A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

A STUDY ON POTENTIAL ANTIOXIDATIVE PIGMENTS OF MICROALGAE IN JEJU ISLAND, KOREA

Karawitage Rohan Samantha Karawita

Department of Marine Biotechnology

GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

2006. 6

A study on potential antioxidative pigments of microalgae in Jeju Island, Korea

Karawitage Rohan Samantha Karawita
(Supervised by Prof. You-Jin Jeon)

A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy

2006. 6

This thesis has been examined and approved by

………………………………………

Thesis director, Joon-Baek Lee, Prof. of Oceanography

………………………………………

Choon-Bok Song, Prof. of Marine Biotechnology

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

………………………………………

Moon-Soo Heo, Prof. of Marine Biotechnology

………………………………………

Jehee Lee, Prof. of Marine Biotechnology

………………………………………

You-Jin Jeon, Prof. of Marine Biotechnology

……………………..

Date

Department of Marine Biotechnology
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

Contents

SUMMARY xi

List of Figures xv

List of Tables xix

INTRODUCTION 1

Part I. Algal characterization and its culture technique for advanced study. 5

1. IMPORTANCE OF ALGAE 6

2. MORPHOLOGICAL CHARACTERISTICS 9
   2.1. Freshwater habitat species 9
   2.2. Tidal pool habitat species 10
   2.3. Jeju coastal benthic habitat species 10

3. SAMPLE COLLECTION 12
   3.1. Freshwater species 12
   3.2. Tidal pool species 13
   3.3. Jeju coastal benthic habitat species 13
   3.4. Isolation of axenic strain 14

4. MASS CULTURE AND FREEZE DRY 15
   4.1. Jeju coastal benthic habitat species 15
   4.2. Tidal pool species 16
   4.3. Freshwater species 16

Part II. Antioxidant properties of microalgae 19

1. ANTIOXIDANT PROPERTIES OF FRESH WATER MICROALGA, *Pediastrum duplex* 20
   1.1. ABSTRACT 20
   1.2. MATERIALS AND METHODS 21
      1.2.1. Materials 21
      1.2.2. Preparation of extract and solvent fractionation 22

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.2.3. Enzymatic hydrolysis procedure 23
1.2.4. Proximate composition 24
1.2.5. Reactive oxygen species inhibition....................................... 25
1.2.5.1. DPPH free radical scavenging assay............................... 25
1.2.5.2. Hydrogen peroxide scavenging assay 25
1.2.5.3. Superoxide anion scavenging assay................................ 25
1.2.5.4. Hydroxyl radical scavenging assay................................. 26
1.2.6. Nitric oxide radical inhibition assay 26
1.2.7. Metal chelating ability 27
1.2.8. Determination of antioxidant activity with the ferric thiocyanate (FTC) method 27
1.2.9. Total polyphenol contents... 27
1.2.10. Scavenging activity/chelating ability calculation 28
1.2.11. Statistical analysis... 28
1.3. RESULTS 29
1.3.1. Proximate composition 29
1.3.2. Reactive oxygen species inhibition....................................... 29
1.3.2.1. DPPH free radical scavenging effect 29
1.3.2.2. Hydrogen peroxide scavenging effects 29
1.3.2.3. Superoxide anion scavenging effects.................................. 30
1.3.2.4. Hydroxyl radical scavenging effects................................... 30
1.3.3. Nitric oxide radical inhibition effects 31
1.3.4. Metal chelating effect 31
1.3.5. Determination of antioxidant activity with the ferric thiocyanate (FTC) method 32
1.3.6. Total polyphenol content 32
1.4. DISCUSSION 38

2. ANTIOXIDANT PROPERTIES OF FRESH WATER BLUE-GREEN MICRO ALGA (CYANOPHYCEAE), *Dactylococcopsis fascicularis* ... 45
2.1. ABSTRACT.. 45
2.2. MATERIALS AND METHODS.. 46
2.3. RESULTS 46
2.3.1. Proximate composition 46
2.3.2. Reactive oxygen species inhibition.. 46

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2.3.2.1. DPPH free radical scavenging effect 46
2.3.2.2. Hydrogen peroxide scavenging effect 47
2.3.2.3. Hydroxyl radical scavenging effect 47
2.3.2.4. Superoxide anion scavenging effect 49
2.3.3. Nitric oxide radical inhibition effect....................................... 49
2.3.4. Metal chelating effect 52
2.3.5. Determination of antioxidant activity with the ferric thiocyanate (FTC) method 52
2.3.6. Total polyphenol content 55
2.4. DISCUSSION 55

3. ANTIOXIDANT PROPERTIES OF TIDAL POOL MICROALGA, *Halochlorococcum porphyrae* 62
3.1. ABSTRACT... 62
3.2. MATERIALS AND METHODS.. 63
3.3. RESULTS 63
3.3.1. Proximate composition 63
3.3.2. Reactive oxygen species inhibition.. 63
3.3.2.1. DPPH free radical scavenging effect 63
3.3.2.2. Hydrogen peroxide scavenging effect 64
3.3.2.3. Superoxide scavenging effect 64
3.3.2.4. Hydroxyl radical scavenging efefct 64
3.3.2.5. Nitric oxide radical inhibition effects 65
3.3.3. Metal chelating effect 71
3.3.4. Determination of antioxidant activity with the ferric thiocyanate (FTC) method 71
3.3.5. Total polyphenol content 71
3.4. DISCUSSION 72

4. ANTIOXIDANT PROPERTIES OF TIDAL POOL MICROALGA, *Oltamannsiellopsis* 78
4.1. ABSTRACT... 78
4.2. MATERIALS AND METHODS.. 79
4.3. RESULTS 79
4.3.1. Proximate composition 79
4.3.2. Reactive oxygen species inhibition.. 79

4.3.2.1. DPPH free radical scavenging effect 79
4.3.2.2. Hydrogen peroxide scavenging effect 80
4.3.2.3. Superoxide scavenging effect 80
4.3.2.4. Hydroxyl radical scavenging activity 81
4.3.3. Nitric oxide scavenging effect 81
4.3.4. Metal chelating effects 87
4.3.5. Determination of antioxidant activity with the ferric thiocyanate (FTC) method 87
4.3.6. Total polyphenol content 87

4.4. DISCUSSION 88

5. ANTIOXIDANT PROPERTIES OF BENTHIC DIATOM, *Achnanthes longipes* 95
5.1. ABSTRACT 95
5.2. MATERIALS AND METHODS 96
5.3. RESULTS 96
5.3.1. Proximate composition 96
5.3.2. Reactive oxygen species inhibition 96
5.3.2.1. DPPH free radical scavenging effect 96
5.3.2.2. Hydrogen peroxide scavenging effect 97
5.3.3.3. Superoxide anion scavenging effect 97
5.3.3.4. Hydroxyl radical scavenging effect 97
5.3.4. Nitric oxide radical inhibition effects 98
5.3.5. Metal chelating effect 98
5.3.6. Determination of antioxidant activity with the ferric thiocyanate (FTC) method 104
5.3.7. Total polyphenol content 104
5.4. DISCUSSION 105

6. ANTIOXIDANT PROPERTIES OF BENTHIC DIATOM, *Navicula* sp. 111
6.1. ABSTRACT 111
6.2. MATERIALS AND METHODS 112
6.3. RESULTS 112
6.3.1. Proximate composition 112

6.3.2. Reactive oxygen species inhibition 112
6.3.2.1 DPPH free radical scavenging effect 112
6.3.2.2. Hydrogen peroxide scavenging effect 116
6.3.2.3. Superoxide scavenging effect 116
6.3.2.4. Hydroxyl radical scavenging efefct 116
6.3.3. Nitric oxide radical inhibition effect 119
6.3.4. Metal chelating effect 119
6.3.5. Determination of antioxidant activity with the ferric thiocyanate (FTC) method 119
6.3.6. Total polyphenol content 120
6.4. DISCUSSION 120
7. ANTIOXIDANT PROPERTIES OF BENTHIC DIATOM, *Amphora coffeaeformis* 126
7.1. ABSTARCT 126
7.2. MATERIALS AND METHODS 127
7.3. RESULTS 127
7.3.1. Proximate composition 127
7.3.2. Reactive oxygen species inhibition 127
7.3.2.1. DPPH free radical scavenging effect 127
7.3.2.2. Hydrogen peroxide scavenging effect 128
7.3.2.3. Superoxide anion scavenging assay 128
7.3.2.4. Hydroxyl radical scavenging assay 128
7.3.3. Nitric oxide radical inhibition effects 129
7.3.4. Metal chelating effect 129
7.3.5. Determination of antioxidant activity with the ferric thiocyanate (FTC) method 135
7.3.6. Total polyphenol content 135
7.4. DISCUSSION 135
Part III. Protective effect of microalgae against DNA damage induced by H_2O_2 143
1. ABSTRACT 144
2. MATERIALS AND METHODS 144
2.1. Enzymatic hydrolysis procedure 144

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2.2. Hydrogen peroxide scavenging assay 145
2.3. Isolation and cryoconservation of human peripheral
lymphocytes 145
2.4. Incubation of lymphocytes.. 146
2.5. Determination of DNA damage 146
3. RESULTS 147
4. DISCUSSION 163
Part IV . Isolation and furification of pigments from microalgae............. 165
1. ISOLATION AND PURIFICATION OF FUCOXANTHIN FROM A BENTHIC DIATAM, *Achnanthes longipes* 166
1.1. ABSTRACT.. 166
1.2. MATERIALS AND METHODS.. 166
1.2.1. Materials 166
1.2.2. Extraction and chromatographic analysis 166
1.3. RESULTS 168
1.4. DISCUSSION... 171
2. ISOLATION AND PURIFICATION OF LUTEIN FROM THE MICROALGA, *Halochlorococcum porphyrae* 173
2.1. ABSTRACT.. 173
2.2. MATERIAL AND METHODS 173
2.2.1. Saponification and extraction of Lutein............................... 173
2.2.2. Determination of Lutein.. 174
2.3.RESULTS 175
2.4. DISCUSSION... 178
REFERENCES 180
ACKNOWLEDGEMENT 188

제주대학교 중앙도서관

국문초록

지역 고유의 미세조류는 오래전부터 이용되어져 왔다. 특히, *Spirulina* 나 *Nostoc* 같은 식용 남녹조류는 수천년 전부터 음식으로 이용되어져 왔다. 그러나 미세조류의 생물공학 분야는 20 세기 중반에 와서야 진전되기 시작 하였다. 미세조류는 살아 있는 유기물에 여러 다른 종류를 포함하고 광합성을 통하여 무기물을 에너지원으로 포획한 빛을 이용하여 단순당을로 전환한다. 조류는 단순한 식물이며, 몇몇 종만 고등식물과 유사하다.

조수 웅덩이에서 발견되는 미세조류 (*Oltamannsiellopsis unicellularis, Hallochlorococcum porphyrae*), 제주 연안의 저서가 서식처인(*Achnanthes longipes, Amphora coffeaeformis, Navicula* sp.) 그리고 담수미세조류(*Pediastrum duplex, Dactylococopsis faciculari*)가 제주도에 넓게 분포되어 있다. 이러한 미세조류의 생리활성 성분의 효능은 항산화 활성이 연구되었다. 그렇기 때문에, 생리활성 물질을 가지는 미세조류를 선택하여 색소를 분리하고 특정화 하여야 한다.

80% methanol, *n*-hexane, chloroform, EtOAC 등의 미세조류 분획물에 대한 항산화 측정은 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$), nitric oxide ($NO^{\cdot}$) inhibition, metal chelating and total antioxidant (FTC)를 평가하여 측정하였다.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

대부분의 분획물에서 DPPH 자유기 라디칼소거, $NO^{\cdot}$ 와 metal chelating 활성이 시판 항산화제인 butylated hydroxytoluene (BHT) 와 α-tocopherol 보다 높은 활성을 나타내었다. DPPH 라디칼 소거는 *O.unicellularis* 의 80% methanol 추출물과 n-hexane 분획물에서 각각 90.6 과 89.6%의 활성을 나타내었다. by *A. longipes* chloroform 과 n-hexane 분획물에서 각각 53.4 와 53.1%로 비교적 높은 H_2O_2 저해활성을 보여주었다. *Navicula* sp.(47.6%), *O.unicellularis* (44.1%), *H.porphyrae* (41.3%)의 EtOAC 분획물에서 $O_2^{\cdot -}$ 소거 활성을 나타내었다. *P.duplex* 의 80% methanol 추출물과 *A. longipes* 의 n-hexane 분획물에서는 각각 73.1 과 56.5%의 $HO^{\cdot}$ 소거활성을 나타내었다. Nitric oxide 활성은 of *A. longipes* 와 *D. faciculari* 의 n-hexane 분획물과 *P.duplex* 의 수용성 잔사에서 75.6, 45.7, 57.7%로 각각 나타났다. *P.duplex* 의 EtOAC 분획물에서는 활성이 82.1%, *A. coffeaeformis* 의 수용성 잔사에서 75.6%, *H.porphyrae* 의 chloroform 분획물에서 72.4%로 높은 metal chelating 활성을 나타내었다.

미세조류 추출물의 항산화 활성을 측정하기 위해서 ferric thiocyanate (FTC)방법을 이용하여 지질과산화를 측정한 결과 *Navicula* sp, *A. longipes*, *A. coffeaeformis* 의 Chloroform 분획물, *P.duplex* 의 EtOAC 분획물에서 항산화 활성이 나타난 것을 유의적으로 확인 할 수 있었다

미세조류의 가수분해물은 5 종류의 carbohydrases (Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo)와

제주대학교 중앙도서관

5 종류의 proteases (Protamax, Kojizyme, Neutrase, Flavourzyme, and Alcalase)로 가수분해 하였고, 이 미세조류 가수분해물의 항산화 활성을 측정하였다. 조수 우덩이와 담수 미세조류에서는 DPPH 활성이 유의적으로 나타났다. *H.porphyrae* 의 Viscozyme 과 Neutrase 가수분해물에서 자유기 라디칼 소거 활성이 다른 가수분해물보다 높개 나타났다. *D. faciculari* 의 Kojizyme 가수분해물과 *P.duplex* Termamyl 가수분해물에서 높은 H_2O_2 소거 활성을 나타내었다. *O.unicellularis* (57.7%)의 Flavozyme 과 *P.duplex* (52.8%)의 Termamyl 가수분해물에서는 $O_2^{\cdot-}$ 소거활성을 나타내었다. *P.duplex* (46.3%)의 Neutrase, *A. coffeaeformis* (45.9%)의 Viscozyme, *A.longipes* (43.9%)의 Flavozyme 가수분해물에서 높은 $HO^{\cdot}$ 소거활성을 나타내었다. $NO^{\cdot}$ 와 metal chelating 은 거의 대부분의 가수분해물에서 높은 활성을 나타내었다. *H.porphyrae*(51.4%)의 Termamyl, *A. coffeaeformis* (75.6%)의 Viscozyme, *P.duplex* (44.8%)의 Celluclast 가수분해물에서 현저한 $NO^{\cdot}$ 저해활성을 나타내었다. Metal chelation 활성에서는 *P.duplex* 의 Celluclast 와 Neutrase 가수분해물에서 각각 86.8 과 80.1% 의 저해 활성을나타내었고, *A. longipes* 의 Ultraflo 와 Kojizyme 가수분해물에서 73.5 와 75.8% 의 저해 활성을 나타내었다.

미세조류 추출물의 항산화 활성을 측정하기 위해서 ferric thiocyanate (FTC)방법을 이용하여 지질과산화를 측정한 결과 *H.porphyrae* 의 Celluclast 와 Protamax, *Navicula* sp.의

제주대학교 중앙도서관

Termamyl, *D. faciculari* 의 Kojizyme 가수분해물에서 항산화 활성이 유의적으로 나타났다.

미세조류에서 과산화수소에의한 DNA 손상 보호효과도 확인 할 수 있었다. *P.duplex* 의 Termamyl, *D. fascicu* 의 Kojizyme, *H .porphyrae* 의 alcalase 가수분해물에서 현저하게 나타났다. 특히, *P.duplex* 의 Termamyl, *D. fascicu* 의 Kojizyme, *H .porphyrae* 의 alcalase 가수분해물에서 높은 저해활성을 나타내었다.

최종적으로, *A.longipes* 와 *H. porphyrae* 에서 항산화성을 가지는 색소인 fucoxanthin 과 lutein 을 분리정제하였다. Fucoxanthin 과 lutein 은 인체에서 잠재적으로 유해한 자유기 라디칼을 중화하고 돌연변이를 줄이기 때문에 암과 심장병을 예방한다.

제주대학교 중앙도서관

총체적으로, 미세조류의 색소, 폴리페놀, 다당류, 단백질 등의 생리 활성 성분은 전도 유망한 물질임을 암시하고 있다. 게다가, 미세조류는 식품과 의약품산업에 있어서 잠재적인 자원의 가치를 가지고 있다.

SUMMARY

Microalgal use by indigenous population has occurred for centuries. Specially, edible blue green algae such as *Spirulina* and *Nostoc* have been used for foods for thousands of years. However, microalgal biotechnology only began to develop in the middle of the last centaury. Microalgae encompass several different groups of living organisms that capture light energy through photosynthesis converting inorganic substances into simple sugars using the captured energy. Algae have been traditionally regarded as simple plants, and some are closely related to the higher plants.

Microalgae in tidal pools (*Oltamannsiellopsis unicellularis* and *Hallochlorococcum porphyrae*), Jeju coastal benthic habitat (*Achnanthes longipes, Amphora coffeaeformis* and *Navicula* sp.), and fresh water streams (*Pediastrum duplex* and *Dactylococopsis facicularis*) are widely spreaded in Jeju Island, Korea. The efficacy of the bioactive constituents of those micralage was investigated for their antioxidant, activity. Thereafter, the pigments with potential bioactivities were purified and characterized in selected microalgae.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

The antioxidative potential of different fractions (80% methanol extract and its organic fractions of *n*-hexane, chloroform and ethyl acetate) of microalgae was evaluated by 1,1-diphenyl–2-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), nitric oxide ($NO^{\cdot}$) inhibition, metal chelating and total antioxidant (FTC)

assays. Most of the fractions have exhibited good antioxidant efefct on DPPH scavenging, $NO^{\cdot}$ and metal chelating compared to commercial antioxidants like butylated hydroxytoluene (BHT) and α-tocopherol. Among them, the higher DPPH activities were recorded from the 80% methanol extracts and n-hexane fraction of *O.unicellularis* (90.6 and 89.6%, respectively). The higher H_2O_2 activity was presented by *A. longipes* fractions (53.4 % from chloroform fraction and 53.1% from n-hexane fraction). The higher $O_2^{\cdot -}$ scavenging activity was exhibited by ethyl acetate fraction of *Navicula* sp.(47.6%), *O.unicellularis* (44.1%) and *H.porphyrae* (41.3%). 80% methanol extract from *P.duplex* (73.1%) and n-hexane fraction (56.5%) of *A. longipes* showed the highest $HO^{\cdot}$ scavenging activity. Significant nitric oxide activities were recorded from the n-hexane fraction of *A. longipes* and *D. faciculari* and aqueous residue of *P.duplex* (75.6, 45.7 and 57.7%, respectively). Higher metal chelating activity was shown by the ethyl acetate fraction of *P.duplex* (82.1%) followed by aqueous residue of *A. coffeaeformis* (75.6%) and chloroform fraction of *H.porphyrae* (72.4%).

To evaluate the antioxidant effects of microalgae extracts, their lipid peroxidation was compared with standard antioxidants using ferric thiocyanate (FTC) method. Chloroform fraction of *Navicula* sp, *A. longipes*, *A. coffeaeformis* and ethyl acetate farction of *P.duplex* exhibited significant antioxidant activities.

제주대학교 중앙도서관

Microalgae hydrolysates prepared by five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo) and five proteases (Protamax, Kojizyme, Neutrase, Flavourzyme, and Alcalase) were also investigated for their antioxidative activity assays. Tidal pool and fresh water microalgae showed significant DPPH activity. Among them extracts of Viscozyme and Neutrase of *H.porphyrae,* reported higher free radical effect than these of the other digests. Kojizyme extract of *D. faciculari* and Termamyl extract of *P.duplex* exhibited higher H_2O_2 activity. Higher $O_2^{\cdot -}$ activity was presented from the Flavozyme extract of *O.unicellularis* (57.7%) and Termamyl extract of *P.duplex* (52.8%). The Neutrase extract of *P.duplex* (46.3%), Viscozyme extract of *A. coffeaeformis* (45.9%) and Flavozyme extract of *A.longipes* (43.9%) showed higher $HO^{\cdot}$ scavenging activity. Most of extracts exhibited significant $NO^{\cdot}$ and metal chelating activity. Among them Termamyl extract of *H.porphyrae* (51.4%), Viscozyme extract of *A. coffeaeformis* (75.6%) and Celluclast extract of *P.duplex* (44.8%) was prominent in $NO^{\cdot}$ inhibition. In metal chelation, Celluclast and Neutrase extract of *P.duplex* (86.8 and 80.1%, respectively) and Ultraflo and Kojizyme extarcts of *A. longipes* (73.5 and 75.8%, respectively) exhibited significant results.

To evaluate the antioxidant effects of microalgae extracts, their lipid peroxidation was compared with standard antioxidants using ferric thiocyanate (FTC) method. Celluclast and Protamax extracts of *H.porphyrae*,

제주대학교 중앙도서관

Termamyl extract of *Navicula* sp. and Kojizyme extract of *D. faciculari* exhibited significant antioxidant activities.

Microalgae showed a protective effect against DNA damage induced by H_2O_2. Among them Termamyl extract of *P.duplex,* Kojizyme extract of *D. fascicu* and alcalase extract of *H .porphyrae* were prominent. Especially Termamyl extract of *P.duplex* exhibited the highest inhibitory effect followed by Kojizyme extract of D. *fascicu* and alcalase extract of *H. porphyrae.*

Finally, fucoxanthin and lutein was purified as antioxidant pigments from *A.longipes* and *H. porphyrae.* Those pigments as antioxidants neutralize potentially harmful reactive free radicals in body cells, and may reduce potential mutations and thereby help to prevent cancer and heart diseases.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Overall, it can be suggested that microalgae possess promising antioxidant bioactive compounds such as pigments, polyphenols, polysaccharides, and protein. Thus, microalgae can be a potential source in food and pharmaceutical industry.

List of Figures

Fig. 1. *P. duplex* (A) and *D. fascicularis* (B) isolated from the freshwater environment.

Fig. 2. *O. unicellularis* (a) and *H.porphyrae*(b) isolated from the tidal pool environment.

Fig. 3. Axenic strain of benthic diatom *Achnanthes longipes* (A), *Navicula* sp. (B) and *A. coffeaeformis* (C) used for antioxidants properties study.

Fig. 4. Scheme of solvent fractionation of *P.duplex*

Fig. 5. Antioxidant activity of different solvent fractions from *P. duplex* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

Fig. 6 Antioxidant activity of different enzymatic extracts from *P. duplex* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

Fig. 7. Antioxidant activity of different solvent fractions from *D. fascicularis* compared to BHT and α- Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

Fig. 8 Antioxidant activity of different enzymatic extracts from *D. fascicularis* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

Fig. 9 Antioxidant activity of different solvent fractions of *H.porphyrae* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

Fig. 10 Antioxidant activity of different enzymatic extracts from *H. porphyrae* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

Fig. 11 Antioxidant activity of different solvent fractions from *O. unicellularis* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid

Fig. 12 Antioxidant activity of different enzymatic extracts from *O. unicellularis* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 13 Antioxidant activity of different solvent fractions from *A. longipes* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

Fig. 14. Antioxidant activity of different enzymatic extracts from *A. longipes* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

Fig. 15. Antioxidant activity of different slovent fractions from *Navicula sp* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

Fig. 16. Antioxidant activity of different enzymatic extracts from *Navicula sp* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

Fig. 17. Antioxidant activity of different solvent extracts from *A. coffeaeformis* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid

Fig. 18. Antioxidant activity of different enzymatic extracts from *A. coffeaeformis* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

Fig. 19. Comet images of L 5178 cells for Termamyl extract of *P.duplex* (a) negative control; (b) cells treated with 50 μM H_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 lM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

Fig. 20. The effect of supplementation *in vitro* with different concentrations of Termamyl extract of *P.duplex* on DNA damage of H_2O_2 induced L 5178 cells.

Fig. 21. Comet images of L 5178 cells for Kojizyme extract of *D. fascicularis*: (a) negative control; (b) cells treated with 50 μM H_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

Fig. 22. The effect of supplementation *in vitro* with different concentrations of Kojizyme extract of *D. fascicularis* on DNA damage of H_2O_2 induced L 5178 cells.

Fig. 23. Comet images of L 5178 cells for Alcalase extract of *H. porphyrae*: (a) negative control; (b) cells treated with 50 μM H_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

Fig. 24 . The effect of supplementation *in vitro* with different concentrations of Alcalase extract of *H .porphyrae* on DNA damage of H_2O_2 induced L 5178 cells.

Fig. 25. Comet images of L 5178 cells for Alcalase extract of *O.unicellularis*:(a) negative control; (b) cells treated with 50 μM H_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

Fig. 26. The effect of supplementation *in vitro* with different concentrations of Alcalase extract of *O.unicellularis* on DNA damage of H_2O_2 induced L 5178 cells.

Fig. 27. Comet images of L 5178 cells for Neutrase extract of *A. longipes*: (a) negative control; (b) cells treated with 50 μM H_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

Fig. 28. The effect of supplementation *in vitro* with different concentrations of Neutrase extract of *A. longipes* on DNA damage of H_2O_2 induced L 5178 cells.

Fig. 29. Comet images of L 5178 cells for Ultraflo extract of *Navicula* Sp. (a) negative control; (b) cells treated with 50 lM H_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

Fig. 30. The effect of supplementation *in vitro* with different concentrations of Ultraflo extract of *Navicula* Sp. on DNA damage of H_2O_2 induced L 5178 cells.

Fig. 31. Comet images of L 5178 cells for Neutrase extract of *A. coffeaeformis*: (a) negative control; (b) cells treated with 50 μM H_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.
Fig. 32. The effect of supplementation *in vitro* with different concentrations of Neutrase extract of *A. coffeaeformis* on DNA damage of H_2O_2 induced L 5178 cells.
Fig. 33. Chemical structures of fucoxanthin
Fig. 34. Chromatogram of acetone extract of *A.longipes*
Fig. 35. Chromatogram of purified fucoxanthin
Fig. 36. Chemical structure of lutein
Fig. 37. Chromatogram of crude lutein obtained by extraction after sponification.
Fig. 38. Chromatogram of purified lutein.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

List of Tables

Table 1. Systematic list of studied phytoplankton from Jeju Island, Korea
Table 2. Optimum hydrolysation conditions and compositions of enzymes
Table 3. Antioxidants activity of 80% methanol extract and its different solvent extarcts from *P. duplex*
Table 4. Antioxidants activity of enzymatic extracts from *P. duplex*
Table 5. Total polyphenolic, polysaccharide and protein contents of 80% methanol extract and its different solvent frctions from *P. duplex*
Table 6. Total polyphenolic, polysaccharide and protein content of different enzymatic extracts from *P. duplex*
Table 7. Antioxidants activity of 80% methanol extract and its different solvent extarcts from *D. fascicularis*
Table 8. Antioxidants activity of enzymatic extracts from *D. fascicularis*
Table 9. Total polyphenolic, polysaccharide and protein contents of 80% methanolic extract and its different solvent frctions from *D. fascicularis*
Table 10. Total polyphenolic, polysaccharide and protein content of different enzymatic extracts from *D. fascicularis*
Table 11. Antioxidants activities of 80% methanol extract and its different solvent extracts from *H.porphyrae*
Table 12. Antioxidants activity of enzymatic extracts from *H.porphyrae*
Table 13. Total polyphenolic, polysaccharide and protein contents of 80%methanil extarct and its different fractions of *H. porphyrae*
Table 14. Total polyphenolic, polysaccharide and protein content of different enzymatic extracts from *H. porphyrae*
Table 15. Antioxidants activity of 80% methanol extract and its different solvent extarcts from *O.unicellularis*
Table 16. Antioxidants activity of enzymatic extracts from *O.unicellularis*
Table 17. Total polyphenolic, polysaccharide and protein contents of 80% methanol extract and its different solvent frctions from *O.unicellularis*
Table 18. Total polyphenolic, polysaccharide and protein content of different enzymatic extracts from *O. unicellularis.*

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 19. Antioxidants activity of 80% methanolic extract and its different solvent fractions from *A. longipes*
Table 20. Antioxidants activity of enzymatic extracts from *A. longipes*
Table 21. Total polyphenolic, polysaccharide and protein content of 80% methanol extract and different solvent fractions from *A. longipes.*
Table 22. Total polyphenolic, polysaccharide, and protein content of different enzymatic extracts from *A. longipes*.
Table 23. Antioxidants activity of 80% methanol extract and its different solvent frcations from *Navicula* sp.
Table 24. Antioxidants activity of enzymatic extracts from *Navicula* sp.
Table 25. Total polyphenolic, polysaccharide and protein content of 80% methanol extract and its different solvent fractions from *Navicula sp.*
Table 26. Total polyphenolic, polysaccharide and protein content of different enzymatic extracts from *Navicula sp*
Table 27. Antioxidants activity of 80% methanolic extract and its different solvent extracts from *A. coffeaeformis*
Table 28. Antioxidant activity of enzymatic extracts from *A. coffeaeformis*
Table 29. Total polyphenolic, polysaccharide and protein content of 80% methanol extract and its different solvent extracts from *A. coffeaeformis*
Table 30. Total polyphenolic, polysaccharide and protein content of different enzymatic extracts from *A. coffeaeformis*
Table 31. H_2O_2 scavenging activity of enzymatic extracts from microalgae

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

INTRODUCTION

The chemical diversity of nature is immeasurable as a great resource of bioactive lead entities. Recent advances in the identification of new natural bioactive compounds from marine environment have been drawing promising attention of current researchers. Numerous phycotechnological studies on bioactive materials revealed that algae are potential sources of pharmacological compounds and food additives potentially exert beneficial health effects such as antioxidative, anticoagulative, antihyaluronidase, antibacterial, and anti-carcinogic. The algae, as a group, represent the third-largest aquacultured crop (after freshwater fishes and mollusks) in the world today. The definition of ‘‘algae’’ includes cyanobacteria, green, red, and brown algae encompassing photosynthetic prokaryotic and eukaryotic organisms as well as their heterotrophic derivatives. By far, most of this production is located in Asia, where brown and red seaweeds dominate the production. Macroscopic marine algae or seaweeds for human consumption, especially Nori (*Porphyra* spp.), Wakame (*Undaria pinnatifida*), and Kombu *(Laminaria japonica)*, are widely cultivated as algal crops, but the list of taxa used as human foods is relatively divers. Use of cultivated seaweeds in Europe and the United States is less although phycocolloids (carrageenan, agar, alginates) are used widely in processed foods.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Marine microalgae have been reported as valuable new sources of pharmacologically active compounds. However, their metabolites have not been studied extensively because of difficulties in the isolation and

cultivation of microalgae. However, nutritional supplements produced from microalgae have been the primary focus of microalgal biotechnology for many years. Human use of single-celled microalgae is much less developed than that ofmacroalgae. Few examples of human foods from microalgae can be cited, although the cyanobacterium *Spirulina* sp. has a limited "health-food" market. Similarly, cultured *Chlorella* has a limited but lucrative health-food market in Japan.

Intaking of antioxidants or foods containing antioxidants exert beneficial health effects particularly against the risks of carcinogenesis, coronary/artery disease, platelet aggregation, neurodegenerative and atherosclerosis. Antioxidants can scavenge biologically toxic ROS such as superoxide, hydroxyl radicals, peroxyl radicals, hydrogen peroxide, singlet oxygen, nitric oxide, and peroxy nitrate (Halliwell, 1991). It also can effectively retard the onset of lipid oxidation in food products. In fact, antioxidants have become an indispensable group of food additives mainly because of their unique properties of enhancing the shelf life of food products without any damage to sensory or nutritional qualities (Madhavi et al., 1996). There are numerous types of harmful free radicals (with one or more unpaired electrons) that are continuously generated in the body. The most common includes superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), nitric oxide radical ($NO^{\cdot}$), peroxynitrate ($ONOO^{-}$) and non free radical species like H_2O_2, singlet oxygen ($^1O_2$) and hypochlorous acid (Halliweill 1991; Brand, 2000; Sanchez-Moreno, 2002). Free radicals (oxidants) are able to damage a

wide range of essential biomolecules including nucleic acids (DNA/RNA), proteins, lipids and carbohydrates (Halliwell and Gutteridge, 1990). Hydrogen peroxide (H_2O_2) is an oxidant belongs to reactive oxygen species (ROS) but not a radical. It is a long-lived molecule, which has a great potential to diffuse hence capable of long distance diffusion. It can produce other ROS like hypochlorous acid (HOCl) by enzymatic (myeleperoxidase) oxidation of chloride ion. HOCl can lead to produce highly reactive singlet oxygen $^1O_2$ or even hydroxyl radicals. Cleavage of DNA, DNA-protein cross-links and oxidation of purines are the main causes of DNA alterations due to ROS effects. Generally, DNA-repair systems are able to regenerate immediately altered DNA without leading to mutations from erroneous base pairing during replication. Under severe oxidative stress conditions, such alterations may leads to altered gene expression apoptosis, carcinogens, and aging (Ames et al., 1995 and Beckman and Ames, 1997).

제주대학교 중앙도서관

Some microalgae are cultured for extractable compounds (e.g. the pigment beta-carotene, fucoxanthin, xanthophyll pigments such as lutein and astaxanthin, and the fatty acid docosahexaenoic acid [DHA]), nutritional supplements, or food additives. Among pigments carotenoids, including fucoxanthin have been demonstrated to have beneficial effects on health, such as antioxidative, anticarcinogenesis and antimutagenesis (Nishino, 1998). Specially, fucoxanthin one the most abundant carotenoids in nature mainly in brown algae and diatoms, and its chemopreventive effects are well established. Fucoxanthin attenuated neuronal cell injury in hypoxia and re-

oxygenation, which is an experimental ischaemic model *in vitro*. Although fucoxanthin is important as a radical scavenger, less attention has paid to extract it in commercial scale from diatom. Lutein is one of the most abundant caroteneoid in the diet and in human blood. There has been recent evidence to suggest that lutein possesses strong antioxidant capabilities and may be useful in reducing the incidence of cancer. Lutein is one of main constituents of green vegetables and fruits. Compared with higher plants, algae have an advantage because they can be cultivated in bioreactors on a large scale and thus are a continuous and reliable source of the product (Shi et al., 1999).

The objective of this study is to describe the potential bioactive compounds of microalgae and identification, extraction purification, and characterization to understand the usefulness of those microalgae as a potential candidate in food and pharmacological industry.

제주대학교 중앙도서관

Part I.

Algal characterization and its culture technique for advanced study

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1. IMPORTANCE OF ALGAE

Algae (singular *alga*) encompass several different groups of living organisms that capture light energy through photosynthesis converting inorganic substances into simple sugars using the captured energy. Algae have been traditionally regarded as lower plants, and some are closely related to the higher plants. Others appear to represent different protist groups, alongside other organisms that are traditionally considered more animal like (protozoa). Thus algae do not represent a single evolutionary direction or line, but a level of organization that may have developed several times in the early history of life on earth. All algae have photosynthetic machinery and produce oxygen as a by-product of photosynthesis. Algae are usually found in damp places or bodies of water and thus are common in terrestrial as well as aquatic environments. However, terrestrial algae are usually rather inconspicuous and far more common in moist, tropical regions than in dry ones, because algae lack vascular tissues and other adaptions to live on land. Algae can endure dryness and other conditions in symbiosis with a fungus as lichen.The various sorts of algae play significant roles in aquatic ecology.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Seaweeds or macroalgae grow mostly in shallow marine waters. Some are used as human food or harvested for useful substances such as agar or fertilizer. The name plankton is derived from the Greek term phyavktov, meaning "wanderer" or "drifter". While some forms of plankton are capable of independent movement and can swim up to several hundreds of meters vertically in a single day, their horizontal position is primarily determined by

currents in the body of water. By definition, organisms classified as "plankton" are unable to resist ocean currents.

Phytoplankton or microalgae is a microscopic organisms that drifts in the water column. Most phytoplanktons are too small to be individually seen with naked eye. However, when present in high numbers, their presence may appear as discoloration of the water (the color of which may vary with the phytoplankton present). In very high densities (so-called algal blooms) these algae may discolor the water, and outcompete or poison other life forms. Phytoplankton, like plants, obtain energy through a process called photosynthesis and so must live in the well-lit surface layer (termed the euphotic zone) of an ocean, or lake or pond. Through photosynthesis, phytoplankton (and terrestrial plants) is responsible for much of the oxygen present in the Earth's atmosphere. Their cumulative energy fixation in carbon compounds (primary production) is the basis for the vast majority of oceanic and some freshwater food chain (chemosynthesis is a notable exception).

제주대학교 중앙도서관

The marine algae perform a major part of primary production, being responsible for 46% of global productivity (Field et al., 1998) and supporting food webs in waters from pond to oceans. Diatoms are the major component of phytoplankton, one of the major groups of primary producers in aquatic ecosystems and an essential food source for aquaculture (St John et al., 2001) which have seldom been found a source of ecological problems in nature (Officer and Ryther, 1980). Physiological adaptations are key processes that

allow phytoplankton to survive and to grow in variable environment. Like all other marine organisms, diatoms ecophysiology is influenced by water temperature, salinity, light intensity and nutrient concentrations (Tomas 1996; Tomas et al., 1998 and Thessen et al., 2005), estimating their seasonal abundance fluctuations and growth rate have been and will be, an important component of marine science studies. Salinity is known to be an important abiotic factor affecting phytoplankton growth. Wide ranges of salinity and temperature may explain frequent appearance of phytoplankton throughout the year in the ocean (Hoshiai et al., 2003).

Jeju is a volcanic Island of South Korea where the benthic diatoms are used as a live feed for shellfish in commercial hatchery. The coastal water temperature and salinity of this island are fluctuated widely and benthic diatoms are often found in a high abundance in this coastal water throughout the year (Affan and Lee, 2004). However, microalgae are paying more attention as nutraceutical and food health in markets. Extract of *Chlorella* sp. and *Spirulina* sp. are being proposed to use with noodles, bread, green tea, beer, and candy (Liang et al., 2004). Several microalgae, such as species of *Chlorella, Spirulina* and *Dunaliella*, are grown commercially and algal products such as β-carotene and phycocyanin are available. The antioxidative activity of phycocyanobilin from *Spirulina platensis* was evaluated against oxidation of methyl linoleate in a hydrophobic system or with phosphatidylcholine liposomes (Hirata, 2000). Phycocyanobilin effectively inhibited the peroxidation of methyl linoleate and produced a

제주대학교 중앙도서관

prolonged induction period. *Aphanizomenon flos-aquae* (cyanophyta) rich in phycocyanin, a photosyntheticpigmen with antioxidant and anti-inflammatory properties (Benedetti, 2004). Diatoms were identified among other thermophilic microorganisms and the main effects of the diatom growth are the progressive enrichment of mud extracts in chlorophyll a parallel to the building up of a sulfoglycolipid endowed with an anti-inflammatory action. Much effort has been expended on the search for new compounds of therapeutic potential, demonstrated in microalgae of all classes, possessing antibacterial, antifungal and anticancer (Richmond, 2004).

2. MORPHOLOGICAL CHARACTERISTICS

2.1. Freshwater habitat species

제주대학교 중앙도서관

There are very few natural freshwater resources in Jeju Island except Mu-Soo stream though this stream shows the water abundance during raining period. It is well known that natural water resources like lakes, reservoirs, streams and rivers contain diversified plankton community seasonally. However, *Pediastrum duplex* and *Dactylococcopsis fascicularis* were collected from Mu-Soo stream of Jeju, Korea. Wild sample was directly collected from the stream into a 1000 ml bottle (polyethylene) and 25 ml flask (Corning, tissue culture treated). Environmental factors such as water temperature and pH were measured on the sampling spot with (YSI 85) and with potable pH meter (Orion 920A), and the water temperature and pH were 28.5°C and 7.86, respectively.

2.2. Tidal pool habitat species

There are many tidal pools in coastal area of Jeju Island. Tidal pool is a semi-closed environmental water body. The environmental condition of the tidal pool is different from the sea. Phytoplankton sample was collected from the tidal pool areas at Gangjung in Jeju coast in November 2004. Water samples were collected in a 1000 ml bottle (polyethylene) and 25 ml flask (Corning, tissue culture treated). Environmental factors were measured on the sampling spot with Termo-Salinometer (YSI 85) and with potable pH meter (Orion 920A). Water temperature, pH and salinity were 12.7°C, 35.4‰, and 8.22, respectively. The samples were immediately moved into the plant growth chamber (Vision, VS-3D) and were kept in the condition of 20℃, 12:12(L: D cycle) for isolation. For the identification of the cultured phytoplankton, samples were observed under the phase-contrast microscope (Zeiss Axioplan) at a magnification of 400X, and the identification was done based on monograph of Tomas (1997). *Oltmannsiellopsis unicellularis* and *Halochlorococcum porphyrae* were identified from the collected sample, which belonged to Chlorophyceae.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2.3. Jeju coastal benthic habitat species

Diatoms (Gr. dia 'through'; tomos 'cutting', i.e., "cut in half") are a major group of eukaryotic algae and are one of the most common types of phytoplankton. Most diatoms are unicellular, although some form chains or simple colonies. A characteristic feature of diatom cells is that they are

encased within a unique cell wall made of silica. These walls show a wide diversity in form, some quite beautiful and ornate, but usually consist of two symmetrical sides with a split between them, hence the group name. Diatoms are indisputably the major component of many food webs, estimating their seasonal abundance fluctuations and growth rate have been, and will be, an important component of marine science studies.

Like all other marine organisms, diatoms ecophysiology is influenced by water temperature, salinity, light intensity and nutrient concentrations (Tomas, 1996; Tomas et al., 1998 and Thessen et al.,. 2005). Salinity is known to be an important abiotic factor affecting phytoplankton growth (Brand, 1984). Wide ranges of salinity and temperature may explain frequent appearance of phytoplankton throughout the year in the ocean (Hoshiai et al., 2003).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Jeju is a volcanic island of Korea, which belongs to the subtropical region where the benthic diatoms are used as a live feed for shellfish in commercial hatchery. The coastal water temperature and salinity of this island fluctuated widely and *Achnanthes longipes, Amphora coffeaeformis* and *Navicula* sp. are being often found in a high abundance in this coastal water throughout the year (Affan and Lee, 2004). Wild mixed-strain of benthic diatoms attached papan (undulated plastic sheet) was collected from the abalone culture hatchery from NFRDI (National Fisheries Research and Development Institute) Jeju. Environmental factors were measured on the sampling spot with Termo-Salinometer (YSI 85) and with potable pH meter

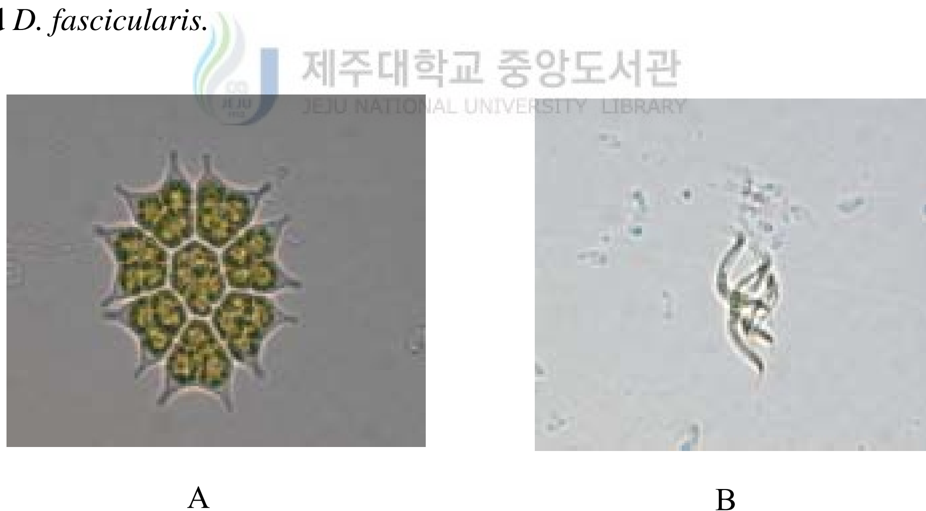
(Orion 920A). Water temperature, pH and salinity were 25°C, 30 ‰, and 8.02, respectively.

3. SAMPLE COLLECTION

3.1. Freshwater species

Freshwater sample was kept into the incubation with adding F/2 media for 3 days and the sample was observed under inverted microscope (Olympus, IX71). Single cell of *P. duplex* and *D. fascicularis* were picked up from the S-R cell by using micropipette and transferred into the multi well which contained culture media of F/2 nutrients, soil extraction and distilled water. Subculture was carried out up to getting the mono-strain of *P. duplex* and *D. fascicularis*.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

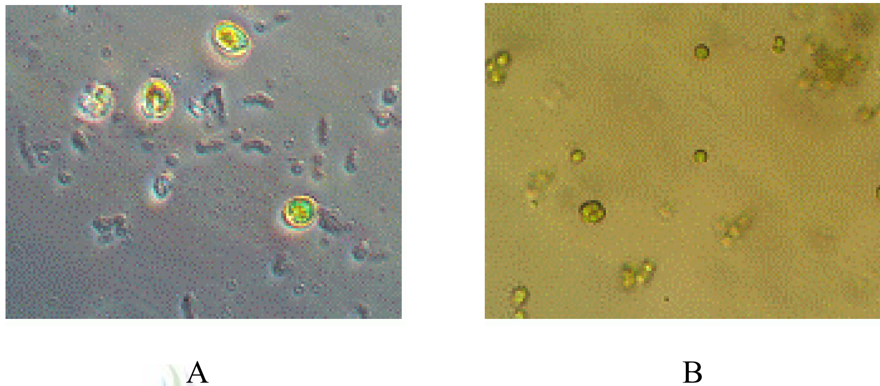


A B

Fig. 1. *P. duplex* (A) and *D. fascicularis* (B) isolated from the freshwater environment.

3.2. Tidal pool species

Oltmannsiellopsis unicellularis and *Halochlorococcum porphyrae* was collected as a mono-strain from the sampling station and for getting axenic specie antibiotics were applied to the subculture media.



A B

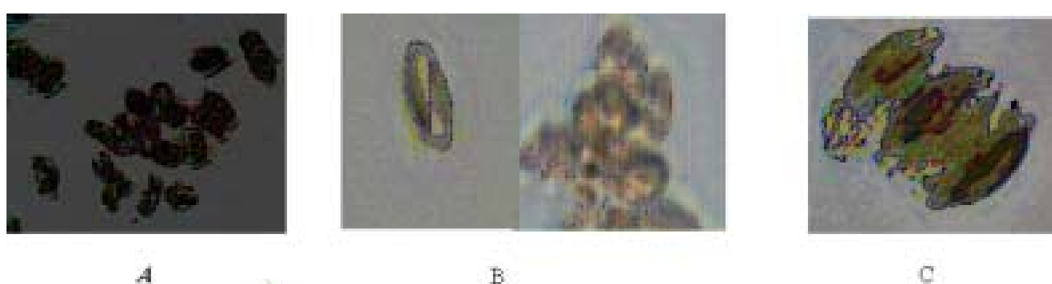
제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 2. *O.unicellularis* (a) and *H.porphyrae* (b) isolated from the tidal pool environment.

3.3. Jeju coastal benthic habitat species

The attached diatoms were removed from the papan (wavy plastic plate, so called papan) and diluted with the same seawater. The sample was again diluted and 1 ml was transferred to the S-R cell. Single cell of benthic diatoms was picked up from the S-R cell by using micropipette under inverted microscope (Olympus, IX71). The single cell was transferred into multi-well for subculture. Subculture of the isolated species was done with autoclaved seawater which was filtered through 0.4 ㎛ filter membrane (Millipore Co., Bedford, MA) and enriched with F/2 nutrients media

(Aquacenter Ltd. USA), trace metals and metasilicate anhydrous crystals ($Na2SiO3$). The isolation process was carried out until to get the mono-strain of the benthic diatoms. For the identification of the cultured benthic diatoms, samples were observed under the phase-contrast microscope (Zeiss Axioplan) at a magnification of X 400, and the identification was done as described by Tomas (1997).



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 3. Axenic strain of benthic diatom *Achnanthes longipes* (A), *Navicula* sp. (B) and *A. coffeaeformis* (C) used for antioxidants properties study.

3.4. Isolation of axenic strain

Bacteria were found to grow with plankton during subculture and mass cultured time and it was decomposing phytoplankton cells, and it was suspected that bacteria might play a vital role for the determination of antioxidants properties of phytoplankton by providing its own cell compounds. For obtaining the axenic strain, antibiotics were used with subculture of plankton at different dosages. The mono-strain of phytoplankton was again streaked on agar plate that was prepared with 2%

agar (w/v) and F/2 (0.04% v/v) media and autoclaved seawater. Mono-strain phytoplankton colony was transferred from the agar plate into the 250 ml flask, which contained 100 ml of F/2 enriched culture media and antibiotics. Seven different doses of antibiotics solution (penicillin 100-250 units/ml, streptomycin 100-250 μg/ml and neomycin 200-500 μg/ml) at 25 units penicillin, 25 μg/ml streptomycin and 50 μg/ml neomycin dozes were used (Sigma; P 4083). About 10 ml cultured Benthic diatoms sample was transferred from the antibiotic mixed media to a 250 ml flask having 100 ml media for the culture of this species and the cultured sample was again streaked on the bacto-agar media for the observation of the presence of bacteria. Finally, axenic species of benthic diatoms was obtained for advanced study, like growth characteristics, mass culture, biochemical composition, antioxidant activities

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

4. MASS CULTURE AND FREEZE DRY

4.1. Jeju coastal benthic habitat species

The mass culture of benthic diatoms was done for 3 weeks in 10 liters Nalgene bottle with autoclaved artificial seawater media, which was enriched with F/2 nutrients media, (Na_2SiO_3) and trace metals solution. Mass culture condition was maintained with salinity, temperature, pH, L: D cycle and fluorescent light intensity of 30‰, 25°C, 8, 12:12 and 180 μE m-1. s-1, respectively.. The standing crop was separated from the culture media by filtering (GF-47 nm). The collected biomass was kept in the Petri dish and

kept into the deep freeze for 24 hours at -70°C, and freeze-dried.

4.2. Tidal pool species

Mass culture of tidal pool phytoplankton was done with autoclaved artificial seawater media which was enriched with F/2 nutrients media, and the culture condition was maintained with salinity, temperature, pH, L: D cycle and fluorescent light intensity of 32‰, 22°C, 8.20, 12:12 and 180 μE m^{-1}. s^{-1}, respectively and the culture was done in 20 liter Nalgene bottle. Separation of standing crops and freeze-drying were same as benthic diatoms.

4.3. Freshwater species

Freshwater phytoplankton was cultured in 20 l Nalgene bottle which contained culture media of F/2 nutrients, soil extraction and distilled water, and the plankton growing condition was maintained with the temperature, pH and light intensity of 28°C, 8.0 and 180 μE m-1. s-1, respectively. Separation of standing crops and freeze-drying were same as benthic diatoms.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 1. Systematic list of studied phytoplankton from Jeju Island of Korea.

Division Cyanophyta

Class Cyanophyceae

Order Chroococcales

Family Chroococcaceae

Genus *Dactylococcopsis*

Dactylococcopsis fascicularis Lemmermann

Division Chrysophyta

Class Bacillariophyceae

Order Pennales

Family Achnanthaceae

Genus *Achnanthes*

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Achnanthes longipes Agardh

Family Naviculaceae

Genus *Amphora*

Amphora coffeaeformis Kützing

Genus *Navicula* Bory

Division Chlorophyta

Class Chlorophyceae

Order Volvocales

Family Dunalielaceae

Genus *Oltamannsiellopsis*

Oltamannsiellopsis unicellularis

Order Chlorococcales

Family Chlorococcaceae

Genus *Halochlorococcum*

Halochlorococcum porphyrae Setchell & Gardner

Family Hydrodictyaceae

Genus *Pediastrum*

Pediastrum duplex Lagerheim

Part II.

Antioxidant properties of microalgae

1. ANTIOXIDANT PROPERTIES OF FRESH WATER MICROALGA, *Pediastrum duplex*

1.1. ABSTRACT

The antioxidative properties of different organic fractions obtained by solvent fractionation of 80% methanolic extract and enzymatic extracts from five proteases and carbohydrases from *Pediastrum duplex* was investigated for DPPH free radical, reactive oxygen species scavenging ($O_2^{\cdot -}$,H_2O_2, $HO^{\cdot}$,$NO^{\cdot}$), metal chelating, and total antioxidant assays. Of the organic fractions tested, ethyl acetate (79.8%) and n-hexane (74.5%) fractions were effective in DPPH scavenging. Fractions of chloroform (47.2%) and ethyl acetate (48.7%) exhibited higher activities on H_2O_2 scavenging. Aqueous residue (57.7%) showed the highest $NO^{\cdot}$ inhibition activity. Ethyl acetate (82.1%) fraction demonstrated a significant activity in metal chelating. In enzymatic extracts all extracts showed significant DPPH activities. Termamyl extracts exhibited highest activities in both H_2O_2 and $O_2^{\cdot}$ radical scavenging. Extracts of Celluclast (44.8%), AMG (42.6%) and Ultraflo revealed significant activities in $NO^{\cdot}$ inhibition. Kojizyme (79.1%), Neutrase (71.7%), and AMG (77.1%) had strong metal chelating activities. These data suggest that both methanolic and enzymatic extracts are rich in antioxidative compounds with different antioxidative properties.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

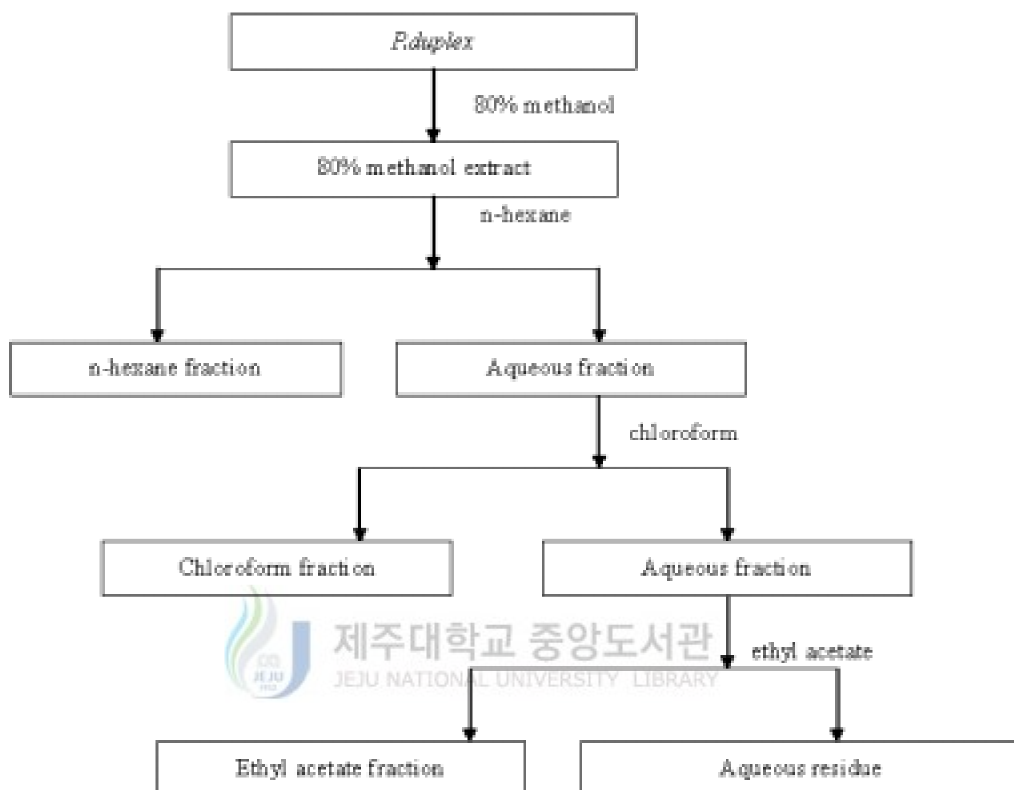
1.2. MATERIALS AND METHODS

1.2.1. Materials

1, 1-Diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium nitroprusside, sulphanilic acid, naphthylethylenediamine dihydrochloride, xanthine, xanthine oxidase from butter milk, nitro blue tetrazolium salt (NBT), butylated hydroxytoluene (BHT), α-tocopherol, 3-(2-Pyridyl)-5,6-di (p-sulfophenyl)-1, 2, 4-triazine, disodium salt (ferrozine), ferric chloride ($FeCl_3.6H_2O$), potassium ferricyanide ($K_3Fe(CN)_6$), ferrous chloride ($FeCl_2.4H_2O$), $FeSO_4.7H_2O$, α-tocopherol, Folin-Ciocalteu reagent and linoleic acid were purc-hased from Sigma Co. (St Louis, USA). 2, 2-Azino-bis (3-ethylbenz-thiazolin)-6-sulfonic acid (ABTS), peroxidase and 2-deoxyribose were purchased from Fluka Chemie (Buchs, Switzerland). Viscozyme, Celluclast, AMG, Termamyl, Ultraflo, Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase were purchased form Novo Co. (Novozyme Nordisk, Bagsvaerd Denmark).All the other chemicals used were analytical grade.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.2.2. Preparation of extract and solvent fractionation



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 4. Scheme of solvent fractionation of *P.duplex.*

Freeze-dried sample were ground into a fine powder. Powdered samples (5 g) were immersed in 80% methanol (500 ml) and placed in a shaking incubator for 24 h at 25° C. The macerated mixture was filtered and the methanol extract was collected and concentrated. The extracts obtained were in sequence fractionated in a separatory funnel with different organic solvents,

such as n-hexane, chloroform, and ethyl acetate (Fig. 4). Each fraction was concentrated and redissolved in methanol to a concentration of 2 mg/ ml. All activities of fractions were compared with standard antioxidants (BHT and α-tocopherol) dissolved in methanol.

1.2.3. Enzymatic hydrolysis procedure

Table 1. Optimum hydrolysation conditions and compositions of enzymes.

Enzyme	Optimum pH	Optimum T(°C)	Enzyme composition
Viscozyme	4.5	50	arabanase, cellulase,hemi-cellulase and xylanase
Celluclast	4.5	50	beta-glucanases
AMG	4.5	60	amyloglucosidase
Termamyl	6.0	60	alpha-amylases
Ultraflo	7.0	60	beta-glucanases
Protamax	6.0	40	endo-proteases
Kojizyme	6.0	40	endo/exopeptidase
Neutrase	6.0	50	neutral beta amyloliquefaciens
Flavozyme	7.0	50	endo/exopeptidase
Alcalase	8.0	50	alcalase

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Freeze-dried *P. duplex* was ground into a fine powder and 1 g was mixed with 100 ml of distilled water. The optimum pH of the each reaction mixtures were adjusted with 1M HCl / NaOH. Optimum pH and temperature conditions for the respective enzymes used were similar to the conditions shown in Table 2. Enzymes were then added at the dosage (enzyme/substrate ratio) of 1%. The mixtures were placed in a shaking incubators for 24 adjusted to optimum temperatures of the respective enzymes used. Resultant mixtures were filtered and the enzymes activity of hydrolysates was inactivated by heat (100 ^{o}C for 10 min). Finally, the pH of each hydrolysate was adjusted to pH 7 with 1M HCl / NaOH.

1.2.4. Proximate composition

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Proximate chemical composition of freeze-dried *P. duplex* was determined according to the AOAC methods (AOAC, 1990). 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 and the moisture content was determined keeping in a dry oven at 105° C for 24 h. In fractions and extracts, the crude protein content was determined by the Lowry spectrophotometric method and the polysaccharide content was determined by phenol-sulfuric method.

1.2.5. Reactive oxygen species inhibition

1.2.5.1. DPPH free radical scavenging assay

Free radical scavenging activity *P. duplex* was determined according to the modified method of Brand-Williams et al. (1995). Sample (2 ml) was mixed thoroughly with 2 ml of freshly prepared DPPH solution (3X 10^{-5} M). The reaction mixture was incubated for 1 h and absorbance was recorded at 517 nm using UV–VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea).

1.2.5.2. Hydrogen peroxide scavenging assay

This assay was carried out according to the method of Muller (1995). Sample (80 µl) and 20 µl of 10 mM hydrogen peroxide were mixed with 100 µl of phosphate buffer (0.1M, pH 5.0) in a 96-microwell plate and incubated at 37ºC for 5 min. Thereafter, 30 µl of freshly prepared 1.25 mM ABTS and 30 µl of peroxidase (1 U/ml) were mixed and incubated at 37º C for 10 min and the absorbance was measured at 405 nm.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.2.5.3. Superoxide anion scavenging assay

This assay was carried out according to Nagai et al. (2003). A mixture of 0.48 ml of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 ml of 3 mM xanthine, 0.02 ml of 3 mM EDTA, 0.02 ml of 0.15% bovine serum albumin, 0.02 ml of 0.75 mM NBT and 0.02 ml of *H. porphyrae* sample was incubated at 25 °C for 10 min. Thereafter the reaction was started by adding

6 mU XOD and kept at 25 °C for 20 min. The reaction was stopped by adding 0.02 ml of 6 mM CuCl. The absorbance was measured in microplate reader (Sunrise; Tecan Co. Ltd., Austria) at 560 nm.

1.2.5.4. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was determined according to Chung et al. (1977). The Fenton reaction mixture consisted of 200 µl of 10 mM $FeSO_4.7H_2O$, 200 µl of 10 mM EDTA and 200 µl of 10 mM 2-deoxyribose was mixed with 1.2 ml of 0.1 M phosphate buffer (pH 7.4) mixed with 200 µl of sample. Thereafter, 200 µl of 10 mM H_2O_2 was added and incubated at 37 °C for 4 h. After incubation, 1 ml of 2.8% trichloroacetic acid (TCA) and 1 ml of 1% thiobarbituric acid (TBA) were mixed and placed in a boiling water bath for 10 min. After cooling, the mixture was centrifuged (5 min, 395 x g) and absorbance was measured at 532 nm.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.2.6. Nitric oxide radical inhibition assay

Nitric oxide radical inhibition was determined according to the method reported by Garrat (1964). Two milliliter of 10 mM sodium nitroprusside in 0.5 ml of phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of *P. duplex* sample and incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into1.0 ml sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml naphthylethylenediamine dihydrochloride (0.1 % w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm.

1.2.7. Metal chelating ability

Metal chelating ability was determined according to the method by Decker and Welch (1990). *P. duplex sample* (5 ml) was added to a solution of 0.1 ml of 2 mM $FeCl_2$. The reaction was started by the addition of 0.2 ml of 5 mM ferrozine solution and reaction mixture was incubated for 10 min at room temperature under shaking condition. After incubation, the absorbance of reaction mixture was measured at 562 nm.

1.2.8. Determination of antioxidant activity with the ferric thiocyanate (FTC) method

Ferric thiocyanate (FTC) method was described in detail by Kikuzaki and Nakatani (1993). *P. duplex* sample (100 mg/l) was mixed with 2 ml of 2.51% linoleic acid in ethanol, 4 ml of 0.05 M of phosphate buffer (pH 7) and 2 ml of distilled water and kept at 40 C in dark. A total of 0.1 ml of above mixture was added to 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate and after 5 min 0.1 ml of 0.02M ferrous chloride in 3.5% HCl was mixed. Absorbance was measured every 24 h for 7 days.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.2.9. Total polyphenol contents

Total polyphenol contents were determined according to the protocol similar to Chandler and Dodds (1993). Sample (1 ml) was mixed with 1ml 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. Thereafter, it was thoroughly mixed and placed in dark for 1 hr and absorbance was measured at 725 nm by the UV–VIS spectrophotometer. A Gallic acid standard curve was obtained for the calculation of polyphenol content.

1.2.10. Scavenging activity/chelating ability calculation

Scavenging activity/chelating ability calculated using the following equation. $[1-(A_i - A_j)/Ac]$ X100.

where

A_i - the absorbance of microalgae sample mixed with active compound

A_j - the absorbance of microalgae sample without active compound

A_c- the absorbance of control with particular solvent (without extract)

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.2.11. Statistical analysis

Statistical analyses were conducted with the SPSS 11.5 version software package on the triplicate (n=3) test data. The mean values of each treatment were compared using one-way analysis of variance (ANOVA) followed by Turkeys tests. P-value of less than 0.05 was considered significantly.

1.3. RESULTS

1.3.1. Proximate composition

Proximate composition of freeze dried *P.duplex* collected along Jeju Island was analyzed and median values were; moisture 6.1%, ash content 15.2%, carbohydrates 30%, proteins 46.3% and fat 2.4%. Polysaccharide and protein content in 80% methanol extract and its solvent fractions and enzymatic extracts is depicted in Table 5 and 6.

1.3.2. Reactive oxygen species inhibition

1.3.2.1. DPPH free radical scavenging effect

According to Table 3, 80% methanol extract and all fractions showed remarkable activities and among them ethyl acetate and n-hexane fractions showed higher activities (79.8 and 74.5%, respectively). As shown in Table 4, all enzymatic extracts exhibited higher DPPH scavenging effects and among them activities showed from Ultraflo (94.2%), Flavozyme (94.1%), and Neutrase (93.7%) even statistically similar with commercials.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.3.2.2. Hydrogen peroxide scavenging effects

Fractions of chloroform (47.2%) and ethyl acetate (48.7%) demonstrated higher activities (Table 1). In addition, 80% methanol extract showed 38.9% scavenging activity. However, other fractions showed less effect on hydrogen peroxide scavenging. The H_2O_2 scavenging activities on

enzymatic extracts are shown in Table 4. Among the recorded results, Termamyl extract (60.6%) exhibited significantly similar H_2O_2 scavenging effects compared to commercial antioxidants followed by Kojizyme extract (50.2%). In addition, Alcalase (48.3%) and Neutrase (42.2%) also showed considerable effect on H_2O_2 scavenging.

1.3.2.3. Superoxide anion scavenging effects

As can be seen in Table 3, among the all fractions, ethyl acetate and chloroform showed the higher activities (40.5 and 31.5%, respectively). In addition, no significant activities were indicated from other fractions. Further, all those activities were significantly lower than that of commercial antioxidants. According to Table 4, among all the enzymatic extracts Termamyl (52.8%) seemed significantly ($P<0.05$) effective in superoxide scavenging effect (Table 4). In addition, all extracts of *P. duplex* showed activities not less than 40%. Of these extracts alcalase (46.2%), Flavozyme (43.9%) and Celluclast (43.2%) were considerable.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.3.2.4. Hydroxyl radical scavenging effects

Aqueous residue of *P. duplex* (57.7%) exhibited highest Hydroxyl radical scavenging effect followed by hexane (28.3%) and ethyl acetate (27.6%). However, no significant effect was shown from other fractions (Table 3). Enzymatic extracts of Neutrase (46.3%) exhibited highest hydroxyl radicals effect followed by Celluclast (31.1%) and Flavozyme

(29.6%). However, Rest of extracts did not demonstrate considerable activities in scavenging of hydroxyl radicals (Table 4).

1.3.3. Nitric oxide radical inhibition effects

As can be seen in Table 3, aqueous residue (57.7%) n-hexane (28.3%) and ethyl acetate (27.6%) from *P. duplex* exhibited significant results even higher than that of commercial antioxidants ($P<0.05$). However, other fractions did not exhibit significant activities on nitric oxide scavenging. Nitric oxide scavenging effect of enzymatic extracts is shown in Table 4, and almost all extracts have shown significantly higher activities compared to commercial antioxidants. All the enzymatic extracts except alcalase showed scavenging effects even significantly higher than that of commercial antioxidants ($P<0.05$). Among these extracts Celluclast (44.8%), AMG (42.6%), Termamyl (41.8%), Kojizyme (40.4%) and Ultraflo (40.1%) were more significant.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.3.4. Metal chelating effect

As shown in Table 3, only ethyl acetate fraction (82.1%) showed significantly higher effect of metal chelating compared to commercial antioxidants. Rest of fraction exhibited very low activities. However, as shown in Table 4, all enzymatic extracts except Celluclast have exhibited very strong metal chelating effects even significantly ($P<0.05$) higher than the activities in commercial antioxidants. Among them Kojizyme, (79.1%),

AMG (77.1%), Protamax (75.2%), Ultraflo (73.6%), Neutrase (71.7%) and Viscozyme (71.4%) were most significant.

1.3.5. Determination of antioxidant activity with the ferric thiocyanate (FTC) method

As shown in Fig 2 and 3, the absorbance of linoleic acid emulsion without the addition of *P. duplex* extracts (control) increased significantly ($P<0.05$). Among the solvent fractions, chloroform and ethyl acetate had significant antioxidant activity compared to commercials (Fig 2). Enzymatic extracts of Viscozyme, Termomyl, and Kojizyme expressed notable antioxidant activity (Fig 3). No significant antioxidant activity was present with other extracts compared to commercial antioxidants.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.3.6. Total polyphenol content

The total phenolic content in the extracts and the fractions varies with the solvent used according to their polarity and the enzymes used (Table 3 and 4). Methanol extract indicated the highest polyphenol content (2104 mg/100g) among the solvent extracts and fractions (Table 5). Of the carbohydrases, Ultraflo extract exhibited highest polyphenol content (1229 mg/100 g) and among proteases, neutrase showed highest polyphenol (1337.6 mg/100 g) contents (Table 6).

Table 2. Antioxidants activity of 80% methanol extract and its different solvent extarcts from *P. duplex.*

Fractions	DPPH	H_2O_2	SOD	OH	NO	MC
Methanol	65.9d±3.2	38.9c±1.6	9.6f±0.3	73.1c±5.8	13.3d±0.6	26.6b±1.7
n-Hexane	74.5c±5.2	32.1d±1.3	22.4d±1.6	5.5f±2.3	28.3b±0.7	3.9d±0.1
Chloroform	65.9d±4.3	47.2b±2.4	40.5b±2.7	13.5d±0.4	2.7e±0.1	11.5c±0.2
Ethyl acetate	79.8b±5.7	48.7b±2.6	31.5c±2.1	7.4ef±0.3	27.6bc±1.1	82.1a±6.4
Aqu. residue	59.9e±1.3	32.5d±1.1	15.5±0.6	8.7e±0.4	57.7a±2.7	11.8c±0.4
BHT	94.6a±6.4	60.1a±4.2	63.2a±4.3	76.6b±4.6	26.1bc±0.9	11.5c±0.1
Tocopherol	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2c±0.6	10.3c±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates.

Table 3. Antioxidants activity of enzymatic extracts from *P. duplex.*

Extracts	DPPH	H_2O_2	SOD	OH	NO	MC
Viscozyme	81.5c±5.4	32.5e±1.3	41.1c±1.6	28.4c±0.9	39.1b±1.1	71.4b±4.3
Celluclast	88.5b±5.9	33.2e±1.6	43.2c±2.4	31.1c±1.1	44.8a±2.2	24.8f±1.2
AMG	77.3d±4.7	33.1e±1.7	42.1c±1.8	22.7d±0.3	42.6a±2.4	77.1a±4.2
Termamyl	90.5b±6.2	60.6a±3.7	52.8b±2.1	21.4d±0.7	41.8a±2.5	45.7e±1.1
Ultraflo	94.2b±6.4	30.3e±1.4	40.3c±1.9	28.5d±0.6	40.1a±1.7	73.6b±4.7
Protamax	91.2b±6.5	34.1e±2.1	40.3c±2.3	19.3e±0.4	39.6b±1.1	75.2b±5.1
Alcalase	89.4b±6.2	48.3b±1.9	46.2c±2.4	18.7e±0.9	20.7d±0.8	64.8c±3.4
Flavozyme	94.1a±6.3	37.1d±1.3	43.9c±1.1	29.6e±1.3	38.7b±1.1	55.5d±2.7
Neutrase	93.7a±6.1	42.2c±1.5	42.1c±2.1	46.3b±1.7	37.9b±1.5	71.7b±5.7
Kojizyme	83.1c±5.3	27.2f±0.9	41.2c±1.4	22.4d±1.3	40.4a±1.8	79.1a±6.1
BHT	94.6a±6.4	60.1a±4.2	63.2a±4.3	76.6a±4.6	26.1c±0.9	11.5g±0.1
Tocopherol	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2c±0.6	10.3g±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 4. Total polyphenolic, polysaccharide and protein contents of 80% methanol extract and its different solvent frctions from *P. duplex.*

Fractions	Polyphenol [a] (mg/100 g)[d]	Polysaccharide [b] (g/100 g) [e]	Protein [c] (g/100 g) [e]
80%metahnol	2104.5±98	26.6±1.4	6.6±0.3
n-Hexane	470.2± 21	9.4±0.0	4.4±0.2
Chloroform	1394.2±75	17.5±0.9	5.3±0.3
Ethyl acetate	1528.7±79	26.2±1.1	7.3±0.3
Aqueous	740.1±42	26.2±1.3	3.8±0.1

M±SE of determinations was made in triplicates

[a] As equivalent gallic acid
[b] As equivalent to glucose
[c] As equivalent to bovine serum albium
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 5. Total polyphenolic, polysaccharide and protein content of different enzymatic extracts from *P. duplex*

Extarcts	Polyphenol [a] (mg/100 g) [d]	Polysaccharide [b] (g/100 g) [e]	Protein[c] extracts (g/100 g) [e]
Viscozyme	1101.5 ±52	7.52±0.3	6.7 ±0.3
Celluclast	1214.8±64	2.33±0.1	5.5±0.2
AMG	1077.9 ±58	8.81±0.1	8.6±0.3
Termamyl	1167.6 ±61	7.77±0.1	6.1±0.2
Ultraflo	1229.0 ±72	1.88±0.1	5.7±0.2
Protamax	1092.1 ±68	2.11±0.1	4.7±0.2
Alcalase	1082.6 ±69	2.28±0.1	4.9±0.3
Flavourzyme	1247.9 ±61	9.01±0.1	7.5±0.3
Neutrase	1337.6 ±84	7.52±0.3	6.7 ±0.3
Kojizyme	1082.6 ±69	2.01±0.1	5.5±0.3

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Values are means of three replicates ± SD

[a] As equivalent gallic acid
[b] As equivalent to glucose
[c] As equivalent to bovine serum albium
[d] mg per 100 g solid of the extracts.
[e]g per 100 g solid of the extracts.
.

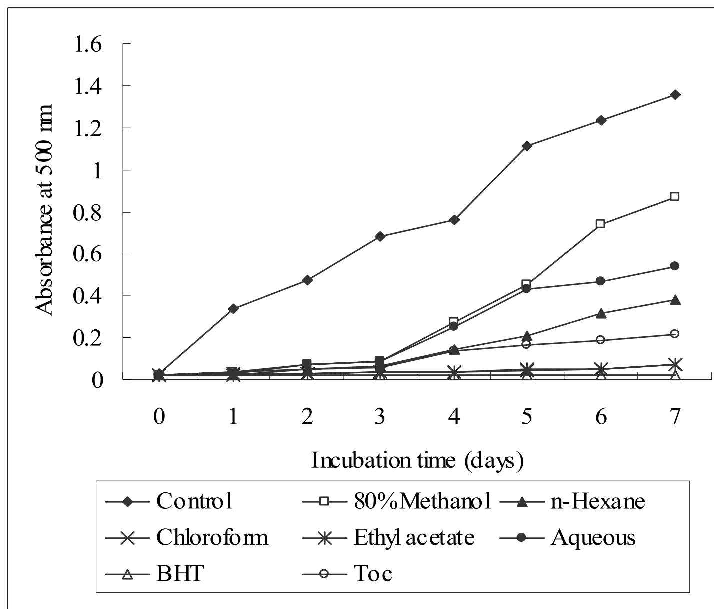
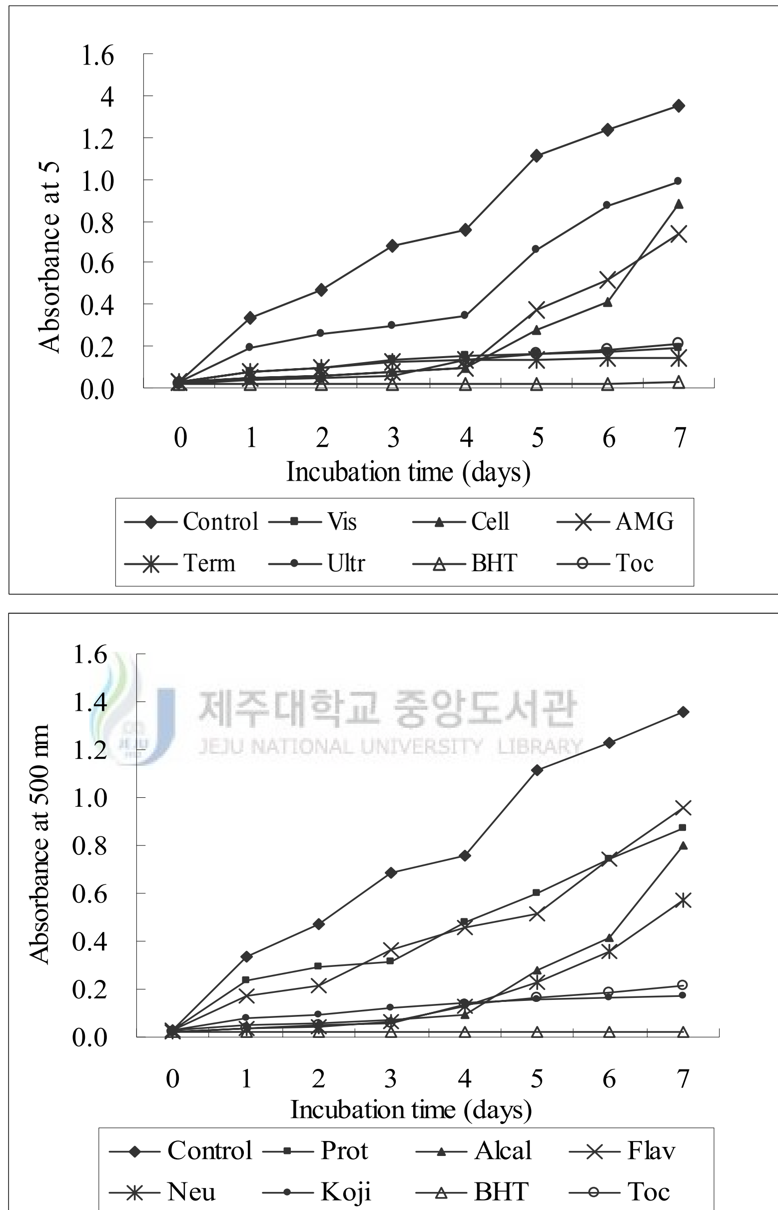


Fig. 5. Antioxidant activity of different solvent fractions from *P. duplex* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 6 Antioxidant activity of different enzymatic extracts from *P. duplex* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

1.4. DISCUSSION

Recently there has been increasing interest in the beneficial potential of natural plants as antioxidants in reducing ROS since they produce compounds such as polyphenolics, and nitrogen containing compounds, phytosterols, carotonoids, and chlorophyll derivatives. In the present study, the interest was focused on antioxidant potential of solvent fractions of 80% methanol extract and enzymatic extracts from a microalga *P.duplex.*

The model of scavenging the stable DPPH is an extensively used method to evaluate antioxidant activities in a comparatively short period compared with other relative methods. In this study, all solvent fractions and all enzymatic extracts exhibited free radical activities indicating the higher efficiency of scavenging of free radicals in *P. duplex*. Therefore, it was clear those biochemical compounds that responsible for DPPH scavenging activity were dispersed in aqueous extracts and organic fractions showing both hydrophilic and hydrophobic characters. Radical scavengers may protect cell tissues from free radicals, thereby preventing diseases such as cancer (Nakayama et al.,. 1993) Even though it is unclear whether active constituents in extracts are active against free radicals after being absorbed and metabolized by cells in the body, free radical-scavenging assays have gained acceptance for their capacity to screen rapidly materials of interest.

The higher superoxide activities were founded in the organic solvent fractions than aqueous fractions. Thus, it can be assume that active

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

compounds may have dispersed in to organic fractions. On the other hand, activity of enzymatic extracts was in higher than solvent fractions. It is suggested that active compounds that not participated to give results for solvent fractions have been released because of enzymatic hydrolysis. Thus, it was clear that biochemical compounds that responsible for superoxide activity had both hydrophobic and hydrophilic characteristics. Superoxide radical generally form first and its effects can be exaggerated as it produces other kinds of cell damage inducing free radicals and oxidizing agents (Spitz et al., 2005). It is a precursor to active free radicals that have potential of the reaction with biological macromolecules and thereby inducing tissue damage (Halliwell and Gutteridge, 1989). Therefore, superoxide radical scavenging by antioxidants has physiological implications.

제주대학교 중앙도서관

In this study, all solvent fractions and all enzymatic extracts exhibited activities indicating the efficiency of scavenging of H_2O_2 in *P. duplex*. Therefore, it was clear those biochemical compounds that responsible for H_2O_2 scavenging activity was dispersed in aqueous extracts and organic fractions showing both hydrophilic and hydrophobic characters. In addition, no much difference between the activities given by both carbohydrases and proteases and those activities even grater than solvent fractions. H_2O_2, a reactive non-radical compound is very important as it can penetrate biological membranes. Although H_2O_2 itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl radicals (Halliweill, 1991). Thus, removing H_2O_2 is very important for the

protection of living systems. Since phenolic compounds (good electron donors) present in *P. duplex* they may accelerate the conversion of H_2O_2 to H_2O (Ruch et al., 1984.).

Hydroxyl radical scavenging activity of the enzymatic extracts and solvent fractions from *P. duplex* was determined as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. Among the solvent fractions, only 80% methanolic extract showed comparatively higher activity. Thus, it can be assumed that those responsible biochemical compounds have hydrophilic properties. In addition, both carbohydrases and protease had same effect on hydrolyzing of *P. duplex* because; there was no much difference between activities showed by these extracts. Hydroxyl radical is the most reactive oxygen species among all ROS due to its strong ability to react with various biomolecules. Hydroxyl radical ($HO^{\cdot}$) reacts with several biological materials oxidatively by hydrogen withdrawal, double bond addition, electron transfer and radical formation, and initiates autoxidation, polymerization and fragmentation (Liu and Ng, 2000).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Not only reactive oxygen species, nitric oxide is also implicated in cancer, inflammation, and other pathological conditions. Nitric oxide ($NO^{\cdot}$) is a gaseous free radical, which has important roles in physiological and pathological conditions. Therefore, the scavenging ability of $NO^{\cdot}$ may also help to disrupt the chain reactions initiated by excessive generation of $NO^{\cdot}$ that are detrimental to human health. In solvent fractions, $NO^{\cdot}$ inhibition

effects were deviated towards hydrophilic status as aqueous residue exhibited significantly higher activities ($P<0.05$). Overall, compared to solvent fractions both carbohydrases and proteases exhibit considerable results. It means those biochemical compounds exhibit hydrophilic properties. Marcocci et al. (1994) has reported that scavengers of nitric oxide compete with oxygen leading to reduce production of nitric oxide.

Ferrous is known as the most important lipid oxidizing prooxidant among the transition metals due to its high reactivity. Ferrozine can make complexes with ferrous ions. In presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, chelating effect of the coexisting chelator can be determined measuring the rate of color reduction. The formation of the ferrozine–Fe^{2+} complex was interrupted in the presence of *P. duplex* extracts and solvent fractions indicating significant chelating ability. It was notable that bioactive compounds that give metal chelating effects were dispersed in ethyl acetate (moderately polar). However, enzymatic extracts showed higher metal chelating ability and it is apparent that hydrophilic components are responsible for metal chelating. In addition, higher activities were present in both carbohydrases and proteases extracts equally. Hence, different extracts and fractions of *P. duplex* demonstrated a noticeable capacity for iron binding, suggesting their ability as peroxidation protector that relates to its ferrous binding capacity (Gülçin et al, 2004).

제주대학교 중앙도서관

To evaluate the antioxidant effects of *P. duplex* extracts, their lipid peroxidation were compared with commercial antioxidants using ferric thiocyanate (FTC) method by determining the amount of peroxide formed in emulsion in during incubation period. High absorbance is an indication of high concentration of formed peroxides. In this experiment, fractions of chloroform and ethyl acetate, enzymatic extracts of Kojizyme, Termamyl and Viscozyme extracts provided higher antioxidant effects among the all extracts and fractions showing that those extracts were able to reduce the formation of peroxides. It suggests that according to enzyme digestion and solvent fractionation different antioxidant components were released from the inside of microalgae cells. These extracts contained high level of polysaccharides, proteins and polyphenols and it could be assumed that those components may influence on antioxidant effects although not higherly correlated with antioxidant effect in this study.

Total phenolic content in both 80% methanol extracts and different enzymatic extracts varies with the solvent used according to their polarity. Extracts of Neutrase, Ultraflo, and 80% methanol extarct consisted of the higher contents of polyphenol. Although in this study it was not revealed according to the recent researches, a highly positive correlation between total phenols and antioxidant activity was found in various plant species (Vinson et al., 1998; Oktay et al., 2003). Therefore, it can be suggested that not only polyphenol contents but other factors also may effect for the antioxidant activity. Further, there are some other bioactive components such as proteins,

polysaccharides and different kinds of pigments are consisted in this microalga. For example it was found that oligosaccharides, sulfate and glycoprotein components have exhibited antioxidant activities in red microalga *Porphyridium sp*. (Spitz et al., 2005). Moreover, the polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans (Tanaka et al., 1998). Phenols are very significant plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano, 1995).

In this study, extracts was prepared using 80%methanolic extract and its solvent fractions and different kinds of enzymes to evaluate antioxidant effects. Methanol is a good solvent to extract varies kind of hydrophilic and hydrophobic bioactive compounds but extraction yield is less compared to enzymatic extractions. By solvent fractionation, biological compounds can be separated according to its polarity. In this way, some water insoluble compounds also can be extracted. On the other hand, plants cells originally contain large amount of soluble polysaccharides and insoluble fibers such as made of cellulose. Both insoluble and soluble fiber together with the other cell wall material performs as a physical barrier for the extraction of desired bioactive materials. Such barriers might make the desired compounds possible to be extracted in high yields. Enzymatic hydrolysis of raw material tissues or cells has reported significant yields of desired compounds and convenient industrial approaches in extraction and purification (Jeon et al., 2000: Nagai and Suzuki, 2000). Also, the

제주대학교 중앙도서관

breakdown and releasing of high molecular weight polysaccharides and proteins themselves may contribute to enhance the antioxidative activities (Ramos and Xiong, 2002; Ruperez et al., 2002). Additionally, enzymatic extracts possesses innovative advantages and characters over conventional extraction procedures. Of the several advantages, water solubility, higher extraction efficacy, greater variation of constituents, minimized environmental pollution/ toxicity and comparatively inexpensiveness are obvious.

In conclusion, it was obvious that *P. duplex* contains considerable antioxidant potential against ROS. Thus, these results propose that *P. duplex* may good candidate as a natural antioxidant source, which could be applied in functional food field and pharmaceutical industry. Therefore, further investigation of extracting and isolation of bioactive compounds, their in vivo antioxidant activity, and the different antioxidant mechanisms relating those active compounds are needed to address.

제주대학교 중앙도서관

2. ANTIOXIDANT PROPERTIES OF FRESH WATER BLUE-GREEN MICRO ALGA (CYANOPHYCEAE), *Dactylococcopsis fascicularis*

2.1. ABSTRACT

Freeze-dried fresh water blue-green microalga (Cyanophyceae), *Dactylococcopsis fascicularis* was extracted with 80% methanol (and sequentially partitioned with solvents in an order of increasing polarity) and with five proteases and carbohydrases to examine, its various antioxidant properties. Of the organic fractions tested, ethyl acetate fraction (39.8%) and aqueous residue (38.9%) were effective in DPPH scavenging. Fractions of n-hexane (47.5%) and chloroform (47.2%) exhibited higher activities on H_2O_2 and hydroxyl radical scavenging (47.5% and 47.2% and 36.4% and 34.1%, respectively). n-Hexane (45.7%) and ethyl acetate fractions (42.7 %%) showed the highest $NO^{\cdot}$ inhibition activity. Aqueous residue (57.7%) and ethyl acetate fraction (37.6%) demonstrated a significant activity in metal chelating ($P<0.05$). In enzymatic extracts, Protamax (67.9%) and Viscozyme (58.4%) showed higher DPPH activities. Kojizyme extract (50.2%) exhibited highest activity followed by Protamax (48.6%) in H_2O_2 radical scavenging. Extracts of Kojizyme (56.9%), Celluclast (58.1%), and Termamyl (54.6%) possessed higher effects in superoxide radical scavenging. All extracts exhibited significant effects in both $NO^{\cdot}$ inhibition and metal chelating ($P<0.05$). Among them, extracts of Flavozyme (43.9%) and Ultraflo (42.8%) revealed significant activities in $NO^{\cdot}$ inhibition while Celluclast (86.8%),

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Neutrase (80.1%), and Termamyl (7.7%) had strong metal chelating activities.

These data suggest that both organic and aqueous fractions as well as enzymatic extracts are rich in hydrophobic and hydrophilic antioxidative compounds with different antioxidative properties.

2.2. MATERIALS AND METHODS

As described in chapter 1.2 "Antioxidant properties of *Pediastrum duplex*'

2.3. RESULTS

2.3.1. Proximate composition

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Proximate composition of freeze dried *D. fascicularis* collected from Mu-Soo stream was analyzed and values were; moisture 5.5%, ash content 47.4%, carbohydrates 33.7%, proteins 8.9% and fat 4.5%. Polysaccharide and protein content in 80% methanol extract and its solvent fractions and enzymatic extracts is depicted in Table 9 and 10.

2.3.2. Reactive oxygen species inhibition

2.3.2.1. DPPH free radical scavenging effect

According to Table 7, 80% methanolic extract and most of its solvent fractions indicated substantial DPPH free radical scavenging effect and among them chloroform and ethyl acetate fractions showed 49.4% and 39.8% activities respectively. Further, enzymatic extracts did not exhibit

significant effect on DPPH radical scavenging compared to commercial antioxidants (Table 8). However, extracts of protamax, Viscozyme and Neutrase exhibited activities as 67.9, 58.4, and 57.4%, respectively.

2.3.2.2. Hydrogen peroxide scavenging effect

The scavenging activities of organic solvent fractions from 80% methanol and enzymatic extracts of *D. fascicularis* is depicted in Table 7. Chloroform fraction (47.2%), 80% methanol extract (47.7%), and n-hexane fraction (42.9%) demonstrated higher activities. However, other fractions showed less effect on hydrogen peroxide scavenging.

The H_2O_2 scavenging activities on enzymatic extracts are shown in Table 8. Among the recorded results, Kojizyme extract (50.2%) and Protamax extract (48.6%) exhibited considerable H_2O_2 scavenging effects. However, rest of extracts showed less effect on hydrogen peroxide scavenging except Neutrase extract (40.6%).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2.3.2.3. Hydroxyl radical scavenging effect

Hydroxyl radical scavenging activity of different solvent fractions of 80% methanolic extract and enzymatic extracts was determined. Fractions of n-hexane (34.1%) and chloroform (36.1%) exhibited higher hydroxyl radical scavenging effects (Table 7). However, no significant effect was shown from other fractions. Enzymatic extracts of Viscozyme (33.8%) exhibited highest

Table 6. Antioxidants activity of 80% methanol extract and its different solvent extarcts from *D. fascicularis.*

Fractions	DPPH	H_2O_2	SOD	HO	NO	MC
80%Methanol	23.1d±1.3	47.7b±2.3	3.5d±0.1	23.3d±0.9	2.7e±0.1	14.1de±0.7
n-Hexane	39.8c±1.7	42.9c±2.1	15.5c±0.3	34.1c±1.4	45.7a±1.8	14.5d±0.8
Chloroform	49.4b±2.1	49.7b±1.7	19.7b±0.7	36.4c±1.3	2.3e±0.1	18.8c±0.8
Ethyl acetate	39.8c±2.4	25.2d±0.8	18.9d±0.3	11.4e±0.6	42.7b±1.8	37.6b±1.6
Aqu. residue	38.9c±2.7	15.2e±0.7	5.3d±0.4	11.2e±0.3	6.5d±0.4	49.2a±1.9
BHT	94.6a±6.4	60.1a±4.2	63.2a±4.3	76.6b±4.6	26.1e±0.9	11.5ef±0.1
Tocopherol	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2e±0.6	10.3f±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

Table 7. Antioxidants activity of enzymatic extracts from *D. fascicularis.*

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

Extarcts	DPPH	H_2O_2	SOD	OH	NO	MC
Viscozyme	58.4c±2.3	32.4d±1.7	49.6b±2.1	33.8b±1.3	39.8b±1.3	65.4cd±3.2
Celluclast	41.2e±1.6	24.8e±1.3	58.1ab±2.3	23.2c±0.9	32.2d±1.6	86.8a±5.7
AMG	38.3e±1.4	34.6d±1.3	47.1b±3.1	17.1e±0.4	40.4b±0.9	58.6cd±3.3
Termamyl	46.7d±2.4	32.5d±1.5	54.6ab±2.4	18.2e±0.3	35.2c±0.7	77.7ab±4.5
Ultraflo	43.2e±2.1	31.3d±1.2	50.1b±2.9	19.3e±0.7	42.8a±0.5	77.1ab±5.7
Protamax	67.9b±3.7	48.6b±2.2	34.3c±1.7	20.9d±1.1	39.8ab±0.7	75.6ab±6.2
Alcalase	49.5d±2.4	27.6c±0.7	39.5c±1.4	15.7e±0.3	36.8c±0.3	76.6ab±5.1
Flavozyme	42.7e±1.2	24.9e±0.4	47.6b±2.4	23.2c±0.4	43.9a±1.2	53.3d±3.7
Neutrase	57.4c±2.4	40.6c±1.4	41.9c±2.3	23.2c±0.9	41.5a±1.6	80.1ab±6.2
Kojizyme	41.4e±1.4	50.2b±2.1	56.9ab±2.1	26.1c±0.3	41.2a±1.2	74.2ab±4.9
BHT	94.6a±6.4	60.1a±4.2	63.2a±4.3	76.6a±4.6	26.1e±0.9	11.5d±0.1
Tocopherol	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2e±0.6	10.3d±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

hydroxyl radical effect. However, rest of extracts did not show considerable activities in scavenging of hydroxyl radicals (Table 8).

2.3.2.4. Superoxide anion scavenging effect

According to Table 7, among the all fractions and 80% methanolic extract, Chloroform fraction, 80% methanolic extract and n-hexane fraction showed the higher activities (49.7, 47.7 and 42.9%, respectively). In addition, no significant activities were indicated from other fractions. Further, all those activities were significantly lower than that of commercial antioxidants. As depicted in Table 3, almost all the enzymatic extracts seemed considerable ($P<0.05$) effect in superoxide scavenging. Of the extracts Kojizyme, Celluclast, Termamyl and Ultraflo indicated remarkable Superoxide anion scavenging activities (56.9, 58.1, 54.6 and 50.1, respectively). In addition Viscozyme, AMG, Flavozyme showed as 49.6, 47.1 and 47.6% respectively.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2.3.3. Nitric oxide radical inhibition effect

As shown in Table 7, n-hexane (45.7%) and ethyl acetate (42.7%) from exhibited significant results even higher than that of commercial antioxidants ($P<0.05$). However, other fractions did not show significant activities on nitric oxide scavenging. Nitric oxide scavenging effect of enzymatic extracts is shown in Table 8 and all the enzymatic extracts exhibited inhibition effects even significantly higher than that of commercial antioxidants ($P<0.05$). Among these extracts activities of Flavozyme (43.9%),

Table 8. Total polyphenolic, polysaccharide and protein contents of 80% methanol extract and its different solvent frctions from *D. fascicularis*

Solvent fractions	Polyphenol [a] (mg/100 g)[d]	Polysaccharide [b] (g/100 g) [e]	Protein [c] (g/100 g) [e]
80%metahnol	1658.1±98.2	27.6±1.2	10.9±0.1
n-Hexane	474.3±18.3	9.1±0.4	2.1±0.1
Chloroform	343.9±11.6	14.3±0.1	7.8±0.2
Ethyl acetate	1318.7±74.4	14.9±0.1	7.6±.0.2
Aqueous	1125.1±61.2	26.9±0.7	6.0±0.2

M±SE of determinations was made in triplicates

[a] As equivalent gallic acid
[b] As equivalent to glucose
[c] As equivalent to bovine serum albumin
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

Table 9. Total polyphenolic, polysaccharide and protein content of different enzymatic extracts from *D. fascicularis*

Enzymatic extracts	Polyphenol [a] (mg/100 g) [d]	Polysaccharide [b] (g/100 g) [e]	Protein [c] (g/100 g) [e]
Viscozyme	978.8±32	3.2±0.1	7.4 ±0.2
Celluclast	941.0 ±34	9.7±0.1	5.1 ±0.3
AMG	922.1 ±48	2.7±0.1	5.1 ±0.3
Termamyl	931.5 ±31	6.1±0.1	10.1 ±0.2
Ultraflo	870.2 ±32	6.8±0.1	10.1±0.5
Protamax	1384.8 ±28	8.4±0.3	9.8 ±0.3
Alcalase	1342.3±39	9.8±0.1	7.8±0.2
Flavourzyme	1092.1 ±21	8.6±0.1	1.3 ±0.2
Neutrase	1068.5 ±24	7.1±0.1	8.8 ±0.3
Kojizyme	983.5 ±19	5.3±0.1	8.2 ±0.2

Values are means of three replicates ± SD

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

[a] As equivalent gallic acid
[b] As As equivalent to glucose
[c] As equivalent to bovine serum albium
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts.

Ultraflo (42.8%), Neutrase (41.5%), Kojizyme (41.2%), and AMG (40.4%) were more significant.

2.3.4. Metal chelating effect

As shown in Table 7, all the fractions and 80% methanolic extract (82.1%) showed significantly higher (P<0.05) effect of metal chelating compared to commercial antioxidants (α-tocopherol: 11.5% and BHT:10.3%). Of the fractions, activities from aqueous residue (49.2%) and ethyl acetate (37.6%) were more significant. In addition, according to Table 8, all enzymatic extracts have exhibited exceptionally strong metal chelating effects even significantly (P<0.05) higher than the activities in commercial antioxidants. Among them Celluclast (86.8%), Neutrase (80.1%), Termamyl (77.7%), Ultraflo (77.1%) and alcalase (76.6%) exhibited strong chelating effects.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2.3.5. Determination of antioxidant activity with the ferric thiocyanate (FTC) method

As shown in Fig. 7 and Fig. 8, the absorbance of linoleic acid emulsion without the addition of *P. duplex* extracts (control) increased significantly (P<0.05). Among the all the solvent farctions n-hexane presented antioxidant activity statistically similar with α –tocopherol (Fig. 7). Enzymatic extracts of Ultraflo, Protamax, Kojizyme and Alcalase exhibited statistically significant (P<0.05) antioxidant activity (Fig 8). No notable antioxidant activity was present with other extracts compared to commercial antioxidants.

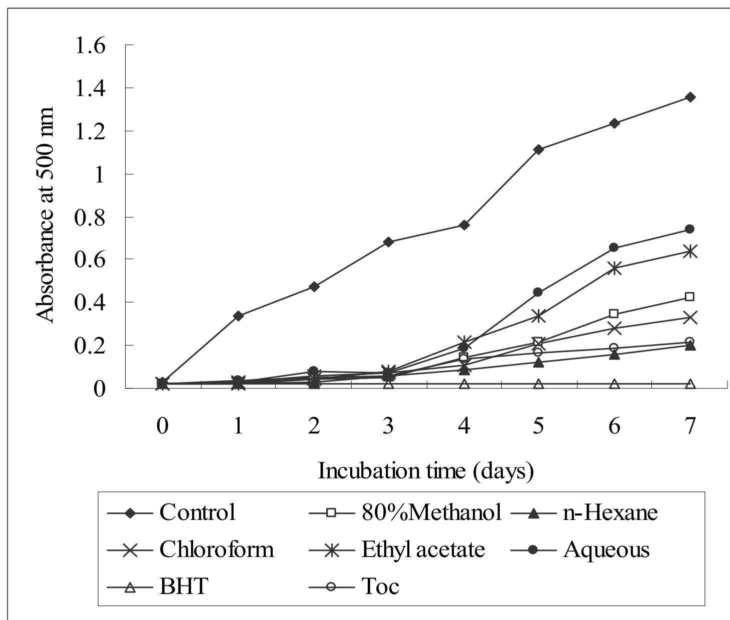
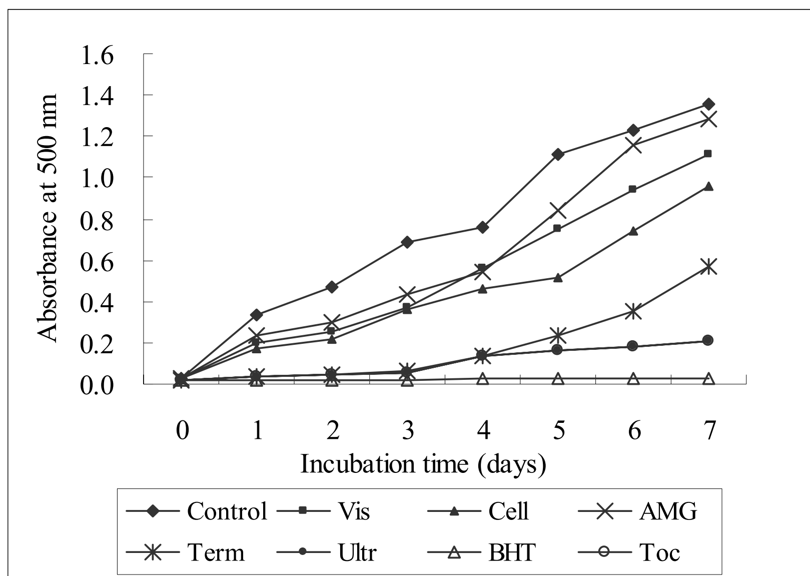


Fig. 7. Antioxidant activity of different solvent fractions from *D. fascicularis* compared to BHT and α- Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.



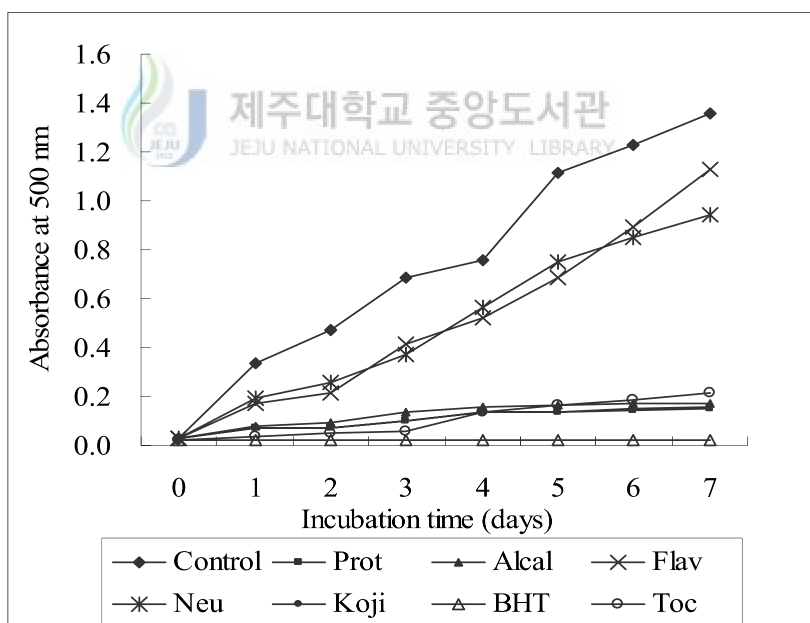


Fig. 8 Antioxidant activity of different enzymatic extracts from *D. fascicularis* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2.3.6. Total polyphenol content

The total phenolic content in the extracts and the fractions varies with the solvent used according to their polarity and the enzymes used (Table 9 and 10). Among solvent extracts and fractions 80% Methanol extract indicated the highest polyphenol content (1658.1 mg/100 g) (Table 9). Of the carbohydrases Viscozyme extract exhibited highest polyphenol content (978.8 mg/100 g) and of the proteases, Protamax showed highest polyphenol (1934 mg/100 g) contents (Table 10).

2.4. DISCUSSION

There are a numerous antioxidant methods and modifications for evaluation of antioxidant activity. Of these, DPPH assay, total antioxidant activity, metal chelating, ractive oxygen species such as H_2O_2, $O_2^{\cdot -}$, and $OH^{\cdot}$ quenching assay are most commonly used for the determination of antioxidant activities of extracts. In the present study, the interest was focused on antioxidant potential of solvent fractions of 80% methanol extract and enzymatic extracts from fresh water cyanophyceae, *D. fascicularis*

The model of scavenging the stable DPPH is an extensively used method to evaluate antioxidant activities in a comparatively short period compared with other relative methods. In this study, all solvent fractions and all enzymatic extracts exhibited a moderate efficiency of scavenging of free radicals in *D. fascicularis*. Therefore, it is apparent those biochemical compounds that responsible for DPPH scavenging activity were dispersed in aqueous extracts and organic fractions showing both hydrophilic and

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

hydrophobic characters. Radical scavengers may protect cell tissues from free radicals, thereby preventing diseases such as cancer (Nakayama et al., 1993) Even though it is unclear whether active constituents in extracts are active against free radicals after being absorbed and metabolised by cells in the body, free radical-scavenging assays have gained acceptance for their capacity to screen rapidly materials of interest.

The higher superoxide activities were founded in the enzymatic extracts than solvent fractions. Enzymatic extraction has played a key role to liberate responsible compounds that not participated for solvent fractionation. Thus, it was apparent that biochemical compounds that responsible for superoxide activity has hydrophilic characteristics. Superoxide radical generally form first and its effects can be exaggerated as it produces other kinds of cell damage inducing free radicals and oxidizing agents (Spitz et al., 2005).

제주대학교 중앙도서관

In this study, all solvent fractions, and all enzymatic extracts exhibited free radical activities indicating the efficiency of scavenging of H_2O_2 in *D. fascicularis* Therefore, it was noticeable those biochemical compounds that responsible for H_2O_2 scavenging activity was soluble in aqueous extracts showing relevant characters. In addition, it could have been some proteases extracts has little higher activities than carbohydrases. H_2O_2, a reactive non-radical compound is very important as it can penetrate biological membranes. Although H_2O_2 itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl

radicals (Halliweill, 1991). Thus, removing H_2O_2 is very important for the protection of living systems.

Hydroxyl radical scavenging activity of the enzymatic extracts and solvent fractions from *D. fascicularis* determined as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. Among the solvent fractions, n-hexane and chloroform fractions showed comparatively higher activities among other counterparts. Thus, it was clear that those responsible biochemical compounds exhibited activities towards hydrophobic properties. In addition, both carbohydrases and protease had same effect on hydrolyzing and giving effects in scavenging from *D. fascicularis* because; there was no much difference between activities showed by these extracts. Hydroxyl radical is the most reactive oxygen species among all ROS due to its strong ability to react with various biomolecules.

Not only reactive oxygen species, nitric oxide is also implicated in cancer, inflammation, and other pathological conditions. Nitric oxide ($NO^{\cdot}$) is a gaseous free radical, which has important roles in physiological and pathological conditions. Therefore, the scavenging ability of NO.may also help to disrupt the chain reactions initiated by excessive generation of NO.that are detrimental to human health. In solvent fractions, NO.inhibition effects were deviated towards hydrophilic and hydrophobic status. In overall, both carbohydrases and proteases exhibit considerable results and no much difference could be seen between these activities. Therefore, the scavenging

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

ability of NO.may also help to interrupt the chain reactions initiated by excessive generation of NO.that are detrimental to human health.

Ferrous is known as the most important lipid oxidizing pro-oxidant among the transition metals due to its high reactivity. Ferrozine can make complexes with ferrous ions. In presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, chelating effect of the coexisting chelator can be determined measuring the rate of color reduction. The formation of the ferrozine–Fe^{2+} complex is interrupted in the presence *of D. fascicularis* enzymatic extracts and solvent fractions indicating significant chelating ability. It is notable that bioactive compounds that give metal chelating effects were dispersed in ethyl acetate and aqueous fraction supposing that those compounds were deviated toward hydrophilic status. However, enzymatic extracts showed strong metal chelating effects and it was apparent that hydrophilic components are responsible for metal chelating. In addition, higher activities were exhibited by both carbohydrases and proteases extracts evenly. Ferrous accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Fridovich, 1995, Halliweill, 1991). Hence, different extracts of *D. fascicularis* demonstrate a noticeable capacity for iron binding, suggesting their ability as peroxidation protector that relates to its ferrous binding capacity (Gülçin et al, 2004).

To evaluate the antioxidant effects of *D. fascicularis* extracts, their lipid peroxidation were compared with standard antioxidants using ferric

제주대학교 중앙도서관

thiocyanate (FTC) method by determining the amount of peroxide formed in emulsion in during incubation period. High absorbance is an indication of high concentration of formed peroxides. In this experiment, fraction of n-hexane, enzymatic extracts of Kojizyme, Alcalase, Protamax, and Ultraflo provided higher antioxidant effects among the all extracts and fractons showing that those extracts were able to reduce the formation of peroxides. It suggests that according to enzyme digestion and solvent fractionation different antioxidant components are released from the inside of microalgae cells. These extracts contained high level of polysaccharides, proteins and polyphenols and it could be assumed that those components may influence on antioxidant effects.

Total phenolic content in both 80% methanolic extracts and different enzymatic extracts varies with the solvent used according to their polarity. Although the ethyl acetate fraction, extracts of Flavozyme, Neutrase consisted of the higher contents of polyphenol it was not shown the posivite correlation with antioxidant activity. Further, there are some other bioactive components such as proteins, polysaccharides and different kinds of pigments are consisted in this microalga. For example it was found that oligosaccharides, sulfate and glycoprotein components have exhibited antioxidant activities in red microalga, *D. fascicularis* (Spitz et al., 2005). Therefore, it can be suggested that not only polyphenol content but other factors also may effect for the activity.

제주대학교 중앙도서관

In this study, both methanolic and enzymatic extraction performed to determine antioxidant activities. Methanol is a good solvent to extract varies kinds of hydrophilic and hydrophobic bioactive compounds but extraction yield is less compared to enzymatic extracts. By solvent fractionation, biological compounds can be segregated according to its polarity and in this way; several water insoluble compounds can be extracted. On the other hand, plants cells originally contain large amount of soluble polysaccharides and insoluble fibers such as made of cellulose. Both insoluble and soluble fiber together with the other cell wall material performs as a physical barrier for the extraction of desired bioactive materials. Enzymatic hydrolysis of raw material tissues or cells has reported significant yields of desired compounds and convenient industrial approaches in extraction and purification (Jeon et al., 2000: Nagai and Suzuki, 2000). In addition, the breakdown and releasing of high molecular weight polysaccharides and proteins themselves may contribute to enhance the antioxidative activities (Ramos and Xiong 2002; Ruperez et al., 2002). Additionally, enzymatic extracts possesses innovative advantages and characters. Of the several advantages, water solubility, higher extraction efficacy, greater variation of constituents, minimized environmental pollution/ toxicity and comparatively inexpensiveness are obvious.

제주대학교 중앙도서관

In conclusion, it was obvious that this species contained antioxidant potential against ROS. Thus these results can be proposed that *D.*

fascicularis may good candidate as a natural antioxidant source, which might be applied in food and pharmaceutical field.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

3. ANTIOXIDANT PROPERTIES OF TIDAL POOL MICROALGA, *Halochlorococcum porphyrae*

3.1. ABSTRACT

The antioxidative properties of different organic fractions obtained by solvent fractionation of 80% methanolic extract and enzymatic extracts from five proteases and carbohydrases from tidal pool microalgae, *Halochlorococcum porphyrae* was investigated for free radical, reactive oxygen species scavenging ($O_2^{\cdot-}$,H_2O_2, $HO^{\cdot}$,$NO^{\cdot}$), metal chelating and total antioxidant assays**.** Chloroform fraction (78%), n-hexane fraction (72%), and water extract (69.4%) exhibited higher effect on DPPH free radical scavenging. Chloroform fraction (72%) and 80% methanol extract (65.4%) showed metal chelating activity that was significantly higher than that of the standard antioxidants. Ethyl acetate fraction (44%) showed the highest $O_2^{\cdot-}$ scavenging activity among all fractions. Both n-hexane (38%) and chloroform (31%) fractions exhibited higher H_2O_2 activities. Among enzymatic extracts, all extracts exhibited remarkable DPPH scavenging activities and out of those Viscozyme (95.1%), Neutrase (94.5%), Celluclast (93.2%), and AMG (87.4%) were more significant. Alcalase (42.1%) and Viscozyme (38.4%) showed higher effects in H_2O_2 scavenging. In $NO^{\cdot}$ inhibition, all extracts recorded significantly higher effects that that of commercial antioxidants and among them Termamyl (51.4%), AMG (51.2%) and Viscozyme (47.1%) exhibited significant results. Flavozyme (52.3%),

제주대학교 중앙도서관

Neutrase (52.1%) and alcalase (41.2) showed significant effect in metal chelating. Thus, these results indicate that active components of *H. porphyrae* that responsible for antioxidant activities exhibit both hydrophilic and hydrophobic properties.

3.2. MATERIALS AND METHODS

As described in chapter 1.2 “Antioxidant properties of *Pediastrum duplex’*

3.3. RESULTS

3.3.1. Proximate composition

Proximate composition of freeze dried *H. porphyrae* was analyzed and values were; moisture 3.3%, ash content 21.3%, carbohydrates 66.4%, proteins 5.1% and fat 3.9%. Polysaccharide and protein content in water extract and 80% methanol extract and its solvent fractions is depicted in Table 13 and 14, respectively.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

3.3.2. Reactive oxygen species inhibition

3.3.2.1. DPPH free radical scavenging effect

As shown in Table 11, the chloroform fraction showed a highest radical scavenging activity (78%) followed by the n-hexane fraction, and aqueous residue (72 and 68%, respectively). According to Table 12, for *H porphyrae* Viscozyme extract (95.1%) and Neutrase (94.5%) exhibited the higher radical scavenging activities followed by Celuclast (93.2%) and AMG

(87.4). In addition, AMG (87.4%), Termamyl (85.7%), Protamax (77.8%), and Ultraflo (76.8%) showed considerable effect in DPPH scavenging.

3.3.2.2. Hydrogen peroxide scavenging effect

Both n-hexane and chloroform fractions exhibited relatively higher H_2O_2 scavenging effects (38% and 31%, respectively) as can be seen in Table 11. However, both superoxide and H_2O_2 scavenging effects values were significantly lower ($P<0.05$) than those of standard antioxidants. As shown in Table 12, among enzymatic extracts alcalase (42.1%), viscozyme (38.4%) and protease (34.8%) demonstrated higher hydrogen peroxide scavenging activities. However, no satisfactory activities provided from the rest of extracts.

3.3.2.3. Superoxide scavenging effect

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

As shown in Table 11, the ethyl acetate fraction (41.3%) showed higher ($P<0.05$) superoxide scavenging activitity through all fractions. Rest of fractions possessed less effect in superoxide scavenging. Among enzymatic extracts, Viscozyme (46.9%), Celluclast (44.3%), and Termamyl (42.2%) exhibited considerable superoxide scavenging activity (Table 12). In addition, Neutrase, AMG, and Ultraflo showed activities as 38.5, 36.8, and 32.1%, respectively.

3.3.2.4. Hydroxyl radical scavenging efefct

As shown in Table 12, the aqueous residue (24%) exhibited significantly higher ($P<0.05$) scavenging activity compared to rest of

fractions. However, these values were significantly ($P<0.05$) less than standard antioxidants tested. As well, enzymatic extracts also failed to present significant activities in Hydroxyl radical scavenging (Table 13). Among enzymatic extracts, Flavozyme (30.7%) indicated higher scavenging activity followed by Kojizyme (22.8%).

3.3.2.5. Nitric oxide radical inhibition effects

Nitric oxide scavenging effect of *H. porphyrae* is shown in Table 11. Among all the fractions aqueous residue (30.1%) exibited significantly higher ($P<0.05$) scavenging effect than those of commercial antioxidants. However, rest of the other fractions and the extracts exhibited less effect on the nitric oxide scavenging. According to the Table 12, all enzymatic extracts indicated significant nitric oxide inhibition effects compare to commercial antioxidants. Among them extracts of Termamyl (51.4%), AMG (51.2%), Celluclast (48.5%), protamax (48.1%), Viscozyme (47.1%) and Alcalase were more significant.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 10. Antioxidants activities of 80% methanol extract and its different solvent extracts from *H.porphyrae*

Fractions	DPPH	H_2O_2	SOD	OH	NO	MC
Methanol	64.9d±2.3	20.2d±1.3	26.3c±0.9	21.3d±0.7	12.4±0.2	65.4b±3.5
n-Hexane	68.2c±3.4	38.1b±1.6	18.2d±0.7	24.5c±0.6	1.4d±0.1	23.2d±1.1
Chloroform	78.5b±4.2	31.4c±1.5	20.3d±1.1	16.2e±0.3	1.2d±0.1	72.4a±4.2
Ethyl acetate	42.1e±2.1	13.1e±0.4	41.3b±2.7	14.6e±0.4	30.1a±1.2	35.2c±1.8
Aqu. residue	68.4c±2.8	10.2f±0.4	24.3c±0.8	16.3e±0.7	2.3d±0.1	21.4d±1.2
BHT	94.6a±6.4	60.1a±4.2	63.2a±4.3	76.6b±4.6	26.1b±0.9	11.5e±0.1
Tocopherol	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2b±0.6	10.3e±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

Table 11. Antioxidants activity of enzymatic extracts from *H.porphyrae*

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Extarct	DPPH	H_2O_2	SOD	OH	NO	MC
Viscozyme	95.1a±5.3	38.4c±1.3	46.9b±2.1	8.6e±0.6	47.1b±1.6	38.4b±2.1
Celluclast	93.2a±5.7	19.4f±0.9	44.3b±2.3	8.7e±0.4	48.5b±2.1	24.9c±1.6
AMG	87.4b±4.2	26.4d±1.2	36.8d±2.1	12.1e±0.7	51.2a±2.3	22.8c±1.7
Termamyl	85.7b±5.4	27.1d±1.6	42.2c±2.6	12.9e±0.6	51.4a±2.3	14.1d±0.5
Ultraflo	76.8c±6.1	22.9e±1.4	32.1e±1.3	12.1e±0.7	45.3c±1.7	11.3d±0.3
Protamax	77.8c±4.1	34.8c±1.7	27.7±f1.1	10.1e±0.2	48.1b±1.5	7.5d±0.1
Alcalase	61.2e±3.2	42.1b±2.1	31.8e±1.3	15.6d±0.2	47.8b±1.7	41.2b±1.4
Flavozyme	78.7c±4.8	17.9f±0.7	28.1f±1.3	30.7b±1.6	37.7±d1.3	52.3a±2.7
Neutrase	94.5±5.9	25.2d±1.1	38.5d±1.4	14.4d±0.1	38.5d±1.1	52.1a±2.8
Kojizyme	69.4d±4.1	16.1f±0.6	27.1f±0.7	22.8c±0.3	33.1d±0.7	3.5e±0.1
BHT	94.6±a6.4	60.1a±4.2	63.2a±4.3	76.6a±4.6	26.1f±0.9	11.5d±0.1
Tocopherol	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2f±0.6	10.3d±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

Table 12. Total polyphenolic, polysaccharide and protein contents of 80%methanol extract and its different fractions of *H. porphyrae*

Fractions	Polyphenol [a] (mg/100 g)[e]	Polysaccharide [c] (g/100 g) [e]	Protein [d] (g/100 g) [e]
80% Methanol	652±54	3.4 ±0.27	5.4±0.4
n-Hexane fraction	5573±285	1.1±0.09	21.3±1.5
Chloroform fraction	1247±114	1.2±0.12	15.4±1.3
Ethyl acetate fraction	891± 98	1.1±0.08	3.7±0.3
Aqueous residue	324± 29	2.2±0.19	2.2±0.1

Values are means of three replicates ± SD

[a] As equivalent gallic acid
[b] As equivalent to glucose
[c] As equivalent to bovine serum albumin
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

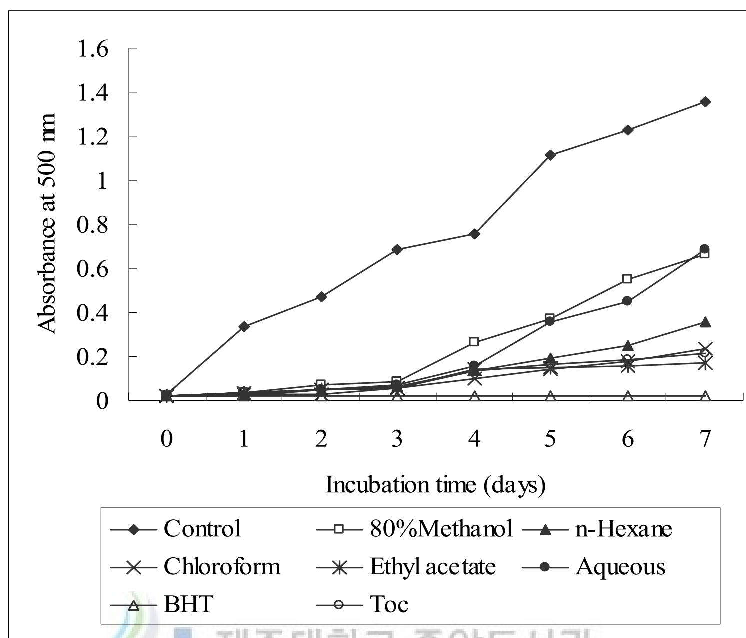
Table 13. Total polyphenolic, polysaccharide and protein content of different enzymatic extracts from *H. porphyrae*

Extracts	Polyphenol [a] (mg/100 g) [d]	Polysaccharide [b] (g/100 g) [e]	Protein [c] (g/100 g) [e]
Viscozyme	615.1±32	19.4±0.9	12.7±0.7
Celluclast	429.7±44	16.9±0.7	10.2±0.4
AMG	326.9±28	17.5±0.9	10.5±0.5
Termamyl	364.9±25	19.8±1.9	11.4±0.4
Ultraflo	617.7±34	19.3±1.2	11.8±0.8
Protamax	647.7±38	12.8±0.7	19.5±1.2
Alcalase	811.4±49	19.2±0.6	21.4±1.3
Flavourzyme	425.6±24	6.8±0.3	8.4±0.4
Neutrase	252.9±14	3.4±0.2	6.9±0.3
Kojizyme	277.9±11	3.2±0.2	4.8±0.2

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

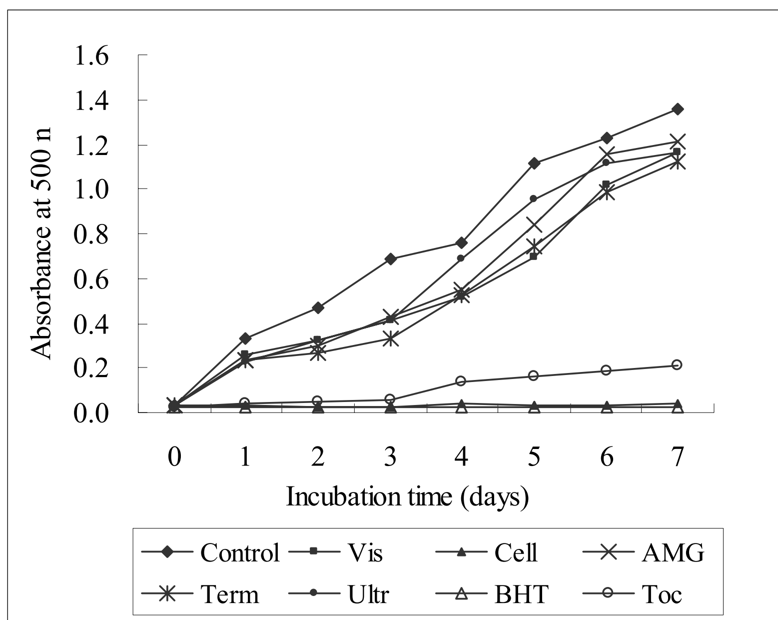
Values are means of three replicates ± SD

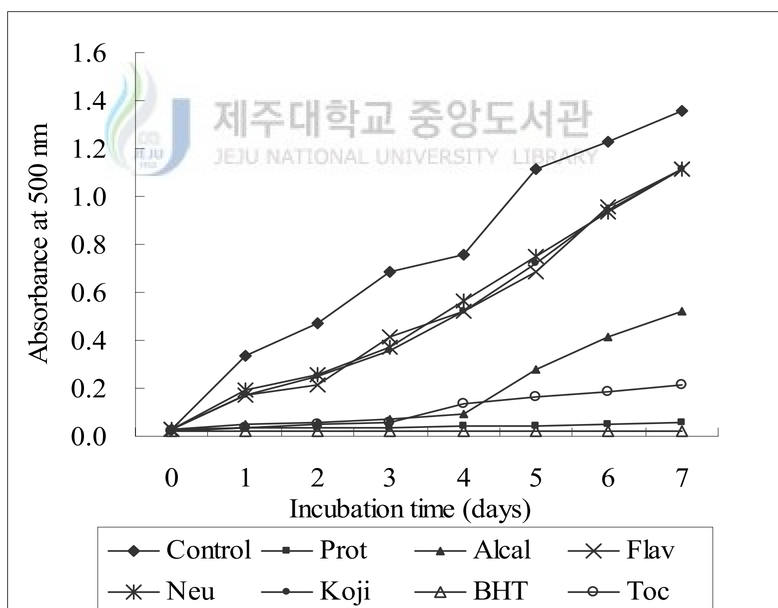
[a] As equivalent gallic acid
[b] As equivalent to glucose
[c] As equivalent to bovine serum albumin
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 9 Antioxidant activity of different solvent fractions of *H.porphyrae* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.





제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 10 Antioxidant activity of different enzymatic extracts from *H. porphyrae* compared to BHT and α-Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

3.3.3. Metal chelating effect

According to Table 11, 80% methanol extract and its solvent fractions indicated higher metal chelating effects and among them the chloroform fraction (72.4%), 80% methanol extract (65.4%) and the ethyl acetate fraction (35.2%) was significantly ($P<0.05$) higher than the activities of commercials. Of the enzymatic extracts of Flavozyme (52.3%), Neutrase (52.1%), Viscozyme (38.4%) and Celluclast (24.9%) exhibited significant effects in metal chelating (Table 12).

3.3.4. Determination of antioxidant activity with the ferric thiocyanate (FTC) method

. As shown in Fig. 9 and Fig. 10 the absorbance of linoleic acid emulsion without the addition of *H.porphyrae* extracts (control) increased significantly ($P<0.05$). Among the all the solvent fractions, ethyl acetate exhibited notable antioxidant activity that significantly similar with α-tocopherol (Fig. 9). Enzymatic extracts of Viscozyme and protease also exhibited notable antioxidant activity even significantly similar with BHT (Fig. 3). No notable antioxidant activity was present with other extracts compared to commercial antioxidants.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

3.3.5. Total polyphenol content

The total phenolic content in the extracts and the fractions varies with the solvent used according to their polarity and the enzymes used (Table 13 and 14). Fraction of n-Hexane indicated the highest polyphenol content

(5573 mg/100 g) among solvent fractions (Table 13). Of the carbohydrases, Ultraflo extract exhibited highest polyphenol content (617.7mg/100 g) while among proteases, Alcalase showed highest polyphenol (811.4 mg/100 g) contents (Table 14).

3.4. DISCUSSION

Since plants are especially susceptible to environmental stress and because of that, it contains very potent antioxidant such as phenolics, polyphenolics, and nitrogen containing compounds, phytosterols, carotonoids and chlorophyll derivatives. Focusing the attention on natural and bioavailable sources of antioxidants, *H. porphyrae,* a tidal pool unicellular microalga was undertaken to investigate the antioxidant properties.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

The effect of antioxidants on DPPH radical scavenging was considered to be due to their hydrogen donating ability. In this study the chloroform fraction, n-hexane fraction and the enzymatic extracts demonstrated notable free radical activities indicating the higher efficacy for scavenging of free radicals in *H. porphyrae*. In addition, these results demonstrated that free radical scavenging compounds had both hydrophilic and hydrophobic properties.

The higher super oxide activity was observed in the ethyl acetate fraction. In addition, the results from enzymatic extracts also exhibited considerable activities. Therefore, it was obvious that the superoxide

scavenging compounds present in these fractions have both hydrophilic and hydrophobic properties. Superoxide radical generally form first and its effects can be exaggerated as it produces other kinds of cell damage inducing free radicals and oxidizing agents (Liu and Ng, 2000).

Both fractions of n-hexane and chloroform showed highest H_2O_2 scavenging activity compare to the other extract and fraction tested. Therefore, it was noticeable that the H_2O_2 hydrogen peroxide scavenging compounds that present in these extracts and fractions have both hydrophilic and hydrophobic characteristics. In addition, it could be seen that enzymatic hydrolysis (especially, Viscozyme and Alcalase) also effective in releasing of compound that responsible in H_2O_2 scavenging. H_2O_2, a reactive non-radical compound is very important as it can penetrate biological membranes. Thus, removing H_2O_2 is very important for the protection of living systems. Addition of H_2O_2 to cells in culture can lead to transition metal ion-dependent $OH^{\cdot}$ mediated oxidative DNA damage (Spencer et al.,1996).

제주대학교 중앙도서관

Except the both water and 80% methanol extracts and the aqueous residue other fractions was not shown significant hydroxyl radical activities. Therefore, it was noticeable that the hydroxyl radical scavenging compounds exhibit more hydrophilic properties. In addition, except Flavozyme other enzymatic extracts did not exhibit satisfactory effect in hydroxyl radical scavenging. Therefore, it can be assumed that the biochemical compounds responsible for activities were in less available form even after enzymatic hydrolysis. Hydroxyl radical is the most reactive oxygen species among all

ROS due to its strong ability to react with various biomolecules. Hydroxyl radical reacts with several biological materials oxidatively by hydrogen withdrawal, double bond addition, electron transfer and radical formation, and initiates autoxidation, polymerization and fragmentation (Liu and Ng, 2000).

In nitric oxide scavenging inhibition, the enzymatic extracts showed the higher activities than organic fractions and those activities were even significantly higher than standard antioxidants. Therefore, it can be assumed that those bioactive compounds were relatively hydrophilic. In addition, enzymatic hydrolysis was effective to extract biochemical compounds that responsible for $NO^{\cdot}$ effects. Nitric oxide is a gaseous free radical, which has important roles in physiological and pathological conditions.

제주대학교 중앙도서관

Ferrozine can make complexes with ferrous ions. In presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, chelating effect of the coexisting chelator can be determined measuring the rate of color reduction. The formation of the ferrozine–Fe^{2+} complex is interrupted in the presence *H. porphyrae* indicating significant chelating ability. Both solvent fractions and enzymatic extracts had significant metal chelating effects. It was apparent that components that cause for the metal chelating has dispersed in both aqueous and organic phase. Ferrous can initiate lipid peroxidation by Fenton reaction as well as accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Fridovich, 1995;

Halliweill, 1991). Thus, different fractions of *H. porphyrae* demonstrate a marked capacity for iron binding, suggesting their ability as peroxidation protector that relates to its iron binding capacity (Gülçin et al., 2004).

To evaluate the antioxidant effects of *H.porphyrae* extracts, their lipid peroxidation was compared with standard antioxidants using ferric thiocyanate (FTC) method by determining the amount of peroxide formed in emulsion in during incubation period. High absorbance is an indication of high concentration of formed peroxides. In this experiment, fraction of ethyl acetate, enzymatic extracts of Protamax and Celluclast provided high antioxidant effects among the all extracts and fractions showing that those extracts were able to reduce the formation of peroxides. It suggests that according to enzyme digestion and solvent fractionation different antioxidant components were liberated from the inside of microalgae cells. These extracts contained high level of polysaccharides, proteins and polyphenols and it could be assumed that those components may influence on antioxidant effects although not correlate with antioxidant effect.

Total phenolic content in both 80% methanolic extracts and different enzymatic extracts varies with the solvent used according to their polarity. Although the fractions of n-hexane and chloroform, the Alcalase extract consisted of the higher contents of polyphenol it was not shown the posivite correlation with antioxidant activity. Further, there are some other bioactive components such as proteins, polysaccharides and different kinds of pigments are consisted in this microalga. For example it was found that

oligosaccharides, sulfate and glycoprotein components have exhibited antioxidant activities in red microalga, *H.porphyrae* (Spitz et al., 2005). Therefore, it is suggested that not only polyphenol content but other factors also may effect for the activity.

In this study, extraction was performed using 80%methanolic and different kinds of enzymes to determine antioxidant activities Methanol is a good solvent to extract varies kind of hydrophilic and hydrophobic potential bioactive compounds but extraction yield is less compared to enzymatic extraction. By solvent fractionation, biological compounds may distribute according to its polarity. In this way, some water insoluble compounds also can be extarcted. Further, plants cells originally contain large amount of soluble polysaccharides and insoluble fibers such as made of cellulose. These fibers together with the other cell wall material act as a physical barrier for the extraction of desired bioactive materials. Enzymatic hydrolysis of raw material tissues or cells has reported significant yields of desired compounds and convenient industrial approaches in extraction and purification (Jeon *et al.* 2000: Nagai and Suzuki 2000). In addition, the breakdown and releasing of high molecular weight polysaccharides and proteins themselves may contribute to enhance the antioxidative activities (Ramos and Xiong, 2002; Ruperez et al., 2002). Of the several advantages of enzymatic extraction, water solubility, higher extraction efficacy, greater variation of constituents, minimized environmental pollution and comparatively inexpensiveness is obvious.

제주대학교 중앙도서관

In conclusion, it was obvious that *H. porphyrae* contains antioxidant potential against ROS. Especially in DPPH radical scavenging effect, Superoxide anion scavenging effect, metal chelating effect and antioxidant activity (linoleci acid peroxidation) it showed higher effects. Thus, these results propose that *H. porphyrae* may good candidate as a natural antioxidant source, which can be applied in food and pharmaceutical industry

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

4. ANTIOXIDANT PROPERTIES OF TIDAL POOL MICROALGA, *Oltamannsiellopsis Unicellularis*

4.1. ABSTRACT

The antioxidative properties of different organic fractions obtained by solvent fractionation of 80% methanolic extract and enzymatic extracts from five proteases and carbohydrases from *O.unicellularis* was investigated for free radical, reactive oxygen species scavenging ($O_2^{\cdot -}$,H_2O_2, $HO^{\cdot}$,$NO^{\cdot}$), metal chelating and total antioxidant assays**.** Almost All fractions exhibited considerable activities on DPPH free radical scavenging. Chloroform fraction (64%) revealed metal chelating activity that is significantly higher than that of the commercial antioxidants. Ethyl acetate fraction showed the highest $O_2^{\cdot -}$ scavenging activity (44%) among all fractions. Both chloroform and n-hexane fractions exhibited higher H_2O_2 activities. n-Hexane fraction (44.4%) exhibited significantly higher $NO^{\cdot}$ inhibition effects. Of the enzymatic extracts viscozyme (79.2%), AMG (78.7%), Alcalase (75.1%) and Kojizyme (74.8%) revealed higher DPPH scavenging activiteis. Flavozyme extract (57.7%) exhibited a higher $O_2^{\cdot -}$ scavenging effect. All extracts showed significantly higher $NO^{\cdot}$ inhibition activities and among them protamax (37.1%), Celluclast (31.4%) and Kojizyme (31.2) was notable. In metal chelating extracts of Termamyl (66.1%), Alcalase (52.9%), Kojizyme (42.6%) and Neutrase (41.1%) had exceptional activities. Thus, these results indicate that active components of *O. unicellularis* that responsible for antioxidant activities exhibit both hydrophilic and hydrophobic properties.

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

4.2. MATERIALS AND METHODS

As described in chapter 1.2 “Antioxidant properties of *Pediastrum duplex*’

4.3. RESULTS

4.3.1. Proximate composition

Proximate composition of freeze dried *O. unicellularis* was analyzed and the values were; moisture 3.1%, ash content 18.0%, carbohydrates 50.1%, protein 4.3% and fat 3.4%. Polysaccharide content and protein content in enzymatic extracts, 80% methanol extract, and its solvent fractions shown in Table 17 and 18.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

4.3.2. Reactive oxygen species inhibition

4.3.2.1. DPPH free radical scavenging effect

As shown in Table 15, 80% methanol extract exhibited the highest radical scavenging activity (90.6%) followed by the n-hexane fraction and the chloroform fraction (89 and 76.4 %, respectively). It is notable that the free radical scavenging activity has been decreased significantly ($P<0.05$) from 80% methanol extract toward final aqueous fraction according to fractionation procedure. Enzymatic extracts of Viscozyme (79.2%), AMG (78.7%), Alcalase (75.1%), Kojizyme (74.8%), and Flavozyme (74.2%) exhibited strong DPPH scavenging effects (Table 16). In addition, protamax,

Celluclast and Termamyl extracts demonstrated activities as 73.6, 70.1, and 66.7%, respectively.

4.3.2.2. Hydrogen peroxide scavenging effect

The scavenging activities of the water extract and the 80% methanol extract and its solvent fractions of *O. unicellularis* on hydrogen peroxide are shown in Table 15. Among the recorded results, the n-hexane and the chloroform fractions exhibited higher H_2O_2 scavenging effects (28% and 24% respectively) through all the fractions. In enzymatic extractions, low effects could be observed. However, alcalase (27.1%) and AMG (20.9%) exhibited higher activities among enzymatic extracts and very less effect was observed from the rest of extracts.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

4.3.2.3. Superoxide scavenging effect

As shown in Table 15, the ethyl acetate fraction (44%) showed significantly higher ($P<0.05$) results through all the fractions in superoxide scavenging effect. Among the other fractions the 80%methanol extract (26%) seemed relatively effective in superoxide scavenging effect followed by the n- hexane and the aqueous residue. Most of enzymatic extracts has given considerable activities in superoxide scavenging. Specially, Flavozyme (57.7%) and Celluclast (51.3%) exhibited higher activities among enzymatic extracts (Table 16). In addition, extracts of AMG, Viscozyme, and Termamyl reported activities as 44.5, 44.3, and 43.2%, respectively.

4.3.2.4. Hydroxyl radical scavenging activity

As shown in Table 15, the aqueous residue (24%) exhibited significantly higher (P<0.05) scavenging activity compared to the rest of fractions. Less effect was observed from enzymatic extracts also and among recorded results extracts of Celluclast (27.6%), Viscozyme (24.8%) and Kojizyme (24.4%) showed higher scavenging effects (Table 16).

4.3.3. Nitric oxide scavenging effect

Nitric oxide scavenging effect of the different extract from *O. unicellularis* is shown in Table 15. Among the all extracts and the fractions 80% methanol extract exibited highest scavenging effect (49%) even significantly (P<0.05) higher than those of commercial antioxidants. However, the rest of other fractions exhibited little effect on the scavenging. Unlike solvent fractions enzymatic extracts exhibited significant activities compared to commercial antioxidants. Of the enzymatic extracts protamax (37.1%), Celluclast (31.4%) and Ultraflo (30.2%) were more significant (Table 16).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 14. Antioxidants activity of 80% methanol extract and its different solvent extarcts from *O.unicellularis*

Fractions	DPPH	H_2O_2	SOD	OH	NO	MC
Methanol	90.6b±6.5	13.5e±0.4	26.7c±1.6	18.4c±0.7	49.3a±1.6	40.3c±2.3
n-Hexane	89.2b±6.7	28.1b±1.3	22.5d±0.8	13.4d±0.6	1.2e±0.1	14.1e±0.5
Chloroform	76.4c±5.2	24.7c±1.1	5.4f±0.1	15.6d±0.7	1.4e±0.1	64.3a±3.7
Ethyl acetate	66.1d±4.2	18.6d±0.4	44.1b±2.6	17.7c±0.3	14.1c±0.4	22.2d±1.2
Aqu. residue	63.4d±4.3	14.1e±0.6	16.2e±0.3	24.3b±0.9	8.7d±0.4	44.4b±2.4
BHT	94.6a±6.4	60.1a±4.2	63.2a±4.3	76.6a±4.6	26.1b±0.9	11.5f±0.1
Tocopherol	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2b±0.6	10.3f±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

Table 15. Antioxidants activity of enzymatic extracts from *O.unicellularis*

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Extarcts	DPPH	H_2O_2	SOD	OH	NO	MC
Viscozyme	79.2b±2.9	11.3f±0.6	44.3d±1.5	24.8b±1.5	27.1c±1.2	10.1f±0.8
Celluclast	70.1c±2.8	17.6e±0.7	51.3c±2.1	27.6b±1.6	31.4b±1.4	6.8f±0.2
AMG	78.7b±1.7	20.9d±1.1	44.5d±1.8	7.7d±0.2	26.1c±1.2	14.f3±0.3
Termamyl	66.7d±2.2	20.5d±0.9	43.2d±1.9	4.7d±0.6	27.1c±1.4	66.1a±2.6
Ultraflo	61.7d±3.9	12.8f±0.7	35.2f±1.7	4.2d±0.3	30.2bc±1.6	40.7c±2.6
Protamax	73.6c±3.2	10.1f±0.11	38.1e±1.2	6.3d±0.2	37.1a±1.2	26.7e±0.4
Alcalase	75.1c±5.2	27.1b±0.5	32.1g±1.1	13.8c±0.2	28.2c±1.1	52.9b±2.1
Flavozyme	74.2c±4.1	5.1g±0.04	57.7b±1.8	11.1c±0.2	25.3c±1.3	31.4d±0.7
Neutrase	72.1c±4.6	24.2c±1.1	25.2h±1.1	9.1d±0.4	28.3c±1.1	41.1c±1.2
Kojizyme	74.8c±5.1	12.2f±0.14	23.2h±1.2	24.4b±0.2	31.2b±1.3	42.6c±2.1
BHT	94.6a±6.4	60.1a±4.2	63.2a±4.3	76.6a±4.6	26.1c±0.9	11.5f±0.1
Tocopherol	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2c±0.6	10.3f±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

Table 16. Total polyphenolic, polysaccharide and protein contents of 80% methanol extract and its different solvent frctions from *O.unicellularis*

Fractions	Polyphenol [a] (mg/100 g)[e]	Polysaccharide[c] (g/100 g) [e]	Protein [d] (g/100 g) [e]
80% Methanol	638±54	7.6±7	3.9.±0.3
n-Hexane fraction	3731±152	1.04±0.0	13.9.±1.2
Chloroform fraction	1585±114	1.31±0.1	18.9.±1.3
Ethyl acetate fraction	1167±098	1.28±0.1	5.9.±0.3
Aqueous residue	394±024	1.53±0.1	3.8.±0.3

Mean±SE of determinations was made in triplicate.

Values are means of three replicates ± SD

[a] As equivalent gallic acid
[b] As equivalent to glucose
[c] As equivalent to bovine serum albumin
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

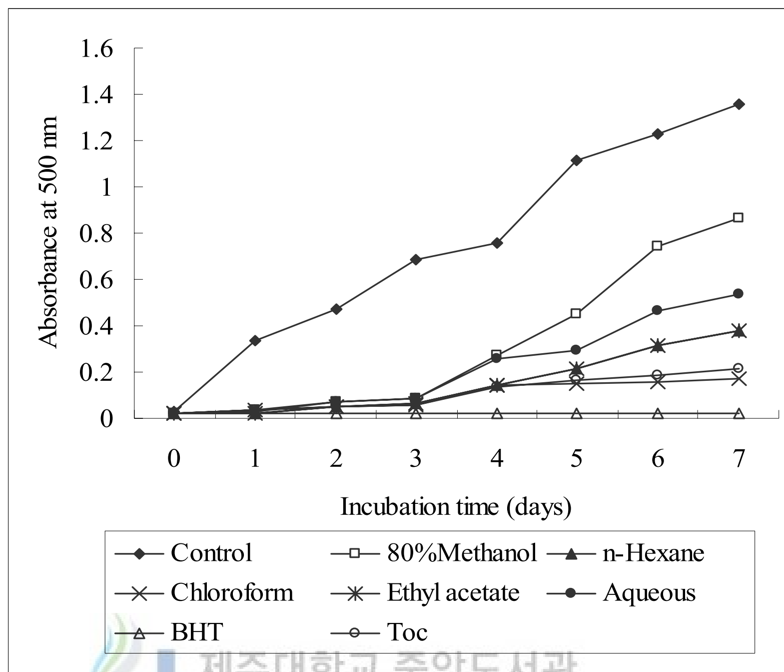
Table 17. Total polyphenolic, polysaccharide and protein content of different Enzymatic extracts from *O. unicellularis*.

Extarcts	Polyphenol [a] (mg/100 g)[d]	Polysaccharide [b] (g/100 g) [e]	Protein [c] (g/100 g) [e]
Viscozyme	397.9±22	17.5±0.1	6.7±0.2
Celluclast	515.5±24	9.3±0.1	7.8±0.3
AMG	365.7±18	9.9±0.1	5.9 ±0.3
Termamyl	574.8±21	7.2±0.3	8.1 ±0.3
Ultraflo	817.7±32	1.1±0.1	11.6 ±0.5
Protamax	591.7±18	9.3±0.1	13.5±0.2
Alcalase	554.1±29	6.3±0.1	11.2 ±0.2
Flavourzyme	598.1±21	10.1±0.1	14.5±0.2
Neutrase	606.3±24	8.1±0.1	7.7±0.3
Kojizyme	470.9±29	7.2±0.1	7.3±0.2

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

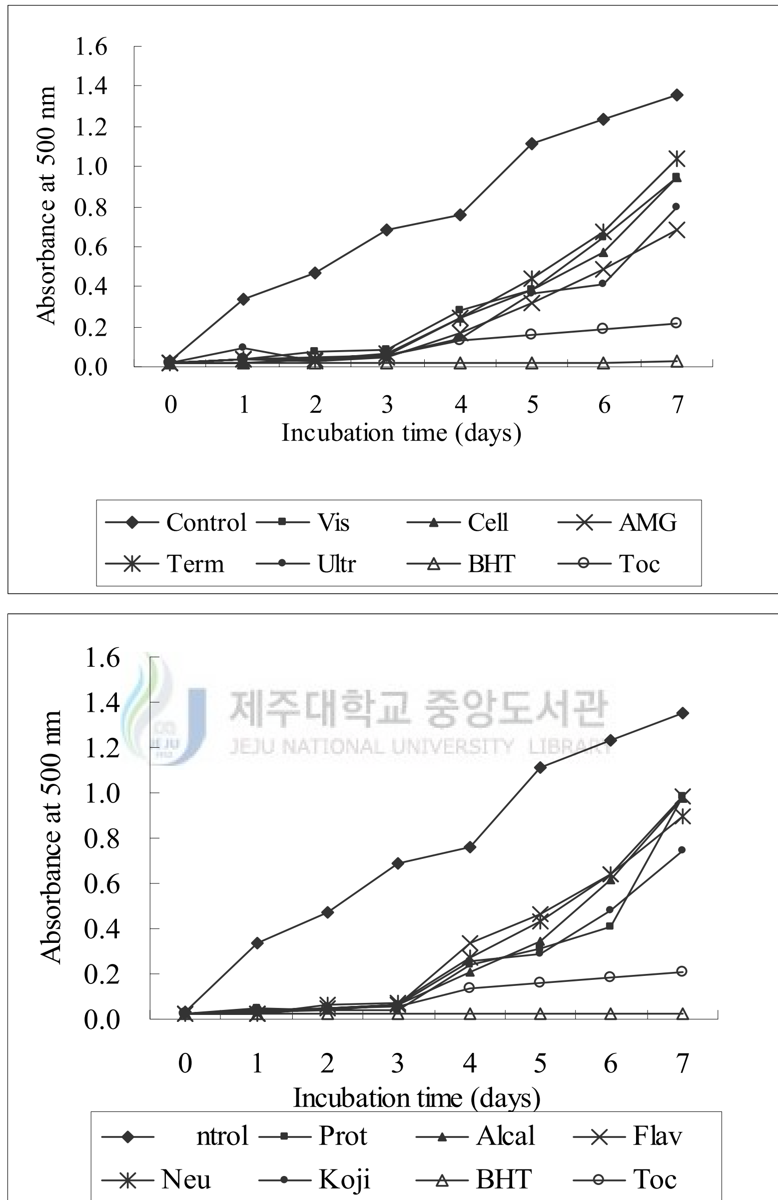
Values are means of three replicates ± SD

[a] As equivalent gallic acid
[b] As equivalent to glucose
[c] As equivalent to bovine serum albium
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 11 Antioxidant activity of different solvent fractions from *O. unicellularis* compared to BHT and α-Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 12 Antioxidant activity of different enzymatic extracts from *O. unicellularis* compared to BHT and α-Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

4.3.4. Metal chelating effects

As shown in Table 15, the higher metal chelating effects ware found in the the chloroform fraction (64%) 80% methanol extract (40%) and aqueous residue (44%) were significantly ($P<0.05$) higher than the activities in α-tocopherol and BHT. All enzymatic extracts except Celluclast and Viscozyme indicated significantly higher ($P<0.05$) metal chelating effects and among them activities of Termamyl (66.1%), alcalase (52.9%), Neutrase (41.1%), kojizyme (42.6) and Ultraflo (40.7%) were very significant (Table 16).

4.3.5. Determination of antioxidant activity with the ferric thiocyanate (FTC) method

As shown in Fig. 11 and Fig. 12, the absorbance of linoleic acid emulsion without the addition of *O. unicellularis* extracts (control) increased significantly ($P<0.05$). Among the all the solvent fractions, chloroform exhibited antioxidant activity significantly similar with α- tocopherol. Enzymatic extracts of Viscozyme, Termomyl, and Kojizyme expressed notable antioxidant activity (Fig. 11). No significant antioxidant activity was present with other extracts compared to commercial antioxidants.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

4.3.6. Total polyphenol content

The total phenolic content in the extracts and the fractions varies with the solvent used according to their polarity and the enzymes used (Table 15 and 16). n-Hexne fraction indicated the highest polyphenol content (5577 mg/100 g) among solvent extracts and fractions (Table 15). Of the

carbohydrases Ultraflo extract exhibited highest polyphenol content (517.7 mg/100 g) and among proteases, Neutrase showed highest polyphenol (60.6.3mg/100 g) content (Table 16).

4.4. DISCUSSION

Recently there has been increasing interest in the beneficial potential of natural plants as antioxidants in reducing ROS since they produce compounds such as phenolics, polyphenolics, and nitrogen containing compounds, phytosterols, carotonoids and chlorophyll derivatives. In the present study, the interest was aimed on antioxidant potential of solvent fractions of 80% methanol extract and enzymatic extracts from a micro alga *O. unicellularis*

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

The model of scavenging the stable DPPH is an extensively used method to evaluate antioxidant activities in a comparatively short period compared with other relative methods. $DPPH^{\bullet}$ has the advantage of being unaffected by certain side reactions that brought by various additives. In this study the 80% methanol extract and organic solvent fractions of *O. unicellularis* showed notable activities. In addition, these results demonstrate that most of free radical scavenging compounds tends to be concentrated in both hydrophilic and hydrophobic solvent fractions. In addition, it can be seen that DPPH scavenging activity of enzymatic extracts also have strong activities. Thus, both carbohydrases and proteases have capability to liberate bioactive compounds for radical scavenging. Radical scavengers may protect

cell tissues from free radicals, thereby preventing diseases such as cancer (Nakayama et al., 1993) Even though it is unclear whether active constituents in extracts are active against free radicals after being absorbed and metabolised by cells in the body, free radical-scavenging assays have gained acceptance for their capacity to screen rapidly materials of interest.

In this study, the higher superoxide scavenging activities were founded in the ethyl acetate fraction when compared to rest of fractions. Further, it was noticeable that the superoxide scavenging compounds present in these fractions had both hydrophilic and hydrophobic properties. In addition, scavenging activity of enzymatic extracts also had moderate activities and those activities were mainly concentrated in carbohydrases extracts. Therefore, it can be assumed that carbohydrases had more ability to release those compounds in superoxide scavenging. Superoxide radical generally form first and its effects can be exaggerated as it produces other kinds of cell damage inducing free radicals and oxidizing agents (Spitz et al., 2005). Although superoxide anion is itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to the oxidative stress (Dahl and Richardson, 1978). It is a precursor to active free radicals that have potential of the reaction with biological macromolecules and thereby inducing tissue damage (Halliwell and Gutteridge, 1989).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

It was apparent that components that cause for the H_2O_2 scavenging has dispersed in both enzymatic extracts and organic fractions showing both

hydrophilic and hydrophobic characteristics. However, a little effect can be seen on scavenging of hydrogen peroxide in this study. H_2O_2, a reactive non-radical compound is very important as it can penetrate biological membranes. Although H_2O_2 itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl radicals (Halliweill, B., 1991). Thus, removing H_2O_2 is very important for the protection of living systems.

Hydroxyl radical scavenging activity of the enzymatic extracts and solvent fractions from *O. unicellularis* was determined as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. Among the solvent fractions, except aqueous residue, the rest of fractions and the 80% methanol extract did not show significant hydroxyl radical activities. Therefore, it is obvious that the hydroxyl radical scavenging exhibited more hydrophilic properties. However, in enzymatic extracts activities also was not in significant level. Hydroxyl radical is the most reactive oxygen species among all ROS due to its strong ability to react with various biomolecules. Hydroxyl radicals are known to be capable of abstracting hydrogen atoms from phospho lipid membranes thus bring about peroxidic reactions of lipids (Kitada et al., 1979).

제주대학교 중앙도서관

Not only reactive oxygen species, nitric oxide is also implicated in cancer, inflammation and other pathological conditions. Nitric oxide is a gaseous free radical, which has important roles in physiological and pathological conditions. Therefore, the scavenging ability of $NO^{\bullet}$ may also help to disrupt the chain reactions initiated by excessive generation of $NO^{\bullet}$

that are detrimental to human health. In solvent fractions, since n-hexane fraction possessed significant activity, $NO^{\bullet}$ inhibition effects were deviated towards hydrophobic status. Thus, it supposed those were fat-soluble compounds such as pigments and steroids. Also, compared to solvent fractions both carbohydrases and proteases extracts exhibited considerable results. It means those biochemical compounds exhibit both hydrophilic and hydrophobic properties. Marcocci et al. (1994) has reported that scavengers of nitric oxide compete with oxygen leading to reduce production of nitric oxide. The rectivities of the $NO^{\cdot}$ and $O_2^{-\cdot}$ were found to be relatively low, but their metabolites ONOO- (peroxynitrite) is extremely reactive and directly induce toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modifications (Moncada et al 1991; Radi et al1991).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Ferrous is known as the most important lipid oxidizing pro-oxidant among the transition metals due to its high reactivity. Ferrozine can make complexes with ferrous ions. In presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, chelating effect of the coexisting chelator can be determined measuring the rate of color reduction. The formation of the ferrozine–Fe^{2+} complex is interrupted in the presence of water extract, the 80% methanol extract, and its solvent fractions of *O. unicellularis* indicating that both fractions had chelating ability. Enzymatic extracts showed higher metal chelating ability and it is apparent that hydrophilic components are

responsible for metal chelating. Ferrous can initiate lipid peroxidation by Fenton reaction as well as accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Fridovich, 1995, Halliweill, 1991). Hence, different extracts of *O. unicellularis* demonstrate a noticeable capacity for iron binding, suggesting their ability as peroxidation protector that relates to its ferrous binding capacity (Gülçin et al., 2004).

To evaluate the antioxidant effects of *O.* unicellularis solvent fractions and enzymatic extracts, their lipid peroxidation were compared with standard antioxidants using ferric thiocyanate (FTC) method by determining the amount of peroxide formed in emulsion in during incubation period. High absorbance is an indication of high concentration of formed peroxides. In this experiment, only chloroform fraction provided high antioxidant effects among the all extracts and fractions showing that those extracts were able to reduce the formation of peroxides. It suggests that according to solvent fractionation different antioxidant components were released from the inside of microalgae cells. These extracts contained high level of polysaccharides, proteins and polyphenols and it could be assumed that those components may influence on antioxidant effects although not correlate with antioxidant effect.

The scavenging activity is not correlated with polyphenol and flavonoids contents as described in published literatures (Lu and Foo, 2000; Kim and Chung, 2002). Therefore, it can be assumed that not only polyphenol and flavonoid contents but other factors also may effect for the

제주대학교 중앙도서관

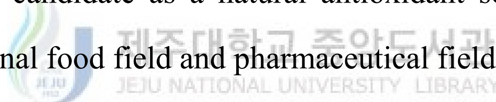
activity. Further, there are some other bioactive components such as proteins, polysaccharides and different kinds of pigments are consisted in this microalga. For example it was found that oligosaccharides, sulfate and glycoprotein components have exhibited antioxidant activities in red microalga, *Porphyridium sp*. (Spitz et al., 2005). Not only polyphenolic but these bio chemical components also may have some positive effect on scavenging activity in *O. unicellularis.* In addition, the polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans (Tanaka et al 1998). Phenols are very significant plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano, 1995).

In this study, extraction was performed using methanol and different kinds of enzymes to evaluate the antioxidant potentioal. Considering methanol, it is a good solvent to extract varies kind of hydrophilic and hydrophobic bioactive compounds but extraction yield is less compared to enzymatic extractions. By solvent fractionation, biological compounds can be separated according to its polarity. In this way, some water insoluble compounds also can be extracted. However, both insoluble and soluble fiber together with the other cell wall material performs as a physical barrier for the extraction of desired bioactive materials. Enzymatic hydrolysis of raw material tissues or cells has showed significant yields of desired compounds and convenient industrial approaches in extraction and purification (Jeon et al., 2000; Nagai and Suzuki, 2000). Also, the breakdown and releasing of

제주대학교 중앙도서관

high molecular weight polysaccharides and proteins themselves may contribute to enhance the antioxidative activities (Ramos and Xiong 2002; Ruperez et al., 2002). Additionally, enzymatic extracts possesses innovative advantages and characters over conventional extraction procedures. Of the several advantages, water solubility, higher extraction efficacy, greater variation of constituents, minimized environmental pollution/ toxicity and comparatively inexpensiveness are obvious comparatively inexpensiveness are obvious.

In conclusion, it is obvious that *O. unicellularis* contains antioxidant potential against ROS. Thus, these results proposed that *O. unicellularis* might be a good candidate as a natural antioxidant source, which can be applied in functional food field and pharmaceutical field.


제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

5. ANTIOXIDANT PROPERTIES OF BENTHIC DIATOM, *Achnanthes longipes*

5.1. ABSTRACT

Freeze-dried benthic diatom, *Achnanthes longipes* was extracted with 80% methanol (and sequentially partitioned with solvents in an order of increasing polarity) and with five proteases and carbohydrases to examine, its various antioxidant properties. Among the organic fractions tested, n-hexane (80.4%) and 80% methanolic extract (76.6%) were effective in DPPH scavenging. Fractions of chloroform (53.4%) and n-hexane (53.1%) exhibited higher activities on H_2O_2. Fraction of n-Hexane was highest in presenting hydroxyl radical scavenging activity and $NO^{\cdot}$ inhibition activity (53.1% and 75.6%) respectively. In enzymatic extracts, Ultraflo (36.4%) and Protamax (31.1%) showed higher DPPH activities. AMG (48.3%) extract exhibited highest activity followed by Viscozyme (44.6%) and Celluclast (43.7%) in superoxide radical scavenging. Extracts of Flavozyme (43.9%), Ultraflo (42.8%), Neutrase (41.5%) and Kojizyme (41.2%) possessed higher effects in hydroxyl radical scavenging. Extracts of kojizyme (29.9%), Viscozyme (28.4) and Ultraflo (24.9%) revealed significant activities ($P<0.05$) in $NO^{\cdot}$ inhibition while Ultraflo (75.8%), Kojizyme (73.5%) and Alcalase (51.5%) had strong metal chelating activities. These data suggested that *Achnanthes longipes* was rich in hydrophobic and hydrophilic antioxidative compounds with different antioxidative properties.

제주대학교 중앙도서관

5.2. MATERIALS AND METHODS

As described in chapter 1.2 "Antioxidant properties of *Pediastrum duplex*'

5.3. RESULTS

5.3.1. Proximate composition

Proximate composition of freeze dried *A. longipes* was analyzed and the values were; moisture 8.1%, ash content 67.8%, carbohydrates 16.6%, proteins 6.4% and fat 1.1%. Polysaccharide content and protein content in enzymatic extracts and 80% methanol extract and its solvent fractions are shown in Table 21 and 22.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

5.3.2. Reactive oxygen species inhibition

5.3.2.1. DPPH free radical scavenging effect

According to Table 19, n-hexane farction (80.4%) indicated highest effect on DPPH radical scavenging followed by 80% methanol extract (76.6%) and chloroform fraction (65.1%). As well, ethyl acetate fraction (59.4%) and aqueous residue showed 59.4% and 46.5% activities respectively. However, enzymatic extracts exhibit less significant effect on DPPH radical scavenging compared to commercial antioxidants (Table 20). Extracts of Ultraflo, Kojizyme and protamax exhibited 36.4, 34.5, and 31.1 % activities respectively.

5.3.2.2. Hydrogen peroxide scavenging effect

As depicted in Table 19, fractions of Chloroform (53.4%), n-hexane (53.1%) and ethyl acetate (52.9%) demonstrated higher H_2O_2 scavenging effect. However, other fractions showed less effect on hydrogen peroxide scavenging. Enzymatic extracts also exhibited less scavenging effect (Table 20). Among them Neutrase extract (12.1%) and Flavozyme extract (11.3%) showed higher activities.

5.3.3.3. Superoxide anion scavenging effect

Superoxide anion scavenging effect of *A. longipes* is depicted in Table 19. Among the all fractions, 80% methanolic extract, and its solvent fractions exhibited less activates in superoxide scavenging. According to Table 20, almost all the enzymatic extracts presented substantial effect. Of the extracts AMG, Viscozyme, Celluclast, and Protamax indicated remarkable superoxide anion scavenging activities (48.3, 44.6, 43.7, and 41.8, respectively). In addition, Ultraflo and Termamyl showed activities as 40.5% and 36.8% respectively (Table 20).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

5.3.3.4. Hydroxyl radical scavenging effect

Fraction of n-hexane (56.5%) exhibited higher hydroxyl radical scavenging effects. However, no significant effect was shown from other fractions (Table 19). Enzymatic extracts of Flavozyme (43.9%), Ultraflo (42.8%) Neutrase (41.5%) and Kojizyme (41.2%) exhibited higher hydroxyl

radical effect. In addition, AMG, Viscozyme and Protamax showed activities as 40.4, 39.8, and 39.8%, respectively (Table 20).

5.3.4. Nitric oxide radical inhibition effects

As shown in Table 19, fractions of n-hexane (75.6%) and ethyl acetate (26.3%) exhibited significant results even higher than that of commercial antioxidants ($P<0.05$). However, no significant activity was shown by other fractions on nitric oxide scavenging. Nitric oxide scavenging effect of enzymatic extracts is shown in Table 20, and extracts of Kojizyme (29.9%) and Viscozyme (39.8%) exhibited significant inhibition effects compared to commercial antioxidants ($P<0.05$). Among the other extracts, Ultraflo (24.9%) and Termamyl (23.8%) possessed considerable nitric oxide scavenging inhibition.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

5.3.5. Metal chelating effect

As shown in Table 19, 80% methanolic extract (11.6%) and chloroform (12.9%) fraction exhibited significantly similar effect of metal chelating compared to commercial antioxidants ($P<0.05$). No significant effect was reported from rest of fractions. In addition, according to Table 20, all enzymatic extracts except Viscozyme, Celluclast, and AMG rest of extracts exhibited

Table 18. Antioxidants activity of 80% methanol extract and its different solvent fractions from *A. longipes*

Fractions	DPPH	H_2O_2	SOD	HO	NO	MC
Methanol	76.6c±4.7	32.3c±1.2	17.3b±1.1	9.3e±0.8	12.8d±0.7	11.6a±0.3
n-Hexane	80.4b±6.3	53.1b±2.3	7.7d±0.4	56.5c±2.8	75.6a±4.7	6.4b±0.3
Chloroform	65.1d±4.3	53.4b±2.4	3.5e±0.1	19.3d±0.9	6.3e±0.1	12.9a±0.7
Ethyl acetate	59.4e±3.7	52.9b±2.5	2.8e±0.1	7.3e±0.4	26.3b±1.3	3.8c±2.9
Aqu. residue	46.5f±2.1	8.1d±0.1	10.7c±0.7	2.9f±0.7	20.6c±1.1	7.5b±0.6
BHT	94.6a±6.4	60.1a±4.2	63.2a±4.3	76.6b±4.6	26.1b±0.9	11.5a±0.1
Toco	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2b±0.6	10.3a±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

Table 19. Antioxidants activity of enzymatic extracts from *A. longipes*

Extracts	DPPH	H_2O_2	SOD	HO	NO	MC
Viscozyme	24.3d±0.3	10.8b±0.2	44.6c±1.1	39.8b±1.2	28.4a±0.7	8.8f±0.4
Celluclast	23.6d±1.3	4.5d±0.1	43.7c±0.9	32.2c±1.6	17.1c±0.5	10.2f±0.2
AMG	21.8d±1.6	5.3d±0.3	48.3b±0.6	40.4b±2.1	16.1c±0.6	9.4f±0.2
Termamyl	23.6d±1.3	7.1c±0.4	36.8d±1.3	35.2c±1.1	23.8b±0.8	15.1e±0.4
Ultraflo	36.4b±1.6	10.1b±0.7	40.5d±1.6	42.8b±2.4	24.9b±1.2	75.8a±3.7
Protamax	31.1c±1.4	10.9b±0.7	41.8d±1.4	39.8b±1.6	14.6c±0.4	49.3b±1.3
Alcalase	30.8c±1.3	8.2b±0.6	36.1d±1.7	36.8b±1.7	17.7c±1.1	51.5b±2.7
Flavozyme	26.1d±0.3	11.3b±0.7	35.4e±0.6	43.9b±2.4	22.1b±0.9	27.3c±1.6
Neutrase	28.8c±1.4	12.1b±0.9	28.6f±1.4	41.5b±1.7	17.1c±0.9	17.3d±1.4
Kojizyme	34.5b±1.6	7.7c±0.2	29.1f±0.4	41.2b±1.4	29.9a±1.1	73.5a±4.9
BHT	96.6a±6.4	60.1a±4.2	63.2a±4.3	76.6a±4.6	26.1ab±0.9	11.5e±0.1
Toco	97.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2b±0.6	10.3e±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 20. Total polyphenolic, polysaccharide and protein contents of 80% methanol extract and its different solvent fractions from *A. longipes.*

Fractions	Polyphenol [a] (mg/100 g) [e]	Polysaccharide [c] (g/100 g) [f]	Protein [d] (g/100 g) [f]
80%metahnol	890.4±42.3	13.8±0.9	5.1±0.3
n-Hexane	1243.8±74.8	12.6±1.1	5.7±0.3
Chloroform	996.4±54.3	21.9±1.2	10.4±0.7
Ethyl acetate	1254.2±78.3	15.8±1.1	11.3±0.5
Aqueous	1501.1± 87.6	10.5±0.7	6.9±0.1

Values are means of three replicates

[a] As equivalent gallic acid

[b] As equivalent to glucose

[c] As equivalent to bovine serum albumin

[d] mg per 100 g solid of the extracts.

[e] g per 100 g solid of the extracts

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

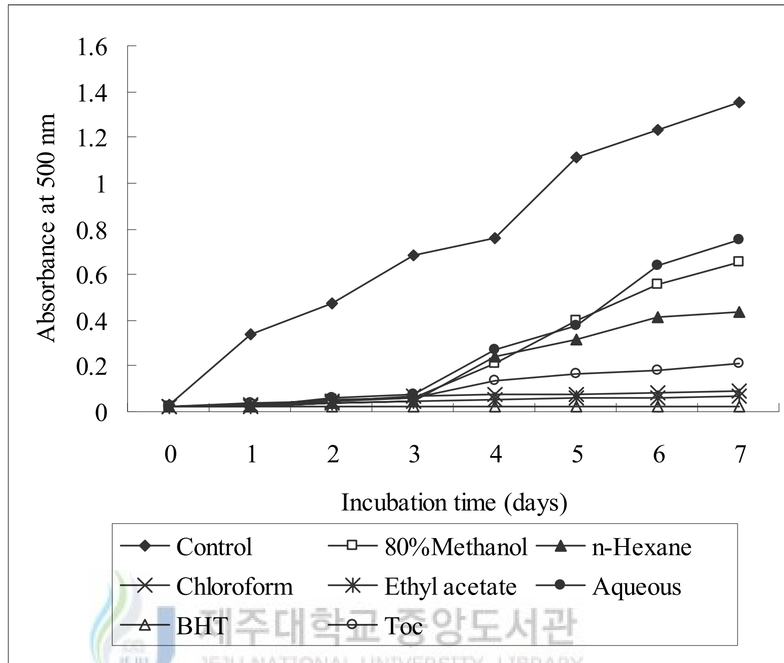
Table 21. Total polyphenolic, polysaccharide, and protein content of different enzymatic extracts from *A. longipes*.

Extracts	Polyphenol [a] (mg/100 g) [d]	Polysaccharide [b] (g/100 g) [e]	Protein [c] (g/100 g) [e]
Viscozyme	742.7 ±23	4.2±0.1	7.4 ±0.2
Celluclast	719.1 ±24	5.3±0.1	8.2 ±0.2
AMG	700.2 ±28	6.6±0.1	11.3 ±0.2
Termamyl	709.6 ±21	7.1±0.1	8.8 ±0.3
Ultraflo	714.4 ±22	7.8±0.1	7.8±0.2
Protamex	739.6±28	6.6±0.1	11.3 ±0.2
Alcalase	756.8 ±39	7.1±0.1	8.8 ±0.3
Flavourzyme	758.5±31	6.6±0.1	11.3 ±0.2
Neutrase	728.5 ±34	9.7±0.1	5.1 ±0.3
Kojizyme	775.7 ±39	6.7±0.1	14.9 ±0.3

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

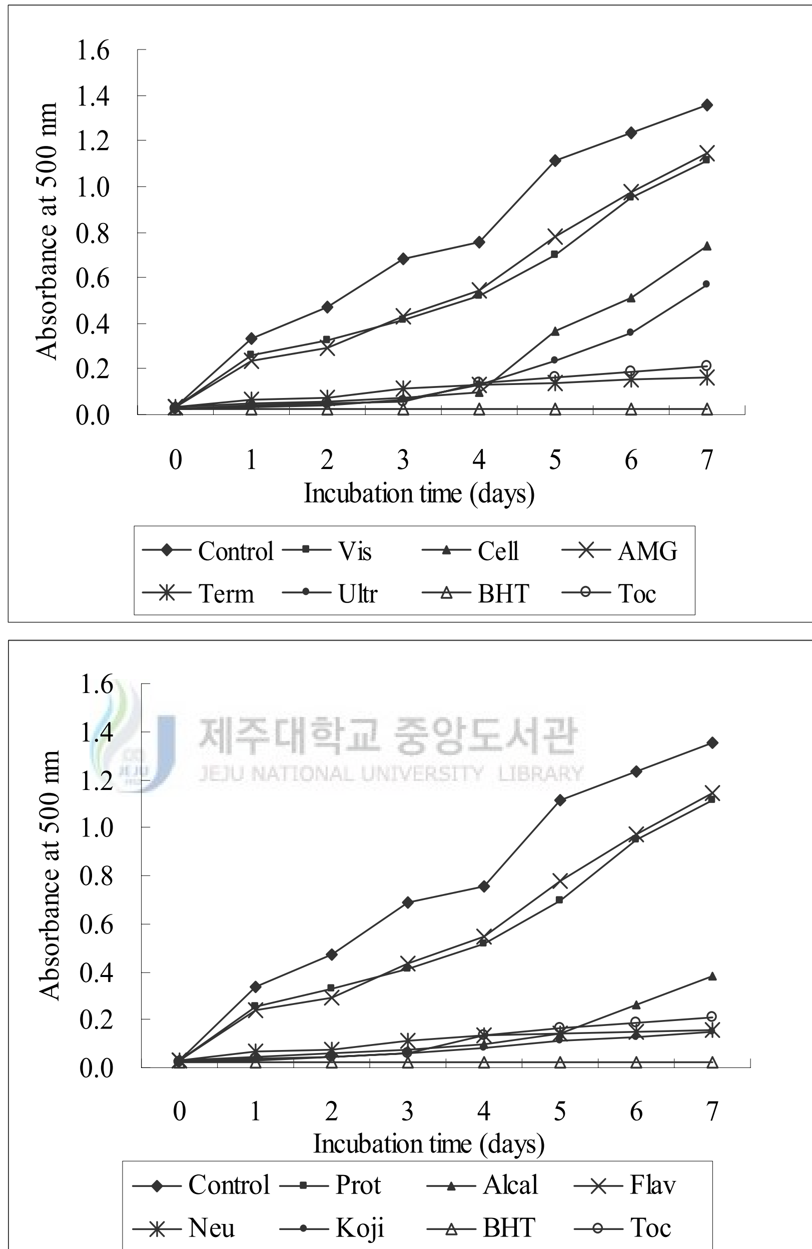
Values are means of three replicates ± SD

[a] As equivalent gallic acid
[b] As As equivalent to glucose
[c] As equivalent to bovine serum albium
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 13 Antioxidant activity of different solvent fractions from *A. longipes* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 14. Antioxidant activity of different enzymatic extracts from *A. longipes* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

metal chelating effects even significantly (P<0.05) higher than the activities in commercial antioxidants. Among them Ultraflo (75.8%), Kojizyme (73.5%), Alcalase (51.5%) and Protease (49.3%) exhibited strong chelating effects.

5.3.6. Determination of antioxidant activity with the ferric thiocyanate (FTC) method

As shown in Fig. 13 and Fig. 14 the absorbance of linoleic acid emulsion without the addition of *A. longipes* extracts (control) increased significantly (P<0.05). Among the all the solvent fractions, chloroform and ethyl acetate exhibited activities notable antioxidant activity even significantly higher than α- tocopherol (Fig. 13). Enzymatic extracts of Termamyl and Kojizyme also exhibited notable antioxidant activity even higher than tocopherol (Fig. 14). No notable antioxidant activity was present with other extracts compared to commercial antioxidants.

제주대학교 중앙도서관

5.3.7. Total polyphenol content

The total phenolic content in the extracts and the fractions varies with the solvent used according to their polarity and the enzymes used. Ethyl acetate fraction indicated the highest polyphenol content (1254.2 mg/100 g) among solvent extracts and fractions (Table 21). Of the carbohydrases Viscozyme extract exhibited the highest polyphenol content (742.7 mg/100 g) and among proteases, Kojizyme showed the highest polyphenol (775.7 mg/100 g) contents (Table 22).

5.4. DISCUSSION

The model of scavenging the stable DPPH is an extensively used method to evaluate antioxidant activities in a comparatively short period compared with other relative methods. $DPPH^{\bullet}$ has the advantage of being unaffected by certain side reactions that brought by various additives. In the present study, the 80% methanol extract and organic solvent fractions of *A. longipes* showed notable activities when compared to the commercial antioxidants. These results demonstrated that most of free radical scavenging compounds tends to be concentrated in both hydrophilic and hydrophobic fractions. Also, there was no significant difference between activities of carbohydrases and proteases treated extracts. Radical scavengers may protect cell tissues from free radicals, thereby preventing diseases such as cancer (Nakayama et al., 1993)

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

The higher H_2O_2 activity founded in organic solvent fractions showing hydrophobic characteristics of active compounds. Further, it was clearly depicted that significantly less activities shown by enzymatic extarcts. Thus, it was clear that biochemical compounds that responsible for superoxide activity are hydrophobic. There was no significant difference between activities given by carbohydrases and proteases treated extracts. H_2O_2, a reactive non-radical compound is very important as it can penetrate biological membranes. Although H_2O_2 itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl

radicals (Halliwell, B., 1991). Thus, removing H_2O_2 is very important for the protection of living systems. Addition of H_2O_2 to cells in culture can lead to transition metal ion-dependent $OH^{\cdot}$ mediated oxidative DNA damage (Spencer et al., 1996).

In this study, the superoxide scavenging activities were spreaded among the all fractions showing both hydrophilic and hydrophobic properties. Other important phenomenon was carbohydrases treated extracts exhibited higher effects than proteases treated extracts. Therefore, it could be assumed that protease have more ability to liberate those compounds in superoxide scavenging. Superoxide radical generally form first and its effects can be exaggerated as it produces other kinds of cell damage inducing free radicals and oxidizing agents (Spitz et al., 2005). Although superoxide anion is itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to the oxidative stress (Dahl and Richardson, 1978). Therefore, superoxide radical scavenging by antioxidants has physiological implications.

제주대학교 중앙도서관

Hydroxyl radical scavenging activity of the enzymatic extracts and solvent fractions from *A. longipes* determined as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. Among the solvent fractions, only n-hexane fraction showed comparatively higher activities. Therefore, it was obvious that those biochemical compounds that responsible for activity are concentrated in n-hexane. In addition, it was supposed those were fat-soluble compounds such as pigments and steroids.

On the other hand, protease treated extracts exhibited higher effects than carbohydrases treated. Therefore, it could be assumed that protease have more capability to liberate those active compounds in hydroxyl radical scavenging. However, in enzymatic extracts activities was not in significant level because those compound showed less hydrophilic properties. Hydroxyl radical is the most reactive oxygen species among all ROS due to its strong ability to react with various biomolecules. Hydroxyl radicals known to be capable of abstracting hydrogen atoms from phospholipid membranes thus bring about peroxidic reactions of lipids (Kitada et al. 1979).

Nitric oxide is also implicated in cancer, inflammation, and other pathological conditions. Among the solvent fractions, only n-hexane fraction showed comparatively higher activities. Therefore, it was clear those biochemical compounds that responsible for activity concentrated in n-hexane farction. In addition, it was supposed those are fat-soluble compounds such as pigments and steroids. Compared to solvent fractions (except n-hexane) both carbohydrases and proteases have shown equivalent effects because of compounds hydrophobic properties. The scavenging ability of $NO^{\cdot}$ may facilitate to interrupt the chain reactions initiated by excessive generation of $NO^{\cdot}$ that are detrimental to human health.

Ferrozine can make complexes with ferrous ions. In presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, chelating effect of the coexisting chelator can be determined measuring the rate of color reduction.

제주대학교 중앙도서관

The formation of the ferrozine–Fe^{2+} complex was interrupted in the presence of *A. longipes*. Solvent fractions indicated a less level of chelating ability unlike enzymatic extracts. Thus, it was notable that bioactive compounds that give metal chelating effects were dispersed in all fractions although those showed less activity. Enzymatic extracts showed higher metal chelating ability and it is apparent that hydrophilic components are responsible for metal chelating. Enzymatic extracts showed higher metal chelating ability and it was apparent that hydrophilic components are responsible for metal chelating. In addition, higher activities are present in both carbohydrases and proteases extracts equally. Ferrous can initiate lipid peroxidation by Fenton reaction as well as accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Fridovich, 1995, Halliweill, 1991). Hence, different extracts of *A. longipes* demonstrate a noticeable capacity for iron binding, suggesting their ability as peroxidation protector that relates to its ferrous binding capacity (Gülçin et al., 2004).

To evaluate the antioxidant effects of *A. longipes* extracts, their lipid peroxidation was compared with standard antioxidants using ferric thiocyanate (FTC) method by determining the amount of peroxide formed in emulsion in during incubation period. High absorbance is an indication of high concentration of formed peroxides. Fractions of ethyl acetate and chloroform, enzymatic extracts of kojizyme and Termamyl provided high antioxidant effects among the all extracts and fractions showing that those

제주대학교 중앙도서관

extracts and fractions were able to reduce the formation of peroxides. It suggested that according to enzyme digestion and solvent fractionation different antioxidant components are released from the inside of microalgae cells. These extracts contained high level of polysaccharides, proteins and polyphenols and it could be assumed that those components may influence on antioxidant effects although not correlate with antioxidant effect.

Total phenolic content in both 80% methanol extracts and its solvent fractions and different enzymatic extracts varies with the solvent used according to their polarity. Although the Aqueous residue and Kojizyme extract consisted of the higher contents of polyphenol it wasn't shown the highest activities for every assay because not only the phenolic content but also other kinds of bioactive compounds also may contribute to antioxidant activitiy. For example it was found that oligosaccharides, sulfate and glycoprotein components have exhibited antioxidant activities in red microalga, *Porphyridium sp*. (Spitz et al., 2005). In addition, the polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans (Tanaka et al., 1998). Phenols are very significant plant constituents on account of their scavenging ability due to their hydroxyl groups (Hatano, 1995).

In this study, 80%metahnol and enzyme (carbohydrases and proteases) used for extraction of antioxidant compounds. Methanol is a good solvent to extract varies kind of hydrophilic and hydrophobic bioactive compounds but extraction yield is less compared to enzymatic extractions.

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

By solvent partition, potential biological compounds can be distributed according to their polarity. In this way, some water insoluble compounds also can be extracted. On the other hand, insoluble and soluble fiber materials of the algal cells may interfere the proper extraction of bioactive compounds and leads to the poor solubility in aqueous medium. Enzymatic hydrolysis of raw material tissues or cells has reported significant yields of desired compounds and convenient industrial approaches in extraction and purification (Jeon et al., 2000; Nagai and Suzuki, 2000). Additionally, enzymatic extracts possesses innovative advantages and characters over conventional extraction procedures. Of the several advantages, water solubility, higher extraction efficacy, greater variation of constituents, minimized environmental pollution/ toxicity and comparatively inexpensiveness are obvious.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

In conclusion, since antioxidants effects observed from *A.longipes,* it can be proposed as a good candidate in the natural antioxidant source, which can be applied in the food and pharmaceutical field.

6. ANTIOXIDANT PROPERTIES OF BENTHIC DIATOM, *Navicula sp.*

6.1. ABSTRACT

Recently, interest in plant-derived food additives has grown, mainly because synthetic antioxidants suffer from several drawbacks. In the present study, different organic fractions from solvent partition of 80% methanolic extract and enzymatic extracts from five proteases and five carbohydrases from *Navicula* sp. was assessed. Among the organic fractions tested chloroform (63.2%) and ethyl acetate (57.3%) fractions were effective in DPPH scavenging. 80% methanolic extract (43.2%) and n-hexane fraction (37.9%) exhibited higher activities on H_2O_2. highest $O_2^{\cdot -}$ radical activity (37.6%) was given by 80% methanolic extract. n-Hexane was highest in presenting hydroxyl radical scavenging activity and $NO^{\cdot}$ inhibition effect (42.2% and 28.8%, respectively). Both ethyl acetate fraction (58.1%) and 80% methanolic extract (50.1%) exhibited significant metal chelating activity. Among all enzymatic extracts, Neutrase (72.6%) showed highest DPPH activity. Flavozyme (48.7%), Viscozyme (47.4%) and Celluclast (45.7%) extracts exhibited higher $O_2^{\cdot -}$ radical scavenging activity. All enzymatic extracts revealed significant activities for both $NO^{\cdot}$ inhibition and metal chelating ($P<0.05$). Out of those extracts Kojizyme (41.7%), Flavozyme (38.9%) and AMG (32.7%) showed significant activities ($P<0.05$) in $NO^{\cdot}$ inhibition while Neutrase (45.2%), Termamyl (41.2%), and

제주대학교 중앙도서관

Ultraflo (33.3%) had strong metal chelating activities. These data suggested that *Navicula* sp. is rich in hydrophobic and hydrophilic antioxidative compounds with different antioxidative properties.

6.2. MATERIALS AND METHODS

As described in, chapter 1.2 "Antioxidant properties of *Pediastrum duplex'*

6.3. RESULTS

6.3.1. Proximate composition

Proximate composition of freeze dried *Navicula* sp. was analyzed and median values were determined as follows; moisture 3.5 %, ash content 63.9 %, carbohydrates 13.5%, proteins 16.9% and lipid 2.1%. Polysaccharide and protein content in water extract and 80% methanol extract and its solvent fractions is depicted in Table 25 and 26.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

6.3.2. Reactive oxygen species inhibition

6.3.2.1 DPPH free radical scavenging effect

Fractions of chloroform (63.2%) and ethyl acetate (57.3%) revealed higher activities among their counterparts (Table 23). As shown in Table 24, Neutrase extract of *Navicula* sp. exhibited the highest radical scavenging activity (72.6%) followed by the Kojizyme and Ultraflo extracts (46.7, and 44.6%, respectively). However, rest of extracts showed moderate results

Table 22. Antioxidants activity of 80% methanol extract and its different solvent frcations from *Navicula* sp.

Fractiions	DPPH	H_2O_2	SOD	HO	NO	MC
Methanol	31.6e±1.3	43.2b±1.3	37.6c±1.3	4.9e±0.2	19.5b±0.7	50.1b±2.8
n-Hexane	39.2d±1.4	37.9c±1.9	26.7d±0.8	42.2c±2.5	28.8a±1.7	4.9e±0.2
Chloroform	63.2b±2.7	32.1d±1.2	22.1e±0.9	8.3d±0.7	12.3d±0.4	7.1e±0.3
Ethyl acetate	57.3c±2.5	23.6e±0.7	47.6b±2.1	2.7e±0.2	27.6ab±1.3	58.1a±2.7
Aqu. residue	31.9e±1.3	14.7f±0.3	4.1f±0.1	4.2e±0.1	15.2c±0.7	14.2c±0.7
BHT	94.6a±6.4	60.1a±4.2	63.2a±4.3	76.6b±4.6	26.1a±0.9	11.5d±0.1
Tocopherol	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2b±0.6	10.3d±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

Table 23. Antioxidants activity of enzymatic extracts from *Navicula* sp.

Extracts	DPPH	H_2O_2	SOD	OH	NO	MC
Viscozyme	32.1f±1.3	16.2c±0.6	47.4b±1.2	24.3b±1.1	30.1b±1.6	22.1e±0.6
Celluclast	37.5f±1.4	15.1c±0.4	45.7b±1.8	19.3c±0.6	27.4d±1.3	25.3e±0.7
AMG	30.3f±1.4	19.4c±0.5	43.1b±1.7	5.3f±0.4	32.7b±1.1	25.3e±0.7
Termamyl	38.8±f1.1	16.4d±0.7	40.9c±1.6	2.4f±0.1	29.3cb±1.6	40.8b±1.9
Ultraflo	44.6cd±1.2	24.9b±0.5	34.3d±1.4	13.d8±0.7	40.6a±1.7	34.3c±1.7
Protamax	41.3e±1.8	12.4d±0.2	38.1d±0.7	11.1e±0.4	24.3e±0.6	28.6d±1.3
Alcalase	42.9de±2.1	15.4d±0.9	36.4d±1.6	12.1e±0.7	32.9b±1.4	27.3d±0.9
Flavozyme	41.1e±1.7	11.6d±0.1	48.7b±1.7	9.5e±0.7	39.5a±1.8	44.7a±0.7
Neutrase	72.6b±5.4	23.4b±0.2	24.3e±0.7	16.6d±0.7	31.3b±1.3	45.2a±2.1
Kojizyme	46.7c±1.2	18.9c±0.8	23.2e±0.6	12.4e±0.7	41.7a±2.4	26.9d±0.9
BHT	94.6a±6.4	60.1a±4.2	63.2a±4.3	76.6b±4.6	26.1e±0.9	11.5f±0.1
Tocopherol	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2e±0.6	10.3f±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

Table 24. Total polyphenolic, polysaccharide and protein content of of 80% methanol extract and its different solvent frcations from *Navicula* sp.

Fractions	Polyphenol [a] (mg/100 g)[d]	Polysaccharide [b] (g/100 g) [e]	Protein [c] (g/100 g) [e]
80%metahnol	332.5± 15.2	27.6±1.7	10.9±0.2
n-Hexane	1116.0±65.2	9.1±0.3	2.1±0.1
Chloroform	1847.5±74.6	14.3±0.7	7.8 ±0.3
Ethyl acetate	397.3±18.6	14.9±0.7	7.6 ±0.2
Aqueous residue	175.7±11.3	26.9±1.3	6.0 ±0.1

Values are means of three replicates

[a] As equivalent gallic acid
[b] As equivalent to glucose
[c] As equivalent to bovine serum albumin
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 25. Total polyphenolic, polysaccharide and protein content of different enzymatic extracts from *Navicula sp*

Extracts	Polyphenol [a] (mg/100 g)[d]	Polysaccharide [b] (g/100 g) [e]	Protein [c] (g/100 g) [e]
Viscozyme	614.5±22	4.8±0.1	8.4 ±0.2
Celluclast	467.8±24	4.7±0.1	7.7±0.3
AMG	367.8±18	4.1±0.1	6.1 ±0.3
Termamyl	500.7±21	9.6±0.3	9.1 ±0.3
Ultraflo	742.1±32	7.3±0.1	14.1 ±0.5
Protamax	652.7±18	5.6±0.1	2.1 ±0.2
Alcalase	592.3±29	5.1±0.1	1.9±0.2
Flavourzyme	581.4±21	5.1±0.1	2.1 ±0.2
Neutrase	951.2±24	7.3±0.1	14.1±0.3
Kojizyme	886.1±29	2.7±0.1	9.8±0.2

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Values are means of three replicates ± SD

[a] As equivalent gallic acid
[b] As equivalent to glucose
[c] As equivalent to bovine serum albium
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts.

6.3.2.2. Hydrogen peroxide scavenging effect

The scavenging activities of the 80% methanol extract (43.2%) and n-hexane farction (37.9%) exhibited higher activities (Table 23). The H_2O_2 scavenging activity decreased from 80%methanol extract to aqueous residue. Ultraflo (29.4%) and Neutrase (23.4%) showed higher activities among enzymatic extracts (Table 24). No significant activity observed from the rest of extarcts.

6.3.2.3. Superoxide scavenging effect

As can be seen in Table 23, ethyl acetate farction and 80% methanol extract exhibited higher activity in all of solvent farctions.. Among the all enzymatic extracts the Flavozyme extract (48.7%) seemed significantly ($P<0.05$) effective in superoxide scavenging effect followed by the Celuclast (45.7%) AMG (43.1%) and Termamyl (40.9%) extract (Table 24).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

6.3.2.4. Hydroxyl radical scavenging efefct

Among the different solven fractions and extracts, n-hexane (42.2%) presented the highest radical scavenging activity (Table 23). However, rest of farctions exhibited less activity. As shown in Table 24, in the hydroxyl radical scavenging assay the Viscozyme extract (24.3%) exhibited significantly higher scavenging activities compared to rest of fractions. Rest of fractions did not show significant activities in scavenging of hydroxyl radicals.

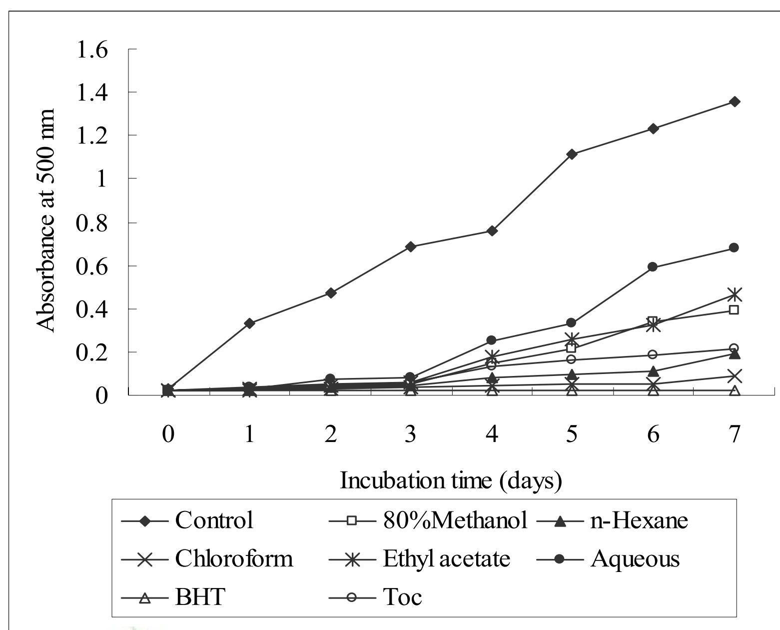
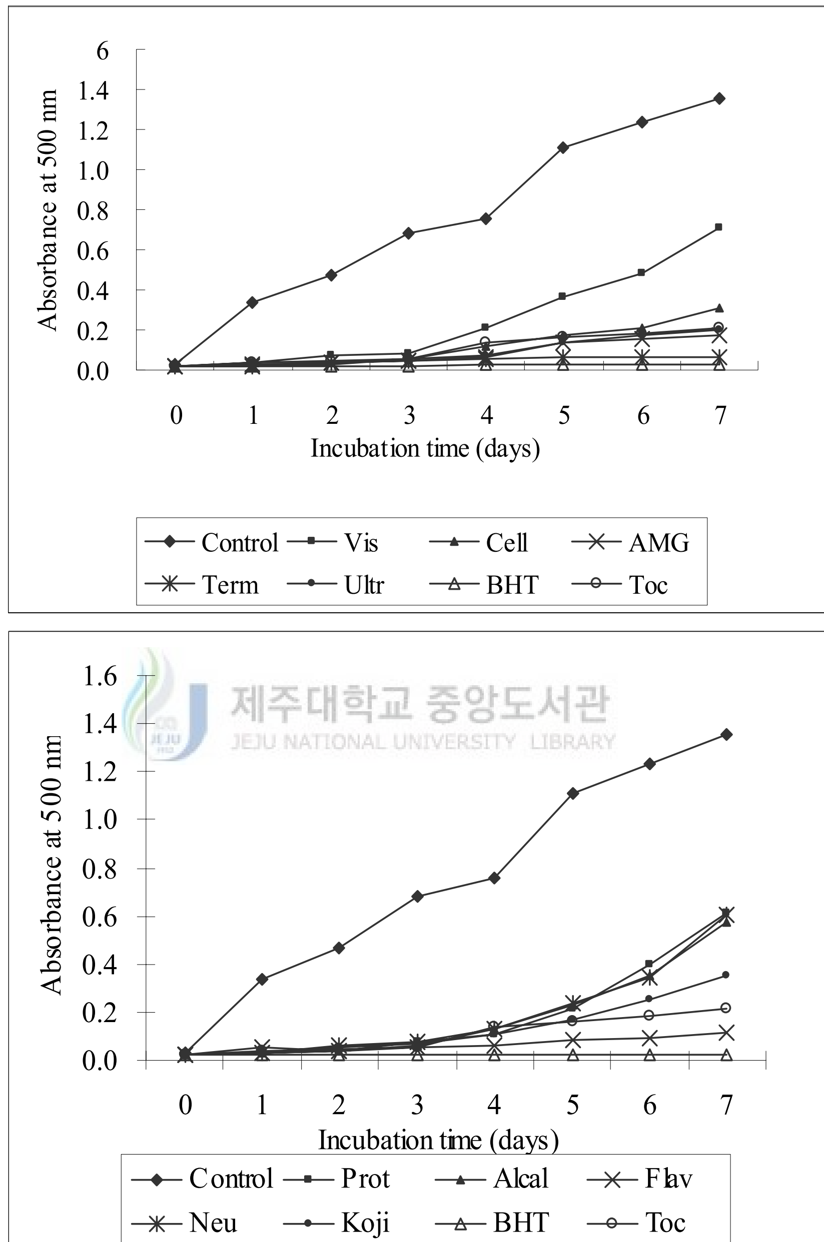


Fig. 15. Antioxidant activity of different slovent fractions from *Navicula sp* compared to BHT and α -Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 16. Antioxidant activity of different enzymatic extracts from *Navicula sp* compared to BHT and α-Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

6.3.3. Nitric oxide radical inhibition effect

Nitric oxide scavenging effect of *Navicula* sp. is presented in Table 23. In this assay, fractions of n-hexane (28.8%) and ethyl acetate (27.6%) exhibited activity, which was higher than those of commercial antioxidants. All enzymatic extracts showed significant nitric oxide inhibition activities ($P<0.05$) and among the all the extracts Kojizyme extract (41.7%), Ultraflo (40.2%) Flavozyme (38.9%) and Neutrase (31.3%) revealed strong effects in nitric oxide radical inhibition (Table 24).

6.3.4. Metal chelating effect

As shown in Table 23, ethyl acetate fraction, 80% methanolic extract (50.1%) and aqueous residue (15.2%) has exhibited significantly strong metal chelating effects even higher than the activities of α-tocopherol and BHT ($P<0.05$). All enzymatic extracts revealed significantly higher activities compared to commercials and among them Neutrase (44.7%), Termamyl (40.8%), and Ultraflo (34.3%) was notable (Table 24).

6.3.5. Determination of antioxidant activity with the ferric thiocyanate (FTC) method

As shown in Fig 15, the absorbance of linoleic acid emulsion without the addition of *Navicula* sp. extracts (control) increased significantly. Among the all the solvent fractions, chloroform and ethyl acetate exhibited antioxidant activity significantly similar with α- tocopherol. Enzymatic extracts of AMG and Termamyl had strong antioxidant effects. No

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

remarkable antioxidant activity was present with other extracts compared to comercials.

6.3.6. Total polyphenol content

The total phenolic content in the extracts and the solvent fractions vary with the solvent used according to their polarity and the enzymes used. Chloroform farction indicated the highest polyphenol content (1847.5 mg/100 g) among solvent extracts and fractions (Table 25). Of the carbohydrases, ultraflo extract exhibited highest polyphenol content (742.1 mg/100 g) and among proteases, neutrase showed highest polyphenol (951.2 mg/100 g) contents (Table 26).

6.4. DISCUSSION

제주대학교 중앙도서관

DPPH is a useful reagent for investigating the free radical-scavenging activities of compounds. In the present study, fractions of chloroform and ethyl acetate had higher scavenging effects. Rest of fractions presented moderate activities. Thus, it revealed that active compound has dispersed according to its polarity. Of the enzymatic extracts, Neutrase extract demonstrated notable free radical activities indicating the higher efficiency of scavenging of free radicals in *Navicula sp*. In addition, it that was indicated that DPPH scavenging activity of proteases treated extracts was relatively higher than the extracts treated with carbohydrases. Radical scavengers may protect cell tissues from free radicals, thereby preventing diseases such as cancer (Nakayama et al., 1993) Even though it is unclear

whether active constituents in extracts are active against free radicals after being absorbed and metabolized by cells in the body, free radical-scavenging assays have gained acceptance for their capacity to screen rapidly materials of interest.

It was interested that H_2O_2 scavenging activity decreased with partition. Thus, it was indicated that active compounds that responsible for H_2O_2 scavenging activity had hydrophobic properties. Carbohydrases and proteases have shown similar impact in enzymatic hydrolyzing. According to these results, it was revealed that *Navicula* Sp. was a moderate hydrogen peroxide scavenger compared with standards. H_2O_2, a reactive non-radical compound is very important as it can penetrate biological membranes. Addition of H_2O_2 to cells in culture can lead to transition metal ion-dependent $OH^{\cdot}$ mediated oxidative DNA damage (Spencer et al., 1996).

제주대학교 중앙도서관

The higher superoxide activity was in the 80% methanol extract and the ethyl acetate fraction. Therefore, it was obvious that the superoxide scavenging compounds presented in these fractions had both hydrophilic and hydrophobic properties. In addition, carbohydrases treated extracts exhibited higher effects than protease treated. It seemed carbohydrases had more ability to release those compounds in superoxide scavenging. Thus, it could be assumed that the potential biological compounds mainly exhibited hydrophilic characteristics in enzymatic extracts. Superoxide radical generally form first and its effects can be exaggerated as it produces other kinds of cell damage inducing free radicals and oxidizing agents (Spitzet, et

al., 2005). Although superoxide anion is by itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to the oxidative stress (Dahl and Richardson, 1978). Therefore, superoxide radical scavenging by antioxidants has physiological implications.

Among the solvent fractions, only n-hexane fraction showed comparatively higher activities. Therefore, it was clear those biochemical compounds that responsible for activity were concentrated in n-hexane farction. In addition, it was supposed those were fat-soluble compounds such as pigments and steroids. Even in enzymatic extracts activities was not in significant level because those compound show less hydrophilic properties. Hydroxyl radical is the most reactive oxygen species among all ROS due to its strong ability to react with various biomolecules.

제주대학교 중앙도서관

Nitric oxide is a gaseous free radical, which has important roles in physiological and pathological conditions. Therefore, the scavenging ability of NO.may also help to disrupt the chain reactions initiated by excessive generation of NO.that are detrimental to human health. In here, solvent fractions but both carbohydrases and proteases also exhibited significant results compared tocommercials. It suggested those biochemical compounds exhibited both hydrophilic and hydrophobic properties. The rectivities of the $NO^{\cdot}$ and $O_2^{-\cdot}$ were found to be relatively low, but their metabolite ONOO- (peroxynitrite) is extremely reactive and directly induce toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation

and DNA modifications (Moncada et al 199; Radi et al1991). Therefore, the scavenging ability of $NO^{\cdot}$ may also help to interrupt the chain reactions initiated by excessive generation of $NO^{\cdot}$ that are detrimental to human health.

Ferrozine can make complexes with ferrous ions and in presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, chelating effect of the coexisting chelator can be determined measuring the rate of color reduction. The formation of the ferrozine–Fe^{2+} complex is interrupted in the presence of *Navicula* sp. extracts indicating significant chelating ability. Both solvent fractions and enzymatic extracts showed notable metal chelating capability and it was apparent that both hydrophilic and hydrophobic components were responsible for metal chelating. Ferrous can initiate lipid peroxidation by Fenton reaction as well as accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Fridovich, 1995, Halliweill, 1991). Hence, different extracts of *Navicula* Sp. demonstrated a noticeable capacity for iron binding, suggesting their ability as peroxidation protector that relates to its ferrous binding capacity (Gülçin et al., 2004)

To evaluate the antioxidant effects of *Navicula* Sp. extracts, their lipid peroxidation was compared with standard antioxidants using ferric thiocyanate (FTC) method by determining the amount of peroxide formed in emulsion in during incubation period. High absorbance is an indication of high concentration of formed peroxides. In this experiment, fractions of chloroform and ethyl acetate, enzymatic extracts of kojizyme, Termamyl and

제주대학교 중앙도서관

Viscozyme extracts provided high antioxidant effects among the all extracts and fractions showing that those extracts were able to reduce the formation of peroxides. It suggests that according to enzyme digestion and solvent fractionationdifferent antioxidant components were released from the inside of microalgae cells. These extracts contained high level of polysaccharides, proteins and polyphenols and it could be assumed that those components may influence on antioxidant effects although not correlate with antioxidant effect.

Total phenolic content in both 80% methanol extracts and different enzymatic extracts varies with the solvent used according to their polarity. In this study total phenolic content and antioxidant activity not highly correlated. Therefore, it can be suggested that not only the polyphenolic compounds but other bioactive compounds also contribute for the activity. For example it was discovered that oligosaccharides, sulfate and glycoprotein components have exhibited antioxidant activities in red microalga, *Porphyridium* sp. (Spitz et al., 2005). The polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans (Tanaka et al., 1998). Phenols are very significant plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano, 1995).

In this study, both methanolic and enzymatic extraction was carried out to determine the antioxidant effects in each extracts and farctions. Methanol is a good solvent to extract varies kind of hydrophilic and

제주대학교 중앙도서관

hydrophobic bioactive compounds but extraction yield is less compared to enzymatic extractions. By solvent fractionation, potential bioactive compounds can be separated according to its polarity. In this way, some water insoluble compounds also can be extracted. On the other hand, enzymatic extaraction has advantages for better extraction becase plants cells originally contain large amount of soluble polysaccharides and insoluble fibers such as made of cellulose that perform as a physical barrier for the extraction of desired bioactive materials. Enzymatic hydrolysis of raw material tissues or cells has shown significant yields of desired compounds and convenient industrial approaches in extraction and purification (Jeon et al., 2000; Nagai and Suzuki, 2000). Also, the breakdown and releasing of high molecular weight polysaccharides and proteins themselves may contribute to enhance the antioxidative activities (Ramos and Xiong 2002; Ruperez et al. 2002). Additionally, enzymatic extracts possesses innovative advantages and characters over conventional extraction procedures. Of the several advantages, water solubility, higher extraction efficacy, greater variation of constituents, minimized environmental pollution/ toxicity and comparatively inexpensiveness are obvious.

In conclusion, *Navicula* Sp. has exhibited antioxidant potential against ROS and it would be good candidate as a natural antioxidant source, which might be applied in food and pharmaceutical industray.

제주대학교 중앙도서관

7. ANTIOXIDANT PROPERTIES OF BENTHIC DIATOM, *Amphora coffeaeformis*

7.1. ABSTARCT

The study was aimed at evaluating the antioxidant activity of solvent fractions of 80% methanolic extract and enzymatic extracts of proteases and carbohydrases from *Amphora coffeaeformis,* a benthic diatom. Among the solvent fractions, chloroform (63.2%) and ethyl acetate (57.3%) were effective in DPPH scavenging. 80% methanolic extract (43.2%) and n-hexane fraction (37.9%) exhibited higher activities on H_2O_2. highest $O_2{\cdot}^-$ radical activity (37.6%) was given by 80% methanolic extract. Fraction of n-hexane was highest in presenting hydroxyl radical scavenging activity and $NO^{\cdot}$ inhibition effect (42.2% and 28.8%, respectively). Both ethyl acetate fraction (58.1%) and 80% methanolic extract (50.1%) exhibited significant metal chelating activity. Among the all enzymatic extracts, Neutrase (72.6%) showed highest DPPH effect. Flavozyme (48.7%), Viscozyme (47.4%) and Celluclast (45.7%) extracts exhibited higher $O_2{\cdot}^-$ radical scavenging activity. All enzymatic extracts revealed significant activities for both $NO^{\cdot}$ inhibition and metal chelating. Out of those extracts Kojizyme (41.7%), Flavozyme (38.9%) and AMG (32.7%) showed significant activities in $NO^{\cdot}$ inhibition while Neutrase (45.2%), Termamyl (41.2%), and Ultraflo (33.3%) had strong metal chelating activities. These data suggest that both kinds of extractions are rich in hydrophobic and hydrophilic antioxidative compounds with different antioxidative properties that can be applied in food and

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

pharmaceutical industry.

7.2. MATERIALS AND METHODS

As described in chapter 1.2 "Antioxidant properties of *Pediastrum duplex"*

7.3. RESULTS

7.3.1. Proximate composition

Proximate composition of freeze dried *A. coffeaeformis* was analyzed and median values were; moisture 5.9 %, ash content 55.8 %, carbohydrates 16.0%, proteins 15.5% and fat 6.8%. Polysaccharide and protein content in water extract and 80% methanol extract and its solvent fractions is depicted in Table 1.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

7.3.2. Reactive oxygen species inhibition

7.3.2.1. DPPH free radical scavenging effect

To evaluate the scavenging effect of DPPH in different solvent fractions from 80%methanol extract and enzymatic extracts from *A. coffeaeformis*, DPPH radical scavenging assay was used (Table 27). Fraction of n-hexane (67.4%) indicated higher effect on DPPH radical scavenging followed by chloroform (35.5%) and ethyl acetate (23.3%). Among enzymatic extracts Neutrase (75.9%), Flavozyme (65.6%) and Kojizyme (46.2%) revealed higher effect on DPPH radical scavenging. However, rest of extracts exhibit less significant effect on DPPH radical scavenging

compared to commercial antioxidants (Table 30).

7.3.2.2. Hydrogen peroxide scavenging effect

The scavenging activities of organic solvent fractions from 80% methanol from *A. coffeaeformis* are depicted in Table 27. Out of those, fractions of n-hexane (47.5%) and Chloroform (46.1%) demonstrated higher activities. However, other fractions showed less effect on hydrogen peroxide scavenging. Enzymatic extracts also exhibited less significant effect on hydrogen peroxide scavenging (Table 30). The highest activity was shown by Neutrase extract (22.1%) followed by Flavozyme (21.1%) and Alcalase (21.1%).

7.3.2.3. Superoxide anion scavenging assay

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Superoxide anion scavenging effects of 80% methanolic extract and its solvent fractions from *A.coffeaeformis* is depicted in Table 2. Among these fractions of ethyl acetate (34.1%) aqueous residue (31.3%) and 80% methanolic extract (28.1%) exhibited higher effects on superoxide scavenging. According to Table 3, enzymatic extracts demonstrated less effect in superoxide scavenging. Of the extracts Kojizyme and Neutrase indicated activities as 17.8% and 11.1%, respectively.

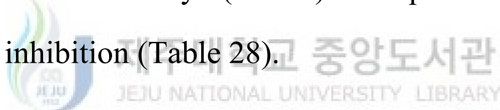
7.3.2.4. Hydroxyl radical scavenging assay

Fraction of n-hexane (55.5%) exhibited higher hydroxyl radical scavenging effects (Table 27). As well, no significant effect was shown from other fractions. Enzymatic extracts of Viscozyme (45.9%), Neutrase (38.5%),

Celluclast (38.2%), and kojizyme (36.4%) exhibited higher hydroxyl radical effects (Table 28). However, rest of extracts showed less effect on hydroxyl radical scavenging.

7.3.3. Nitric oxide radical inhibition effects

As shown in Table 27, n-hexane (75.6%) and ethyl acetate (26.3%) exhibited significant results compare to commercial antioxidants ($P<0.05$). However, no significant effect was shown by other fractions on nitric oxide scavenging. Among the enzymatic extracts, Kojizyme (29.9%) and Viscozyme (39.8%) exhibited inhibition effects even significantly higher than that of commercial antioxidants ($P<0.05$). Among the other extracts, Ultraflo (24.9%) and Termamyl (23.8%) also possess considerable nitric oxide scavenging inhibition (Table 28).


제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

7.3.4. Metal chelating effect

As shown in Table 27, aqueous residue (75.6%), 80% methanolic extract (51.4%) and ethyl acetate fraction (12.9%) exhibited significantly higher effect of metal chelating compared to commercial antioxidants. No significant effect was reported from rest of fractions. In addition, according to Table 28, all enzymatic extracts have exhibited strong metal chelating effects even significantly ($P<0.05$) higher than the activities in commercial antioxidants. Among them Termamyl (89.3%), Protamax (88.8%), Alcalase (86.2%), AMG (60.7%), Viscozyme (56.6) and Ultraflo (55.1%) exhibited strong chelating effects.

Table 26. Antioxidants activity of 80% methanol extract and its different solvent extracts from *A. coffeaeformis*

Fractions	DPPH	H_2O_2	SOD	HO	NO	MC
Methanol	22.7d±0.9	23.3c±0.8	28.1c±1.4	4.1f±0.1	14.8b±0.4	51.4b±2.4
n-Hexane	67.4b±3.2	47.5b±2.3	27.4c±1.3	55.5c±2.1	3.4c±0.1	1.7e±0.1
Chloroform	35.5c±2.3	46.1b±2.1	15.2d±0.7	13.3d±0.2	6.1c±0.2	2.1e±0.1
Ethyl acetate	23.3d±1.2	21.cd±1.2	34.1b±1.7	6.4e±0.3	16.8b±0.7	43.2±2.7
Aqu. residue	18.9e±0.9	19.1d±0.7	31.3b±1.6	7.9e±0.4	29.6a±1.4	75.6c±3.8
BHT	94.6a±6.4	60.1a±4.2	63.2a±4.3	76.6b±4.6	26.1a±0.9	11.5a±0.1
Tocopherol	94.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2a±0.6	10.3d±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

Table 27. Antioxidant activity of enzymatic extracts from *A. coffeaeformis*

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Extarcts	DPPH	H_2O_2	SOD	OH	NO	MC
Viscozyme	31.8f±1.4	19.4b±0.3	8.9b±0.6	45.9b±2.9	46.4a±2.1	56.6c±2.3
Celluclast	24.3h±1.9	17.8c±0.6	4.2c±0.1	38.2c±1.9	44.6a±2.2	24.8e±1.2
AMG	29.7h±1.7	10.5c±0.7	6.5c±0.1	33.6c±1.3	43.6a±2.4	60.7b±4.2
Termamyl	24.3h±1.2	16.7c±0.7	4.2c±0.1	29.1d±1.3	28.2d±0.5	89.3a±4.1
Ultraflo	27.5g±1.4	15.4c±0.4	7.1c±0.2	26.7d±1.6	35.7b±1.7	55.1c±2.7
Protamax	25.6h±1.5	17.3c±0.8	6.8c±0.3	20.5d±1.4	37.5b±2.1	88.8a±5.1
Alcalase	41.8e±2.2	21.1b±1.1	8.9b±0.4	29.8d±1.9	31.1c±1.8	86.2a±3.4
Flavozyme	65.6c±3.3	21.1b±1.2	10.6b±0.7	29.3d±2.1	31.1c±1.9	51.2c±2.7
Neutrase	75.9b±4.1	22.1b±1.3	11.1b±0.3	38.5c±1.7	31.4c±1.3	45.4d±2.7
Kojizyme	46.2d±2.3	13.9c±0.9	17.8b±0.8	36.4c±2.3	23.2c±1.8	52.2c±3.1
BHT	96.6a±6.4	60.1a±4.2	63.2a±4.3	76.6a±4.6	26.1d±0.9	11.5f±0.1
Toco	97.3a±7.1	62.5a±4.9	61.5a±4.7	79.5a±4.7	25.2d±0.6	10.3f±0.1

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

Table 28. Total polyphenolic, polysaccharide and protein contents of 80% methanol extract and its different solvent fraction from *A. coffeaeformis*

Fractiions	Polyphenol [a] (mg/100 g)[d]	Polysaccharide [b] (g/100 g) [e]	Protein [c] (g/100 g) [e]
80%metahnol	124.2±9.4	0.5±0.0	0.3±0.0
n-Hexane	332.5 ±15.7	1.5±0.1	1.0±0.0
Chloroform	1067.6±51.2	1.6±0.1	1.1±0.1
Ethylacetate	3558.2±112	1.4±0.1	1.4±0.1
Aqueous	171.1±9.4	0.7±0.0	0.2±0.0

Values are means of three replicates

[a] As equivalent gallic acid
[b] As equivalent to glucose
[c] As equivalent to bovine serum albumin
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

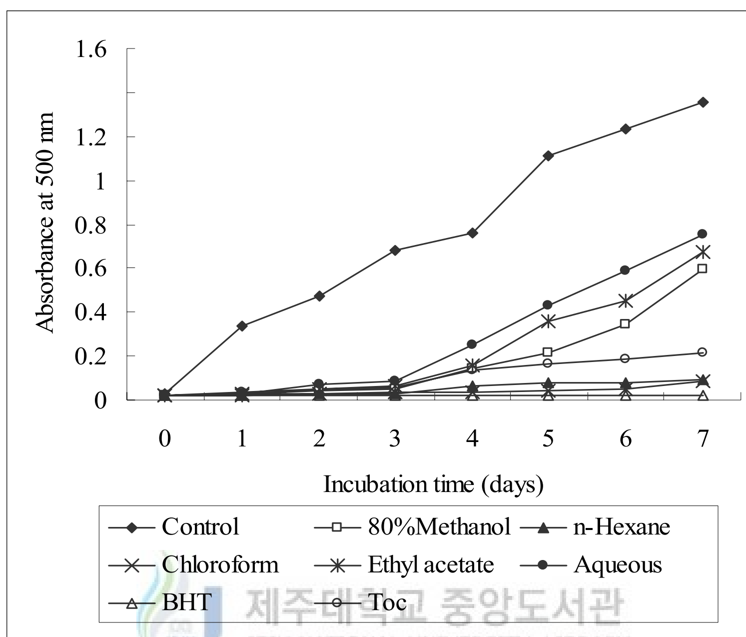
Table 29. Total polyphenolic, polysaccharide and protein content of different enzymatic extracts from *A. coffeaeformis*

Extracts	Polyphenol [a] (mg/100 g) [d]	Polysaccharide [b] (g/100 g) [e]	Protein [c] (g/100 g) [e]
Viscozyme	416.1±12	11.2±0.9	9.7±0.7
Celluclast	429.7±44	15.9±0.7	10.7±0.4
AMG	306.3±18	11.5±0.9	4.9±0.3
Termamyl	364.9±25	9.8±0.1	104±0.4
Ultraflo	597.3±34	9.3±0.0	11.8±0.8
Protamax	657.7±48	2.9±0.0	19.5±0.7
Alcalase	821.4±39	9.2±0.6	4.6±0.1
Flavourzyme	415.6±14	6.3±0.3	9.4±0.4
Neutrase	252.1±14	4.4±0.2	7.9±0.3
Kojizyme	279.7±16	4.2±0.2	7.8±0.2

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

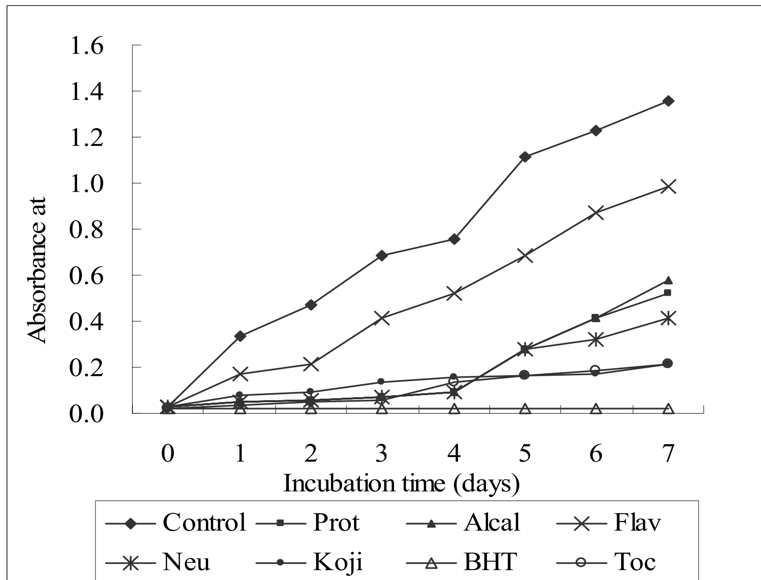
Values are means of three replicates ± SD

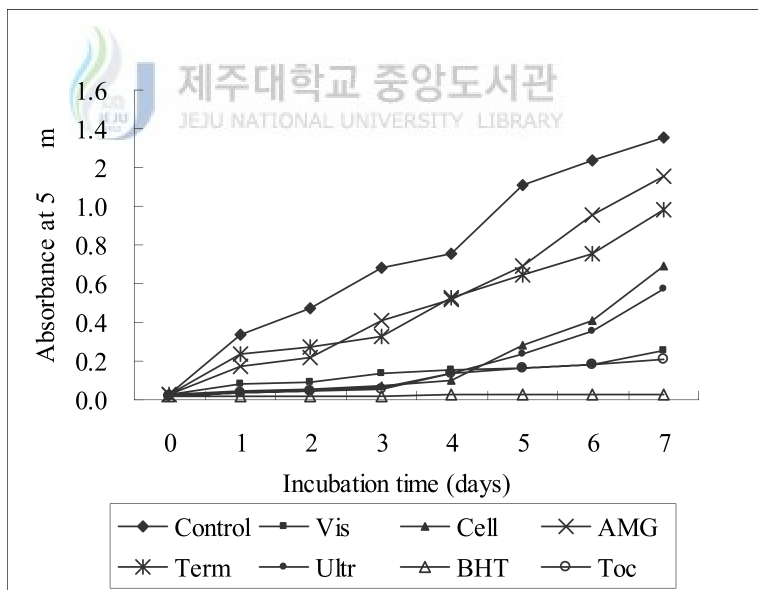
[a] As equivalent gallic acid
[b] As equivalent to glucose
[c] As equivalent to bovine serum albumin
[d] mg per 100 g solid of the extracts.
[e] g per 100 g solid of the extracts



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 17. Antioxidant activity of different solvent extracts from *A. coffeaeformis* compared to BHT and α - Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid





제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 18. Antioxidant activity of different enzymatic extracts from *A. coffeaeformis* compared to BHT and α-Tocopherol at 1 mg/ml of concentration of ethanol as assessed by linoleic acid.

7.3.5. Determination of antioxidant activity with the ferric thiocyanate (FTC) method

As shown in Fig. 18 the absorbance of linoleic acid emulsion without the addition of *A. coffeaeformis* extracts (control) increased significantly ($P<0.05$). Among the all the solvent fractions, chloroform and ethyl acetate exhibited antioxidant activity significantly higher than α-tocopherol. Enzymatic extracts of Kojizyme also exhibited notable antioxidant activity even significantly similar with BHT. No remarkable antioxidant activity was presented from the other extracts compared to commercial antioxidants.

7.3.6. Total polyphenol content

Ethyl acetate fraction indicated the highest polyphenol content (3558 mg/100 g) among solvent extracts and fractions (Table 29). Of the carbohydrases, Celluclast extract exhibited highest polyphenol content (429.7 mg/100 g) and among proteases, alcalase showed highest polyphenol (821.4 mg/100 g) contents (Table 30).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

7.4. DISCUSSION

Research in antioxidant products from natural plants has made significant advances in recent years since plants produce potential antioxidant compounds such as polyphenolics, nitrogen containing compounds, phytosterols, carotonoids, and chlorophyll derivatives. In the present study, the attention was focused on antioxidant potential of solvent

fractions of 80% methanol extract and enzymatic extracts from a micro alga *A. coffeaeformis.*

DPPH$^{\bullet}$ has the advantage of being unaffected by certain side reactions that brought by various additives. In this study, n-hexane fraction demonstrated notable free radical activities indicating the higher efficiency of scavenging of free radicals in *A. coffeaeformis*. Therefore, it was clear those biochemical compounds that responsible for DPPH scavenging activity was concentrated in n-hexane farction. Thus, it was supposed those were fat-soluble compounds such as pigments and steroids. In addition, it could be seen that DPPH scavenging activity of proteases treated extracts was relatively higher than carbohydrases treated. Even though it is unclear whether active constituents in extracts are active against free radicals after being absorbed and metabolized by cells in the body, free radical scavenging assays have gained acceptance for their capacity to screen rapidly materials of interest.

제주대학교 중앙도서관

The highest superoxide activity was founded in the fraction of n-hexane and chloroform. Further, it obviously depicted that significantly less activities by enzymatic extracts also. Thus, it was clear that biochemical compounds that responsible for superoxide activity are strongly hydrophobic. Other important phenomenon was protease treated extracts exhibited higher effects than carbohydrases treated. Therefore, it can be assumed that protease had more ability to release those compounds in superoxide scavenging. Superoxide radical generally form first and its effects can be exaggerated as

it produces other kinds of cell damage inducing free radicals and oxidizing agents (Spitz et al., 2005). Therefore, superoxide radical scavenging by antioxidants has physiological implications.

As depicted in Table 27, an H_2O_2 activity was founded in the fractions of n-hexane and chloroform. Further, it was depicted that significantly less activities are shown by enzymatic also. Thus, it was assumed that biochemical compounds that responsible for superoxide activity are strongly hydrophobic. Other important fact was protease treated extracts exhibited higher effects than carbohydrases treated. Therefore, it can be assumed that protease had more capacity to release those compounds in superoxide scavenging. H_2O_2, a reactive non-radical compound is very important as it can penetrate biological membranes. Thus, removing H_2O_2 is very important for the protection of living systems. Addition of H_2O_2 to cells in culture can lead to transition metal ion dependent $OH^{\cdot}$ mediated oxidative DNA damage (Spencer et al., 1996).

Hydroxyl radical scavenging activity of the enzymatic extracts and solvent fractions from *A. coffeaeformis* was determined as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. Among the solvent fractions, only n-hexane fraction showed comparatively higher activities. Therefore, it was clear those biochemical compounds that responsible for activity are concentrated in n-hexane farction. In addition, it was supposed those were fat-soluble compounds such as pigments and steroids. On the other hand, carbohydrases treated extracts exhibited higher

제주대학교 중앙도서관

effects than protease treated. Thus, it can be assumed that carbohydrases have more capability to liberate those compounds in hydroxyl radical scavenging. However, in enzymatic extracts activities was not in significant level because those compound show less hydrophilic properties. Hydroxyl radical reacts with several biological materials oxidatively by hydrogen withdrawal, double bond addition, electron transfer and radical formation, and initiates autoxidation, polymerization and fragmentation (Liu and Ng, 2000).

Nitric oxide is also implicated in cancer, inflammation and other pathological conditions. Nitric oxide is a gaseous free radical, which has important roles in physiological and pathological conditions. In solvent fractions, NO.inhibition effects were deviated towards hydrophilic status. Also, compared to solvent fractions both carbohydrases and proteases exhibited considerable effect. It supposed those biochemical compounds exhibit both hydrophilic and hydrophobic properties. Further, carbohydrases extracts have shown slightly higher activities than proteases. Marcocci et al., (1994) reported that scavengers of nitric oxide compete with oxygen leading to reduce production of nitric oxide. The rectivities of the $NO^{\cdot}$ and $O_2^{-\cdot}$ were found to be relatively low, but their metabolite ONOO- (peroxynitrite) is extremely reactive and directly induce toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modifications (Moncada et al., 1991; Radi et al., 1991). Therefore, the

제주대학교 중앙도서관

scavenging ability of NO.may also help to interrupt the chain reactions initiated by excessive generation of NO.that are detrimental to human health.

Ferrous is known as the most important lipid oxidizing pro oxidant among the transition metals due to its high reactivity. Ferrozine can make complexes with ferrous ions. The formation of the ferrozine–Fe^{2+} complex was interrupted in the presence of *A. coffeaeformis* extracts and solvent fractions indicating significant chelating ability. It was notable that bioactive compounds that give metal chelating effects were not dispersed properly in non-polar solvents such as n-hexane and chloroform. However, since ethyl acetate is moderately polar solvent, activities were recognized. Enzymatic extracts showed higher metal chelating ability and it was apparent that hydrophilic components are responsible for metal chelating. In addition, higher activities were present in both carbohydrases and proteases extracts equally. Ferrous can initiate lipid peroxidation by Fenton reaction as well as accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Fridovich, 1995, Halliweill, 1991). Hence, different extracts of *A. coffeaeformis* demonstrated a noticeable capacity for iron binding, suggesting their ability as peroxidation protector that relates to its ferrous binding capacity (Gülçin et al, 2004).

제주대학교 중앙도서관

To evaluate the antioxidant effects of *A. coffeaeformis* extracts, their lipid peroxidation was compared with standard antioxidants using ferric thiocyanate (FTC) method by determining the amount of peroxide formed in emulsion in during incubation period. High absorbance is an indication of

high concentration of formed peroxides. In this experiment fractions of hexane and chloroform, enzymatic extracts of AMG, Termomyl, Ultraflow and Flavozyme provided high antioxidant effects among the all extracts and fractions showing that those extracts were able to reduce the formation of peroxides. It suggests that according to enzyme digestion different antioxidant components were released from the inside of microalgae cells. These extracts contained high level of polysaccharides, proteins and polyphenols and it could be assumed that those components may influence on antioxidant effects although not correlate with antioxidant effect.

Total phenolic content in both 80% methanol extracts and different enzymatic extracts varies with the solvent used according to their polarity. Although the ethyl acetate, celluclast and alcalase consisted of the highest contents of polyphenol it was not correlated with polyphenol content as described in previously (Lu and Foo, 2000; Kim and Chung, 2002). Therefore, it can be assumed that not only other bioactive compounds may also effect for the activity. Further, there are some other bioactive components such as proteins, polysaccharides and different kinds of pigments are consisted in this microalga. For example it was found that oligosaccharides, sulfate and glycoprotein components have exhibited antioxidant activities in red microalga, *Porphyridium sp*. (Spitz et al., 2005). Phenols are very significant plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano, 1995).

제주대학교 중앙도서관

In this study, for the extraction 80%methanol and different kinds of enzymes were used. Methanol is a good solvent to extract varies kind of hydrophilic and hydrophobic bioactive compounds but extraction yield is less compared to enzymatic extractions. By solvent fractionation, biological compounds can be separated according to its polarity. In this way, some water insoluble compounds also can be extracted as well as activity also higher in some assays. However, insoluble and soluble fiber in plant cells, together with the other cell wall material performs as a physical barrier for the extraction of desired bioactive materials. Surmounting such barriers might make the desired compounds possible to be extracted in higher yields. Enzymatic hydrolysis of raw material tissues or cells has reported significant yields of desired compounds and convenient industrial approaches in extraction and purification (Jeon et al., 2000; Nagai and Suzuki, 2000). Also, the breakdown and releasing of high molecular weight polysaccharides and proteins themselves may contribute to enhance the antioxidative activities (Ramos and Xiong, 2002; Ruperez et al., 2002). Additionally, enzymatic extracts possesses innovative advantages and characters over conventional extraction procedures. Of the several advantages, water solubility, higher extraction efficacy, greater variation of constituents, minimized environmental pollution/ toxicity and comparatively inexpensiveness are obvious.

제주대학교 중앙도서관

In conclusion, since an antioxidants potential was observed from *A. coffeaeformis* it can be proposed as a good candidate as a natural antioxidant source, which might be applied in food and pharmaceutical industry.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Part III.

Protective effect of microalgae against DNA damage induced by H_2O_2

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1. ABSTRACT

The DNA damage inhibitory effect of Termamyl extract of *P.duplex,* Kojizyme extract of *D. fascicularis,* Alcalase extract of *H. porphyrae*, Neutrase extract of *O. unicellularis*, Neutrase extracts of *A.longipes*, Ultralo extarct of *Navicula* sp.and Neutrase extract of *Amphora coffeaeformis* were investigated using comet assay. With the increased concentrations the inhibitions effect of cell damage increased significantly in the extracts of *P.duplex, D. fascicularis, A.longipes* and *H. porphyrae.* Inhibitory effect of cell damage was 80.2% for *P.duplex*, 69.3% for *D. fascicularis*, 51.6% for *A.longipes,* and 50.1% for *H.porphyrae* and at 100 μg/ml of concentration. Thus, it is suggested that fresh water microalgae have higher DNA damage inhibitory effect.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2. MATERIALS AND METHODS

2.1. Enzymatic hydrolysis procedure

Freeze-dried seven species of microalgae were ground into a fine powder and 1 g was mixed with 100 ml of distilled water. The optimum pH of the each reaction mixtures were adjusted with 1M HCl / NaOH. Optimum pH and temperature conditions for the respective enzymes used were similar to the conditions shown in Table 2. Enzymes were then added at the dosage (enzyme/substrate ratio) of 1%. The mixtures were placed in a shaking incubators for 24 adjusted to optimum temperatures of the respective

enzymes used. Resultant mixtures were filtered and the enzymes activity of hydrolysates was inactivated by heat (100 ^{o}C for 10 min). Finally, the pH of each hydrolysate was adjusted to pH 7 with 1M HCl / NaOH. Each extract was adjusted to a concentration of 2 mg/ ml. All activities of extracts were compared with standard antioxidants (BHT and α- tocopherol) dissolved in methanol.

2.2. Hydrogen peroxide scavenging assay

This assay was carried out according to the method described by Muller (1995). Enzymatic extract of microalgae (80 μl) and 20 μl of 10 mM hydrogen peroxide were mixed with 100 μl of phosphate buffer (0.1M, pH 5.0) in a 96-microwell plate and incubated at 37º C for 5 min. Thereafter, 30 μl of freshly prepared 1.25 mM ABTS and 30 μl of peroxidase (1 U/ml) were mixed and incubated at 37º C for 10 min and the absorbance was measured by microplate reader at 405 nm.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

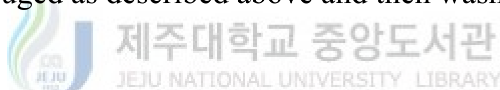
2.3. Isolation and cryoconservation of human peripheral lymphocytes

Mouse lymphoma L5178 obtained from healthy male mouse. A 5 ml of fresh whole blood was added to 5 ml of phosphorous buffered saline (PBS) and layered onto 5 ml of Histopaque 1077. After centrifugation for 30 min at 400g at room temperature, the lymphocytes were collected from the just above the boundary with the Histopaque 1077, washed with 5 ml PBS buffer. Thereafter, they were resuspended in freezing medium (90% fetal calf serum, 10% dimethyl sulfoxide) at 6X10^{6} cells/ml. The cells were frozen to -

80 ºC using a Nalgene Cryo 1 ºC freezing container and then stored in liquid nitrogen.

2.4. Incubation of lymphocytes

Each lyophilized extract was dissolved in PBS and diluted into concentrations 25, 50 and 100 ㎍/ml. A diluted extract aliquot of 1 ml with a lymphocyte suspension containing 2x104 cells/ml was incubated for 60 min at 37 ^{0}C in a dark incubator together with the untreated control sample. After pre incubation, samples were centrifuged at 2000X g for 5 min at 4 ^{0}C. The incubated cells were resuspended in PBS with 50 μM H_2O_2 for 5 min on ice. The untreated control sample was resuspended only in PBS without H_2O_2. Cells were centrifuged as described above and then washed with 1 ml PBS.



2.5. Determination of DNA damage

The alkaline comet assay was performed according to the method recorded by Singh et al. (1995) with a slight modification. The cell suspension was mixed with 75 µl of 0.5% low melting agarose (LMA), and added to the slides precoated with 1.0% ordinary melting agarose (NMA). After solidification of the agarose, slides were covered with other 75 µl of 0.5% LMA followed by immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 h at 4 °C. Thereafter slides were placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. An electric current of 25 V/300 mA

was applied for 20 min at 4 °C for electrophoresis of the DNA. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4 °C, thereafter treated with ethanol for another 5 min before staining with 50 µl of ethidium bromide (20 µg/ml). Measurements were done by image analysis (Kinetic Imaging, Komet 5.0, UK) and fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of 3 replicate slides).

3. RESULTS

The DNA damage inhibitory effect of Termamyl extract of *P.duplex,* Kojizyme extract of *D. fascicularis,* alcalase extract of *H. porphyrae*, Neutrase extract of *O. unicellularis*, Neutrase extracts of *A.longipes*, Ultralo extarct of *Navicula* sp.and Neutrase extract of *Amphora coffeaeformis* were investigated using comet assay based on H_2O_2 scavenging activity (Table 31 and Fig.20-33). With the increased concentrations the inhibitions effect of cell damage increased significantly in the extracts of *P.duplex, D. fascicularis A.longipes* and *H. porphyrae* ($P<0.05$). The highest concentration (100 µg/ml) of the extract indicated considerable defensive effect against DNA damage, as compared to the control group. Inhibitory effect of cell damage was 80.2% for *P.duplex*, 69.3% for *D. fascicularis*, 51.6% for *A.longipes,* and 50.1% for *H.porphyrae* and at 100 µg/ml of concentration. However, rest of enzymatic extracts exhibited less inhibitory

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

effect in this assay. Photomicrographs of different DNA migration profiles obtained from lymphocytes, when treated with different concentrations of extracts are shown in Fig. 20, 22, 24,26,28,30, and 32. In the group treated with only hydrogen peroxide, the DNA was completely damaged but the addition of extracts with hydrogen peroxide reduced the damage. The DNA migration changed with the increment of the concentrations; specially, at 100 μg/ml of the extract indicated protecting effect against DNA damage in contrast to the control.

Table 30. H_2O_2 scavenging activity of enzymatic extracts from microalgae

Extract.	*P.duplex*	*D. fasci*	*H.porpy*	*O.unicell*	*A.longi*	*Navicula*	*A. coffe*
Viscozyme	32.5±1.3	32.4±1.7	38.4±1.3	11.3±0.6	10.8±0.2	16.2±0.6	19.4±0.3
Celluclast	33.2±1.6	24.8±1.3	19.4±0.9	17.6±0.7	4.5±0.1	15.1±0.4	17.8±0.6
AMG	33.1±1.7	34.6±1.3	26.4±1.2	20.9±1.1	5.3±0.3	19.4±0.5	10.5±0.7
Termamyl	60.6±3.7	32.5±1.5	27.1±1.6	20.5±0.9	7.1±0.4	16.4±0.7	16.7±0.7
Ultraflo	30.3±1.4	31.3±1.2	22.9±1.4	12.8±0.7	10.1±0.7	24.9±0.5	15.4±0.4
Protamex	34.1±2.1	48.6±2.2	34.8±1.7	10.1±0.1	10.9±0.7	12.4±0.2	17.3±0.8
Alcalase	48.3±1.9	27.6±0.7	42.1±2.1	27.1±0.5	8.2±0.6	15.4±0.9	21.1±1.1
Flavozyme	37.1±1.3	24.9±0.4	17.9±0.7	5.1±0.04	11.3±0.7	11.6±0.1	21.1±1.2
Neutrase	42.2±1.5	40.6±1.4	25.2±1.1	24.2±1.1	12.1±0.9	23.4±0.2	22.1±1.3
Kojizyme	27.2±0.9	50.2±2.1	16.1±0.6	12.2±0.14	7.7±0.2	18.9±0.8	13.9±0.9

Sample concentration is 2 mg/ml and M±SE of determinations was made in triplicates

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

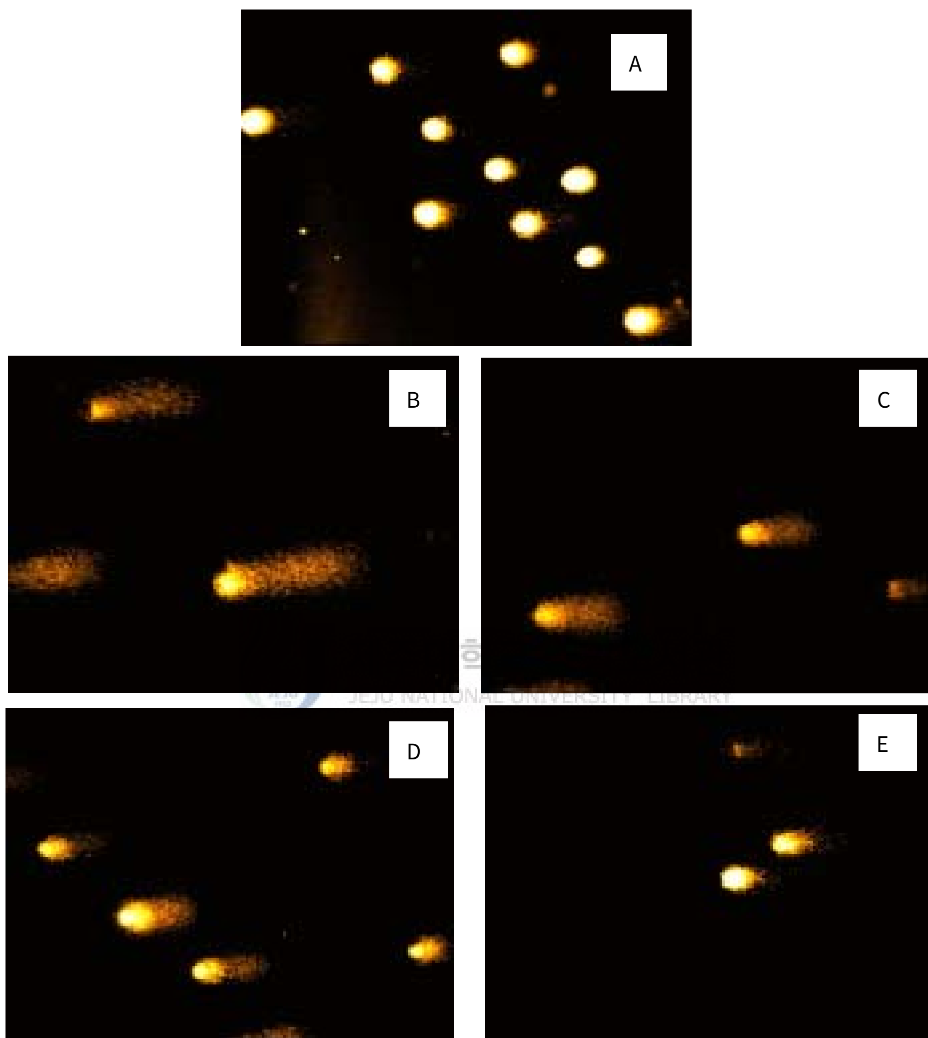


Fig. 19. Comet images of L 5178 cells for Termamyl extract of *P.duplex* (a) negative control; (b) cells treated with 50 μM H_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

JEJU NATIONAL UNIVERSITY LIBRARY

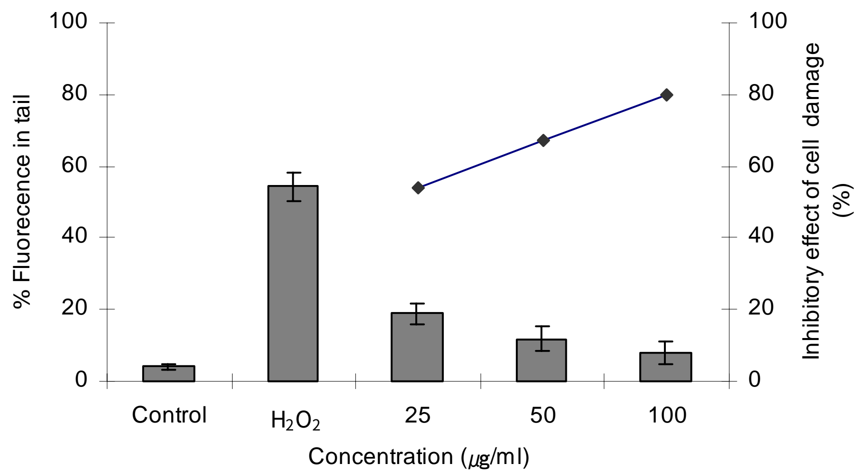


Fig. 20. The effect of supplementation *in vitro* with different concentrations of Termamyl extract of *P.duplex* on DNA damage of H_2O_2 induced L 5178 cells.

JEJU NATIONAL UNIVERSITY LIBRARY

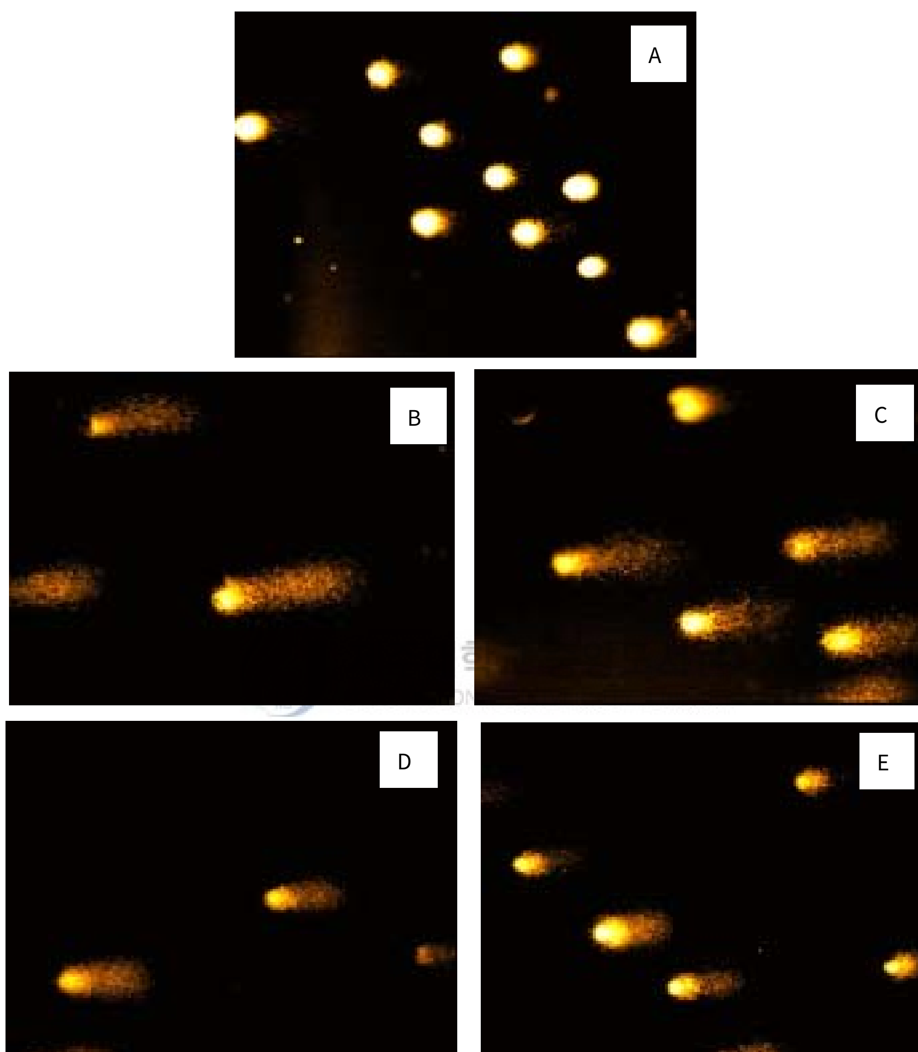


Fig. 21. Comet images of L 5178 cells for Kojizyme extract of *D. fascicularis*: (a) negative control; (b) cells treated with 50 μMH_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

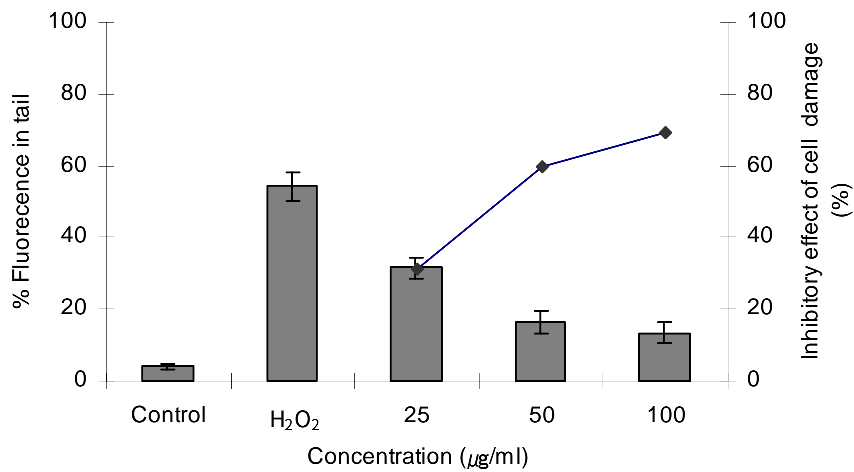
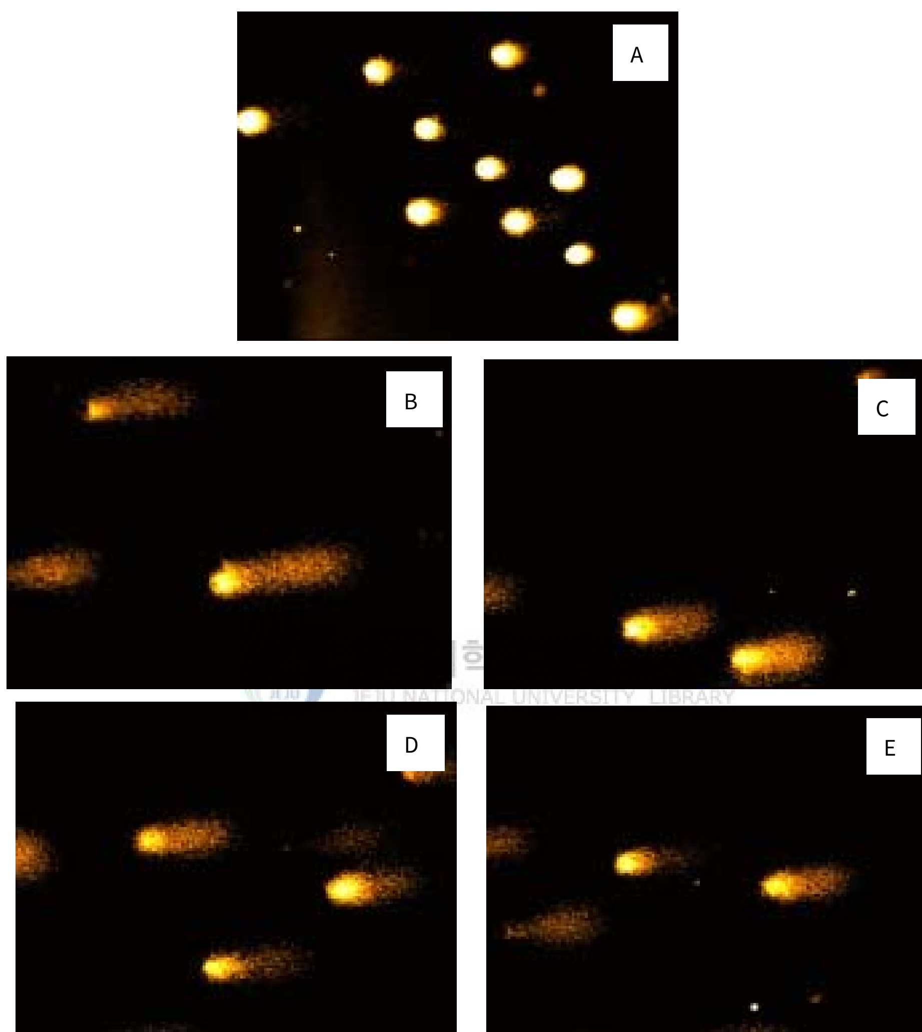


Fig. 22. The effect of supplementation *in vitro* with different concentrations of Kojizyme extract of *D. fascicularis* on DNA damage of H_2O_2 induced L 5178 cells.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY



JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 23. Comet images of L 5178 cells for Alcalase extract of *H. porphyrae*: (a) negative control; (b) cells treated with 50 μM H_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

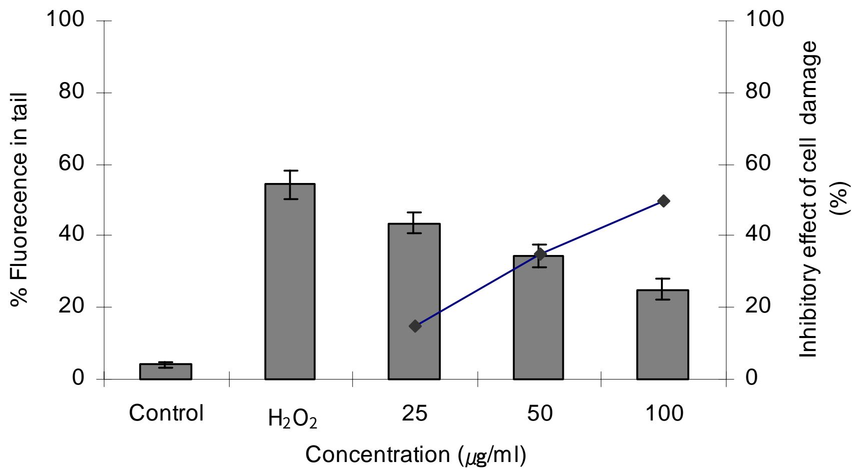


Fig. 24 . The effect of supplementation *in vitro* with different concentrations of Alcalase extract of *H .porphyrae* on DNA damage of H_2O_2 induced L 5178 cells.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

50 μM H_2O_2.

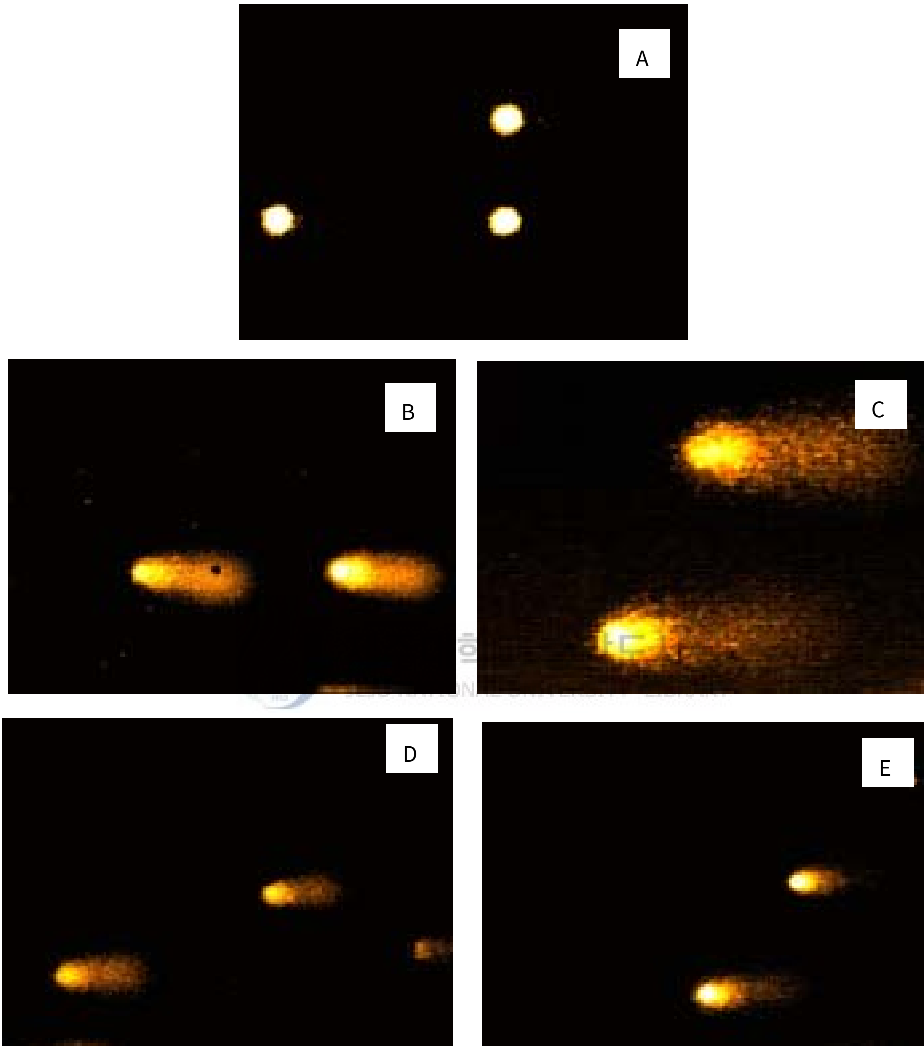


Fig. 25. Comet images of L 5178 cells for Alcalase extract of *O.unicellularis*:(a) negative control; (b) cells treated with 50 μMH_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

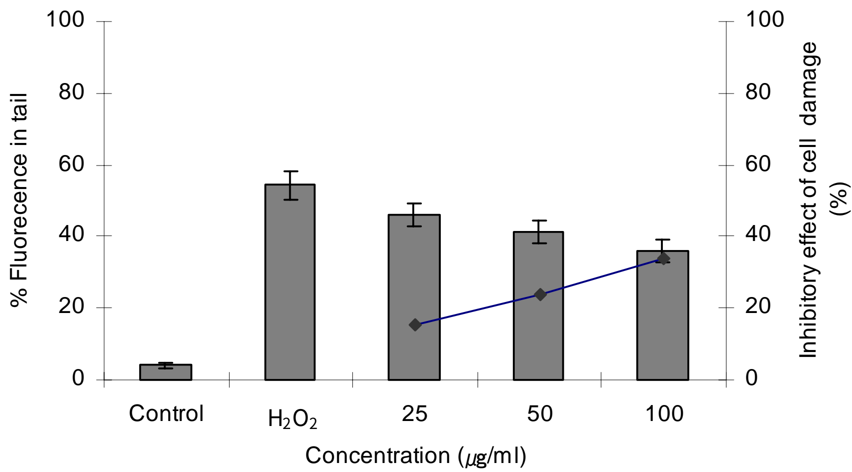


Fig. 26. The effect of supplementation *in vitro* with different concentrations of Alcalase extract of *O.unicellularis* on DNA damage of H_2O_2 induced L 5178 cells.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

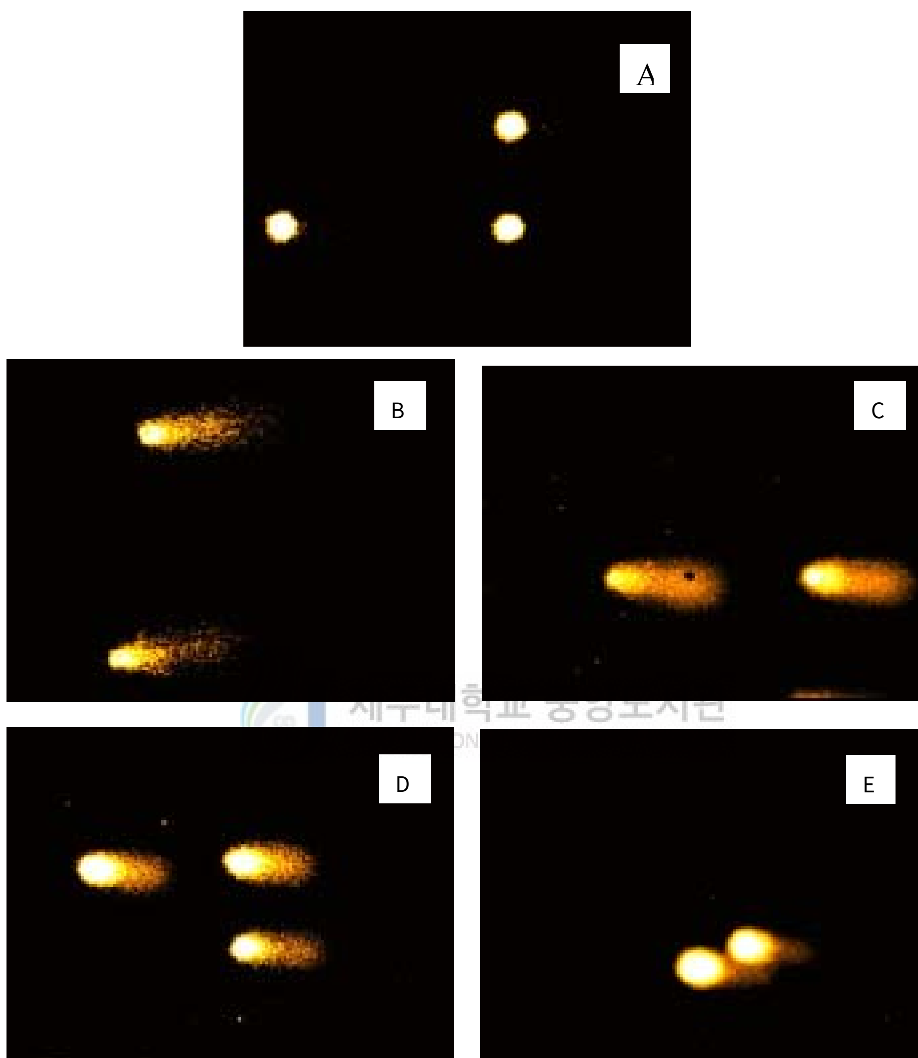


Fig. 27. Comet images of L 5178 cells for Neutrase extract of *A. longipes*: (a) negative control; (b) cells treated with 50 μM H_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μMH_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

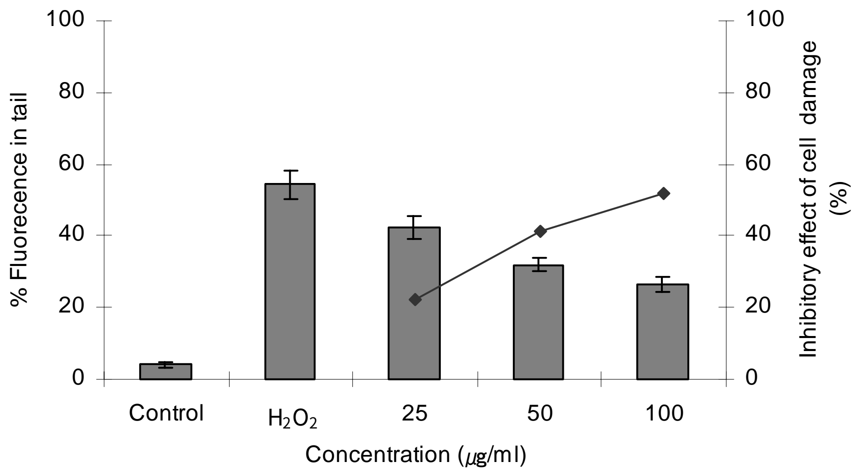


Fig. 28. The effect of supplementation *in vitro* with different concentrations of Neutrase extract of *A. longipes* on DNA damage of H_2O_2 induced L 5178 cells.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

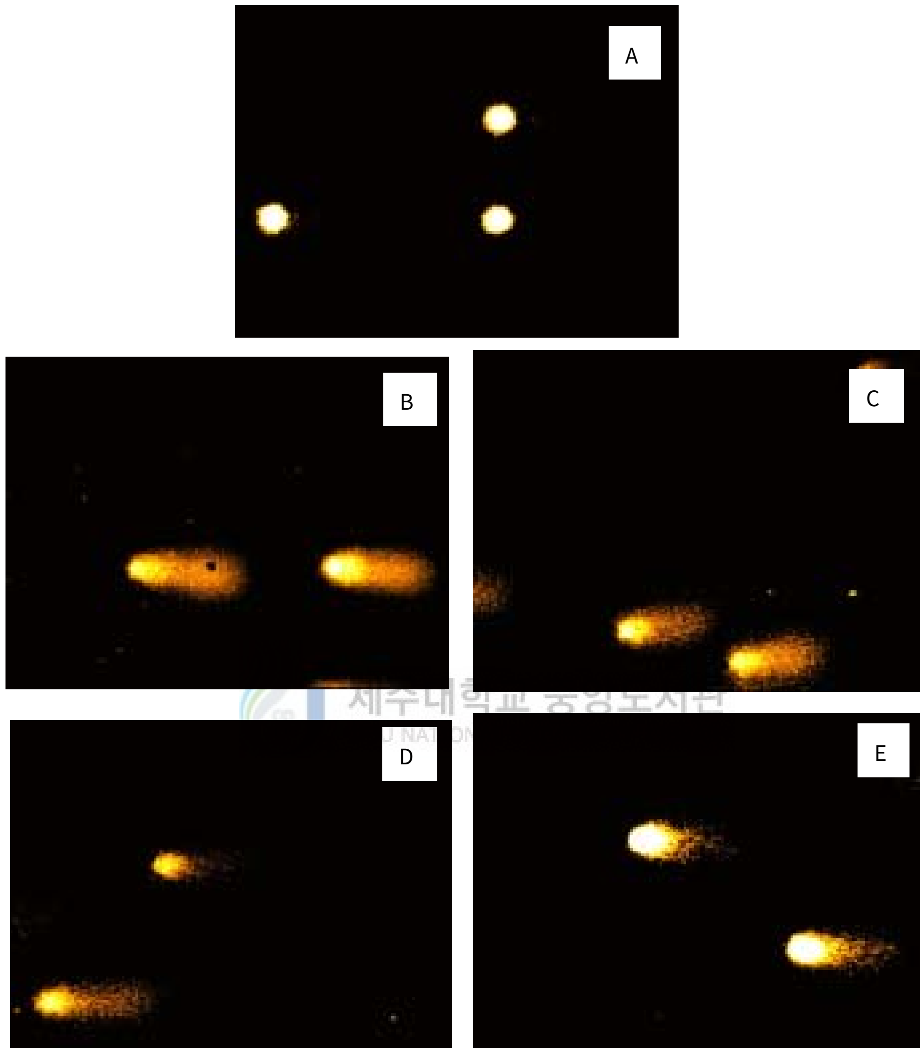


Fig. 29. Comet images of L 5178 cells for Ultraflo extract of *Navicula* Sp. (a) negative control; (b) cells treated with 50 lM H_2O_2 (c) cells treated with 25 µg/ml of extract+ 50 µM H_2O_2 (d) cells treated with 50 µg/ml with extract+ 50 µM H_2O_2 (E) cells treated 100 µg/ml with extract+ 50 µM H_2O_2.

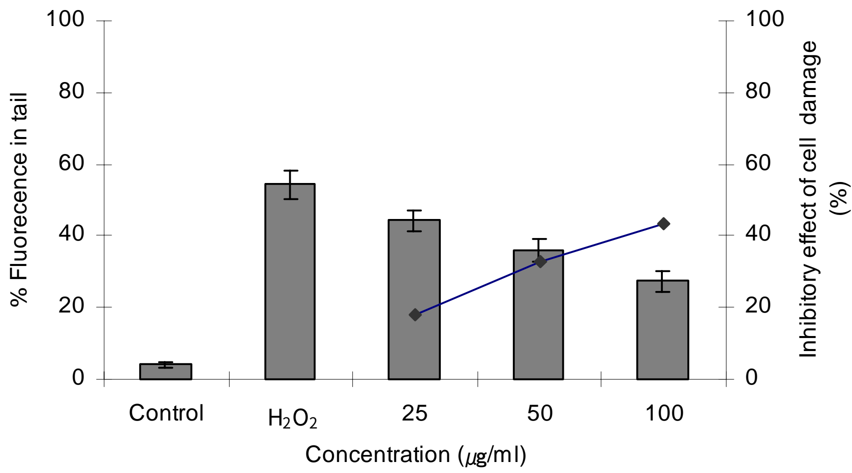


Fig. 30. The effect of supplementation *in vitro* with different concentrations of Ultraflo extract of *Navicula* Sp. on DNA damage of H_2O_2 induced L 5178 cells.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

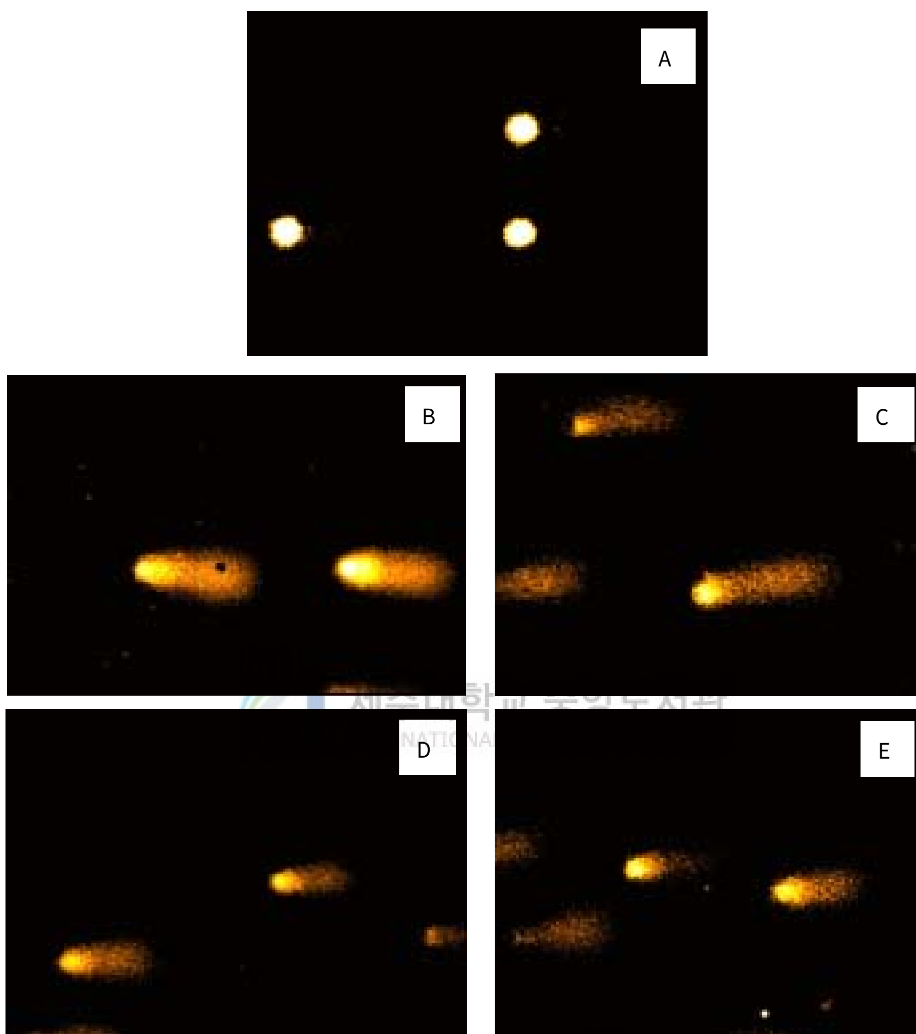


Fig. 31. Comet images of L 5178 cells for Neutrase extract of *A. coffeaeformis*: (a) negative control; (b) cells treated with 50 μM H_2O_2 (c) cells treated with 25 μg/ml of extract+ 50 μM H_2O_2 (d) cells treated with 50 μg/ml with extract+ 50 μM H_2O_2 (E) cells treated 100 μg/ml with extract+ 50 μM H_2O_2.

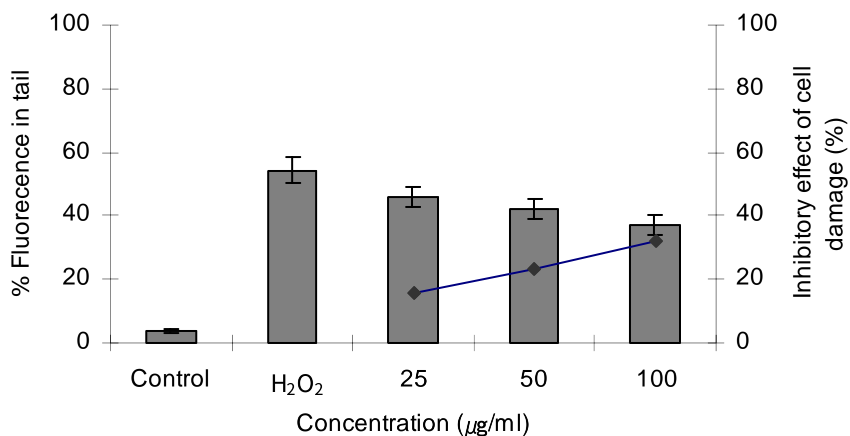


Fig. 32. The effect of supplementation *in vitro* with different concentrations of Neutrase extract of *A. coffeaeformis* on DNA damage of H_2O_2 induced L 5178 cells.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

4. DISCUSSION

DNA damage is well known to be one of the most sensitive biological markers for evaluating oxidative stress caused by ROS, smoke heat, toxic chemicals and ultraviolet light (Kassie et al., 2000). The sequence of the DNA base pares can be changed and leads errors/ disorders in replicating DNA if the damage could not be repaired by the repair mechanisms of existing DNA. The DNA damage of cultured rat lymphocytes was artificially induced by H_2O_2 and the ability of microalgae enzymatic extract to inhibit the damage was extrapolated by the fluorescence intensity of the tail extent movement using comet assay.

Comet assay (single cell gel electrophoresis), which is a rapid and sensitive fluorescence microscopic method for detection of primary DNA damage on the individual cell level, is extensively used to evaluate the genotoxicity of test substances (Olive et al., 1990;Fairbairn et al., 1995).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Several studies have revealed both exogenous and endogenous materials inducing DNA damage, which can lead to the diseases such as cancer and heart disease (Singh et al., 1995). In addition, many studies have been done on inhibition of DNA damage by food materials such as tea (Zhang et al., 2002), juice (Park et al., 2003), plant extract (Zhu and Loft, 2001), and flavonoid (Senthilmohan et al., 2003).

In this study enzymatic extracts from the microalgae was investigated as natural water solube antioxidant sources. In this study, Termamyl extract of *P.duplex,* Kojizyme extract of *D. fascicularis,* alcalase

extract of *H. porphyrae*, Neutrase extracts of *A.longipes*, showed higher inhibition effect compared to rest of extracts. Thus, can be suggested that fresh water microalgae have higher DNA damage inhibitory effect than that of tidal pool and benthic species. Therefore, those enzymatic extracts can be applied in food and pharmaceutical industry. Further studies are needed for the identification of potential antioxidant compounds responsible for those effects.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Part IV.

Isolation and furification of pigments from microalgae

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1. ISOLATION AND PURIFICATION OF FUCOXANTHIN FROM A BENTHIC DIATAM, *Achnanthes longipes*

1.1. ABSTRACT

An effective method for the isolation and purification of fucoxanthin from diatoms was developed. Fucoxanthin was extracted in large quantity from the marine benthic diatom, *Achnanthes longipes*. Concentrated acetone extract subjected to silica gel chromatography and flash chromatography. Purity of purified fucoxanthin was determined by HPLC. Purity was 95%.

1.2. MATERIALS AND METHODS

1.2.1. Materials

Standard fucoxanthin was obtained from The International Agency for 14C Determination DHI Institute for Water and Environment in Denmark, isolated from following algae classes. Sillica gel 60 F_{254} gel and TLC aluminum sheets (silica 60 F_{254} gel-coated) purchased from Merck. All the other chemicals used were in analytical grade.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.2.2. Extraction and chromatographic analysis

Extarction and purification process was performed acrding to Yan et al. (1999) with modifications. The freeze-dried *A. longipes* (50 g) was extracted with acetone by grinding and sonication (20 min., 20 kHz) in darkness at 4 °C for 2 hours, and finally filtered to remove cellular particle debris. The extraction procedure was repeated until the extract was almost colorless. After filtration, the extract was evaporated to small volume and

subjected to chromatographic analysis. Concentrated filtration applied in to a silica gel column (450 mm, 50 mm, Silica 60 F_{254}-gel) and eluted by hexane: ethyl acetate mixture (5.5-4.5, V/V). Thereafter, the fractions with the colored bands developed in the column were collected and active fractions were identified by DPPH free radical scavenging assay. Active fractions bright orange in color was collected and concentrated in a vacuum evaporator and further separated by flash chromatography in a silica gel column (300 mm, 22 mm), eluting with chloroform: acetone (10-1, V/V). The major pigmented bands was collected and combined together according to TLC separations [Silica 60 F_{254}-gel coated aluminum sheets; eluent: hexane: ethyl acetate (5.5-4.5, V/V)]. Thereafter, the major pigmented band was purified once again by the flash chromatography. The purity of fucoxanthin was determined by HPLC.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

HPLC were performed on a Dionex P 680 pumping system with a reverse phase ODS column (C_{18} column: dimention; 250 X 4 mm: partical size; 5, Waters) according to mantoura and Llewellyn (1983) The solvents used for chromatography were filtered and degassed with helium before use. Mobile phases used in the gradient elution consisted of primary eluant (A) consisting of methanol and 1 M ammonium acetate (80:20 v/v), and a secondary eluant (B) consisting of methanol and acetone mixed in the proportion 60:40 (v/v). 1 M ammonium acetate was used as an ion-pairing reagent, and it is recommended that it be present in both the sample and mobile phase to improve pigment separation and suppressed dissociation of

isolated compounds. The separation was carried out by changing the solvent mixture composition according to Barlow (Barlow et al. 1993). 100% solvent A changed, in the linear gradient, to 100% of B 10 minutes after injection, and then during 15 minutes 100% of B was isocratically held at a constant flow rate of 0.8 mL min^{-1}. After 25 minutes of analysis, the solvent composition returned to its initial conditions in 10 minutes.

1.3. RESULTS

Acording to Fig. 35, it was shown that fucoxanthin was the major pigment in acetone extract of *A.longipes.* Further, the carotenoid pigment after purification identifies as fucoxanthin with comparison with standards fucoxanthin. Along with purified fucoanthin, there were three miner components. The purity of purifified fucoxanthin was 95% and yield was 48 μg/g.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

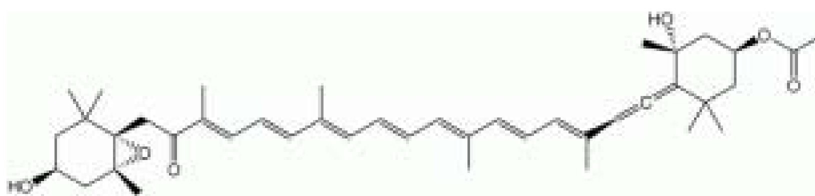


Fig. 33. Chemical structures of fucoxanthin

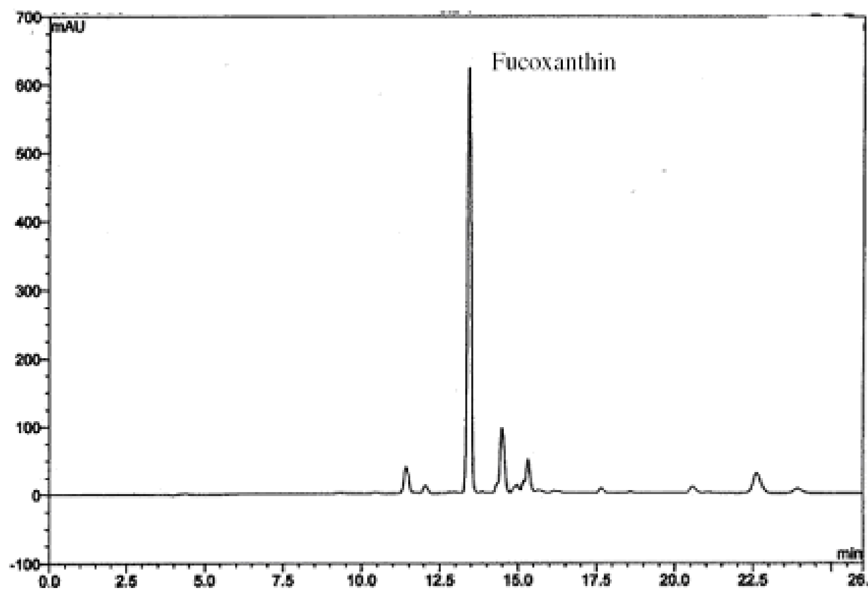


Fig. 34. Chromatogram of acetone extract of *A.longipes*

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

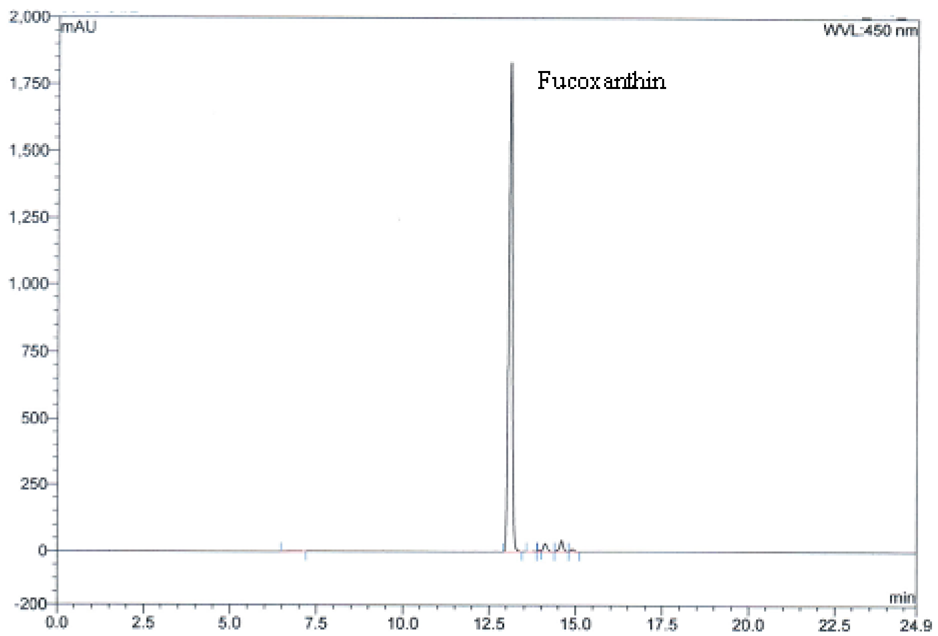
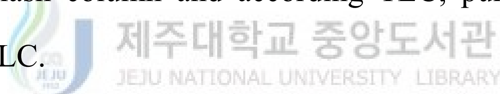


Fig. 35. Chromatogram of purified fucoxanthin from *A.longipes*

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

1.4. DISCUSSION

Acetone was selected for the extraction of pigments because of its higher extraction efficiency. Since fucoxanthin is a medium polarity pigment "hexane: ethyl acetate" mixture (5.5-4.5, V/V) was selected as an eluent for better recovery. Active fractions were selected according to bright orange color and DPPH radical activity. In addition using TLC the presence of fucoxanthin was confirmed in particular fractions. Flash column chromatography was selected for further purification because it was a quick and easy way to separate complex mixtures of compounds. Since fucoxanthin is a medium polarity, pigment eluted with chloroform: acetone (10-1, V/V) for best results. Purified fractions applied two times for further purifications in flash column and according TLC, purified fractions were undergone on HPLC.


제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fucoxanthin (Fig. 1) one the most abundant carotenoids in nature, especially in brown algae and diatoms, and its chemopreventive effects are well established. Carotenoids, including fucoxanthin have been demonstrated to have beneficial effects on health, such as anticarcinogenesis and antimutagenesis. Fucoxanthin attenuated neuronal cell injury in hypoxia and re-oxygenation, which is an experimental ischaemic model *in vitro*. Oxygen radical generation is considered to occur after hypoxia and re-oxygenation, whereby free radicals damage neurons. Nomura et al. (1997) documented that the quenching activity of carotenoids, including fucoxanthin, was greater than that of α-tocopherol under anoxic conditions. A previous study also

demonstrated that tocopherol attenuated neuronal cell injury in hypoxia and re-oxygenation. Therefore, the preventive effect of fucoxanthin against neuronal cell injury may be caused by its radical-scavenging activity.

Ikeda et al. (2003) reported that in cultured neuronal cells from stroke-prone spontaneously hypertensive rats (SHRSP), fucoxanthin isolated from Wakame powder significantly attenuated neuronal cell injury in hypoxia and re-oxygenation. Fucoxanthin in Wakame may be protective against ischaemic neuronal cell death seen in SHRSP with stroke. These results suggest that Wakame protects against the development of neurological events and prevents against the development of stroke in SHRSP. This protective effect is independent of hypertension. Thus, in conclusion, it seems valuable to investigate the industrial utilization of fucoxanthin from diatom sources for pharmaceutical industry.

제주대학교 중앙도서관

2. ISOLATION AND PURIFICATION OF LUTEIN FROM THE MICROALGA, *Halochlorococcum porphyrae*

2.1. ABSTRACT

An effective method for the isolation and purification of lutein from the *Halochlorococcum porphyrae* was developed. Crude lutein was obtained by extraction with dichloromethane from the microalga after saponification. The water-soluble impurities in the crude lutein were removed by washing with 30% aqueous ethanol, and the fat-soluble impurities were removed by extraction with hexane.

2.2. MATERIAL AND METHODS

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Standard lutein, vitamin C, and potassium hydroxide were obtained from Sigma Chemical Co. (St. Louis, MO). Other solvents were in HPLC grade. The tidal pool microalga, *H porphyrae* was provided by the Dept. of oceanography.

2.2.1. Saponification and extraction of Lutein

Preparation of crude lutein was carried out according to the literature with modifications. As mentioned by Li et al., (2001) 500 mL of 5.0M KOH solution containing 2.5% ascorbic acid was added to 50 g of the freeze dried algal cells, and the mixture was incubated at 60 °C for 40 ºC min under shaking condition and then was cooled to room temperature. Dichloromethane (500 ml) was added to the mixture to extract lutein. Lutein

extract was separated by using separately funnel. The extraction procedure was repeated until the extract was almost colorless, and all extracts were combined. The extract was washed with 30% aqueous ethanol (v/v) until the water phase was almost colorless and the pH was near neutral. After separation, the organic phase was dried by rotary vaporization at 40°C. The residue was redissolved in 85% aqueous ethanol (v/v). The fat-soluble impurities were extracted with *n*-hexane. After separation, the concentration of ethanol in the water phase was diluted to precipitate the lutein. Purified Lutein was separated by filtration with a 0.45 ㎛ filter membrane (Millipore Co. Bedford, MA) was dried.

2.2.2. Determination of Lutein

The crude sample and purified lutein were analyzed by HPLC according to the method of Shi and Chen (1999). The HPLC system used throughout this study consisted of a Dionex P 685 pump, a sample injector with a 20㎕ loop. The column used was a reversed phase ultrasphere C_{18} Colum (250X4.6mm, 5µm, Waters, USA) The mobile phase was (Methanol/dichloromethane/acetonitrile/water (67.5:22.5:9.5:0.5, v/v), and the flow rate was 1.0 ml/min. The effluent was monitored at 450 nm.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2.3.RESULTS

The HPLC profile of the crude Lutein and purified Lutein was shown in Fig. 37 and Fig. 38. As revealved Fig. 38, the purified lutein also contains two peaks, as impurities and it was believed those were isomers of Lutein. The purity was 77% and the yield was 412 ㎍/g.

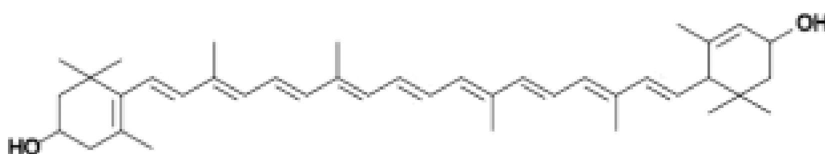
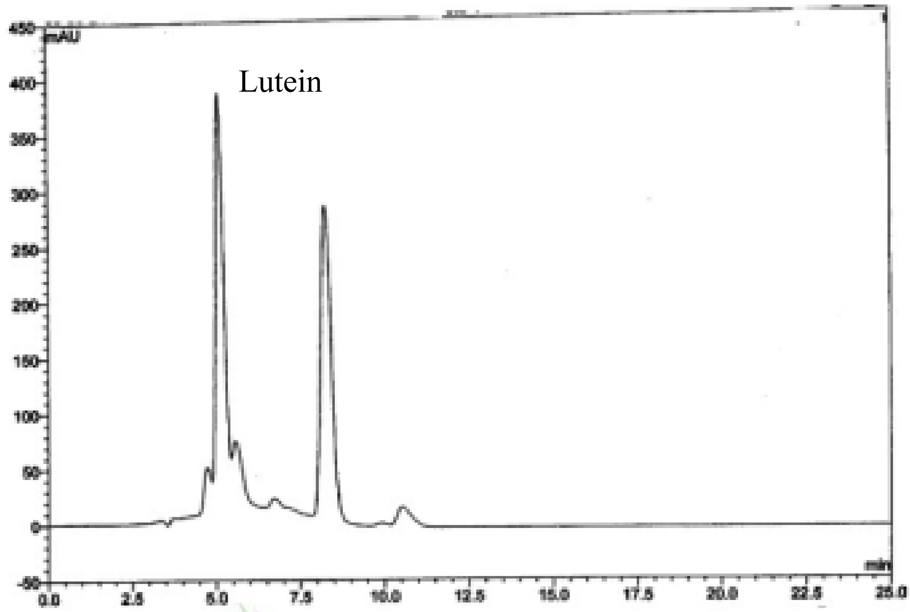


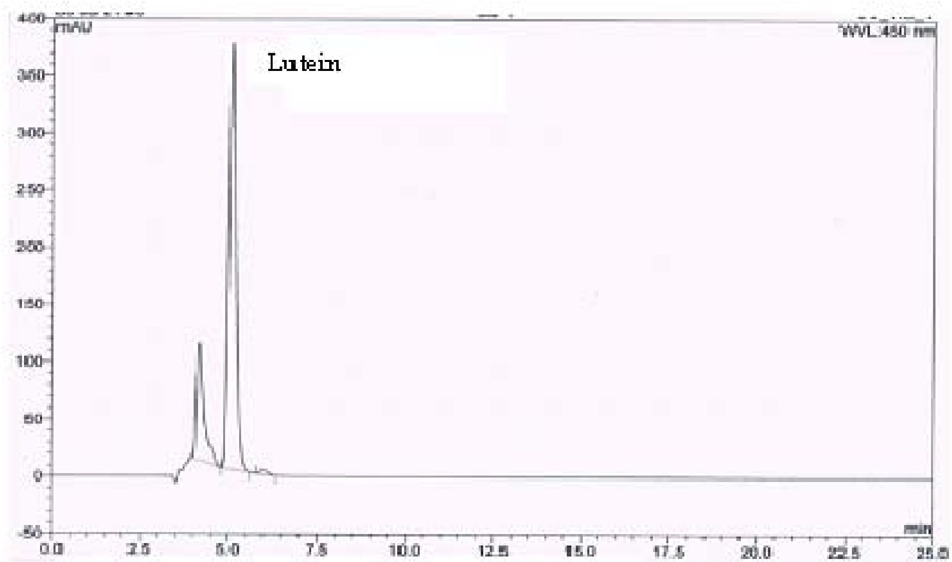
Fig. 36. Chemical structure of lutein.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY



제주대학교 중앙도서관

Fig. 37. Chromatogram of crude Lutein obtained by extraction after sponification.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 38. Chromatogram of purified lutein.

2.4. DISCUSSION

Lutein fatty acid esters were converted to lutein by saponification. As an antioxidant to prevent oxidation of lutein acorbic acid was used in this study. Dichloromethane was chosen because of the excellent solubility of lutein in this solvent. If saponification is carried out after lutein fatty acid esters have been extracted from the material, the algal cells should be dried and ruptured before extraction with organic solvents in order to enhance lutein extractability. Thus, the extraction after saponification was adopted in the present process. The crude extract contained water-and fat-soluble impurities and needed to be purified further. Therefore, the dichloromethane extract was washed with 30% aqueous ethanol until the water phase was almost colorless and the pH was near neutral. After separation, the organic phase was dried by rotary vaporization at 40 °C. Thereafter, the lutein containing fat-soluble impurities was dissolved in 85% aqueous ethanol, and the fat-soluble impurities were extracted theree times with *n*-hexane. After separation, the concentration of ethanol in water phase was diluted to with distilled water in order to precipitate lutein. The lutein obtained by filtration with a 0.45㎛ filter membrane (Millipore Co., Bedford, MA) was lyophilized to dryness, and its purity was determined by HPLC. In addition to the major peak, there were two miner peaks. It is suggested that those peaks were isomers of lutein. Therefore, further purification is need if necessary.

Lutein is a naturally occurring caroteneoid that has no vitamin A activity. There has been recent evidence to suggest that lutein, one of the

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

most abundanr carotenoids in the diet and in human blood posses, strong antioxidant effects, and may usefull in reduction of the incidence of cancer. Lutein is mostly available in green vegitable and fruits such as broccoli, gren beans, green peas, lima beans, cabbage, kale as well in in yellow/orange fruits such as mango, papaya, winter squash and oranges. The lutein in these vegetables and fruits exists naturally in the free non-esterified form and co-exists with other carotenoids.

However, the isolation and purification of pure lutein in large quantities from vegetables and fruits is not economical because it needs many expensive and time-consuming purification steps. As a solution, previously marigold flowers were used as raw material for extracting lutein (*1*, *9*). On the other hand, marigold flowers might contain herbicide and pesticide residues, which are harmful to the health of human beings. In the present method, the alga was adopted as material for extracting lutein, which should not contain any herbicides or pesticidess or any other toxic substances because the nutrient medium for the algae could be well controlled.

제주대학교 중앙도서관

In conclusion, *H. porphyrae* can be a potential substitute for extracting pure lutein without any trace of biohazard chemicals.

REFERENCES

Affan, M. A., and Lee, J.B. (2004), Seasonal characteristics of phytoplankton dynamics and environmental factors in the coast of Mara-do and U-do, Jeju Island, Korea. *Algae*, 9, 235-245.

AOAC .1990. Official Method of Analysis of the Association of Official Analitical Chemists. 15th edn,Washington DC: AOAC.

Barlow, R.G., Mantoura, R.F.G., Gough, M.A. and Fileman T.W. 1993. Pigment signatures of the phytoplankton composition in the north-eastern Atlantic during the 1990 spring bloom. *Deep Sea Res*. 40:459-477.

Brand, L. E. 1984. The salinity tolerance of forty-six marine phytoplankton isolates. *Estuar. Coast. Shelf Sci*. 18:543–56.

Brand-Williams, W., Cuvelier, M. E., and Berset, C. 1995 Use of a free radical method to evaluate antioxidant activity. *Food Sci. Technol*. 28, 25-30.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Chandler, S.F. and Dodds, J.H. 1993. The effect of phosphate, nitrogen, and sucrose on the production of phenolics and solasidine in callus cultures of Solanum laciniatum. *Plant Cell Reports* 2:105-110.

Chandler, S.F. and Dodds, J.H. 1993. The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasidine in callus cultures of *Solanum laciniatum. Plant Cell Reports* 2:105-110.

Chung, S.K., Osawa, T. and Kawakishi, S.1997. Hydroxyl radical-scavenging effects of spices and scavengers from black mustard (*Brassica nigra*) *Bioscience. Biotechnology, Biochemistry* 61:118-123.

Chung, S.K., T. Osawa and S. Kawakishi.1997. Hydroxyl radical-scavenging effects of spices and scavengers from black mustard (*Brassica nigra) Biosci. Biotech, Biochem* 61:118-123.

Dahl, M.B.K. and Richardson, T. 1978. Activated oxygen species and oxidation of food constituents. *Crit Rev Food Sci Nutr*.10: 209–41.

Decker, E. A. and Welch B. 1990. Role of ferritin as a lipid oxidation catalyst in muscle food. *J. Agric. Food Chem* 38:674-677.

Decker, E. A. and Welch, B. 1990. Role of ferritin as a lipid oxidation catalyst in muscle food. *J. Agric. Food Chem.* 38:674-677.

Fairbairn, D.W., Olive, P.L. and O Neill, K.L. 1995. The comet assay: a comprehensive review. *Mutat. Res*. 339:37–59.

Field, C. B., Behrenfeld, M. J., Randerson, J.T., and Falkowski, P. G. 1998. Primary production of the bio-sphere: Integrating terrestrial and oceanic components. *Science* 281: 237-240.

Fridovich, I. 1995. Superoxide Radical and Superoxide Dismutases. *Annual Review Biochemistry* 64:97-112.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fridovich, I. 1995. Superoxide Radical and Superoxide Dismutases. *Annu Rev Bioche*. 64:97-112.

Garrat, D.C. 1964. The Quantitative Analysis of Drugs, pp.456–458, Chapman and Hall, Japan.

Garrat, D.C. 1964. The Quantitative Analysis of Drugs, vol. 3. Chapman and Hall, Japan, pp.456–458.

Gülçin, l., Beydemir, S., Ahmet, H.A., Elmasta, M., Büyükokuroglu, M.E.,2004. In vitro antioxidant properties of morphine. *Pharmacol. Res*. 49:59-66.

Halliweill, B. 1991. Reactive oxygen species in living systems: Source, biochemistry, and role in human disease. *Ame. J. of Medicine*. 91:14-19.

Halliwell, B. and Gutteridge, J.M. 1989. Free radical in biology and medicine. Clarendon Press, Oxford. pp. 23–30.

Hatano, T. 1995. Constituents of natural medicines with scavenging effects on active oxygen species. Tannins and related polyphenols. *Nature Medicine (Tokyo)* 49:357-363.

Hirata, T., Tanaka, M., Ooike, M., Tsunomura, T. and Sakaguchi, M. 2000. Antioxidant activities of phycocyanobilin prepared from *Spirulina platensis. J. Appl. Phycol*. 12**:**435–439.

Hoshiai, G., Suzuki T., Kamiyama, T.,Yamasaki, M. and Ichimi, K. 2003. Water temperature and salinity during the occurrence of Dinophysis fortii and D. acuminate in Kesennuma Bay, northern Japan, *Fisheries Science* 69: 1303-1305.

Ikeda K., Kitamura, A., Machida, H. and Watanabe, M, Negishi, H., Hiraoka, J. and Nakano, T. 2003.Effect of *Undaria pinnatifida* (Wakame) ON the development of cerebrovascular diseases in stroke-prone spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol*. 30:44 - 48.

Jeon, Y.J., Byun, H.G. and Kim, S.K. 2000. Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. *Process Biochem*. 35: 471-478.

Kassie, F., Pqrzefall, W. and Knasmuller, S. 2000. Single cell gel electrophoresis assay: a new technique for human biomonitoring studies. *Mutat. Res*. 463:13–31.

Khachik, F. 1995. Process for isolation, purification, and recrystallization of lutein from saponified marigolds oleoresin and uses thereof. U.S. Patent 5,382,714.

Kikuzaki, H. and Nakatani, N. 1993. Antioxidant effects of some ginger constituents. *J Food Sci*. 58:1407-10.

Kitada, M., Igarashi, K., Hirose, S. and Kitagawa, H. 1979. Inhibition bypolyamines of lipid peroxidase formation in rat liver microsomes. *Biochem. Biophys. Res. Commun***.** 87:388-394.

Li, H. B., Chen, F., Zhang, T. Y., Yang, F. Q., Xu, G. Q. 2001. Preparative isolation and purification of lutein from the microalga *Chlorella* V*ulgaris* by high-speed counter-current chromatography. *J. Chromatogr. A.* 905:151-155.

Liang S., Liu X., Chen, F. and Chen, Z. 2004. Current microalgal health food R and D activities in China. *Hydrobiol.* 512:45-48.

Liu, F. and Ng, T.B. 2000. Antioxidative and free radical scavenging activities of selected medicinal herbs. *Life Sciences* 66:725-735.

Madhavi, D.L., Deshpande, S.S. and Salunkhe, D.K. 1996. Food Antioxidants. New York: Basel. pp. 1-4.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Mantoura, R.F.C. and Llewellyn, C.A. 1983. The rapid determination of algal chlorophyll and caroteneoid pigments and their breakdown products in natural waters by reverse- phase high performance liquid chromatography. *Analyt.chim. acta.* 151:297-314.

Marcocci, P.L., Sckaki, A. and Albert, G.M.1994. Antioxidant action of Ginkgo biloba extract EGb 761. *Methods Enzymol*. 234:462–475.

Moncada, S., Palmer, R.M. and Higgs, E.A.1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev*. 43:109-142.

Muller, H.E. 1995. Zentralbl Bakterio. *Mikrobio Hyg*. 259:151-158.

Murakami, Y., Oshima, Y. and Yasumoto, T. 1982. Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima*. *Bulletin of the Japanese Society of Scientific Fisheries* 1982,:48:69-72.

Mynderse, J. S., Moore, R. E., Kashiwagi, M. and Norton, T. R.1977. Antileukemia activity in the Oscillatoriaceae, isolation of debromoaplysiatoxin from *Lyngbya. Science* 196:538-540.

Nagai, T. and Suzuki, N. 2000. Isolation of collagen from fish waste material-skin, bone and fins. *Food Chem.* 68:277–281.

Nagai, T., Inoue, I., Inoue, H. and Suzuki, N. 2003. Preparation and antioxidant properties of water extract of propolis. *Food Chem.* 80:29-33.
Nakamura, T., Nagayama, K., Uchida, K. and Tanaka, R. 1996. Antioxidant activity of phlorotannins isolated from the brown alga *Eisenia bicyclis. Fish Sci.* 62:923–926.

Nakano, T., Watanabe, M., Sato, M. and Takeuchi, M. 1995. Characterization of catalase from the seaweed *Porphyra yezoensis. Plant Sci.* 104**:**127-133.

Nishino, H. 1995. Cancer prevention by carotenoids. Mutat Res 402, 159-163.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Nomura, T., Kikuchi, M., Kubodera A. and Kawakami, Y. Proton-donative antioxidant activity of fucoxanthin with 1,1-diphenyl-2-picrylhydrazyl (DPPH). *Biochem. Mol. Biol. Int.* 42: 361–70.

Officer, C.B and Ryther, J.H. 1980. The possible importance of silicon in marine eutrophication. *Mar. Ecol. Prog. Ser.*3: 83-91.

Oktay, M., Gülçin, I. and Küfrevioˇglu, Ö.˙I. 2003. Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. *Lebensmittel-Wissenchaft und Technologie* 36**:**263–271.

Olive, P.L., Banath, J.P. and Durand, R.E. 1990. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the ‘‘Comet’’ assay. *Radiat. Res*. 122**:**86–94.

Oyaizu, M. 1986. Products of the browning reaction. Antioxidative activities of products of the browning reaction of glucosamine. *Jpn J. Nutr.* 44:307–315.

Park, Y.K., Park, E.J., Kim, J.S. and Kang, M.H. 2003. Daily grape consumption reduces oxidative DNA damage and plasma radical levels in healthy Koreans. *Mutat. Res*. 529, 77–86.

Radi, R, Beckman, J.S., Bush, K.M. and Freeman, B.A. 1991. Peroxynitrite induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* 288: 481-487.

Ramos, E.A.P. and Xiong, Y.L. 2002. Antioxidant Activity of Soy Protein Hydrolysates in a Liposomal System. *J. Food Sci*.67: 2952-2956.
Ramos, E.A.P. and Xiong, Y.L. 2002. Antioxidant activity of soy protein hydrolysates in a liposomal system. *J. of Food Sci*. 67:2952-2956.

Richmond A. 2004. Handbook of Microalgal Culture Biotechnology and Applied Phycology. Blackwell publishing, Australia, pp. 179-203.

Ruch, R. J., Chung, S. U. and Klaunig, J. E. 1984. Spin trapping of superoxide and hydroxyl radicals. *Methods Enzymol.* 105: 198–209.

Rupérez P., Ahrazem O. and Leal J.A. 2002. Potential antioxidant capacity of sulfated polysaccharides from the edible marine brown seaweed *Fucus vesiculosus*. *J. Agric.Food Chem*. 50: 840-5.

Senthilmohan, S.T., Zhang, J., Stanley, R.A. 2003. Effects of flavonoid extract Enzogenol with vitamin C on protein oxidation and DNA damage in older human subjects. *Nutr. Res*. **23**:1199–1210.

Shi, X. M. and Chen, F. 1999. Production and rapid extraction of lutein and the other lipid-soluble pigments from *Chlorella protothecoides* grown under heterotrophic and mixotrophic conditions. *Nahrung* 43:109-113.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Singh, N.P., Graham, M.M., Singh, V. and Khan, A. 1995. Induction of DNA single-strand breaks in human lymphocytes by low doses of X-rays. *Int. Radiat. Biol*. 68**:**563–569.

Spencer, J. P. E., A. Jenner, O.I., Aruoma, C.E. Cross, R. Wu and B. Halliwell. 1996. Oxidative DNA damage in human respiratory tract epithelial cells. Time course in relation to DNA strand breakage. *Biochemical Research Communications* 224:17–22.

Spencer, J. P. E., Jenner, A., Aruoma, O. I., Cross, C. E., Wu, R. and Halliwell, B. 1996. Oxidative DNA damage in human respiratory tract epithelial cells. Time course in relation to DNA strand breakage. *Biochem. Biophys. Res. Commun*. 224**:**17–22.

Spitz, T.T., Bergman, M., Moppes, D., Grossman, S. and Arad, M.S. 2005. Antioxidant activity of the polysaccharide of the red microalga *Porphyridium* sp. *J. App. Phycol*. 17: 215–222.

John, S. M.A., Clemmesen, C., Lund, T. and Koster, T. 2001. Diatom production in the marine environment: Implications for larval fish growth and condition. *J. Mar. Sci*. 58:1106–1113.

Tanaka, M., Kuei, C.W., Nagashima, Y. and Taguchi, T. 1998. Application of antioxidative maillard reaction products from histidine and glucose to sardine products. *Nippon Suisan Gakkaishi* 54**:**1409-1414.

Tanaka, M., Kuei, C.W., Nagashima, Y. and Taguchi, T. 1998. Application of antioxidative maillard reaction products from histidine and glucose to sardine products. *Nippon Suisan Gakkaishi*. **54,**1409-1414.

Thessen, A.E., Michael, Q. and Mossison, L.P.W. 2005. Effect of salinity on Pseudo-nitzschia species (Bacillariophyceae) growth and distribution. *J. Phycol*. 41: 21-29.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Tomas J. H.1996. Effects of temperature and illuminance on cell division rates of three species of tropical oceanic phytoplankton. *J. App. Phycol*. 2:17-22.

Tyczkowski, J. K. and Hamilton, P. B. 1991**.**Preparation of purified lutein and its diesters from extracts of marigold (*Tagetes erecta*). *Poult. Sci.*, 70:651-654.

Vinson, J.A., Yong, H., Xuchui, S., Zubik, L., 1998. Phenol antioxidant quantity and quality in foods: vegetables. *J. Agric. Food. Chem.* 46: 3630–3634.

Yan, X. Chuda, Y. Suzuki, M. and Nagata, T. 1999. Fucoxanthin as the major antioxidant in *Hijikia fusiformis*, a common edible seaweed. *Biosci. Biotechnol. Biochem*. 63: 605-607.

Zhang, H., Spitz, M.R., Tomlinson, G.E., Schabath, M.B., Minna, J.D. and Wu, X. 2002. Modification of lung cancer susceptibility by green tea extract as measured by the Comet assay. *Cancer Detect. Prev*. 26: 411–419.

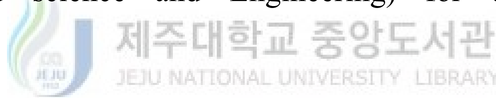
Zhu, C.Y. asd Loft, S. 2001. Effects of Brussels sprout extracts on hydrogen peroxide-induced DNA strand breaks in human lymphocytes. *Food Chem. Toxicol*. 39: 1191–1197.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

ACKNOWLEDGEMENT

First and foremost thanks are due to my supervisor, Prof. You-Jin Jeon, without whose guidance and encouragement this thesis would not be what it is today. His extensive comments, helpful discussions and the interactions cause a direct impact on the final form and quality of this thesis.

I would like to extend my sincere gratitude towards Prof. Joon-Baek Lee (Dept. of Oceanography) for his guidance, invaluable advices and encouragements. Also, I wish to offer my humble gratitude to professors of my department, Prof. Choon-Bok Song, Prof. Moon-Soo Heo, Prof. Jehee Lee and Prof. In-Kyu Yeo for their kind advices and guidance. Further, many thanks to Prof. Ki-Wan Lee (Dept. of Marine Biology), Prof. Soo-Huyn Kim (Dept. of Food science and Engineering) for their kindness and encouragement.



I would like to bow Dr. Nihal Wijesinghe and Dr. Sarananda Hewage for their invaluable advices and kind blessing.

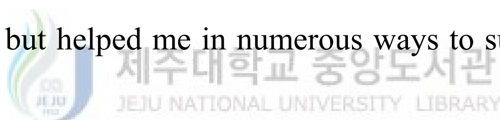
Also, I greatly acknowledge to my lab members: Soo-Jin Heo, Kil-Nam Kim, Yasantha, Seon Heui Cha, Gin-Nae Ahn, Won-Suk Kim, Seong-Hong Lee, Sung-Myung Kang, Hyun- Phyl Yang, Dae-Seung Kim, Won-Woo Lee, Ji-Hyuk Lee, Seok-Chun Ko, Areum Daseul Kim, Eun-San Jo, Suk-Kyoung Lee and Mahinda for their support and kindness.

I feel a deep sense of gratitude for my parents who formed part of my vision and taught me the good deeds that really matter in life. I am

grateful for my sisters and brother for rendering me the sense and the value of brotherhood.

Special thanks to Dr. Nalin Siriwardhana for guiding me to study in Korea and giving much encouragement and invaluable assistance. This work would not have been success without the assistant and helps of Abu Affan (Dept. of Oceanography). Also, I greatly acknowledge all my Korean, Sri Lankan and foreign friends for giving me great assistance and valuable suggestions during my study. Also, Brain Korea and NURI programs are greatly acknowledged for granting me full Ph.D scholarship.

At last but not least I would like to thank all those whom I have not mentioned above but helped me in numerous ways to success my education in CNU.


제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY