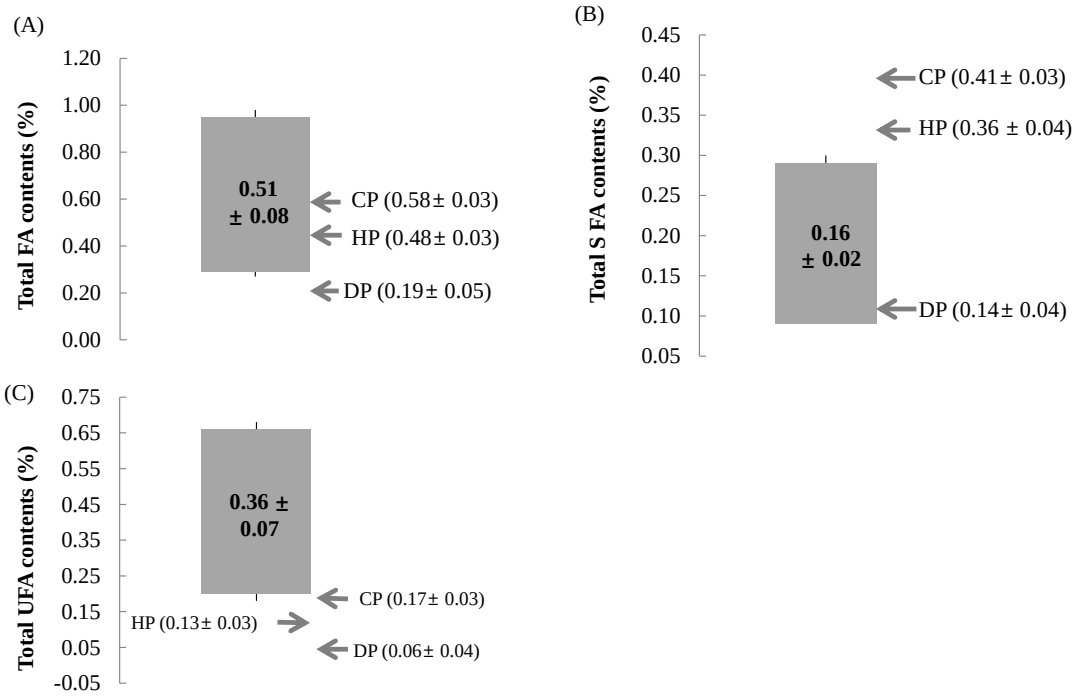

Figure 1-8. Fatty acid (FA) content from control strains (CP), disease resistant strains (DP) and high temperature- resistant strains (HP) of *Pyropia yezoensis* .

(A) Total FA, (B) Saturated FA, and (C) Unsaturated FA.

Data are mean ± standard deviation.

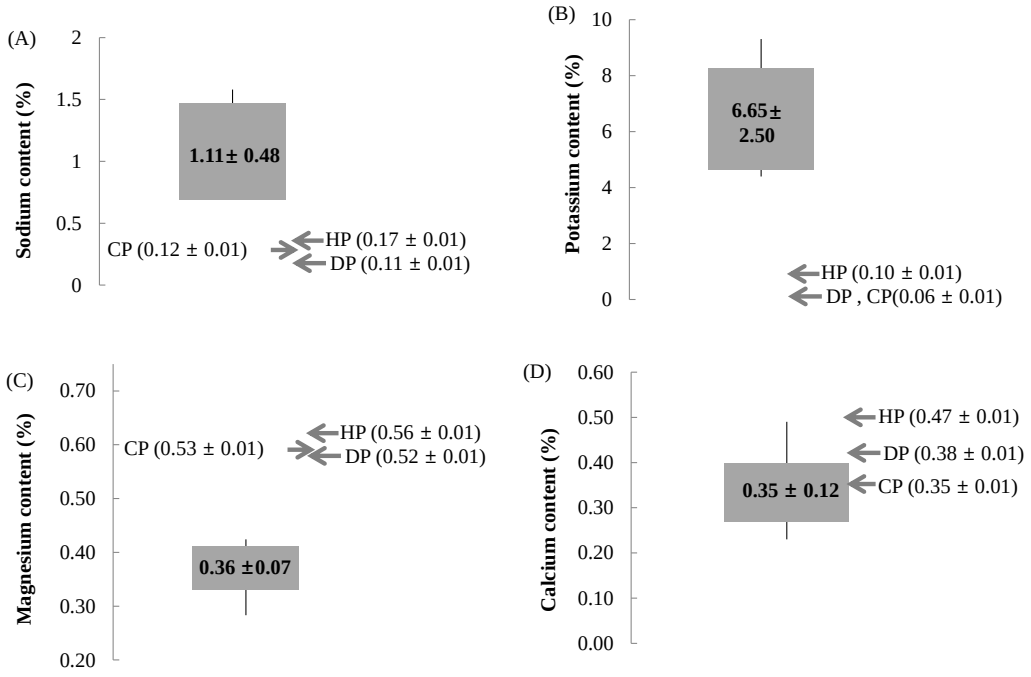

Figure 1-9. Mineral content from control strains (CP), disease resistant strains (DP) and high temperature resistant strains (HP) of *Pyropia yezoensis* :

(A) sodium, (B) potassium, (C) magnesium, and (D) calcium.

Data are mean ± standard deviation.

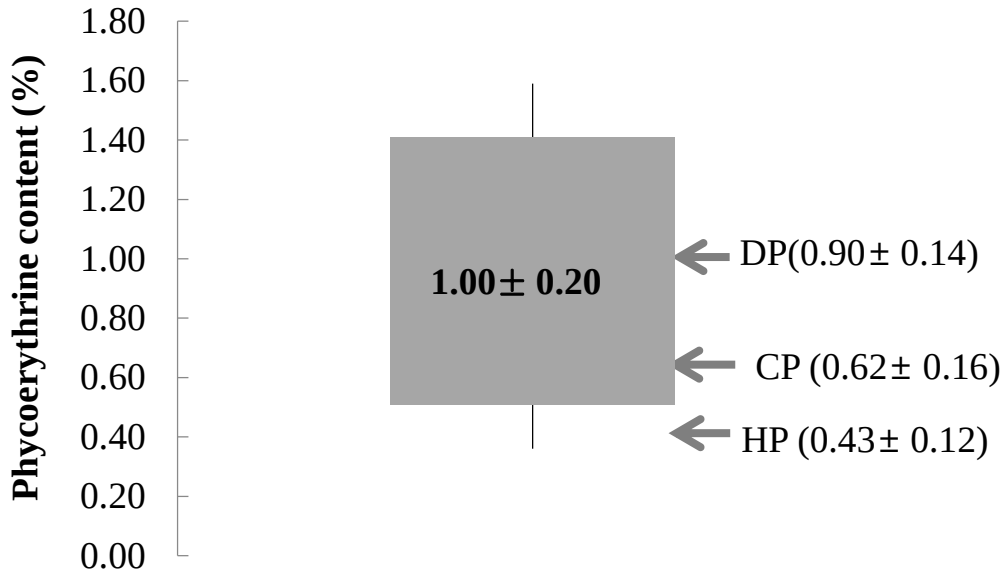

Figure 1-10. Phycoerythrine content from control strains (CP), disease resistant strains (DP) and high temperature resistant strains (HP) of *Pyropia yezoensis* .

Data are mean ± standard deviation.

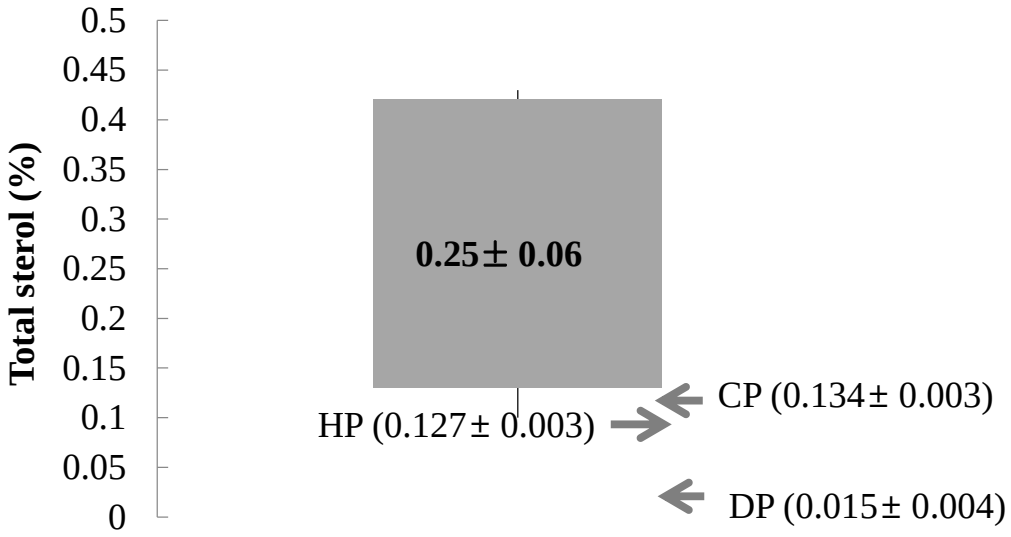

Figure 1-11. Total sterol content from control strains (CP), disease resistant strains (DP) and high temperature resistant strains (HP) of *Pyropia yezoensis* .

Data are mean ± standard deviation.

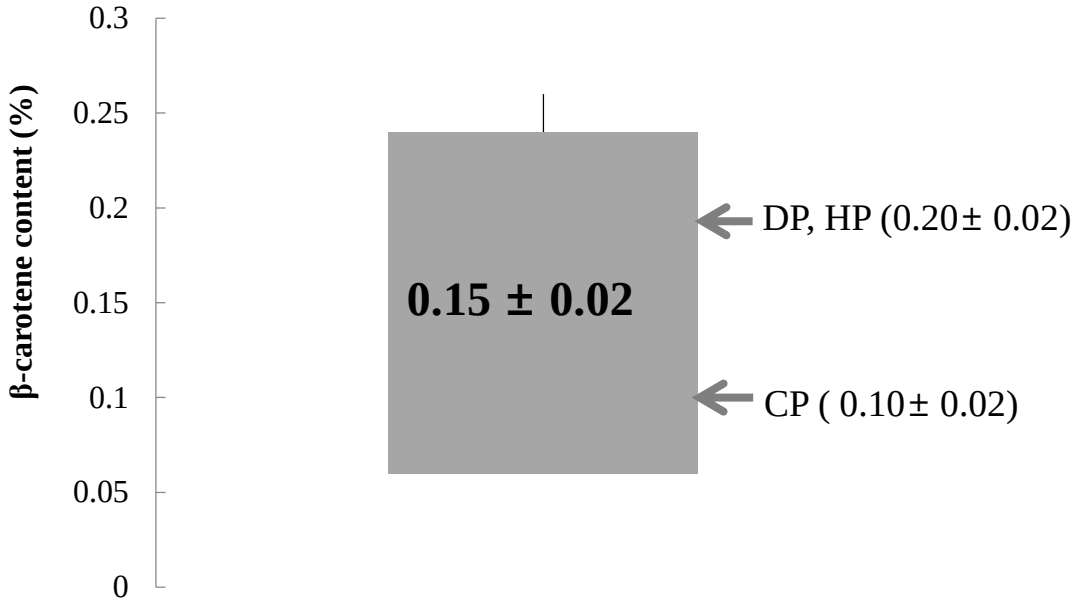

Figure 1-12. β- carotene content from control strains (CP), disease resistant strains (DP) and high temperature resistant strains (HP) of *Pyropia yezoensis*.

Data are mean ± standard deviation.

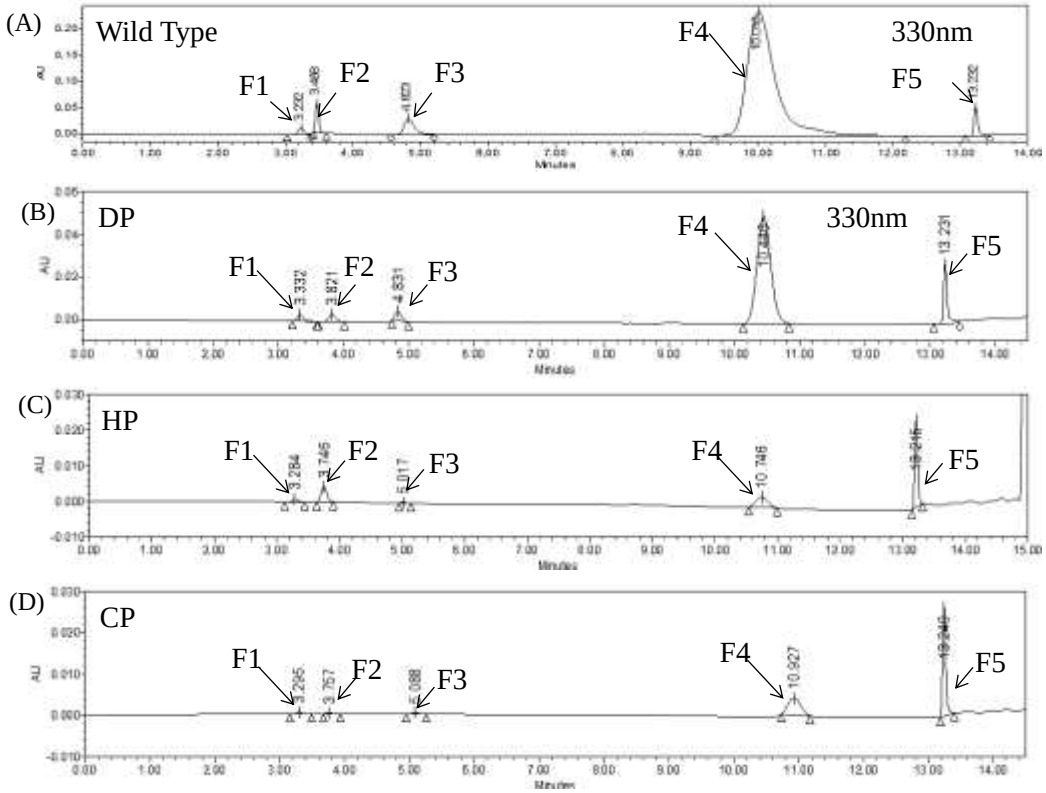

Figure 1-13. HPLC chromatograms (330 nm) for mycosporine-like amino acid (MAA) content:

(A) marine cultivated species, (B) disease resistant strains (DP), (C) high temperature resistant strains (HP) and (D) control strains (CP) of *Pyropia yezoensis*.

F1: Palythine, F2: Shinorine, F3: Asterine-330, F4: Porphyra-334, F5: Usujirene**.**

Table 1-3. Area value of each mycosporine-like amino acid component for marine cultivated species (CP), disease resistant strains (DP) and high temperature resistant strains (HP) of *Pyropia yezoensis* .

Area value(μV*sec)	DP	HP	CP
Palythine (F1)	**20329**	**5194**	**3116**
Shinorine (F2)	**23039**	**27163**	**2110**
Asterina-330 (F3)	**30254**	**1217**	**3070**
Porphyra-334 (F4)	**741281**	**38679**	**54969**
Usujirene (F5)	**113225**	**83478**	**91698**

Area value = Area value of mycosporine – like amino acids in HPLC chromatogram

(Unit: μ V* sec)

Part II.

Evaluation of antioxidant and anti-inflammation activity of enzymatic hydrolysate for disease and high temperature resistant *Pyropia yezoensis* strains

1. Abstract

This part investigated bioavailability which is availability of human gastric enzymatic hydrolysates from disease (DP) and high temperature resistant (HP) *Pyropia yezoensis* (*P. yezoensis*). Enzymatic hydrolysates from DP and HP strains were investigated for antioxidant and anti-inflammation activity using electron spin resonance (ESR), normal cell lines (Vero cell), and mouse macrophages (RAW 264.7). The enzymatic hydrolysates were prepared by 4 gastric hydrolytic enzymes (pepsin, trypsin, α-chymotrypsin, and α-amylase) and commercial hydrolytic enzymes (AMG and alcalase) which are known to hydrolyze carbohydrate and protein from *P. yezoensis.* Antioxidant and anti-inflammation effects were measured from alkyl radical and hydrogen peroxide scavenging activity and pro-inflammatory mediators such as nitric oxide (NO) levels. As a result, HP strains were shown as excellent antioxidant activity than DP and CP strains. And trypsin and α-chymotrypsin assisted hydrolysates of high temperature-resistant strains (HPA, HPT) showed significantly reduced alkyl radicals and hydrogen peroxide. But, NO levels were significantly decreased in AMG hydrolysates. In conclusion, HP strains have better antioxidant and anti-inflammation activity than DP strains and hence have potential as excellent seeds for the global seed industry.

2. MATERIALS AND METHODS

2.1. Materials

Enzymes including pepsin, trypsin, α-amylase, and α-chymotrypsin were purchased from Sigma Aldrich (USA), whereas AMG and Alcalase enzymes were purchased from Novozyme (Denmark). Fetal bovine serum (FBS) was purchased from Welgene (Daegu, Korea). And Dimethyl sulfoxide (DMSO), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 5, 5-Dimethyl-1-pyrroline N-oxide (DMPO) and α-(4-Pyridyl N-oxide)-N-tert-butylnitrone (POBN) reagents were purchased from Sigma Aldrich (USA). Roswell Park Memorial Institute (RPMI)-1640, phosphate buffered saline (PBS), antibiotic and trypsin-EDTA were purchased from Gibson/BRL (Burlington, Ont, Cananda).

2.2. Preparation of enzymatic hydrolysate

Powder of DHP strains were extracted by pepsin, trypsin, α-chymotrypsin, α-amylase, AMG, and Alcalase enzymes under optimal conditions (**Table 4**) for 12 h. The extracts were then inactivated by heating to 100℃ and cooling to 4℃, then filtered (qualitative circles 110 mm, Whatman, UK). Their yields were measured in a dry oven at 105℃, and they were finally freeze dried (Samwon, Korea)

2.3. Carbohydrate contents of enzymatic hydrolysate

Analysis of carbohydrate content was measured by the phenol-sulfuric method (Nielsen, 2009). Glucose standard (0.1 mg/ml) was prepared and the sample and standard placed in tube along with 25 μl 80% phenol solution, and 2.5 ml 95% sulfuric acid (DAE JUNG, Korea) then placed in the dark for 30 min. Measurements were then taken at 480 nm using an ELISA micro plate reader (SYNERGY HT, Bio TeK, USA).

2.4. Protein contents of enzymatic hydrolysate

Analysis of protein contents of their extracts was measured BCA protein Assay kit (protein biology, Thermo Fisher Scientific, Korea). BSA standard (1 mg/ml) and distilled water were added to each wells in 96-well plates. BCA reagents A and B were mixed (50:1) and added to each sample in the plate then the treated samples were kept at 37℃ for 30 min. Finally, the plate was measured at 562 nm by ELISA micro plate reader (SYNERGY HT, Bio TeK, USA).

2.5. Alkyl radical scavenging activity

Alkyl radical scavenging activity was measured using ESR spectrometer (JES-FA200, USA) introduced by Heo et al. (2008). Extraction samples (20 μl) or sample buffer (usually distilled water) were put in a e-tube along with 40 mM 2,2'-Azobis(2-methylpropion-amidine) dihydrochloride (AAPH) (Sigma Aldrich, USA) and 40 mM a-(4-pyridyl N-Oxide)-N-tert-butylnitrone (POBN) (Sigma Aldrich, USA). Then incubate at 37℃ for 30 min.

2.6. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was measured following the modified method of Sroka and Cisowski (2003). Extract samples were placed in a 96-micro-well plate, along with Phosphate buffer (0.1 M), and 10 mM Hydrogen peroxide (20 μl), and incubated at 37℃ for 5 min. After incubation, 1.25 mM ABTS and 1 unit/ml peroxidase was added, and samples were incubates at 37℃ for 10minute. Finally, the plate was measured by ELISA micro plate reader (SYNERGY HT, Bio TeK, USA) at 405 nm.

2.7. Cell culture

Vero cell isolated from kidney epithelial cells extracted from African green monkeys (Chlorocebus sp.) was incubated in a humidified CO_2 incubator (5% CO_2, along with cultured RPMI Medium 1640, containing 10% heat inactivated fetal bovine serum, and 0.1% penicillin streptomycin (100 µg/ml). RAW 264.7 cell line isolated mouse macrophage cells were also incubated in the humidified CO_2 incubator, and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 0.1% penicillin streptomycin (100 µg/ml).

2.8. Measurement of cell viability

Cell viability was measured by MTT assay which is a test for metabolic competence based on assessment of mitochondrial performance (Kim, 2006; Mosmaann, 1983). Vero cells were seeded in a 96 well micro plate at 5.0 X 10^4cells/ml and RAW 264.7 cells were seeded in 24 well micro plate at 2.0 X 10^5cells /ml. After 16–24 h, cells were treated with samples at 15.625, 31.25, 62.5, 125, and 250 µg/ml and incubated for 24 hours at 37℃. MTT solution (50 µl, 2 mg/ml) was then added to each well and incubated for 4 hours. And the plates were then carefully removed media by using suction and added DMSO (dimethyl sulfoxide) and incubated overnight at room temperature. Finally, absorbance was measured using an ELISA micro plate reader (SYNERGY HT, Bio TeK) at 540nm.

2.9. Measurement of intracellular reactive oxygen species (ROS) production

ROS production was measured by DCFH-DA assay. Vero cells ($5.0x10^4$) were pre-incubated for 16–24 hours. Then samples (10 µl) were treated with different concentration of trypsin and α-chymotrypsin – assisted extracts from high temperature- resistant strains (31.25, 62.5, and 125 µg/ml). After 1 h, cells were treated with 0.5 mM hydrogen peroxide

(10 µl) and incubated for 30 min. And DCF-DA (5 µg/ml) was introduced to the cells and incubated for 30min. Finally, the plates were detected at 485 nm excitation wavelength and 535 nm emission wavelength on an ELISA fluorescence spectrometer.

2.10. Measurement of nitric oxide (NO) production

After incubation of RAW 264.7 cell lines ($2.0x10^5$ cells/ml) with LPS (10 µg/ml) were incubated for 24 h, then the amount of nitric oxide accumulated in the culture medium was measured to indicator NO production. Briefly, 100 µl of cell culture medium was transferred to a 96 well plate and mixed with 100 µl of Griess regent [1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochlotide in 2.5% phosphoric acid]. The mixture was incubated at room temperature for 10 min, and was measured using a micro plate reader at 540 nm.

2.11. Statistical analysis

All data are expressed as mean ± standard deviation (SD). Significant differences among the groups were determined using the unpaired Student's-*t* test on SPSS 18. A value of $*p < 0.05$ or $**p < 0.01$ was accepted as an indication of statistical significance.

Table 2-1. Optimal hydrolysis condition of each enzyme (pepsin, trypsin, α-chymotrypsin, α-amylase, AMG, Alcalase) (substrate to enzyme: 100:1)

Enzyme	α-Amylase	AMG	Pepsin	trypsin	α-chymotrypsin	Alcalase
pH	7.0	4.5	2.0	8.0	8.0	8.0
Temperature(℃)	37	60	37	37	37	50

3. Results and Discussion

3.1. Yield

Figure 2-1 shows yield of enzymatic hydrolysate from DHP strains. Most HP extracts show higher yield than DP strains. Especially, trypsin and α-chymotrypsin assisted extracts showed higher yield than other enzymes. Many extracts from control strains (CP) showed higher yields than DHP strains. Protein hydrolytic enzymes (trypsin, α-chymotrypsin and alcalase) assisted extracts showed higher yield than other enzymes. Lee et al. (2016) reported that AMG assisted hydrolysates were shown higher yield than other enzymes. However, this study shows that trypsin and α-chymotrypsin assisted hydrolysates have higher yield than AMG and other enzymes. CP and HP strains shows similar yield for most enzymatic hydrolysates, but DP strains showed lower yields for all enzymatic hydrolysates.

3.2. Carbohydrate contents

Figure 2-2 shows the carbohydrate contents of enzymatic hydrolysate from DHP strains. Carbohydrate contents of pepsin, α-amylase, and AMG assisted extracts were higher than for other enzymes and carbohydrate contents were similar in DHP and CP strains. This result suggests that pepsin and carbohydrase – assisted extracts including α-amylase and AMG assisted extracts have higher carbohydrate content (50–60 %). And DP, HP, and CP strains have similar carbohydrate contents in all extracts. Lee et al. (2016) reported that AMG assisted hydrolysates have higher carbohydrate content than other enzymes but this study shows that pepsin and AMG assisted hydrolysates from DHP strains have higher carbohydrate contents.

3.3. Protein contents

Figure 2-3 shows protein content of enzymatic hydrolysate from DHP strains. Protein hydrolytic enzymes including pepsin and trypsin assisted extracts showed higher protein content than other enzymes (30 %) and HP strains have higher protein content than DP and CP strains. Cian et al. (2015) reported that protein hydrolytic enzyme assisted hydrolysates have higher total amino acid and protein content. This result suggests that all hydrolysates were 10–20% but this study shows that protein hydrolytic enzyme (pepsin, trypsin) assisted hydrolysates have higher protein contents than other enzymatic hydrolysates. HP and CP strains have similar protein contents in pepsin hydrolysates and HP strains have higher protein content than CP strains in trypsin hydrolysates.

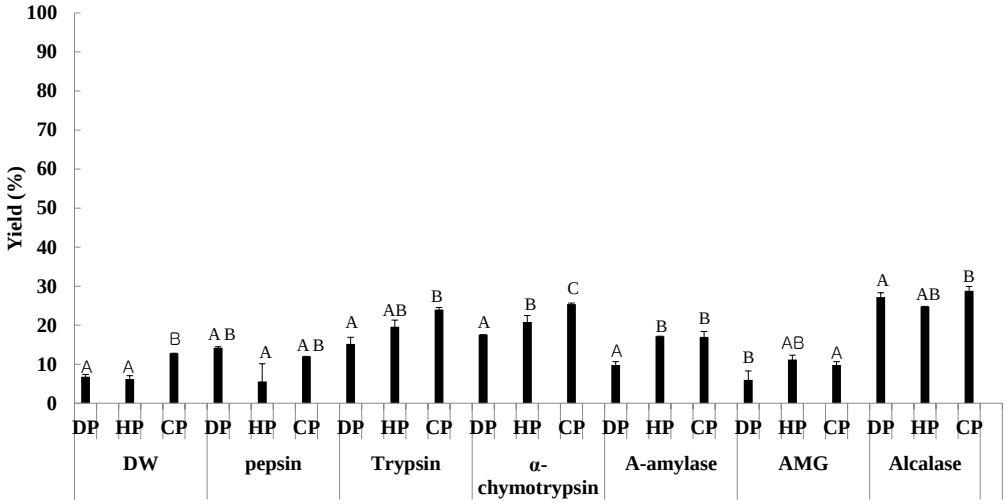

Figure 2-1. Yield of enzymatic hydrolysate from control (CP), disease (DP) and high temperature resistant (HP) strains of *Pyropia yezoensis*.

A, B, C: indicate significant differences between DP, HP, and CP groups (p <0.05).

Data are the means ± standard deviations of triplicate experiments

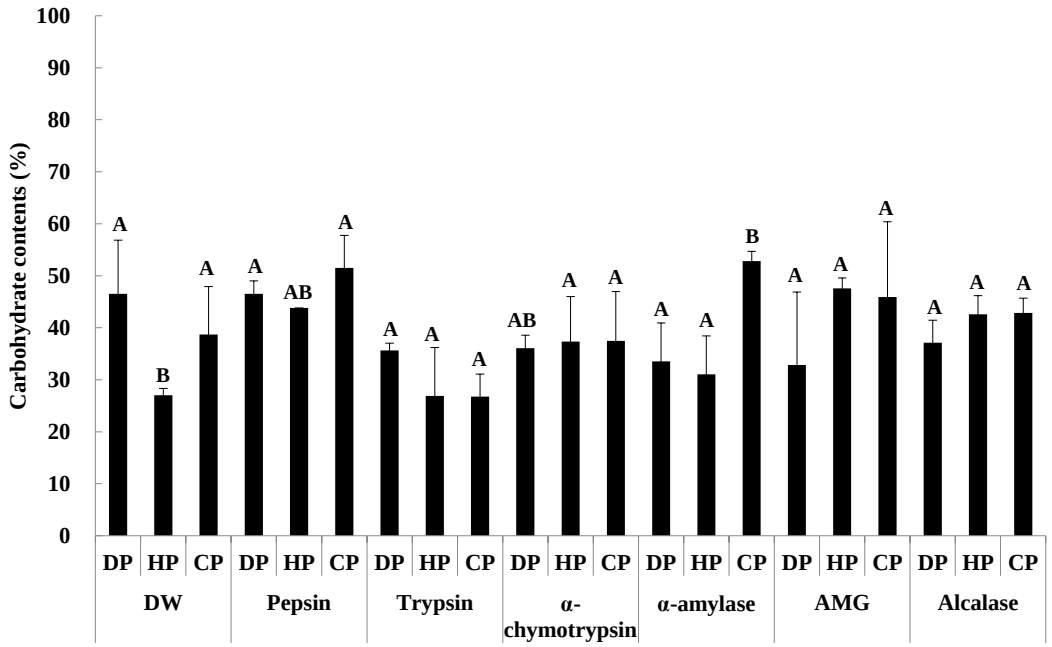

Figure 2-2. Carbohydrate contents of enzymatic hydrolysate from control (CP), disease (DP) and high temperature resistant (HP) strains of *Pyropia yezoensis*.

A, B, C: indicate significant differences between DP, HP, and CP groups (p <0.05).

Data are the means ± standard deviations of triplicate experiments

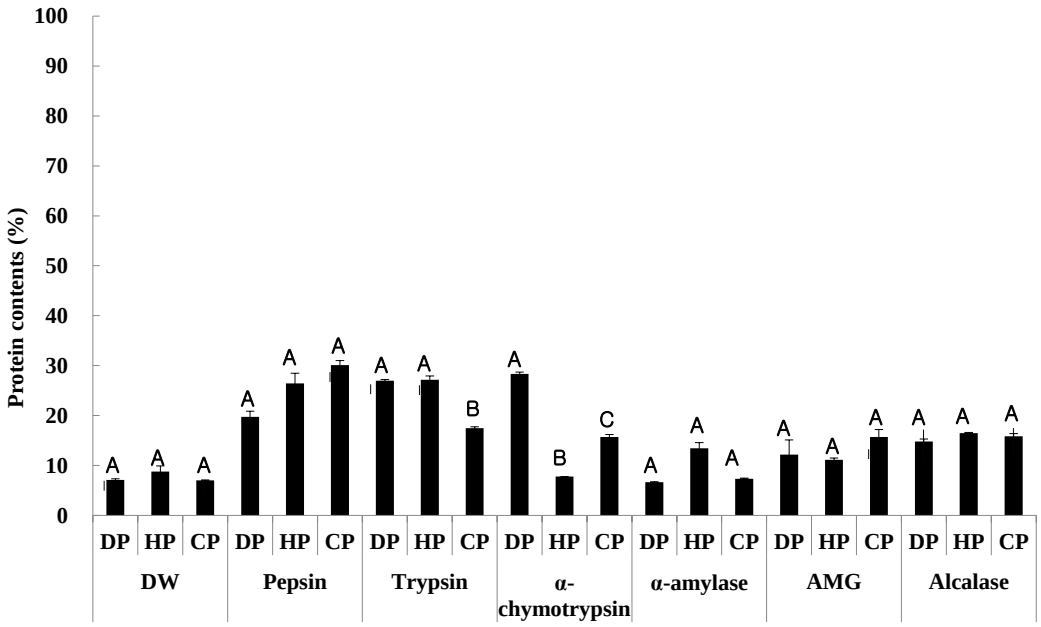

Figure 2-3. Protein contents of enzymatic hydrolysate from control strains (CP), disease resistant strains (DP) and high temperature resistant (HP) strains of *Pyropia yezoensis*.

A, B, C: indicate significant differences between DP, HP, and CP groups (p <0.05).

Data are the means ± standard deviations of triplicate experiments

3.4. Free radical scavenging activity

Table 2-2 shows IC_{50} values of enzymatic hydrolysates from DHP strains against alkyl radical. Heo et al. (2006) showed that lower IC_{50} values were meant better alkyl radical scavenging activity. Most CP extracts show better alkyl radical scavenging activity than DHP strains. However, in protein hydrolytic enzymes including trypsin and α-chymotrypsin HP strains show better alkyl radical scavenging activity than control strains. This suggests that protein hydrolytic enzyme assisted extracts from HP strains (including trypsin and α-chymotrypsin) had better antioxidant activity. **Table 2-3** shows IC_{50} values of enzymatic hydrolysates from DHP strains against hydrogen peroxide. HP strains show better antioxidant activity than DP strains and gastric protein hydrolytic enzyme assisted extracts (trypsin and α-chymotrypsin) show better antioxidant activity than other enzymes. Cian et al. (2015) reported that enzymatic hydrolysates from red seaweeds have strong free radical scavenging activity and Lee et al. (2016) reported that AMG assisted hydrolysates from *P. yezoensis* have strong free radical scavenging activity. In contrast, this study shows that trypsin and α-chymotrypsin assisted hydrolysates have stronger antioxidant activity than AMG and other enzymes.

3.5. Cell viability in H_2O_2 - induced Vero cells

Trypsin and α-chymotrypsin assisted hydrolysates from HP strains have higher antioxidant activity than other enzymes. Therefore, we evaluated trypsin and α-chymotrypsin assisted hydrolysate cytotoxicity in Vero cells as shown in **Figure 2-4**. Trypsin and α-chymotrypsin HP and CP extracts show no cytotoxicity in Vero cells (250, 125, and 62.5 μg/ml). **Figure 2-4** shows the protective effects of trypsin and α-chymotrypsin assisted hydrolysates from HP strains (HPT, HPA) on H_2O_2- induced Vero cells. HPT and HPA extracts show better activity than CPT and CPA. Cian et al. (2015) reported that pepsin and pancreatin (α-chymotrypsin)

assisted hydrolysates showed higher antioxidant activity whereas this study shows that trypsin and α-chymotrypsin assisted hydrolysates from HP strains have higher antioxidant activity than CP hydrolysates. Thus, trypsin and α-chymotrypsin assisted HP hydrolysates have higher antioxidant activity than CP strains and showed no cytotoxicity in Vero cells.

3.6. Effects of DHP in H_2O_2 – induced intracellular ROS productions on Vero cells.

Intracellular ROS scavenging activity of HPA and HPT was measured based on the principle that DCFH-DA reacts with ROS in the cells and is oxidized to DCF, a fluorescent substance. Vero cells were treated with HPA and HPT for 1 h, and then treated with 500 μM H_2O_2 for 30 min. **Figure 2-6** shows that intercellular ROS scavenging activity (40 %) for 125μg/ml HPA. This result shows that HPA has a good intracellular ROS scavenging activity on H_2O_2 - induced Vero cell. Cian et al. (2015) reported that protein hydrolytic enzymes (pepsin, pancreatin) assisted extracts showed higher antioxidant activity whereas this study shows that α-chymotrypsin assisted extracts from HP strains have higher antioxidant activity than other enzymes. Therefore, HP strains showed better cell protective effects and intercellular ROS scavenging activity than CP strains.

Table 2-2. Alkyl radical scavenging activity (IC_{50}) of enzymatic hydrolysates from control (CP), disease resistant strains (DP) and high temperature resistant strains (HP)

IC_{50} Value(mg/ml)	DP	HP	CP
DW	**0.54± 0.02^{A}**	**0.54± 0.03^{A}**	**0.37± 0.01^{B}**
Pepsin	**1.20± 0.18^{B}**	**0.75± 0.19^{AB}**	**0.53± 0.18^{A}**
Trypsin	**0.54± 0.04^{AB}**	**0.46± 0.00^{A}**	**0.54± 0.02^{B}**
α-chymotrypsin	**0.28± 0.11^{A}**	**>0.3125**	**0.17± 0.10^{A}**
α-amylase	**1.00± 0.19^{B}**	**0.62± 0.01^{A}**	**0.42± 0.09^{A}**
AMG	**1.28± 0.09^{A}**	**1.02± 0.07^{A}**	**0.87± 0.48^{A}**

$^{A, B}$: indicate significant differences between DP, HP and CP groups (p <0.05).

Data are mean ± standard deviation of triplicate experiments.

Table 2-3. Hydrogen peroxide scavenging activity (IC_{50}) of enzymatic hydrolysates from control (CP) and disease resistant strains (DP) and high temperature resistant strains (HP)

	DW			Pepsin			Trypsin			α-chymotrypsin		
	DP	HP	CP	DP	HP	CP	DP	HP	CP	DP	HP	CP
IC_{50} Value (mg/ml)	1.29± 0.14^{A}	1.04± 0.13^{A}	1.19± 0.24^{A}	1.09± 0.23^{A}	0.68± 0.20^{B}	0.65± 0.16^{B}	0.15± 0.04^{A}	0.08± 0.03^{A}	0.08± 0.03^{A}	0.15± 0.05^{A}	0.15± 0.09^{A}	0.10± 0.02^{A}
				Alcalase			AMG			α-amylase		
				DP	HP	CP	DP	HP	CP	DP	HP	CP
				0.90± 0.12^{A}	0.40± 0.15^{B}	0.60± 0.13^{A}	0.16± 0.05^{A}	0.12± 0.00^{A}	0.27± 0.06^{B}	0.85± 0.12^{A}	0.65± 0.15^{A}	0.52± 0.14^{A}

$^{A, B}$: indicate significant differences between DP, HP and CP groups (p <0.05).

Data are mean ± standard deviation of triplicate experiments.

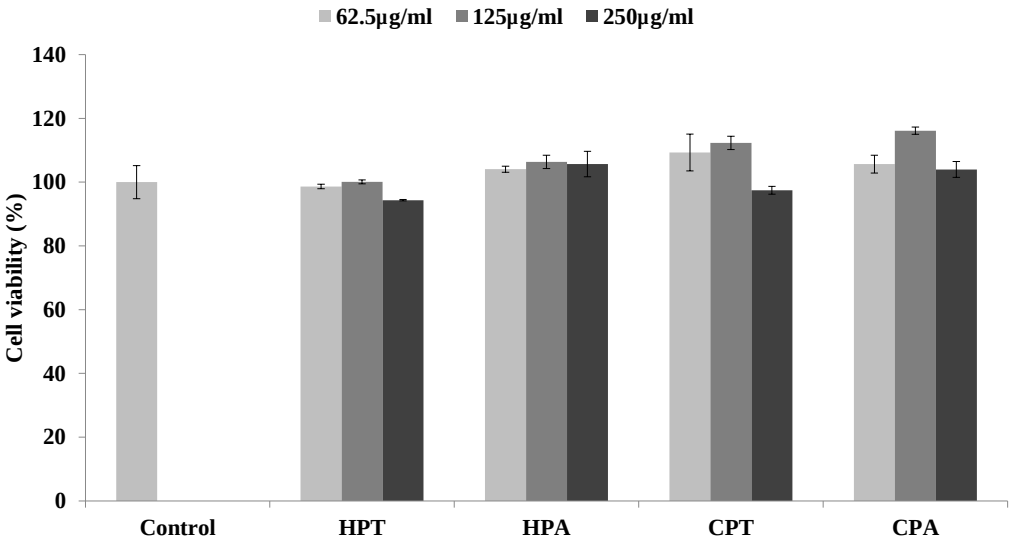

Figure 2-4. Cytotoxicity for trypsin and α-chymotrypsin assisted extracts from high temperature resistant (HPT and HPA) and control (CPT and CPA) in Vero cell.

Data are mean ± standard deviation of triplicate experiment.

**$p<0.01$ and * $p<0.05$ indicate significant differences from the control group.

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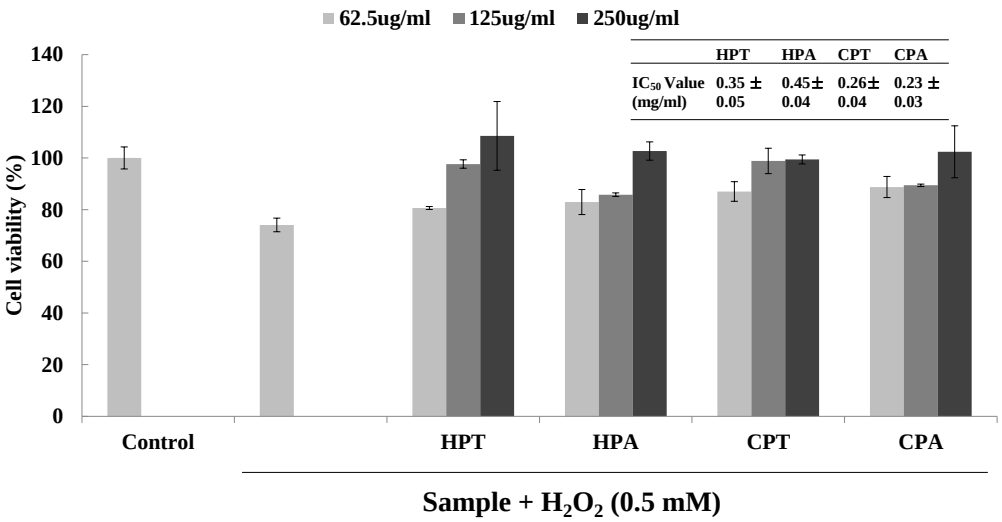

Figure 2-5. Cell viability for trypsin and α-chymotrypsin assisted extracts from high temperature resistant (HPT and HPA) and control (CPT and CPA) on H_2O_2-induced Vero cells.

Data are mean ± standard deviation of triplicate experiment.

**p<0.01 and * p<0.05 indicate significant differences from the control group.

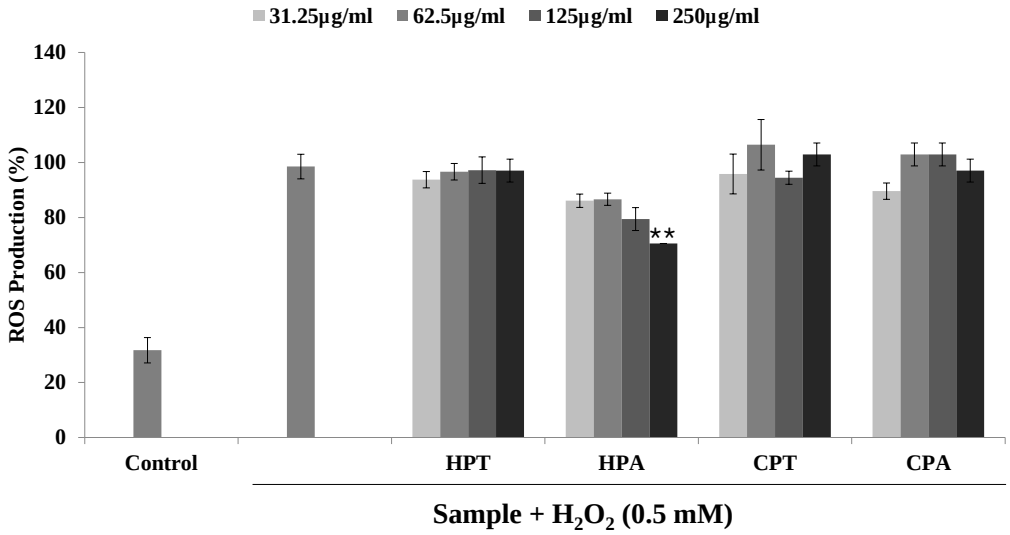

Figure 2-6. Reactive oxygen species (ROS) production for trypsin and α-chymotrypsin assisted extracts from high temperature resistant (HPT and HPA) and control (CPT and CPA) on H_2O_2- induced Vero cells.

Data are mean ± standard deviation of triplicate experiment.

**p<0.01 and * p<0.05 indicate significant differences from the H_2O_2-stimulated group.

3.7. Effects of DHP in LPS - induced NO production on RAW 264.7 cells

We examined potential anti-inflammation properties of enzymatic hydrolysates from DHP on LPS -induced NO production on RAW 264.7 cells. Cells were treated with or without extract samples (125 μg/ml and 250 μg/ml) for 1 h and then treated with LPS (1 μg/ml) for 16–24 h. NO concentrations were measured in the culture supernatants by Griess reactions and ELISA assays. **Figure 2-7** shows that LPS treatment significantly increased NO concentration but extracts from HP strains inhibited LPS induced production of NO in a concentration dependent manner. **Figure 2-8** shows cell viability in RAW 264.7 cells measured by MTT assay. HP extracts show better anti-inflammation activity than other enzymes. Lee et al. (2016) reported that AMG assisted hydrolysates had higher antioxidant and anti-inflammatory activity than other enzymes. Lee et al (2015) reported that bioactive peptides have strong anti-inflammation activity. This study confirms that AMG assisted hydrolysates from CP strains had higher NO scavenging activity than HP and DP strains but trypsin and α-chymotrypsin assisted hydrolysates from CP strains have lower anti-inflammatory activity than HP strains (**Figure 2-7**) and α-chymotrypsin assisted hydrolysates had higher cell viability than other enzymatic hydrolysates (**Figure 2-8**). Thus, AMG assisted hydrolysates have higher anti-inflammatory activity but lower cell viability than other enzymatic hydrolysates.

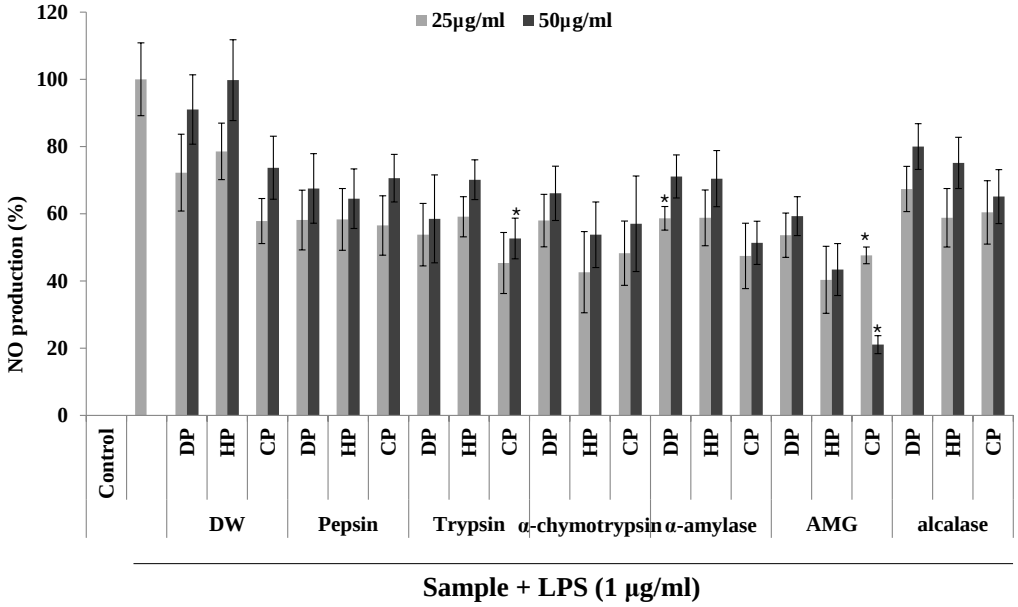

Figure 2-7. Nitric oxide (NO) production for trypsin and α-chymotrypsin assisted extracts from disease and high temperature resistant-strains on LPS-induced RAW 264.7 cells.

Data are mean ± standard deviation of triplicate experiment.

**$p<0.01$ and * $p<0.05$ indicate significant differences from the LPS-stimulated group.

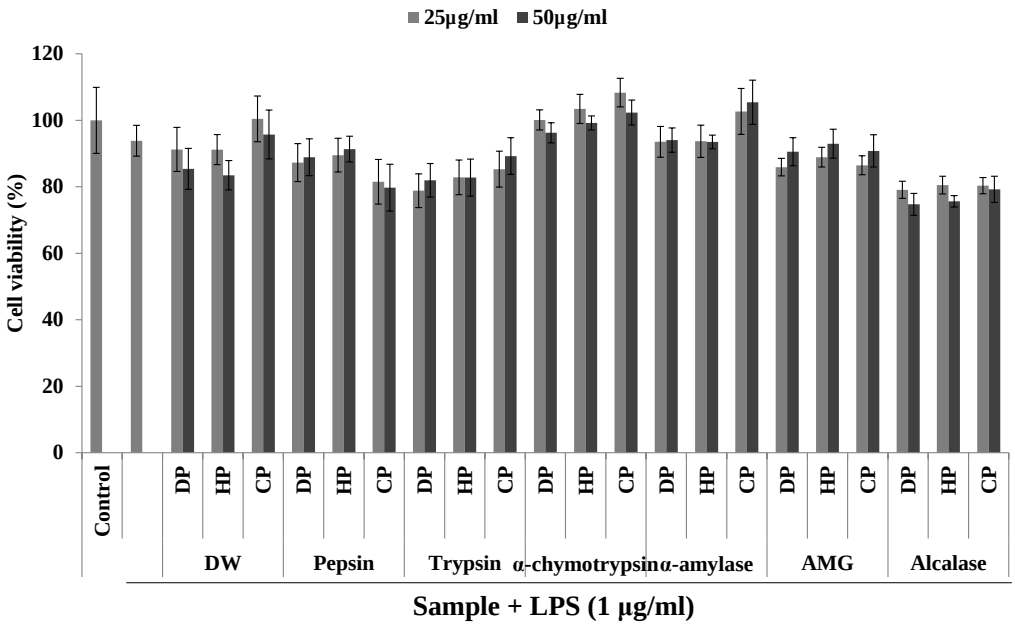

Figure 2-8. Cell viability for trypsin and α-chymotrypsin assisted extracts from disease and high temperature resistant-strains on LPS-induced RAW 264.7 cells.

Data are mean ± standard deviation of triplicate experiment.

**$p<0.01$ and * $p<0.05$ indicate significant differences from the control group.

CONCLUSION

Pyropia yezoensis (*P. yezoensis*) is widely cultivated in East Asia and it is mainly cultivated at low water temperature. However, water temperature is steadily increasing due to global warming and disease and high temperature resistant strains have attracted considerable research interest. We analyzed proximate composition, monosaccharide, amino acid, and mineral contents of disease resistant (DP) and high temperature resistant (HP) strains of *P. yezoensis*. Disease resistant strains showed higher protein and essential amino acid content than HP strains but HP strains have higher carbohydrate and monosaccharide content than DP strains. Functional components from DHP strains were showed lower content than marine cultivated species and control strains (CP) because phycoerythrin, β-carotene, sterols, and MAAs are influenced by environmental factors. And HP strains didn't show cytotoxicity in vero cell and it shows as higher intracellular ROS scavenging activity and NO scavenging activity than DP strains. In conclusion, HP strains showed higher phycoerythrin and sterol content and carbohydrate contents. And they showed as higher anti-oxidant and anti- inflammation activity than DP strains. Therefore, we found that HP strains have a potential for excellent seeds.

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석사학위를 시작한 지가 엊그제 같은데 벌써 2년이라는 시간이 흘러 졸업을 앞두고 있으니 지난 세월이 주마등처럼 지나가며 만감이 교차 합니다. 그 동안 남들보다 부족한 저의 석사학위 논문을 완성하고 마무리할 수 있도록 물심양면으로 도와주신 많은 분들이 있는데, 이 자리를 빌려 그 분들에게 감사의 표현을 하겠습니다. 우선, 제가 석사학위 과정 동안 어려움을 겪지 않도록 물심양면 도와주시고 오로지 제가 자신감을 찾아 더 잘할 수 있도록 많은 가르침을 주신 전유진 교수님께 무한한 감사의 말씀을 드립니다. 교수님의 애정 어린 지도로 인해 제가 석사 학위 과정 처음에 가졌던 두려움도 떨쳐버리고 무사히 학위과정을 마칠 수 있게 되었습니다. 앞으로 교수님의 가르침에 보답하여 교수님과 이 실험실의 명예를 드높이도록 한걸음 더 나아가도록 하겠습니다. 더불어 저의 학위논문 심사를 위해 바쁘신 와중에 시간을 내어 어려운 발걸음을 해주신 송춘복 교수님, 허문수 교수님께도 감사의 말씀을 드리며, 오로지 부족한 학위논문을 더 잘 마무리 하기 위해 심사 중에 제게 해주신 조언들 앞으로 각골난망하며 살아가도록 하겠습니다. 그리고 학부과정과 석사과정에서 전공지식 및 많은 가르침을 주신 해양의생명과학부 의 최광식 교수님, 이영돈 교수님, 김기영 교수님, 이제희 교수님, 이경준 교수님, 이승헌

교수님, 여인규 교수님, 정석근 교수님, 박상율 교수님 그리고 정준범 교수님께도 감사의 말씀을 드립니다. 교수님의 진심 어린 관심과 조언으로 이 학위논문을 완성할 수 있었습니다. 그리고 우리 해양생물자원이용공학 실험실의 여러 박사님과 선배님 들도 대단히 감사합니다. 먼저, 부족함이 많은 제게 여러 가지의 조언을 많이 해주신 이지혁 박사님과 고주영 박사님, 그리고 제가 고민이나 걱정거리가 있을 때마다 가끔씩 조언과 좋은 말씀을 많이 해주시는 이원우 박사님, 그리고 부족한 저의 발표를 위해 많은 조언을 해주신 류보미 박사님, 그리고 제가 학위논문 실험을 진행하는 거에 있어서 많은 도움을 주신 강민철 박사님, 그리고 부족한 저의 학위논문을 처음부터 끝까지 차근차근 지도해 주신 오재영 박사님께 감사의 말씀을 전합니다. 박사님들의 진심 어린 관심과 조언이 제가 한걸음 더 성장하는데 큰 힘이 되었습니다. 그리고 실험실에 있다가 박사를 졸업하고 박사 후 과정을 하고 있는 은아 누나와 나래 누나도 실험실에 있는 동안에 제가 어려움을 겪지 않도록 진심 어린 조언을 아낌없이 해주셔서 제가 무사히 석사학위 과정을 마칠 수 있었다고 감사의 말씀을 드립니다. 그리고 가끔씩 부족한 제가 잘되길 바라는 마음에서 진심 어린 조언을 아낌없이 하며 학위논문 실험을 진행하는 동안 바쁜 시간 쪼개며 많은 도움을 주신 서영이 누나, 그리고 항상 웃으면서 진심 어린 조언을 해주시는 현수형, 그리고 제가

잘되길 바라는 마음에서 진심 어린 조언을 하며 도움을 주신 혜원이 누나, 그리고 지금은 멀리 떨어져 있지만 서로 공감대를 형성하며 서로에게 의지가 되는 둘도 없는 친구이자 석사 동기 수현이, 이제 내년부터는 더 바쁠 예정인 효근이형, 그리고 나보다 후배이지만 가끔씩 조언을 해주며 곁에서 힘을 주는 준건이, 그리고 인생 경험이 많아 항상 내가 배울게 더 많은 지민이 형, 그리고 항상 웃으며 열심히 하는 범석이 그리고 멀리서 온 스리랑카 와 중국 사람들, 제가 옆에서 질문하면서 가끔 귀찮게 해도 웃으면서 잘 대답해주는 샤누라 그리고 항상 아침마다 웃으면서 인사하는 아산카 그리고 온지 얼마 안되어 앞으로 더 열심히 할 틸리나 와 히루니 그리고 항상 웃으면서 편하게 대하는 왕레이, 위린, 윤페이 그리고 교환학생으로 온 유안까지 모두 감사합니다. 여러분 덕분에 제가 많은 것을 배우고 느끼며 한걸음 더 나아갈 수 있었습니다. 그리고 함께 자리가 생길 때마다 제가 더 발전할 수 있도록 늘 좋은 말씀을 많이 해주시고 우리 실험실을 빛내 주신 허수진 박사님, 김길남 박사님, 이승홍 교수님, 그리고 안긴내 교수님 및 다른 박사 졸업하신 선배님 들께 감사의 말씀을 드립니다. 그리고 항상 함께 자리를 할 때마다 웃으면서 분위기가 가라앉지 않게 힘을 주시는 준성이 형 그리고 제게 도움이 될만한 진심 어린 조언을 해주시는 은이 누나 그리고 제가 처음 실험 실에 왔을 때 많은 가르침과

도움을 주신 윤택이 형과 형호 형에게도 감사의 말씀을 드립니다. 그리고 내병성 및 고온내성 방사무늬 김 샘플을 제공해 주신 공주대학교 김광훈 교수님 연구실의 연구원분 들께 감사의 말씀 드립니다. 그리고 멀리 떨어져 있지만 전화통화를 하면서 안부를 주고 받으며 제가 흔들리지 않고 주어진 길을 똑바로 갈 수 있도록 힘을 주는 저의 가족들, 집에서 가장 막내여서 멀리 떨어져 있지만 언제든 전화 통화를 하면 반가운 목소리로 받아 주시는 어머니, 그리고 어머니처럼 표현이 많은 편은 아니지만 그래도 제가 힘든 일이 있으면 같이 고민 해주며 조언도 해주시는 든든한 아버지, 그리고 항상 제가 힘들어 할 때 마다, 내가 자랑스럽다면서 제게 힘을 주는 나의 든든한 비오 형이 있었기 때문에 제가 힘들어 하며 흔들릴 때 마다 흔들리지 않고 무사히 석사학위를 마칠 수 있었던 것 같습니다. 그래서 이 자리를 빌려 우리 가족들에게 사랑하며 감사하다고 말하고 싶습니다. 그리고 제가 타향살이를 하는 동안 정말 가족같이 지냈던 같은 학사 동기이면서 기숙사에서 같이 방을 사용하면서 친하게 지냈던 기숙사 사람들인 형주형, 인재형, 재원이, 진수, 은성이, 그리고 지금은 비록 연락이 되지 않지만 친하게 지냈었던 민철이 형까지 이 사람들이 있었기에 외롭고 힘든 타향살이에서도 흔들리지 않고 지낼 수 있었던 것 같습니다. 그리고 제가 성당에 갈 때마다 항상 웃으며 반갑게 맞이 해주는 저희 김기량 성당

아르코 청년회와 주임 신부님, 수녀님 그리고 제주 가톨릭 대학생 연합회의 회원으로서 활동하며 친하게 지냈었던 제주 교구청 소속 김석주 베드로 신부님 이하의 많은 사람들께 감사의 말씀을 드립니다. 이 사람들 덕분에 제가 힘들고 지치는 학위 과정 동안 다시 일어 설수 있는 힘을 가질 수 있게 되었으며, 그리고 제가 조금 자신감을 되찾을 수 있게 도움을 주었습니다. 끝으로, 이제까지 언급한 이 모든 사람들과 언급하지 못했던 사람들 모두들 덕분에 이 부족한 제가 무사히 석사 학위 과정을 마칠 수 있었습니다. 정말로 감사합니다.