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ISOLATION AND CHARACTERIZATION OF POTENTIAL BIOACTIVE COMPOUNDS FROM BROWN ALGA, TOT (*HIZIKIA FUSIFORMIS)*

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요약문

톳*(Hizikiafusiformis*)은 갈조류 모자반과 (Fucales, Pheophyceae)에 속하는 해조류로서 한국과 일본 그리고 유럽의 몇몇 국가에서 광범위하게 소비되고 있는 유용한 해조류이다. 톳(*H. fusiformis*)의 생리활성성분의 효능은 항산화, 항암, 항고혈압 그리고 항응고활성 등에서 연구되어졌고, 그 이후로 이러한 잠재적 생리활성을 갖는 성분들이 정제되고 특징지어졌다.

톳(*H. fusiformis*)의 다양한 fraction (organic and aqueous fractions of n-hexane, chloroform and ethyl acetate)의 항산화 잠재성을 1,1-diphenyl − 2-picrylhydrazyl (DPPH), superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), nitric oxide ($NO^{\cdot}$) scavenging, ferrous ion chelating, 그리고 reducing power assay 등으로부터 평가한 결과는 대부분의 fraction 이 $NO^{\cdot}$와 DPPH free radical 에서 상업용 항산화제 (BHT, α-tocopherol)보다 우수한 소거활성을 나타내었다. 이 fraction 들 중 $NO^{\cdot}$ (IC_{50} : 0.1 mg/ml)와 DPPH free radical (IC_{50} : 0.144 mg/ml)의 소거활성은 n-hexane 분획물에서 가장 높게 나타났으며 Chloroform 분획물에서는 $HO^{\cdot}$ 소거활성이 가장 높게 나타내었다 (IC_{50} : 0.04 mg/ml). Aqueous chloroform fraction 은 BHT 와 비슷하게 우수한 $O_2^{\cdot -}$소거활성 (IC_{50} : 0.12 mg/ml)과 metal chelating (IC_{50} : 0.131 mg/ml) 활성을 나타내었으며, Reducing power 는 aqueous ethyl acetate fraction 에서 가장 높은 활성을 나타내었다.

또한 톳(*H. fusiformis*)은 다섯 가지 상업용 당분해효소 (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo)와 단백질분해효소 (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase)로 가수분해 시켜 DPPH radical 과 H_2O_2 소거방법으로 그들의 항산화 활성을 검정하였다. 그 중에서 Ultraflo (당분해효소)와 Alcalase (단백질분해효소) 가수분해물은 다른 가수 분해물들에 비해 우수한 항산화 효과를 나타내었다. 최적의 가수분해 조건하에서 Ultraflo 와 Alcalase 의 조합은 *H. fusiformis* 의 antioxidant (특히 phlorotannins) 추출효율을 증가시켰고, 추출효율을 강화하기 위해 효소적 가수분해에 pH 조절과 열처리를 동반하였다. 추출은 heat (100 ^{o}C for 45 min), enzymatic hydrolysis (combination of 2% Alcalase and 3% Ultraflo at pH 8.0 and 54-58 ^{o}C for 24 h) 그리고 pH control (pH 12.0)의 순서로 수행되었는데 이때 항산화물질의 추출 효율은 유의적으로 증대되었다($p<0.05$). 이러한 결과 얻어진

hydrophilic phlorotannin 항산화는 Sephadex LH-20 를 이용한 gel filtration chromatography 로부터 정제하였으며, Hizikia hydrophilic phlorotannin 8 (HHpP8)으로 명명하였다. HHpP8 의 free radical (DPPH) 소거활성은 0.05, 0.1, 0.25, 그리고 0.5 mg/ml 의 농도에서 각각 34.8, 59.2, 71.3 및 84.4%를 나타내었다. ABTS system 에서 측정된 H_2O_2 소거활성은 0.05, 0.1, 0.25 and 0.5 mg/ml 의 농도에서 각각 18.2, 52.2, 64.4 와 74.2%를 나타내었다. 또한 cell system 에서의 H_2O_2 소거활성은 0.005, 0.01, 0.1 mg/ml 의 농도에서 각각 26.9, 40.8 ,8.9%를 나타내었다.

Fish oil 과 linoleic 산에서 톳 메탄올 추출물(*H. fusiformis* methanolic extracts, HME)의 지질과산화 억제효과는 peroxide value (PV), thiobabituric acid reactive substances (TBARS), conjugated diene hydroperoxides (CDH) 와 weight gaining assays 를 이용하여 측정한 결과 0.1% HME 의 항산화 효과는 BHT 와 유사하고 α-tocopherol 보다 우수한 효과를 나타내었다.

톳(*H. fusiformis*)의 crude phlorotannin fraction 의 암세포 성장억제 효과는 B16(흑색종), CT26(대장암), U937(혈액암), Hella(자궁암) 세포주를 대상으로 측정하였으며, 50 μg/ml 의 농도에서 crude phlorotannin 의 세포성장 억제효과는 B16, CT26, U937 그리고 Hela cancer cell 에서 각각 82.4, 73.2, 77.4, 그리고 50.1%를 나타내었다. 이 crude phlorotannin 분획물은 gel filtration chromatography (Wakogel C-300HG)로 부터 추가 분리하여 얻어진 분획물이 CT-26 에 대하여 높은 세포성장억제효과를 나타내었다. 가장 우수한 활성을 갖는 분획물을 선별하여 Hizikia hydrophobic phlorotannin 20 (HHbP20)으로 명명하였다. Hoechst 33342 (Investigation of apoptotic body formation) 처리에 의한 세포독성 연구에서는 HHbP20 의 항암활성이 apoptosis 를 통하여 두드러지게 나타났다.

톳 단백질 추출물(*H. fusiformis* protein extract)의 서로 다른 peptic digest 의 항고혈압 효과는 angiotensin-1 전환효소를 저해하는 방법으로 수행하였다. 다섯 가지 단백질 분해효소 (Protamax, Kojizyme, Neutrase, Flavourzyme and Alcalase)는 peptic digests 를 준비하는데 사용하였다. 다섯 가지 peptic digest 중에 Flavourzyme 처리된 digest 는 ACE 저해 (IC_{50} : 83.2 μg/ml)에 유의적인 효과를 나타내었다 ($p<0.05$). Flavourzyme 으로 처리한 peptic digest 는 pepsin 과 Pepsin ⟶ Trypsin + Chymotripsin 을 처리하여 또다시 가수분해 하였다. 그 후에 Pepsin ⟶ Trypsin + Chymotripsin 처리한 digest 는 3 종류의 ultrafiltration membranes (5, 10

and 30 kDa cut-off membranes)으로 분획되었다. 얻어진 분획물들 중 5 kDa fraction 에서 가장 우수한 ACE 저해활성을 나타내었다 (IC_{50} : 53.2 μg/ml). 이 5 kDa fraction 은 SP-Sephadex C-25 (cation exchange chromatography)와 Sephadex G-10 (gel filtration chromatography for dialysis) columns 으로부터 다시 분리하여 부분적으로 정제된 small molecular peptides 가 우수한 ACE 저해효과를 보였다 (IC_{50} : 36.4 μg/ml).

H. fusiformis hot water fraction 과 다섯 가지 효소적 가수분해물의 항응고활성은 activated thromboplastin time (APTT) assay 로 측정하였다. 항응고활성은 효소적 가수분해물에서 우수한 효과를 나타내었지만, 상업용 항응고제(Heparin)와 비교했을 때 그리 만족할만한 결과를 얻지는 못했다.

종합적으로 톳(*H. fusiformis*)은 항산화, 항암 그리고 항고혈압 물질과 같은 유망한 생리활성 성분을 가지고 있어 기능성 식품 뿐만 아니라 제약산업을 위한 재료로서의 가치가 있는 유용한 해조류이다.

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SUMMARY

Tot (*Hizikia fusiformis*) is a common edible brown seaweed (class Pheophyceae, order Fucales, family Sargassaceae) being widely consumed in Korea, Japan and some European countries. The efficacy of the bioactive constituents of Tot was investigated for their antioxidant, anticancer, antihypertensive and anticoagulative activities. Thereafter, the compounds with potential bioactivities were purified and characterized.

The antioxidative potential of different fractions (organic and aqueous fractions of *n*-hexane, chloroform and ethyl acetate) of Tot was evaluated by 1,1-diphenyl–2-picrylhydrazyl (DPPH), superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), nitric oxide ($NO^{\cdot}$) scavenging, ferrous ion chelating and reducing power assays. Almost all the fractions have exhibited good activities on $NO^{\cdot}$ and DPPH scavenging compared to commercial antioxidants (butylated hydroxytoluene (BHT) and α-tocopherol). Among them, the highest nitric oxide (IC_{50} : 0.10 mg/ml) and DPPH (IC_{50} - 0.144 mg/ml) activities were recorded from the organic *n*-hexane fraction. Organic chloroform fraction showed the highest $HO^{\cdot}$ scavenging activity (IC_{50} : 0. 0.4 mg/ml). Aqueous chloroform fraction demonstrated higher $O_2^{\cdot -}$ scavenging (IC_{50} : 0.12 mg/ml) and metal chelating (IC_{50} : 0.131 mg/ml) activities similar to BHT. The highest reducing power was shown by the aqueous ethyl acetate fraction followed by n- hexane fraction.

Tot 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 in DPPH radical and hydrogen peroxide scavenging assays. Digests prepared with Ultraflo (carbohydrase) and Alcalase (protease) have reported higher antioxidative efficacies than that of the other digests. Combination of Ultraflo and Alcalase under optimum hydrolysis conditions could intensify the antioxidant (specially phlorotannins) extraction efficacy of Tot. In order to enhance the extraction efficacy, the enzymatic digestion was combined with pH control and heat treatments. When the extraction was performed in the sequence of heat (100^{o}C for 45 min), enzymatic hydrolysis (combination of 2% Alcalase and 3% Ultraflo at pH 8.0 and 54-58^{o}C for 24 h) and pH control (pH 12.0), the antioxidant extraction efficacy was significantly (P<0.05) increased. From the resultant extract, a potential hydrophilic phlorotannin antioxidant was purified by gel filtration chromatography using Sephadex LH-20 and named as Hizikia hydrophilic phlorotannin 8 (HHpP8). Its free radical (DPPH) scavenging activity at the concentrations of 0.05, 0.1, 0.25 and 0.5 mg/ml were 34.8, 59.2, 71.3 and 84.4 % respectively. The H_2O_2 scavenging activity determined in ABTS system at 0.05, 0.1, 0.25 and 0.5 HHpP8 mg/ml were 18.2, 52.2, 64.4 and 74.2 % respectively. The H_2O_2 scavenging activities measured in cell system were 26.9, 40.8, 58.4 and 68.9 % at 0.5, 2.5 and 5 HpP8 μg/ml respectively.

The lipid peroxidation inhibitory effects of Tot methanolic extracts (HME) on fish oil and linoleic acid were studied using peroxide value (PV), thiobabituric acid reactive substances (TBARS), conjugated diene hydroperoxides (CDH) and weight gaining assays. In general, the antioxidative effect at 0.1% HME was compatible

with the effect of BHT and slightly higher than that of the α-tocopherol.

The cancer cell growth inhibitory potential of the crude phlorotannin fraction of Tot was initially investigated in 4 cell lines namely B16, CT26, U937 and Hela cells representing melanoma, colon, promonocytic and cervical cancer cells respectively. The cell growth inhibitory activities of the crude phlorotannin at 50 μg/ml were 82.4, 73.2, 77.4 and 50.1 % on B16, CT26, U937 and Hela cancer cells respectively. Thereafter, the crude phlorotannin fraction was further fractionated by gel filtration chromatography (Wakogel C-300HG). Resultant fractions showed higher cell growth inhibition against CT26. The most active fraction was selected and named as Hizikia hydrophobic phlorotannin 20 (HHbP20). The cytotoxicity investigation performed by using nuclear staining with Hoechst 33342 (Investigation of apoptotic body formation) showed that the anticancer activity of the HHbP20 was obviously via apoptosis.

The antihypertensive efficacy of different peptic digests of Tot protein extract was investigated by the angiotensin-1 converting enzyme inhibitory assay. Five proteolytic enzymes (Protamax, Kojizyme, Neutrase, Flavourzyme and Alcalase) were initially used in the preparation of peptic digests. Of the five peptic digests, Flavourzyme treated digest was significantly (P<0.05) effective in ACE inhibition (IC_{50} : 83.2 μg/ml). Flavourzyme treated peptic digest was further hydrolysed with (1) Pepsin and (2) Pepsin⟶Trypsin + Chymotripsin. Thereafter, the Pepsin⟶Trypsin + Chymotripsin treated digest was fractionated with 3 ultrafiltration membranes (5, 10 and 30 kDa cut-off membranes). Of the resultant fractions, the 5 kDa fraction showed the highest significant (P<0.05) ACE inhibitory activity (IC_{50} : 53.2

μg/ml). The 5 kDa fraction was further frac-tionated by SP-Sephadex C-25 (cation exchange chromatography) and Sephadex G-10 (gel filtration chromatography for dialysis) columns. The partially purified small molecular peptides showed a good ACE inhibitory activity (IC_{50} : 36.4 μg/ml).

The anticoagulant activity of Tot hot water fraction and its five enzymatic digests were investigated by activated thromboplastin time (APTT) assay. Even though, the anticoagulant activities were increased with enzymatic digestion, activities were not satisfactory when compared to the commercial anticoagulant compound (Heparin).

Overall, it can be suggested that Tot possess promising bioactive compounds such as antioxidative, anticancer and anti-hypertensive compounds, thus it is not only a functional food but also a raw material for pharmaceutical industry.

INTRODUCTION

The chemical diversity of nature is immeasurable thus 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 seaweeds are potential sources of pharmacological compounds and food additives potentially exert beneficial health effects such as antioxidative, anticoagulative, antihyaluronidase, antibacterial and anti-carcinogenic (Ireland et al., 1993; Okai et al., 1996; Duval et al., 2000; Ruperez and Calixto, 2001; Lim et al., 2002; Shibata et al., 2003; Athukorala et al., 2003). Tot (*Hizikia fusiformis*) is an edible brown seaweed (class Pheo-phyceae, order Fucales, family Sargassaceae) being widely consumed in Korea, Japan (Ohno and Largo, 1998; Shon, 1998) and some European countries. During last two decades, a number of researches on Tot have pointed out a variety of biological benefits including antioxidative and anticoagulative activities (Kim et al., 1998; Yan et al., 1999; Nagai and Yukimoto, 2003).

Tot reported to contain potential bioactive compounds such as pigments (fucoxanthine) and secondary metabolites like phlorotannins. Extraction of hydrophobic compounds like fucoxanthine is possible with organic solvents. However, no significant research attention has been focused on the hydrophilic bioactive compounds of seaweeds. Literature on extraction of hydrophilic bioactive materials from seaweeds is limited. Seaweeds contain large amount of highly viscous polysaccharide that become one of the key impediments in

such extraction processes. Brown seaweeds contain soluble dietary fiber polysaccharides as alginates, fucans and laminarins together with the insoluble fibers made of cellulose (Mabeau and Kloareg 1987; Lahaye and Kaeffer, 1997; Yan et al., 1996). Alginic acid is the main structural component of the brown algal cell wall, which is a linear copolymer of β-1,4-D-mannuronic acid and α-1,4-L-guluronic acid, with the residues organized in blocks of polymannuronic acid and polyguluronic acid, as well as heteropolymeric sequences of guluronic and mannuronic acid (Moen et al., 1997; Kloareg and Quatrano, 1998). Both insoluble and soluble fibers together with other cell wall materials act as a physical barrier for the extraction of desired bioactive materials. Furthermore, the characteristic gelling properties of alginate and laminarin like polysaccharides are greatly interfering in the proper extraction of desired bioactive compounds. The gel formation with the addition of water and further entrapment of desired bioactive compound in the gel matrices interfere the proper extraction of bioactive compounds and leads to the poor solubility in aqueous medium. The enzymes can work primarily by macerating the tissues of the algae and breaking down the cell walls and complex interior storage materials like laminarins to release interior compounds. Under acidic conditions, proteins can bind specially with polyphenols. The brown algal polyphenols (phlorotannins) have been recognized as potential antioxidative materials in seaweeds. Breakdown of such barriers can enhance the extraction of desired bioactive materials located inside cells. Also, the breakdown /releasing of high molecular weight polysaccharides and proteins themselves can contribute to enhance the antioxidative activities

(Ramos and Xiong, 2002; Ruperez et al., 2002; Heo et al., 2003; Ahn et al., 2003).

Polyphenols (e.g. phenolic acids, flavonoids, tannins) being widely distributed in plants are known to act as potential antioxidants. Also, polyphenols in human diet may exert beneficial health effect via protecting against some diseases, including coronary heart disease and some cancers (Hotta et al., 2002). In general, phenolic compounds or polyphenols have a similar basic structural chemistry including an "aromatic" or "phenolic" ring structure. Phytophenolic compounds have been associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Rice-Evans et al., 1995; Jorgensen et al., 1999). The protective effects of plant plolyphenols in biological systems are ascribed to their capacity to transfer electrons to free radicals, chelate metal catalysts, activate antioxidant enzymes and inhibit oxidases. Polyphenols are basically of two classes; condensed tannins and hydrolysable tannins derived from shikimic acid pathway. Polyphenols in marine brown algae are called as phlorotannins, which are formed by the polymerization of phloroglucinol (1, 3, 5 trihydroxybenzene) monomer units and synthesized in the acetate-malonate pathway (Ragan and Glombitza, 1986). Phlorotannins are compounds with a wide range of molecular sizes (126Da ~ 650kDa). Moreover, phlorotannins reported to have potential bioactivities specially as antioxidant, anticancer, antibacterial, antihyaluronidase antiglycosidases, antiplasmin compounds (Nakamura et al., 1996; Shibata et al., 2002; Nagayama et al., 2002).

It is widely accepted that intake of antioxidants or foods containing antioxidants exert beneficial health effects particularly

against the risks of carcinogenesis, coronary/artery disease, platelet aggregation, neurodegenerative and atherosclerosis (Tzeng et al., 1991; Ranelletti et al., 1992; Aruoma 1994; Madsen and Bertelsen, 1995; Wanasundara and Shahidi 1998; Chew et al., 1999; Tsang and Kamei, 2004). 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 (Harris et al., 1992; Wagner et al., 1993). In fact, anti-oxidants 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 conti-nuously 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's 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 immediately regenerate 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).

The world cancer report indicates that cancer rates are set to increase at an alarming rate globally. Cancer is a group of more than 100 different diseases, but most common includes skin, lung, colon, rectal, breast, endometrial, ovarian and prostate cancers. Globally, colorectal cancer (cancer of the colon and rectum) is the third most common human cancer and the second cause of cancer-related deaths in the United States (Greenlee et al., 2000). Colorectal cancer can strike at any age, and its occurrence intensifies over the age of 50. In particular, certain growths that protrude from the inner wall of the colon or rectum (polyps) called as adenomas/neoplastic polyps starts to develop as colon cancer. It takes about 5-10 years for a benign polyp to transform into cancer. The risk of developing cancer from a polyp is related to its gross appearance, histological features, and size (Stryker et al., 1984 and Murakami et al., 1990). However, colon cancer is a curable disease and it is easy when detected at early stages. Colon cancer chemotherapy treatments can be given to destroy cancerous cells that may remain in the body after surgery or to kill/control cell growth. Seaweeds reported to contain anticancer constituents and impart multiple therapeutic benefits (Carper, 1989; Yamamoto and Maruyama, 1985; Funahashi et al., 1999 and

Funahashi et al., 200). However, the anticancer activities of phenolic entities in seaweeds have been poorly investigated.

Hypertension, a well-known risk factor for cardiovascular disease and its sequelae, such as stroke, myocardial infarction, heart failure, and renal diseases associated with high rates of morbidity and mortality. The rennin-angiotensin-aldosteron system (RAAS) plays a central role in blood pressure regulation and has long been a target of pharmacologic approaches in controlling blood pressure (Black, 2003). Renin catalyzes the conversion of angiotensinogen (NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Ser-R) to angiotensin I (NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-COOH) subsequent involvement of angiotensin I converting enzyme (ACE) catalyzes the conversion of angiotensin I (an inactive decapeptide) to angiotensin II (NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH) which is a potent octapeptide, mediates the circulatory homeostasis. The ACE is a zinc-containing exopeptidase enzyme located in the endothelial lining of the vasculature of the lung and cleaves dipeptides at the C-terminus of oligopeptides. In particular, ACE involves cleaving histidyl-leucine of angiotensin I forming angiotensin II, and degrades bradykinin (vasodepressor) to inactive peptides (Curtiss et al 1978, Dzau 2001). To help prevent and to treat hypertension, inhibition of ACE has been a widely using therapeutic strategy and the literature on ACE inhibitory compounds is quite extensive. ACE possesses two active sites, N- and C –terminal with different affinities to different substances (Actis-Goretta et. al 2002). Even though, the treatments with synthetic anti-ACE substances could provide definite and positive inhibitory activities, certain side effects such as cough, taste disturbances, skin rashers have been

reported to occur during treatments with commonly using anti-ACE substances like Captopril (Atkinson and Robertson, 1979). Researchers have been continuously scrutinizing for anti-ACE substances from natural recourses, and their attention on peptides and polyphenolic (specially tannins and flavanoids) compounds have progressed at a higher rate due to their pronounced positive effects. Modes of actions of those natural anti-ACE substances include chelating with metal ion (Zn) in ACE or competitively binding with the active sites of ACE. Chelating agents abolish activity of ACE by removing the metal ion to yield the inactive, metal-free apoenzyme. ACE inhibitory peptides of food proteins and their hydrolysates gained interest of scientists and research for the identification of potential peptides have been increasing significantly. During last few decades, different food protein hydrolysates (beef, milk, cheese, casein, soy sauce, fish tissues, several fermented foods and seaweeds) have been increasingly acknowledged for their ACE inhibitory potential (Miyoshi et al., 1991; Kinoshita et al., 1993; Matsui et al., 1993; Maeno et al., 1996; Abubakar et al., 1998; Kohama et al., 1998; Suh et al., 1999). These investigations could notably contribute to the enhanced knowledge of interrelationships between particular foods and nutritional benefits. Frequent consumption of such peptide containing foods could be beneficial in maintaining blood pressure at a healthy level. Moreover, identification of such potential peptides may overcome the health complications (side effects) of existing synthetic drugs for hypertension. The proteiolytic enzymes can yield peptidic digests of proteins with a great variation of peptides. The obvious fact that different proteases can yield different verities of peptides can be used as an experimental tool for the preparation of

different peptides from Hizikia proteins. The enzymatic digestion of food proteins to obtain bioactive peptides has been a common approach of researches specially in ACE inhibitory peptide investigation research (Miyoshi et al., 1991; Maeno et al., 1996; Abubakar et al., 1998).

Anticoagulant drugs have been widely used for the prevention of venous thromboembolic disorders. Moreover heparin is employed during extracorporeal circulation, such as in major vascular surgery and in hemodialysis (Murano et al., 1999). Various anticoagulant polysaccharide compounds have been isolated and characterized from marine algae. Algal anticoagulant polysaccharides exert their anticoagulant activity through potentiating antithrombin III (AT III) and / or heparin cofactor II (HC II) that are important endogenous inhibitors, called “serpin” (Frank et al., 1989). Fucoidan, a sulfated fucan (L-fucopyranose) is a promising anticuagulative polysaccharide among other polysaccharides present in algae. This compound catalyses thrombin inhibition by antithrombin and by heparin cofactor II. Fucoidan can enhance the heparin cofactor II-thrombin interaction more than 3500-fold. Recently it has been investigated that, fucoidan prevents sulfatide binding to the adhesive proteins trombospondin, laminin, and von Willebrand factor (Matsubara, 2004). Highly sulfated semisynthetic fucoidan has been reported to stimulate t-PA-induced plasma clot lysis by protecting plasmin activity from (alpha) 2-antiplasmin and by decreasing the rate of polymer formation. Fucoidan from *Ecklonia kurome* was reported to enhance the activation of Glu-Plg by high molecular weight urokinase (HMW u-PA) and by single and two-chain t-PA (Nishino et al.,

2000). Therefore, it is obvious that seaweeds polysaccharides are a good alternative source for anticoagulative drug production.

The objective of this study is to describe the potential bioactive compounds of Tot by identification, extraction purification and characterization. Specially, to strengthen the literature on antioxidants, anticancer and antihypertensive drug candidates of Tot.

Part I

Reactive oxygen species scavenging, metal chelating and reduceing power properties of different solvent fractions from Tot (*H. fusiformis*)

1. ABSTRACT

The antioxidative properties of different organic and aqueous fractions obtained by solvent fractionation of 80% methanolic extract of Tot were investigated for free radical, reactive oxygen species scavenging ($O_2^{\cdot -}$,H_2O_2, $HO^{\cdot}$,$NO^{\cdot}$), metal chelating, reducing power and lipid peroxidation (conjugated diene and thiobarbituric acid reactive substances formation) inhibition assays. Of the organic and aqueous fraction tested, the organic ethyl acetate and aqueous chloroform fractions were notably effective. Almost all fractions exhibited significantly higher activities on $NO^{\cdot}$ and DPPH scavenging compared to commercial antioxidants. Organic chloroform fraction showed the highest $HO^{\cdot}$ scavenging activity among other counterparts. Aqueous chloroform fraction demonstrated $O_2^{\cdot -}$ scavenging and metal chelating activities that are similar to BHT. The lipid peroxidation inhibition was significantly higher in organic ethyl acetate fraction than that of α-tocopherol. These data suggest that both organic and aqueous fractions are rich in hydrophobic and hydrophilic antioxidative compounds with different antioxidative properties.

2. MATERIALS AND METHODS

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 fish oil were purc-hased from Sigma Co. (St Louis, USA). 2, 2-Azino-bis (3-ethylbenzthiazolin)-6-sulfonic acid (ABTS), ethylenediamine tetra-acetic acid (EDTA), peroxidase and 2-deoxyribose were purchased from Fluka Chemie (Buchs, Switzerland). All the other chemicals used were analytical grade supplied by Flucka or Sigma Co.

2.2. Preparation of extract and solvent fractionation

Tot was collected in May 2004 from the coastal area of Jeju Island of South Korea. Epiphytes, salt and sand were removed using tap water and the samples were rinsed with deionised water before freeze-drying. Dried samples were ground in to a fine powder. Powdered samples (40 g) were immersed in 80 % methanol (1000 ml) and placed in a shaking incubator for 24 h at 25 ºC. The macerated mixture was filtered using a Buchner funnel and methanol extract was collected. Solvent fractionation of methanol extract was performed and respective fractions were obtained (Fig. 1). All activities

of both aqueous and organic fraction were compared with commercial antioxidants (BHT and α- tocopherol) dissolved in methanol.

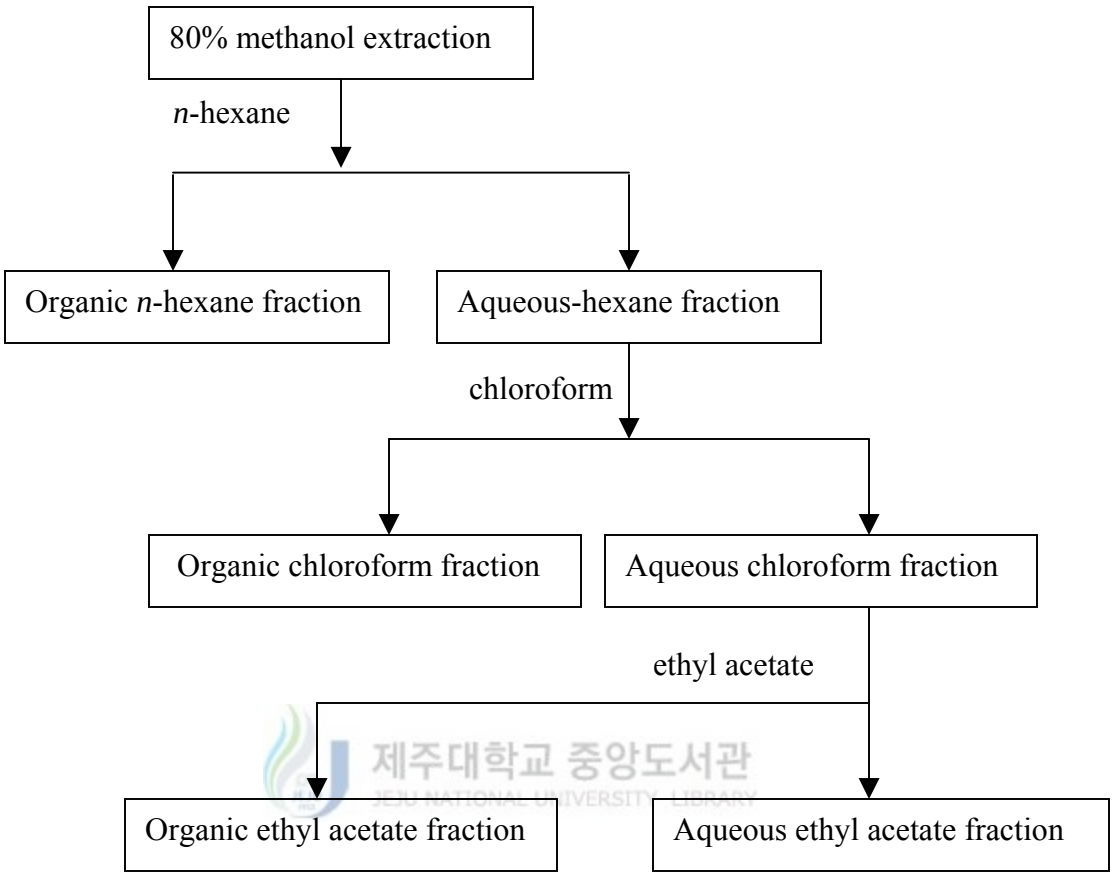

Fig. 1. Scheme of solvent fractionation of Tot.

2.3. Reactive oxygen species (ROS) inhibition

2.3.1. Free radical scavenging assay

Free radical scavenging activity of the different fractions of Tot was measured according to the modified method of Brand-Williams (1995). Each sample fraction (2 ml) was mixed thoroughly with 2ml of freshly prepared DPPH solution (3X 10^{-5} M) dissolved in DMSO and thoroughly mixed. The reaction mixture was incubated for 1 h and absorbance was measured at 517 nm using UV–VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea).

2.3.2. Superoxide anion scavenging assay

This assay was carried out according to the method of Nagai et al., (2003). The reaction mixture consisted 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 Tot sample. After incubation at 25 °C for 10 min, the reaction was started by adding 6 mU XOD and keeping at 25 °C for 20 min. Reaction was stopped by adding 0.02 ml of 6 mM CuCl. The absorbance was measured in ELISA reader (Sunrise; Tecan Co. Ltd., Austria) at 560 nm.

2.3.3. Hydrogen peroxide scavenging assay

This assay was carried out according to the method of Mueller (1995). Tot extract (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 1.25mM ABTS and 30 µl of peroxidase (1 U/ml)

were mixed and incubated at 37 ºC for 10 min and the absorbance was recorded by ELISA reader at 405 nm.

2.3.4. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was determined according to Chung et al., (1997). 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 1.2ml of 0.1 M phosphate buffer (pH 7.4) mixed with 200 µl of Tot fraction. Thereafter, 200 µl of 10 mM H_2O_2 was added and incubated at 37 °C for 4 h. After incubation, 1ml of 2.8% TCA and 1ml of 1% 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 in a UV–VIS spectrophotometer.

2.3.5. Nitric oxide radical inhibition assay

Nitric oxide radical inhibition was determined according to the method reported by Garrat (1964). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of Griess Illosvoy reaction. 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 Tot sample and incubated at 25°C for 150 min. From 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 in ELISA reader.

2.4. Metal chelating ability

Metal chelating ability was determined according to the method by Decker and Welch (1990). 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 solution was measured at 562 nm.

2.5. Reducing power assay

This experiment was conducted according to the method developed by Oyaizu for reducing power test (1986). Tot fractions (2.5 ml) were spiked with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1 potassium ferricyanide. The mixture was then placed in a 50℃ water-bath for 20 min. After cooling rapidly, 2.5 ml of 10 % trichloroacetic acid was added, and centrifuged at 3000 rpm for 10 min. The supernatant (5 ml) was then mixed with 5 ml of distilled water and 1 ml of 0.1 % ferric chloride. The absorbance at 700 nm was recorded for the reaction for 10 min. Higher the absorbance represents stronger reducing power.

2.6. Total polyphenol contents

Total polyphenol contents were determined according to the protocol similar to Chandler and Dodds (1993). Tot (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.

2.7. Scavenging activity/chelating ability calculation

Scavenging activity/chelating ability was calculated using the following equation and IC_{50} value was expressed as the concentration (mg/ml) of the extract that inhibits 50% of the respective ROS. For the calculation of IC_{50} value five different concentrations were tested.

$$[1-(A_i - A_j)/Ac] \times 100.$$

A_i - the absorbance of Tot fraction mixed with active compound

A_j - the absorbance of same Tot fraction without active compound

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

2.8. Statistical analysis

Statistical analyses were conducted with the SPSS 11.5 version software package on the triplicate (n=3) test data. Analysis of variance was performed by ANOVA procedures. Significant difference ($P< 0.05$) between the means of test parameters was determined by least significant differences (LSD values) tests. Pearson correlation coefficient was calculated by using Minitab stastical software according to the Mibnitab Inc. guidelines.

3. RESULTS

3.1. Free radical scavenging effect

To evaluate the scavenging effect of DPPH in aqueous and organic fractions, DPPH radical scavenging assay was performed and these results were compared with commercial antioxidants (Table 1). Organic ethyl acetate fraction showed a significantly higher ($P<0.05$) radical scavenging activity (IC_{50} 0.113±0.02 mg/ml) than that of BHT (IC_{50} 0.36±0.002 mg/ml) while organic *n*-hexane (IC_{50} 0.144±.03 mg/ml) and aqueous chloroform (IC_{50} 0.157±0.03 mg/ml) fractions exhibited strong scavenging activities. The correlation between radical scavenging ability and total polyphenolic content is 0.921 and it is in accordance with results recorded by Siriwardhana et al. using different solvent extraction of Tot antioxidants (Siriwardhana et al., 2003).

3.2. Superoxide anion scavenging effect

The highest $O_2^{\cdot-}$scavenging activity (IC_{50} 0.12±0.02 mg/ml) recorded in the aqueous chloroform fraction is significantly higher ($P<0.05$) than that of BHT (IC_{50} 0.165±0.02 mg/ml). The second highest activity (IC_{50} 1.01±0.02 mg/ml) was recorded in the aqueous ethyl acetate fraction while the other fractions exhibited relatively lower activities.

3.3. Hydrogen peroxide scavenging effect

The scavenging activities of both aqueous and organic fractions of Tot on hydrogen peroxide are shown in Table 1. Among recorded results, aqueous and organic fractions of ethyl acetate

exhibited relatively higher H_2O_2 scavenging effects (IC_{50} 1.9 ±0.02 and 2.54±0.05 mg/ml respectively). But those activities were lower when compared with commercial antioxidants tested BHT and α-tocopherol where IC_{50} values were 0.07±0.004 and 0.127±0.03 mg/ml respectively. Moreover, the polyphenolic content of the highly active ethyl acetate fraction was the highest of all fractions. Therefore, it is obvious that the H_2O_2 scavenging compounds present in these fractions have both hydrophilic and hydrophobic properties.

Table 1. Antioxidative effect of different fractions of Tot

Fraction	Total Polyphenol	IC_{50} (mg/ml)					
	(mg/ml)	DPPH	O_2^-	H_2O_2	OH	NO	Metal chelating
80 %MeOH	0.054 ±0.006	1.41e ±0.03	2.31f ±0.21	3.42d ±0.24	1.42d ±0.04	1.59c ±0.12	1.1b ±0.03
Aqueous *n*-hexane	0.054 ±0.005	1.15d ±0.01	1.81e ±0.05	4.21f ±0.14	1.1c ±0.04	0.14a ±0.002	0.93b ±0.01
Organic *n*-hexane	0.035 ±0.002	0.14b ±.03	2.62g ±0.12	3.36d ±0.11	1.81d ±0.05	0.10a ±0.002	1.94d ±0.21
Aqueous chloroform	0.053 ±0.003	0.15b ±0.03	0.12a ±0.02	3.21d ±0.02	1.41d ±0.21	0.09a ±0.01	0.13a ±0.002
Organic chloroform	0.047 ±0.003	0.21b ±0.02	1.63d ±0.1	3.87e ±0.13	0.4b ±0.11	0.66b ±0.06	1.26b ±0.05
Aqueous ethyl acetate	0.047 ±0.002	0.21b ±0.04	1.01b ±0.02	2.54c ±0.05	1.47d ±0.08	0.1a ±0.004	0.18a ±0.07
Organic ethyl acetate	0.073 ±0.005	0.11b ±0.02	1.9e ±0.04	1.90b ±0.02	2.51e ±0.030	0.51b ±0.09	0.77b ±0.0
BHT		0.36c ±0.02	0.16a ±0.02	0.07a ±0.004	0.02a ±0.004	1.59c ±0.2	0.11a ±0.02
α-tocopherol		0.01a ±0.003	1.3c ±0.03	0.12a ±0.03	0.46b ±0.002	2.1d ±0.6	1.72c ±0.2

The values (mean ±SD n=3)

Significant differences at $P < 0.05$ indicated with different letters.

3.4. Hydroxyl radical scavenging effect

Hydroxyl radical scavenging activity of organic and aqueous fractions of H. fusiformis was determined as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. Organic chloroform fraction exhibited higher scavenging activity (IC_{50} : 0.4 ±0.11 mg/ml) comparable to α-tocopherol (IC_{50} : 0.46±. 0.002 mg/ml) but lower than that of BHT (IC_{50} : 0.023 ±0.004 mg/ml). However, the values shown by other fractions were lower than the commercial antioxidants tested.

3.5. Nitric oxide scavenging effect

All tested organic and aqueous fractions of Tot except methanol fraction exibited significant ($P<0.05$) scavenging effects than that of commercial antioxidants (Table 1). Among them, aqueous fractions of chloroform (IC_{50} 0.093± 0.010 mg/ml), ethyl acetate (IC_{50} 0.1±. 0.004 mg/ml) and organic *n*-hexane fraction (IC_{50} : 0.1± 0.002 mg/ml) showed intensive activities. Furthermore, scavenging activities of aqueous fractions (except aqueous *n*-hexane fraction) showed slightly higher activities than that of organic fraction.

3.6. Metal chelating effect

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 aqueous and organic

fractions of Tot indicating that both fractions have chelating ability. Aqueous fractions of chloroform (IC_{50} 0.131±0.002 mg/ml) and ethyl acetate (IC_{50} :0.18±0.07 mg/ml) exhibited significantly ($P<0.05$) high-er chelating effects compared to that of α-tocopherol (IC_{50} : 1.72±0.2 mg/ml). It is obvious that the metal scavenging activity was higher in aqueous fraction than organic fraction. Moreover, the activity has increased from methanol to ethyl acetate in both aqueous and organic fractions with the progression of hydrophilicity.

3.7. Reducing power

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of Tot. Fig. 2 depicts the reductive effects of Tot fractions compared with BHT and α-tocopherol. Similar to antioxidant activity, the reducing power of Tot fractions increased with increasing dosage. All the fractions showed significantly higher activities than control but less than that of commercial antioxidants tested. Among tested fractions, aqueous fraction except ethyl acetate exhibited higher reducing power indicating that these fractions consist of hydrophilic polyphenolic compounds that cause higher reducing power.

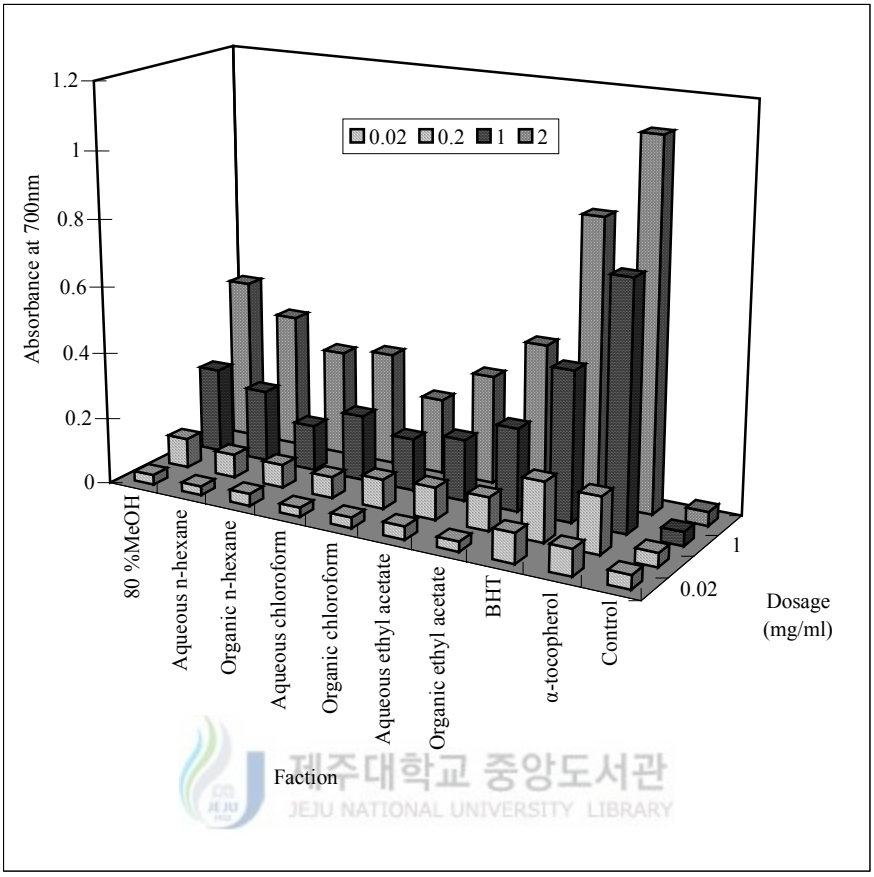

Fig. 2. Reducing power of different fractions of Tot.

3.8. Total polyphenol contents

Polyphenole content in both organic and aqueous fractions of Tot varies with the solvent used according to their polarity. Although ethyl acetate fraction consists of the highest content of polyphenol it doesn't show the highest activity for every assay assuming not only the content but also properties of polyphenolic compounds contribute to different activities in different fractions.

4. DISCUSSION

In cells, free radicals are continuously produced either as by-products of metabolism or deliberately as in phagocytes (Cheeseman and Slater, 1993). DPPH is a free radical generating compound and has been widely used to evaluate the free radical scavenging ability of various antioxidative compounds (Hatano et al., 1989). Polyphenols are particularly effective as antioxidants in polyunsaturated fatty acids; in fact they easily transfer a hydrogen atom to lipid peroxyl radicals and form the aryloxyl, which being incapable of acting as a chain carrier or couples with another radical and thereby quenching the radical process (Ruberto et al., 2001). The results demonstrate that most of free radical scavenging compounds tends to be concentrated in hydrophilic solvent fractions (ethyl acetate). According to this study, for scavenging of free radical fractionated compounds of Tot were even better than that of BHT.

Superoxide and hydroxyl radicals are the two most effective representative free radicals. In cellular oxidation reactions, superoxide radical is normally formed first and its effects can be magnified because it produces other kind of cell damaging free radicals and oxidizing agents (Liu and Ng, 2000). In cellular oxidation reactions $O_2^{\cdot -}$ are formed when oxygen is reduced. $O_2^{\cdot -}$ is normally formed first and its effects can be magnified as it produces other kinds of cell damaging free radicals and oxidizing agents (Liu and Ng, 2000). Nagai et al. (2003) recorded a significant superoxide anion scavenging activity for a beverage made of Tot, and Sekine et al. (1998) reported a good superoxide anion scavenging activity in Shiunko, a traditional herbal medicine, which has been used to treat

burns and hemorrhoids by external applications. Superoxide radicals are not only formed in the body but also the early products of protein glycation, such as the Schiff base and Amadori compound which may be key structural components involved in the generation of superoxide radicals (Ukeda et al., 2002).

Hydrogen peroxide, 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. . The measurement of H_2O_2 scavenging activity can be one of the useful methods determining the ability of antioxidants to decrease the level of prooxidants such as H_2O_2 (Czochra and Widensk, 2002). The activity of the water extract observed in the present study implies that Tot contains water-soluble antioxidants potential to decrease the pro-oxidants like H_2O_2. Therefore the potential components in Tot can attribute to maintain high level of values in H_2O_2 scavenging and attain the sound health benefit and great source of H_2O_2 scavenging hydrophilic phenolic antioxidant**.**

Hydroxyl radical is the most reactive oxygen species among all ROS due to its strong ability to react with various biological materials oxidatively by hydrogen withdrawal, double bond addition, electron transfer and radical formation, and initiates autoxidation, polymerization and fragmentation (Liu and Ng, 2000). 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). Therefore, higher hydroxyl scavenging activity shown in organic chloroform fraction, which is relatively

hydrophobic, can be used to minimize the adverse effects from hydroxyl radical.

Nitric oxide ($NO^{\cdot}$) is a gaseous free radical, which has important roles in physiological and pathological conditions. Marcocci et al. (1994) have 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 induces toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modifications (Moncada et al., 1991; Radi et al., 1991). Furthermore, $HO^{\cdot}$ may be generated after decomposing of these $ONOO^{-}$ and can cause renal injuries (Beckman et al., 1990). Peroxynitrite can oxidize low-density lipoprotein (LDL) directly or via its reaction products, releasing copper ions that cause in destruction of ceruloplasmin, and generally attack tyrosine residues in different proteins, as observed in many inflammatory diseases (Halliwell, 1997). Therefore, the strong $NO^{\cdot}$scavenging effect of both hydrophilic and hydrophobic antioxidants is an advantage and Tot may contain different kinds of $NO^{\cdot}$ scav-enges with different chemical properties. Thus, the $NO^{\cdot}$ scavenging effect observed in different fractions of Tot can be used to minimize or retard the damages from $NO^{\cdot}$ radicals.

Ferrous can initiate lipid peroxidation by Fenton reaction as well as accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Halliweill, 1991). Metal chelating activity can attribute to reduce the concentration of the catalyzing transition metal in lipid peroxidation (Duh et al., 1999). Furthermore, chelating agents that form σ bonds with a metal are

effective as secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion (Gordon, 1990). Thus, different fractions of Tot demonstrate a marked capacity for iron binding, suggesting their ability as peroxidation protector that relates to its iron binding capacity.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). However, the activity of antioxidants have been assigned to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997).

Numbers of researches have pointed out that seaweed polyphenols are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation. Moreover, the polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans (Tanaka et al., 1998).

Part II

Enzymatic Hydrolysis of Tot (*H. fusiformis*) – A Novel approach for effective extraction of antioxidative compounds

1. ABSTRACT

Tot hydrolysates prepared by using five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamax, Kojizyme, Neutrase, Flavourzyme and Alcalase) were investigated for their extraction efficacy (yield and total polyphenolic content) and antioxidative activity (DPPH radical and hydrogen peroxide scavenging activity). Termamyl and Ultraflo of the carbohydrases and Flavourzyme and Alcalase of the proteases were selected due to their high efficacy of extraction and antioxidative activity. Selected enzymes were used to investigate the optimum enzymatic reaction time and dosage (enzyme/substrate ratio) suitable for hydrolysis. Optimum reaction time for the enzymatic hydrolysis was 3 days and optimum dosage of hydrolysis was observed to be 5%. Simultaneously, Ultraflo of the two carbohydrases and Alcalse of the two proteases were selected as the most effective enzymes. Combination of Ultraflo and Alcalase under optimum hydrolysis conditions could intensify the extraction efficacy of antioxidative materials from Tot. The hydrolysate obtained by combining the enzymes was separated into four different molecular weight fractions (< 1, 1-10, 10-30 and > 30 kDa) and recorded the polyphenolic content distribution and respective antioxidative ability. The fraction < 1kDa was identified as less effective and those fractions >

1 kDa indicated comparatively higher antioxidative activities related to their polyphenolic content.

2. MATERIALS AND METHODS

2.1. Materials

Tot was collected from the Jeju Island coast of S. Korea in October 2003. Epiphytes, salt and sand were removed using tap water and the samples were rinsed with deionised water before freeze-drying.

Viscozyme L (containing arabanase, cellulase, beta-glucanase, hemi-cellulase and xylanase), Celluclast 1.5 L FG (β-glucanases), AMG 300 L (1,4-α-D-glucosidase), Termamyl 120 L (α-amylases), Ultraflo L (β-glucanases), Protamex (endo-proteases), Kojizyme 500 MG (endo/exopeptidase), Neutrase 0.8 L (neutral B. amyloli-quefa-ciens proteases), Flavourzyme 500 MG (endo/ exopeptidase) and Alcalase 2.4L FG (alcalase) were purchased form Novo Co. (Novo-zyme Nordisk, Bagsvaerd Denmark). 1,1-Diphenyl-2-picrylh-ydrazyl (DPPH), dimethyl sulfoxide (DMSO), butylated hydrox-ytoluene (BHT), butylated hydroxyanisole (BHA), α-tocopherol and Folin-Ciocalteu reagent were purchased from Sigma Co. (St Louis, USA). 2,2-Azino-bis (3-ethylbenzthiazolin)-6-sulfonic acid (ABTS) and peroxidase were purchased from Fluka Co. All the other chemicals used were analytical grade supplied by Fluka or Sigma Co.

Table 2. Optimum hydrolysation conditions and compositions of enzymes*

Enzyme	Optimum pH	Optimum temperature (^{o}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
Nutrase	6.0	50	neutral beta amyloliquefaciens
Flavozyme	7.0	50	endo/exopeptidase
Alcalase	8.0	50	alcalase

* Enzymes were purchased from Novozyme Nordisk, Bagsvaerd Denmark.

2.2. Enzymatic hydrolysis procedure

Freeze dried Tot 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 5% (except in the optimum concentration investigation experiment). The mixtures were placed in a shaking incubators (for 3 days except in the optimum time investigation experiment) adjusted to optimum temperatures of the respective enzymes used. Resultant mixtures were filtered in vacuum with Watmann No. 1 (Watmann Ltd. England) filter paper 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.

2.3. Primary investigation of hydrolysates

Each hydrolysate was investigated for extraction efficacy and antioxidative activity. Extraction efficacy was investigated by the yield and total polyphenolic content, while the antioxidative activity was investigated by DPPH radical and hydrogen peroxide scavenging activities.

2.4. Measurement of extraction yield

Yields of the hydrolysates obtained by enzymatic hydrolysis of Tot were calculated by dry weight of hydrolyzed filtrate over dry weight of the seaweed sample used.

2.5. Total phenolic compounds

Total phenolic compounds were determined according to the protocol similar to Chandler and Dodds (1993). One mL of Tot hydrolysate was mixed in a test tube containing 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50 % Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 ml of 5% Na_2CO_3 was added. Thoroughly mixed mixture was placed in a dark room for 1 hr and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea). A gallic acid standard curve was obtained for the calculation of phenolic content.

2.6. DPPH radical scavenging assay

This assay was based on the scavenging of stable 1,1-di-phenyl-2-picrylhydrazyl (DPPH) radicals by the radical scavenging components in Tot hydrolysates. Modified method of Brand Williams (1995) was used to investigate the free radical scavenging activity. DPPH solution in dimethyl sulfoxide (DMSO) was prepared at the concentration of 3 $x10^{-5}$ M. A 2 ml fraction of Tot hydrolysate and 2 ml of freshly prepared DPPH solution were thoroughly mixed. The reaction mixture was incubated for 1 hr and absorbance was recorded at 517 nm using UV-VIS spectrophotometer.

2.7. Hydrogen peroxide scavenging assay

This assay was based on the ability of Tot hydrolysates to scavenge the hydrogen peroxide in ABTS-peroxidase medium according to the method of Muller (1995). Twenty μl of Tot hydro-lysate and 20 μl of 10 mM hydrogen peroxide were mixed with 100

μl of phosphate buffer (0.1 M, pH 5.0) in a 96-microwell plate and incubated at 37 ^{o}C for 5 min. Finally, 30 μl of freshly prepared 1.25 mM ABTS and 30 μl of peroxidase (1 U/ml) were mixed and incubated at 37 ^{o}C for 10 min before recording the absorbance in ELISA reader (ELX tek Instruments Inc) at 405 nm.

2.8. Scavenging activity calculation

Scavenging activity was calculated as

$[1-(A_i - A_j)/A_c] \times 100]$.

Where in the DPPH method: A_i= the absorbance of enzymatic extract mixed with DPPH solution; A_j = the absorbance of same extract mixed with 2 ml DMSO; A_c = the absorbance of DPPH solution adding 2 ml DMSO.

And in hydrogen peroxide calculations:

A_i = the absorbance measured with enzymatic extract

Aj = the absorbance measured with same extract but without ABTS.

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

2.9. Effect of enzymatic reaction time and enzyme dosage (enzyme/substrate ratio)

Two most effective enzymes of each carbohydrase and protease were used in this investigation. Different dosages (1, 2.5 and 5%) of enzymes were added at the optimum pH and temperature conditions and incubated for 3 days. Samples were taken at every 24 hrs (every day) and the effects for on yield, total polyphenoiloic content, DPPH radical and hydrogen peroxide scavenging activity were investigated.

2.10. Effect of combination of proteases and carbohydrases

Of the carbohydrases and proteases used in the above experiment (effect of enzymatic reaction time and enzyme dosage), the most active carbohydrase and protease were selected for the combination and each enzyme was applied at the concentration of 2.5% (total enzyme concentration 5%). Hydrolysis was continued for 3 days and the effects on yield, total polyphenoiloic content, DPPH radical and hydrogen peroxide scavenging activities were invest-tigated.

2.11. Fractionation of hydrolysates

Hydrolysates obtained were fractionated in Prep/Scale-TFF cartridges of molecular weight cut-off (MWCO) 30, 10 and 1 kDa. Cartridges were assembled in the ascending order of MWCO increment and hydrolysates were pumped with pressure (Fig. 3). Resulted fractions, according to the molecular weight (<1 , 1-10, 10 - 30 and >30 kDa) of the constituents of hydrolysates were again investigated for yield, total polyphenoiloic content, DPPH radical and hydrogen peroxide scavenging activities.

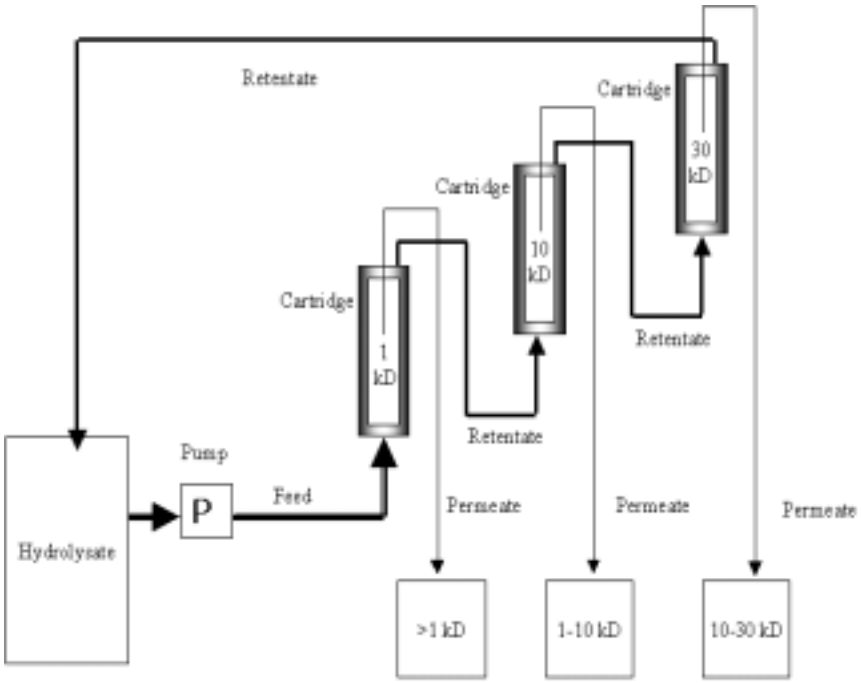

Fig. 3. Ultrafiltration membrane system for fractionation of Tot hydrolysates according to the molecular weight of constituents.

3. RESULTS

3.1. Effect of different enzymes (carbohydrases and proteases) on extraction of antioxidative compounds

Extraction efficacy of Tot constituents by each enzyme was investigated with the parameters of yield and total polyphenolic content of respective hydrolysates. Extracted yield due to Viscozyme, Termamyl and Ultraflo of carbohydrases and all the five enzymes of proteases were more than 35% and sometimes over 40% (Fig. 4). Total polyphenolics of the hydrolysates extracted by Ultraflo of carhydrases and Neutrase, Flavourzyme and Alcalase of proteases were comparatively higher than the hydrolysates by the other enzymes (Fig. 5). The correlation between extracted yield and total poly-phenolic content of the hydrolysates were not significant and all the correlation coefficient values were less than the 0.7 (results not shown). The DPPH radical scavenging activity of the hydrolysates by all the enzymes was higher than 70% and compatible with commercial antioxidants tested (Fig. 6). Hydrolysates obtained by AMG, Termamyl and Ultraflo of carbohydrases and Flavourzyme and Alcalase of proteases were comparatively stronger in hydrogen peroxide scavenging (Fig. 7). The correlation of hydrogen peroxide scavenging activity and total polyphenolic content was comparatively higher than the other correlations (DPPH and total polyphenolic content, yield and DPPH radical scavenging activity or yield and hydrogen peroxide scavenging activity).

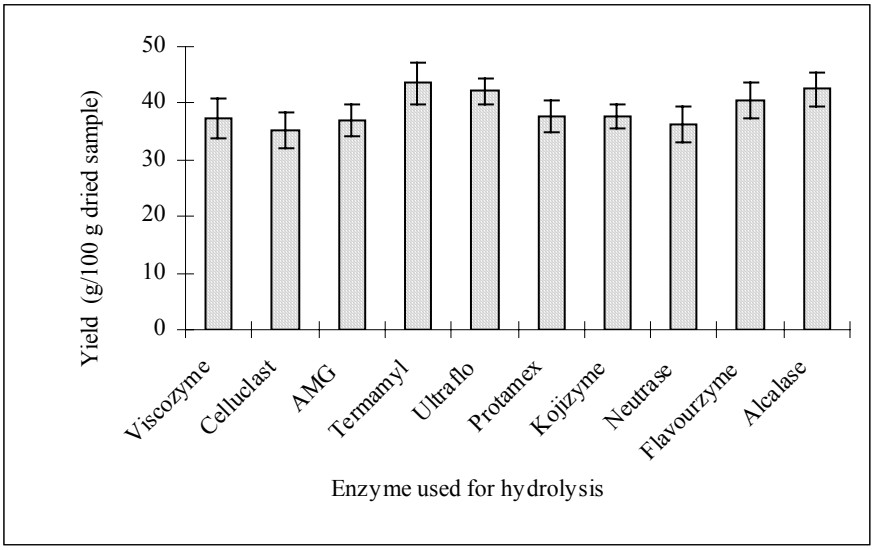

Fig. 4. Yield of Tot hydrolysates obtained by treatments of different enzymes at their optimum pH and temperatures for 3 days.

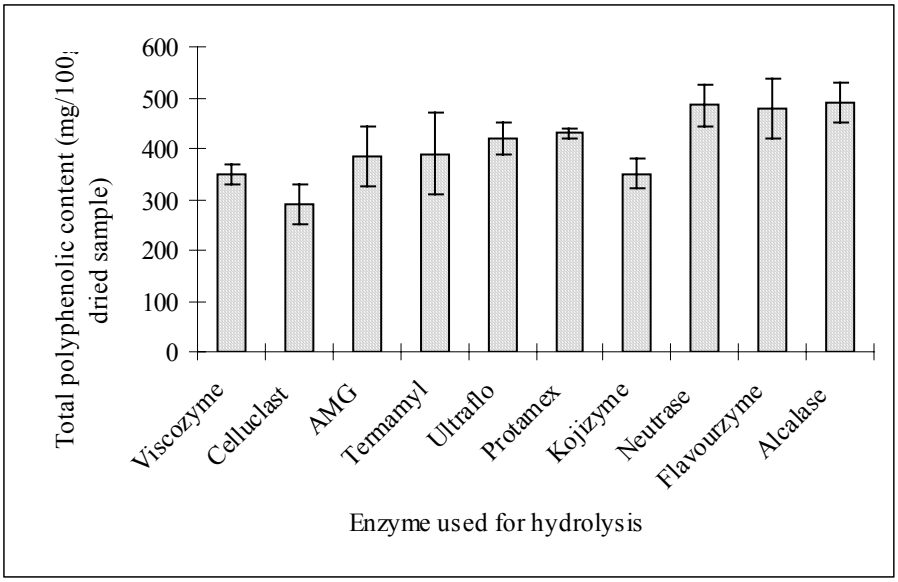

Fig. 5. Total polyphenolic content in the Tot hydrolysates obtained using different enzymes at their optimum pH and temperatures for 3 days.

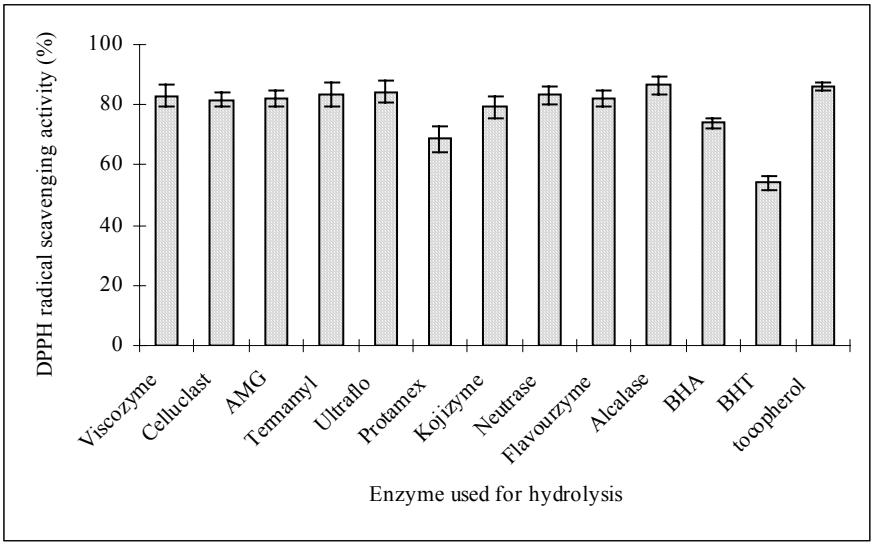

Fig. 6. DPPH radical scavenging activity (%) of Tot hydrolysates obtained using different enzymes at their optimum pH and temperatures for 3 days.

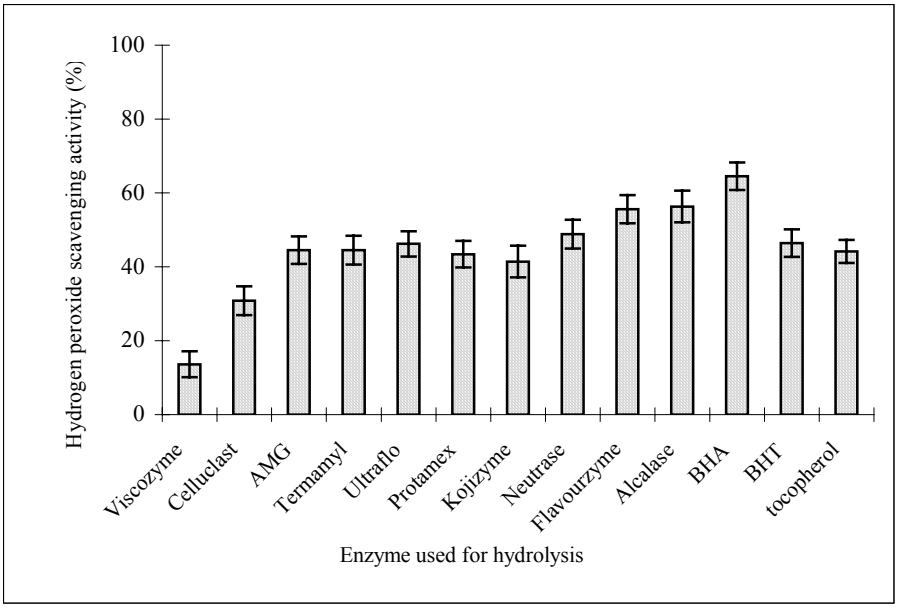

Fig. 7. Hydrogen peroxide scavenging activity (%) of Tot hydrolysates obtained using different hydrolytic enzymes at their optimum pH and temperatures for 3 days.

3.2. Effect of enzymatic extraction time and enzyme dosage

The yield of each hydrolysate treated with all the selected enzymes was increased gradually with the time (Fig. 8). Effect of the enzyme dosage (enzyme/substrate ratio) on yield was dose-dependent. Similar to the effect of time and enzyme dosage on yield, the total polyphenolic contents of hydrolysates were increased with the increment of time and enzyme dosage (Fig. 9). Hydrolysates treated with proteases were relatively higher in total polyphenolics compared to the hydrolysates treated with carbohydrases. Of the two carbohyrases investigated for total polyphenolic content, Ultraflo treated hydrolysate was best over the hydrolysate treated with Termamyl. DPPH radical scavenging activity of the hydrolysates was increased in a dose dependant manner and also increased with the increment of the time (Fig. 10). DPPH radical scavenging activity of proteases treated hydrolysates was comparatively higher than the hydrolysates treated with carbohydrases. In the third day after the enzymatic treatment, Alcalase of the proteases (at 5 % dosage) and Ultraflo of the carbohydrases (at 5 % dosage) reported the highest DPPH radical scavenging activity. Also, the hydrogen peroxide scavenging activity of the hydrolysates was increased in a similar pattern as in the DPPH radical scavenging activity (Fig. 11). Moreover, the hydrogen peroxide scavenging activity of the proteases treated hydrolysates was also higher than the hydrolysates treated carbohydrases.

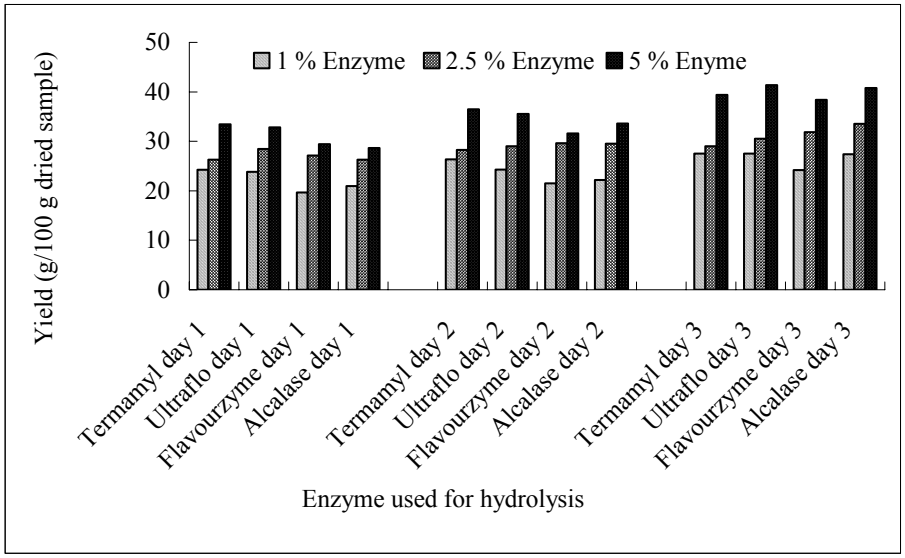

Fig. 8. Yield of Tot hydrolysates obtained using selected four enzymes at their optimum pH and temperatures for 3 days.

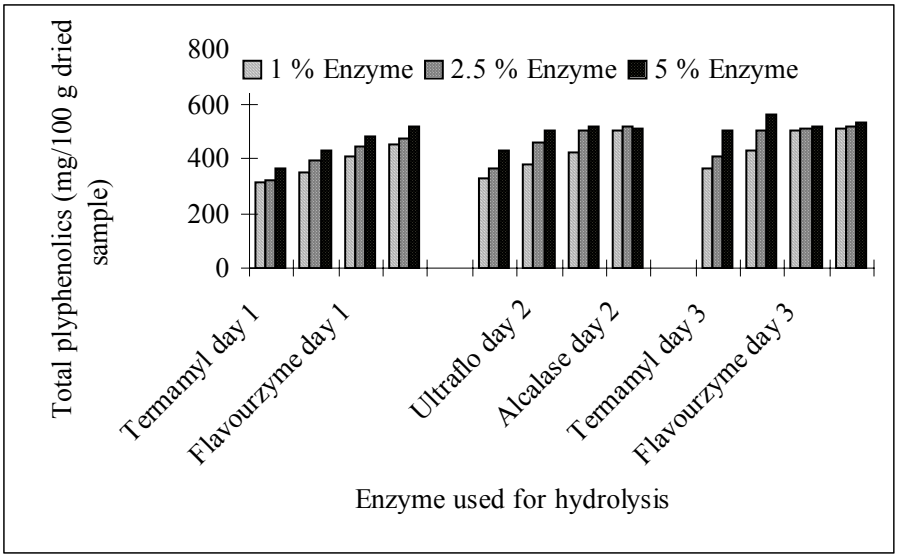

Fig. 9. Total polyphenolic content of Tot hydrolysates obtained using four selected enzymes at their optimum pH and temperatures for 3 days.

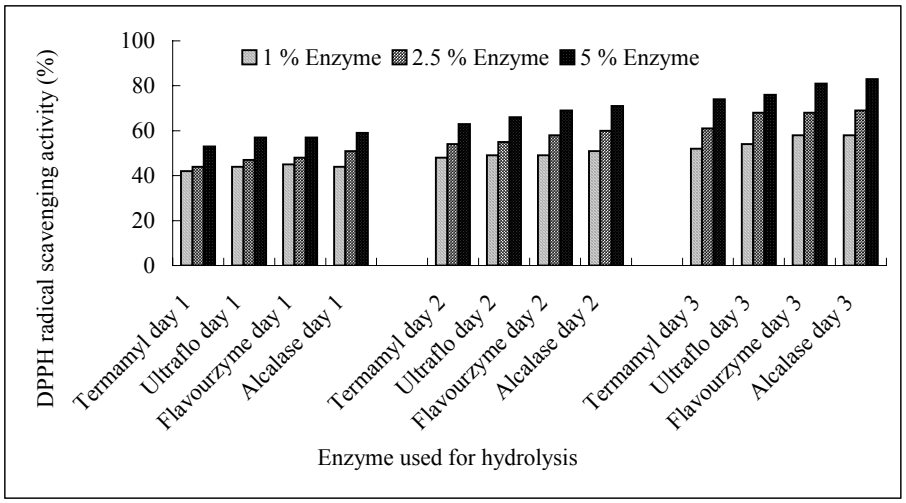

Fig. 10. DPPH radical scavenging activity (%) of Tot hydrolysates obtained using four selected enzymes at their optimum pH and temperatures for 3 days.

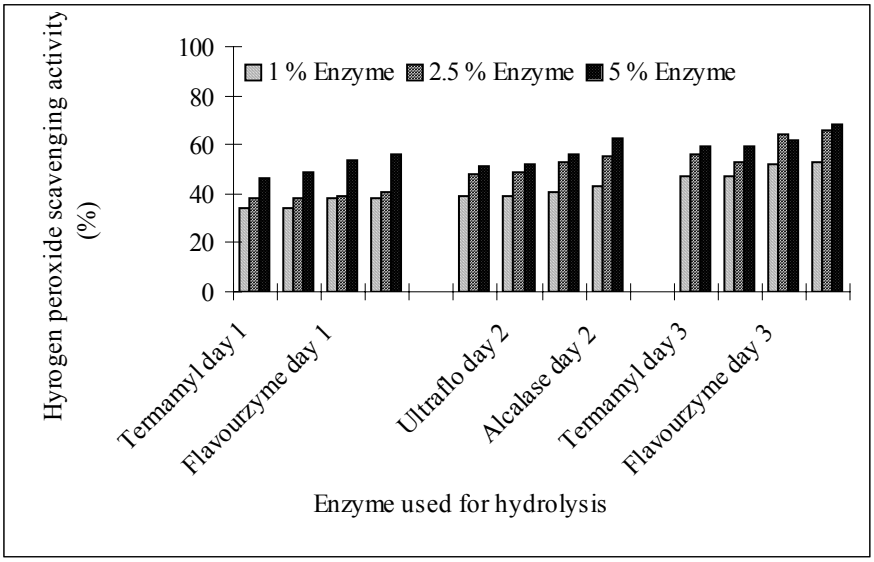

Fig. 11. Hydrogen peroxide scavenging activity (%) of Tot hydrolysates obtained using four selected enzymes at their optimum pH and temperatures for 3 days.

3.4. Effect of combination of protease and carbohydrases

Combination of proteases and carbohydrases to hydrolyze both proteins and carbohydrates expected to be more effective than the single hydrolysis (either by proteases or carbohydrases). In comparison of the results of combined enzymatic hydrolysis and single enzymatic hydrolysis, there were no significant yield increment but the total polyphenolic content was notably increased (Table 3). Investigated antioxidative activities of DPPH radical and hydrogen peroxide scavenging activities were also increased due to combination of enzymes.

Table 3. Results of Tot hydrolysates obtained by mixed enzyme treatment for 3 days

Parameter	Result
Yield	41.3 (g/100 g (dried sample)
Carbohydrates	26.2 (g/100 g (dried sample)
Protein	11.3 (g/100 g (dried sample)
Total polyphenols	820 (mg/100 g (dried sample)
DPPH radical scavenging activity	84.5%
Hydrogen peroxide scavenging activity	71%

Samples were at the original level after hydrolysation (without adjusting their concentration). DPPH concentration; 3 x 10^{-5} M.

3.5. Fractionation of hydrolysates according to the molecular weight of constituents

Distribution of yield in four molecular weight fractions was investigated and particular results showed that fractions of >30 kDa was significantly higher than the other three fraction of <30 kDa (Fig. 11). Of the < 30 kDa fractions, 1-10 kDa fraction contained the highest content and <1 kDa was the lowest. Distribution pattern of total polyphenolic content was slightly similar to the yield distribution except the net total polyphenolic content of <30 kDa fractions was higher than total polyphenolic content of >30 kDa fraction (Fig. 12). The DPPH radical scavenging activity of < 1 kDa fraction was comparatively lower than the other three fractions (Fig. 13). Of the fractions investigated at the concentration of 2 mg/ml, 1 - 10 kDa fraction showed the highest DPPH radical scavenging activity but which was not significantly different form the 10-30 kDa and >30 kDa fractions. Hydrogen peroxide scavenging activity of the fractions was similar to the DPPH radical scavenging activity except the highest hydrogen peroxide scavenging activity observed in the >30 kDa fraction (Fig. 14).

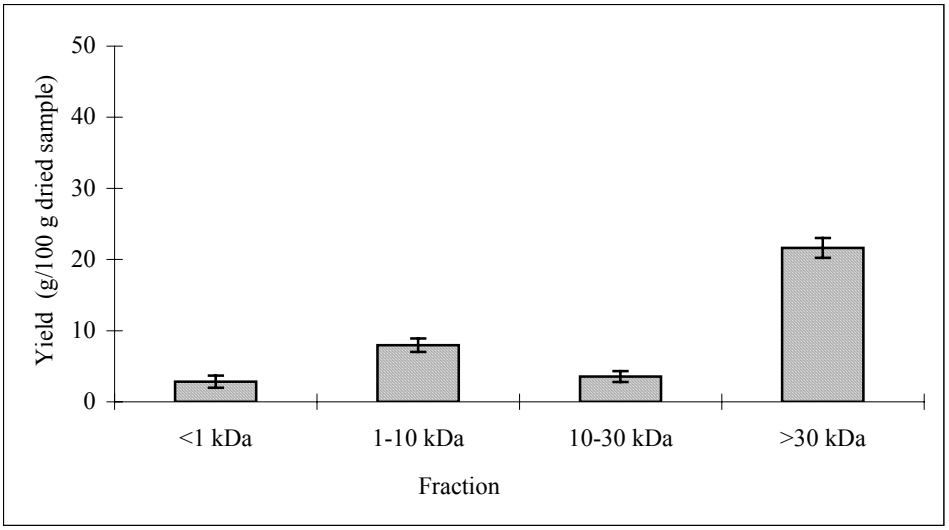

Fig. 12. Yield in the different molecular weight fractions of Tot hydrolysate obtained by mixed enzymatic hydrolysis with Alcalase (at 2.5% dosage) and Ultraflo (at 2.5% dosage) for 3 days.

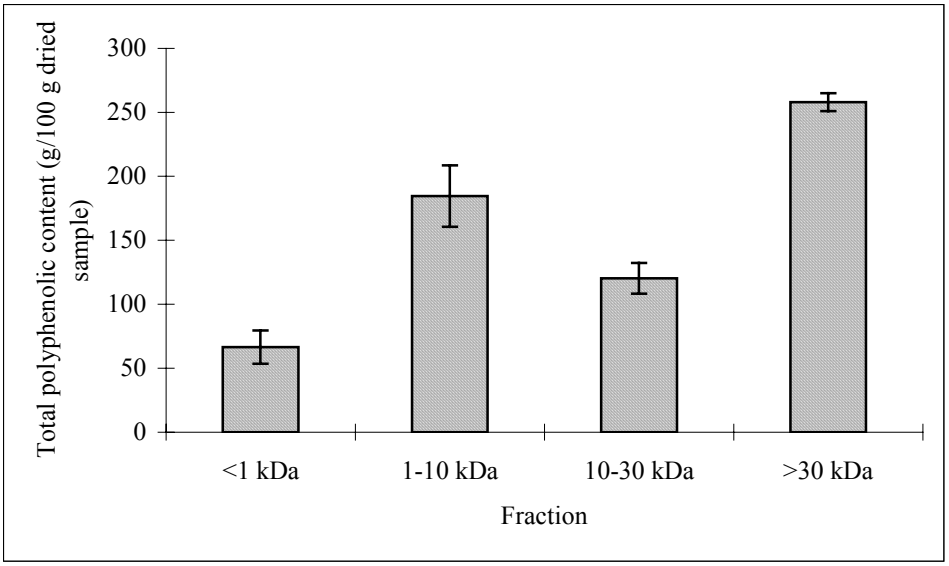

Fig. 13. Total polyphenolic content in the different molecular weight fractions of Tot hydrolysate obtained by mixed enzymatic hydrolysis with Alcalase (at 2.5% dosage) and Ultraflo (at 2.5% dosage) for 3 days.

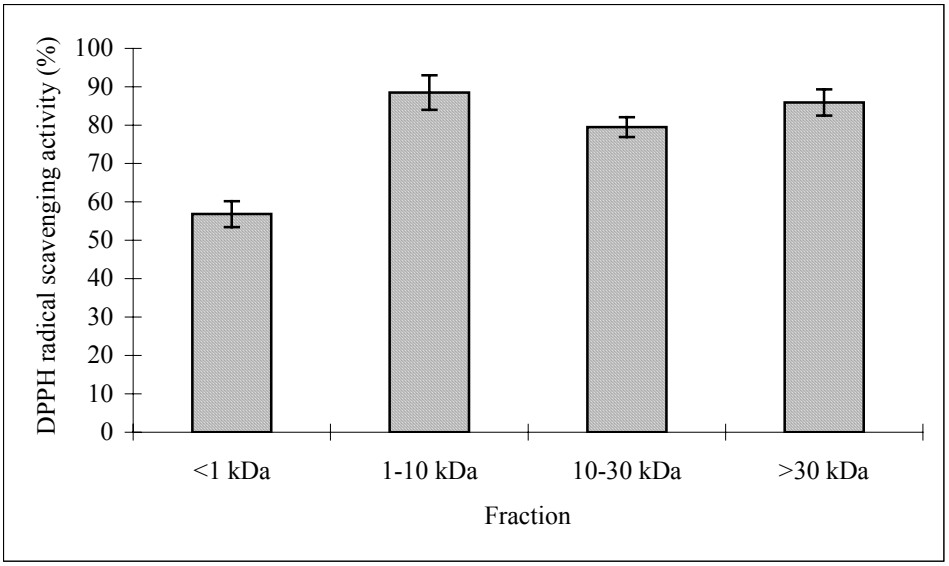

Fig. 14. DPPH radical scavenging activity (%) of the different molecular weight fractions of Tot hydrolysate obtained by mixed enzymatic hydrolysis with Alcalase (at 2.5% dosage) and Ultraflo (at 2.5% dosage) for 3 days.

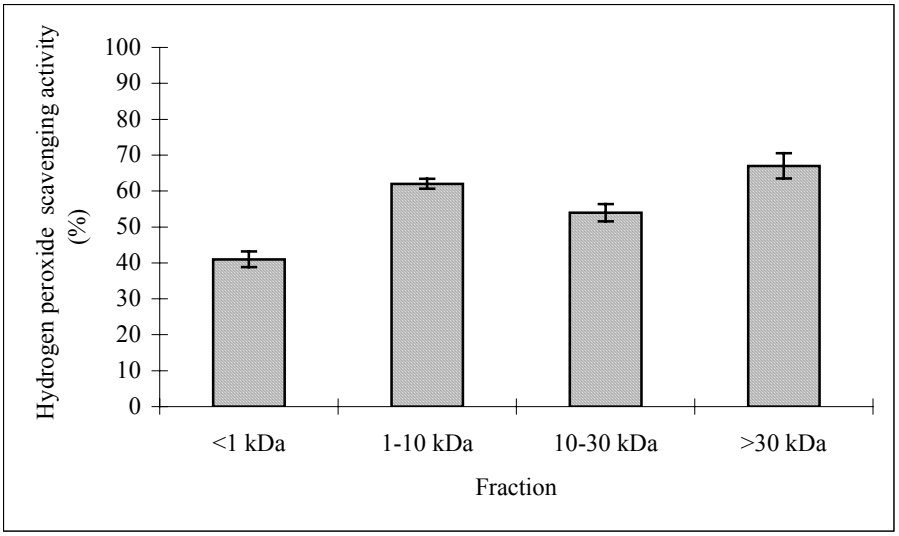

Fig. 15. Hydrogen peroxide scavenging activity (%) of the different molecular weight fractions of Tot hydrolysate obtained by mixed enzymatic hydrolysis with Alcalase (at 2.5% dosage) and Ultraflo (at 2.5% dosage) for 3 days.

4. DISSCUSSION

Considering the notable results recorded in this primary investigation, Termamyl and Ultraflo of carbohydrases and Flavourzyme and Alcalase of proteses were selected for further investigations of their optimum time and dosage. In general, α-amylase containing Termamyl and β-glucanase containing Ultraflo of carbohydrases and endo/exopeptidase containing Flavourzyme and alkaline endopeptidase containing Alcalase of proteases were more effective than other enzymes used. This can be mainly due to the breakdown of laminarin (β-1,3-glucans) by β-glucanase and amylose by α-amylase and peptide bonds by endo/exopeptidase and alcalase.

The observations in enzymatic extraction time and enzyme dosage investigation suggests that the breakdown of protein/protein based materials (breakdown of peptide bonds in the interior of a polypeptide chain or protein molecule) can contributes to the enhanced antioxidative activities of Tot hydrolysates. Moreover, Ultraflo treated hydrolysates showed higher DPPH radical and hydrogen peroxide scavenging activities compared to Termamyl treated hydrolysates. Laminarin (β-1, 3-glucan) is the main storage polysaccharide of brown seaweeds and this observation suggests that breakdown of lamiarin by β-glucanase of Ultraflo effectively contributes to the enhanced antioxidative activity. Flavourzyme contains both endo and exopeptidases but Alcalase contains only endopeptidase. This is an agreement with the highest DPPH radical scavenging activity (in electron spin resonance spectrometrical assay) recorded for the Ultraflo enzymatic extract over AMG, Celluclast, Termamyl, and Vizcozyme by Ahn et al. (2003) The higher activity of

Alcalase over Flavourzyme suggests that the relatively higher amount of endopeptidase in Alcalase could have enhanced the effect. Therefore, it is obvious that Alcalase of proteases and Ultraflo of carbohydrases are more suitable than Termamyl and Flavourzyme.

There are large amount of polysaccharides and proteins in the seaweeds that not only interrupt the effective extraction of desired antioxidative compounds but also complex (formation of complexes) with desired bioactive materials. Proteins are prone to complex with polyphenols and precipitate further (Moen et al., 1997). Moreover, proteins/breakdown products of proteins and carbohydrates/breakdown products of carbohydrates themselves can exert the enhancement of antioxidative activity. A number of studies on brown seaweed polysaccharides reported potential biological activities, specially sulfated polysaccharides reported to have antioxidative, anti-HIV, anticoagulant and anti-mutagenic effects (Beress et al., 1993; Nardella et al., 1996; Durig et al., 1997; Ruperez et al., 2002). Breakdown of proteins disrupts protein-polyphenol complex formation. Ramos and Xiong (2002) reported notable antioxdative activities of enzymaticaly hydrolyzed protein hydrolysates.

The concentration (2mg/ml) used in fractionated samples (<1, 1-10, 10-30 and >30 kDa) were approximately 2 fold less than the concentrations used during the selection of enzymes (Effect of different enzymes on extraction of antioxidative compounds) and standardization of optimum hydrolysis conditions (Effect of enzymatic extraction time and enzyme dosage). Hence, it conforms the promising antioxidative activity of the constituents > 1 kDa moleculer weight. Moreover, the comparatively higher polyphenolic content of 1-10 kDa fraction and its higher antioxidative activity

reports the antioxidative activity of low molecular weight polyphenols. The notable correlation of polyphenolic content of the each fraction and their antioxidative activity confirms the results recorded in our previous study (Siriwardhana et al., 2003).

Antioxidative activity of Tot like brown seaweeds can be due to carotenoid pigments (fucoxanthin), polyphenolics (phlorotannins), vitamin (vitamin C and E), sulfated polysaccharides or their breakdown products (laminarin, fucoidans) and proteins or their breakdown products (Nardella, 1996; Yan et al., 1999; Ramos and Xiong, 2002; Burtin, 2003). In this enzymatic hydrolysis, polyphenolics, vitamin C, sulfated polysaccharides and proteins of the above potential antioxidative materials can be the most effective due to their native water-soluble character. Phlorotannins of brown seaweeds constitute an extremely heterogeneous group of molecules (structure and polymerization degree of heterogeneity) providing a wide range of potential biological activity (Burtin, 2003). This phlorotannins are formed by the polymerization of phloroglucinol (1, 3, 5 trihydroxybenzene) and have molecular weight ranging from 126.5 Da to 650 kDa (Ragan and Glombitza, 1986). Generally, a major part of brown seaweed polyphenols is high molecular weight (>10 kDa) and potentially binds with proteins (Moen, 1997). Therefore, the breakdown of proteins may enhance the availability of free polyphenols to scavenge free radicals or any other oxidative materials.

The promising antioxidative activity of all the fractions >1 kDa suggests the further research work to identify the specific beneficial effects not only the antioxidat activity but also a variety of bioactivities including antimutagenic, anticoagulent and anti-hypertensive activities. Moreover, the different molecular weight poly-

phenolic compounds involved with antioxidative activities to be further investigated related to their structure and functional properties.

Part III

Optimization of hydrophilic antioxidant (phlorotannins) extraction from Tot (*H. fusiformis*) by integrating treatments of enzymes, heat and pH control

1. ABSTRACT

Effective extraction of bioactive compounds can be achieved by treatments such as pH control, heat and enzymatic hydrolysis. Tot antioxidants were extracted with those treatments individually and extraction efficacies were investigated by measuring total phenolic contents and antioxidant activities. Increased pH could successfully improve the extraction and incubation at pH 12.0 for 12 hr was the most effective pH treatment. Incubation at 100 ^{o}C for 45 min was significantly (P<0.05) effective than the other heat treatments tested. Optimum condition for enzymatic treatment was combination of 2% Alcalase (alkaline endopeptidase/protease) and 3% Ultraflo (β–glucanase/carbohydrase) at pH 8.0 and 54-58 ^{o}C for 24 hr. Integration of those optimized treatments in the extraction sequence of heat (H), enzymatic hydrolysis (E) and pH control (P) was the most effective sequence. Compared with other extraction sequences, HEP sequence indicated significantly higher phenolic content (phlorotannin content) and antioxidative activities in DPPH (1, 1-Diphenyl-2-picrylhydrazyl) and H_2O_2 scavenging assays.

2. MATERIALS AND METHODS

2.1. Materials

Tot was collected from the Jeju Island coast of S. Korea in February 2004. Epiphytes, salt and sand were removed using tap water and the samples were rinsed with deionized water before freeze-drying.

Ultraflo L (β-glucanases) and Alcalase 2.4 L FG (alkaline endo-peptidase) enzymes were purchased from Novo Co. (Novozyme Nordisk, Bagsvaerd Denmark). 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO) and Folin-Ciocalteu reagents were purchased from Sigma Co. (St. Louis, USA). 2, 2-Azino-bis (3-ethylbenzthiazolin)-6-sulfonic acid (ABTS) and peroxidase were purchased from Fluka Co. (Buchs, Switzerland). All the other chemicals used were analytical grade supplied by Fluka or Sigma Co.

2.2. Experimental design for optimized production of antioxidant extracts from Tot

Present work was designed to investigate the optimum composition of the enzyme mixture and its optimum hydrolysis conditions (pH and temperature). Simultaneously, two independent experiments were designed to evaluate the effects of pH and heat on extraction of Tot antioxidants. Furthermore, the effects of such treatments on antioxidant stability were also investigated with the same experiments. Finally, the three treatments (heat, pH control, and enzymatic hydrolysis) at their optimum conditions were combined/integrated in all possible processing sequences and determined the best sequence for the effective production of antioxidant extracts.

In the determination of antioxidant extraction efficacy of different treatments, both extraction efficacy (yield and polyphenilic extraction) and antioxidant activities (DPPH radical and H_2O_2 scavenging) were considered and those effects were statistically analyzed.

2.3. Effect of pH on extraction of Tot antioxidants (pH control)

One gram of freeze-dried Tot was mixed with 100 ml of distilled water and incubated at different pH conditions (pH 2.0-12.0) for 1 day without enzymatic hydrolysis. The pH conditions were adjusted with 1M HCl / NaOH. Resultant mixtures were filtered in vacuum with Whatman No. 1 (Whatman Ltd. England) filter paper and final pH was adjusted to 7.0. The best pH treatment (effective pH in liberation of antioxidants) was selected and its effect at different incubation times (0, 0.5, 1, 2, 4, 6, 8, 10, 12, 18, 24 hr) was investigated in order to determine the optimum incubation time.

2.4. Effects of heat on extraction of Tot antioxidants (heat treatment)

One gram of freeze-dried Tot was mixed with 100 ml of distilled water and incubated in shaking incubator at different temperatures (25, 50, 75 and 100 ^{o}C) for different times (0, 15, 30, 45 and 60 min) without enzymatic hydrolysis. Resultant mixtures were allowed to cool and filtered in vacuum. Thereafter, the optimum heat treatment for the antioxidant extraction was also determined.

2.5. Enzymatic hydrolysis of Tot

Freeze dried Tot was ground into a fine powder and 1 g was mixed with 100 ml of distilled water in six separate conical flasks. The pH and temperature of the mixtures were adjusted as 7.5 and 55 ^{o}C respectively. Enzymes were then added at the total of 5% dosages (enzyme/substrate ratio) with different ratios of Alcalase and Ultraflo. After incubating for 1 day the enzyme activity of hydrolysates was inactivated by heat (100 ^{o}C for 10 min) and the mixtures were filtered in vacuum. Of the resultant enzymatic hydrolysates, the best hydrolysate was selected by comparing the antioxidant extraction efficacies. Selected enzyme mixture of Alcalase and Ultraflo was further investigated for its optimum pH and temperature conditions. The pH and temperature conditions of the enzyme reaction mixtures were adjusted in ranges from 7.0 to 8.0 with 0.2 intervals (by using 1M HCl / NaOH) and 50 ^{o}C to 60 ^{o}C with 2 ^{o}C intervals respectively. The hydrolysates obtained at the best enzyme ratio under different pH and temperature conditions were compared for their antioxidant extraction efficacies in the determination of optimum conditions for the enzymatic hydrolysis.

2.6. Separation of hydrophilic antioxidants

In order to obtain hydrophilic antioxidants, all the extracts from each treatment were fractionated with an organic solvent mixture of methylene chloride plus methanol (1:1, v/v) and the resultant aqueous fractions were applied in the assays used for the determination of extraction efficacies.

2.7. Parameters for the determination of efficacy of antioxidant extraction

Hydrolysates obtained in all experimental conditions were investigated for the antioxidant extraction efficacies and those were statistically compared. Antioxidant extraction efficacies were determined by the yield, total polyphenolic content and antioxidative activities such as DPPH radical and hydrogen peroxide scavenging values (SC_{50} : sample concentration required for 50% scavenging of given oxidant). Before starting all the assays, the pH of the extracts was adjusted to 7.0 by using 1M HCl / NaOH.

2.8. Measurement of extraction yield

Yield of the resultant extracts was calculated as a percentage of dried weight of the extract over the dried weight of the raw seaweed sample used.

2.9. Total phenolic content

Content of the total phenolic compounds was determined according to the protocol similar to Chandler and Dodds (1993). One ml of the extract was mixed in a test tube containing 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 ml of 5% Na_2CO_3 was added. Thoroughly mixed mixture was placed in a dark room for 1 hr and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea). A gallic acid standard curve was used for the extrapolation of total phenolic content. Polyphenols in brown algae is called as phlorotannins.

2.10. DPPH radical scavenging assay

The assay was based on the scavenging of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by the radical scavenging components in the extract. Modified method of Brand Williams (1993) was used to determine the free radical scavenging activity. DPPH solution in dimethyl sulfoxide (DMSO) was prepared at the concentration of 3 $x10^{-5}$ M. A 2 ml fraction of Tot extract and 2 ml of freshly prepared DPPH solution were thoroughly mixed. The reaction mixture was incubated for 1 hr and absorbance was recorded at 517 nm using UV-VIS spectrophotometer.

2.11. Hydrogen peroxide (H_2O_2) scavenging assay

The assay was based on the ability of the extract to scavenge the hydrogen peroxide in ABTS-peroxidase medium according to the method of Mueller (1995). Hundred micro liter of Tot hydrolysate and 20 µl of 10 mM hydrogen peroxide were mixed with 100 µl of phosphate buffer (0.1 M, pH 5.0) in a 96-microwell plate and incubated at 37 ^{o}C for 5 min. Finally, 30 µl of freshly prepared 1.25 mM ABTS and 30 µl of peroxidase (1 U/ml) were mixed and incubated at 37 ^{o}C for 10 min before recording the absorbance in ELISA reader (Tecan, Sunrise Co. Ltd. Sweden) at 405 nm.

2.12. Calculation of 50% scavenging concentration (SC_{50})

The concentration of the extract (mg/ml) that required to scavenge 50% of DPPH radical or H_2O_2 (SC_{50}) was calculated by using the percent scavenging activities of five different extract concentrations.

The percent scavenging activity of the extract was calculated by the following equation:

$[1-(A_i - A_j)/A_c] \times 100$

Where in the DPPH assay:

A_i= the absorbance of extract mixed with DPPH solution;

A_j = the absorbance of same extract mixed with 2 ml DMSO;

A_c = the absorbance of DPPH solution adding 2 ml DMSO.

In hydrogen peroxide assay:

A_i = the absorbance of reaction mixture mixed with extract

A_j = the absorbance of reaction mixture (without ABTS) mixed with same extract

A_c = the absorbance of control (without extract).

2.13. Determination of effective processing sequence of treatments

Three optimized treatments (heat, pH control and enzymatic hydrolysis) were integrated to enhance the effect of antioxidant extraction. Thus, Tot antioxidant extracts were prepared in all possible sequences of the three treatments under the optimum conditions determined in the experiments above. The effective sequence was determined for the production of Tot extracts rich in antioxidants.

2.14. Statistical analysis

All the experiments were performed in triplicates (n=3) and an ANOVA test (using SPSS 11.5 statistical software) was used to compare the mean values of each treatment. Significant differences between the means of test parameters were determined by using Duncan test ($P<0.05$).

3. RESULTS

3.1. Effect of pH on antioxidant extraction (effect of pH control)

Extraction of Tot antioxidants under different pH conditions was investigated in order to determine the effect of both acidic and alkaline hydrolysis (Table 4). As we expected, there was a progressive increment in antioxidative activity with the increment of from 8.0 to12.0 pH. Incubation time increased from 0 to12 hr under pH 12 (the optimum pH) exhibited progressively increased antioxidant extraction efficacy but the effect of further incubation time increment from 12 to 24 hr showed no significant effect on extraction efficacy (Table 5). The higher yields and polyphenolic contents (34.5 and 7.59 g/100g dried sample respectively) were recorded at pH 12.0. Furthermore, the higher DPPH radical scavenging activities were recorded at both pH 11.0 and 12.0 (SC_{50} : 2.41 and 2.43 mg/ml respectively), where those activities were even not significantly different each other. The H_2O_2 scavenging activity at pH 12.0 (SC_{50}: 1.87 mg/ml) was significantly higher than those of all the other pH treatments.

Considering the totality in terms of antioxidant extraction efficacy under different pH conditions, incubation at pH 12 for 12 hr were extrapolated as optimum pH control condition.

Table 4. Effect of different pH on the extraction of Tot antioxidants

Incubation pH	Extraction efficacy (g/100g dried sample)		Antioxidant activity SC_{50} (mg/ml)	
	Yield	Total polyphenols	DPPH	H_2O_2
2	27.5^{d} ±2.3	0.47^{a} ±0.01	3.46^{e} ±0.13	4.25^{a} ±0.21
3	18.5^{abc}±2.5	1.11^{b} ±0.08	3.89^{d} ±0.21	3.98^{ab} ±0.22
4	14.6^{a} ±2.7	1.43^{c} ±0.06	4.65^{ab} ±0.16	3.68^{bc} ±0.18
5	16.5^{abc} ±1.6	1.57^{d} ±0.08	4.82^{ab} ±0.09	3.99^{ab} ±0.26
6	18.3^{ab} ±2.3	1.81^{e} ±0.03	4.21^{c} ±0.11	3.21^{d} ±0.14
7	19.5^{bc} ±1.8	1.73^{e} ±0.08	4.61^{b} ±0.09	2.59^{e} ±0.11
8	19.5^{bc} ±2.6	1.47^{cd} ±0.04	4.87^{a} ±0.08	3.51^{cd} ±0.08
9	20.4^{bc} ±1.4	1.72^{e} ±0.06	3.54^{e} ±0.09	3.46^{cd} ±0.21
10	22.5^{ca} ±1.4	1.69^{e} ±0.07	3.21^{f} ±0.12	2.64e ±0.18
11	26.4^{d} ±2.7	3.54^{f} ±0.06	2.41^{g} ±0.22	2.25f ±0.09
12	34.5^{e} ±3.1	7.59^{g} ±0.08	2.43^{g} ±0.14	1.87^{g} ±0.16

All the samples were incubated at 25°C for 1 day.

No enzymatic hydrolysis was performed.

Values within each column followed by a different letter are significantly different (P<0.05).

Table 5. Effect of alkaline treatment at different incubation periods on extraction of Tot antioxidants

Incubation time (hr)	Extraction efficacy (g/100g dried sample)		Antioxidant activity SC_{50} (mg/ml)	
	Yield	Total polyphenols	DPPH	H_2O_2
0	26.3^{a} ±2.13	2.12^{a} ±0.08	3.62^{a} ±0.08	2.23^{a} ±0.01
0.5	28.5^{a} ±1.35	3.72^{b} ±0.06	2.94^{b} ±0.06	2.16^{a} ±0.11
1	29.7^{ab} ±2.16	3.80^{b} ±0.12	2.68^{cd} ±0.04	2.11^{ab} ±0.08
2	31.8^{bc} ±2.11	5.15^{c} ±0.07	2.76^{c} ±0.08	2.06^{ab} ±0.13
6	32.8^{bc} ±1.43	6.51^{d} ±0.08	2.61^{d} ±0.08	2.06^{ab} ±0.14
12	34.8^{c} ±2.21	7.25^{e} ±0.09	2.42^{e} ±0.08	1.92^{b} ±0.11
24	34.4^{c} ±1.84	7.48^{f} ±0.09	2.42^{e} ±0.09	1.88^{b} ±0.16

All the samples were incubated at pH 12 and 25°C.

No enzymatic hydrolysis was performed.

Values within each column followed by a different letter are significantly different (P<0.05).

3.2. Effect of heat (thermal degradation) on antioxidant extraction

The capability of heat to enhance the extraction of antioxidants and its effect on antioxidative compounds was investigated with different heat treatments (Table 6). Specially, the effect of heat on breakdown of algal polysaccharides (both inter and intracellular matrix constituents) and liberation of target antioxidative compounds (mainly polyphenols) was investigated. Interestingly, there was no significant destruction of anitoxidative components but a notable significant degradation in matrix constituents resulting higher extraction efficacy under elevated thermal conditions. There was a significant difference in extraction of polyphenolic compounds between the heat treatments of 25 and 100 ^{o}C (1.4 and 3.74 g/100g dried sample) starting from their shortest time (15 min) of incubation. The extraction efficacy (yield and polyphenolic liberation) was progressively increased with the increment of extent of heat applied. Likewise, the antioxidative capabilities of the resultant extracts were also increased. In case of antioxidative activity of the extracts at 100 ^{o}C heat treatment (the highest heat treatment), the DPPH radical scavenging activity was observed to be progressively increased with the increasing of heating time but there was no difference in H_2O_2 scavenging activities. Hence, the treatment of 100 ^{o}C for 45 min was extrapolated as the optimum temperature-time combination.

Table 6. Effect of different heat treatments on extraction of Tot antioxidants

Incubation Temp (°C)	Time (min)	Extraction efficacy (g/100g dried sample) Yield	Total polyphenols	Antioxidant activity SC_{50} (mg/ml) DPPH	H_2O_2
25	60	17.5^{a} ±1.12	1.4^{a} ±0.21	2.50^{a} ±0.08	3.82^{a} ±0.12
50	15	24.5^{def} ±0.98	2.15^{bd} ±0.66	2.42^{ab} ±0.04	2.56^{b} ±0.22
	30	24.3^{def} ±1.3	2.03ac ±0.23	2.42ab±0.06	2.41^{bc} ±0.96
	45	26.2^{efg}±1.6	2.14^{bd} ±0.65	2.36^{bcd} ±0.06	2.31^{bc} ±0.12
	60	20.6^{b} ±2.2	2.02^{ac} ±0.44	2.28^{cde} ±0.08	2.22b^{c} ±0.04
75	15	21.4^{bc} ±1.4	1.91ab±0.31	2.40^{ab} ±0.07	2.38^{bc} ±0.06
	30	23.1^{bc} ±0.94	2.79^{de} ±0.42	2.38^{bc} ±0.05	2.36^{bc} ±0.13
	45	23.5^{cde}±1.5	3.04^{e} ±0.14	2.25^{de} ±0.06	2.11^{bc} ±0.08
	60	22.4^{bc} ±0.99	2.74^{ce} ±0.44	2.24^{e} ±0.09	1.94^{c} ±0.09
100	15	23.4^{cd} ±1.45	3.74^{f} ±0.32	2.19^{e} ±0.06	2.13^{bc} ±0.07
	30	27.4^{g} ±1.32	3.41^{ef} ±0.37	2.01^{f} ±0.06	1.95^{c} ±0.11
	45	27.5g±2.21	3.78^{f} ±0.26	1.86^{g} ±0.08	2.04^{bc} ±0.08
	60	26.5f^{g}±1.86	4.65^{g} ±0.28	1.84^{g} ±0.02	2.06^{bc} ±0.11

No enzymatic hydrolysis was performed.

Values within each column followed by a different letter are significantly different (P<0.05).

3.3. Optimum ratio of protease and carbohydrase for combination

In the present experiment, the efficacy of the extraction of antioxidants was investigated with 6 different mixtures of Alcalase and Ultraflo (Table 7). The hydrolysates treated with the both enzymes could significantly enhance the extraction of anitoxidative compounds compared to the control where the Tot was not subjected to enzymatic hydrolysis. Of the hydrolysates treated with different enzyme combinations, the combination made by mixing 2 % of Alcalase and 3% of Ultraflo (2:3 A/U) reported the maximum yield (42.5 g/ 100 g dried sample). The highest polyphenolic extraction was reported for 2:3 and 5:0 A/U combinations (4.35 and 4.58 g/100 g dried sample respectively). The DPPH radical scavenging activities of both 2:3 and 3:2 A/U combinations were significantly higher than those of other combinations tested. Compared to the other A/U combinations, a significantly higher H_2O_2 activity was reported in the 2:3 A/U combination (SC_{50}: 0.98 mg/ml).

Table 7. Determination of optimum ratio of Alcalase (A) and Ultraflo (U) for the combination in order to produce antioxidant rich extracts from Tot

Enzyme Dosage	Extraction efficacy (g/100g dried sample)		Antioxidant activity SC_{50} (mg/ml)	
A/U	Yield	Total polyphenols	DPPH	H_2O_2
0/5	33.5^{b} ±2.2	3.39^{ab} ±0.78	2.7^{b} ±0.2	1.35^{b} ±0.05
1/4	36.5^{b} ±3.1	3.89^{ab} ±0.54	2.57^{b} ±0.26	1.27^{b} ±0.04
2/3	42.5^{c} ±2.5	4.35^{ab} ±0.62	2.24^{c} ±0.26	0.98^{d} ±0.06
3/2	37.5^{b} ±2.1	4.14^{ab} ±0.48	2.16^{c} ±0.15	1.15^{c} ±0.03
4/1	34.0^{b} ±2.7	4.17^{ab} ±0.68	2.49^{bc}±0.1	1.32^{b} ±0.07
5/0	37.3^{b} ±1.6	4.58^{b} ±0.64	2.43^{bc}±0.32	1.32^{b} ±0.08
NE	19.2^{a} ±2.9	3.67^{a} ±0.94	4.41^{a} ±0.29	2.61^{a} ±0.06

Samples were incubated at pH 7.5 and 55 ^{o}C during a hydrolysis of 1 day.

Values within each column followed by a different letter are significantly different (P<0.05). NE; No enzyme

3.4. Optimum pH and temperature for the enzyme combination

The enzyme combination (2:3 A/U) selected above was further investigated for its optimum pH and temperatures for hydrolysis. Since optimum pH and temperature for Alcalase and Ultraflo were different (Alcalase; pH 8.0 and 50 ^{o}C and Ultraflo; pH 7.0 and 60 ^{o}C), conditions were optimized for the selected enzyme mixture (2:3 A/U). Therefore, the pH range of 7.0-8.0 and temperature range of 50-60 ^{o}C was investigated for the determination of optimum pH and temperature (Table 8). Unlike in other experiments, there was no any notable variation between different pH and temperature treatments tested. This can be explained that the combined enzyme activity is optimum not only at a definite pH or a temperature but also in a range of both pH and temperature. Extraction of polyphenolics at the ranges of pH 7.0-7.2 and 7.8-8.0 was comparatively higher than that of other pH ranges investigated. But in terms of antioxidant activity, both DPPH radical and H_2O_2 scavenging activities were highest at pH 8.0. In comparison, there was a tendency of liberating polyphenolics at the temperatures range of 54-58 ^{o}C when compared to the other temperatures tested. Simultaneously, the antioxidative activities were also comparatively higher in the same range (54-58 ^{o}C). Considering the antioxidative activities and extraction efficacies, pH 8.0 and temperature range of 54-58 ^{o}C were selected as the optimum conditions of the combined enzyme mixture for Tot hydrolysis.

Table 8. Determination of optimum pH and temperature for the combined enzyme mixture

Incubation conditions		Extraction efficacy (g/100g dried sample)		Antioxidant activity SC_{50} (mg/ml)	
pH	Temp.(°C)	Yield	Total polyphenols	DPPH	H_2O_2
7.0	50	27.8^{a-g} ±1.2	3.85^{gh} ±0.08	2.62^{a-e} ±0.12	1.98^{a} ±0.08
	52	31.4^{e-k} ±1.6	3.99^{h-k} ±0.10	2.61^{a-e} ±0.11	1.86^{b} ±0.06
	54	35.2^{k-l} ±2.4	4.08^{h-l} ±0.31	2.54^{a-f} ±0.05	1.68^{bc} ±0.05
	56	25.6^{a-c} ±1.6	4.44^{mn} ±025	2.40^{d-I} ±0.20	1.65^{cd} ±0.06
	58	32.1^{h-l} ±2.2	4.09^{h-l} ±0.05	2.54^{a-f} ±0.11	1.45^{ef} ±0.02
	60	39.1^{m} ±1.5	4.07^{h-l} ±0.13	2.62^{a-e} ±0.32	1.51^{de} ±0.11
7.2	50	31.5^{f-k} ±1.0	4.30^{k-n} ±0.02	2.31^{f-j} ±0.08	1.30^{gh} ±0.05
	52	34.6^{k-l} ±1.4	4.60^{n-o} ±0.07	2.42^{c-h} ±0.12	1.29^{gh} ±0.04
	54	31.5^{f-k} ±1.4	4.89^{o} ±0.08	2.44^{c-g} ±0.21	1.34^{fg} ±0.06
	56	32.4^{i-l} ±3.1	4.86^{o} ±0.16	2.23^{g-k} ±0.04	1.54^{c-e} ±0.09
	58	28.6^{c-I} ±1.5	4.21^{im} ±0.07	2.34^{f-I} ±0.04	1.47^{ef} ±0.11
	60	28.6^{c-I} ±2.4	4.85^{o} ±0.06	2.30^{f-j} ±0.13	1.52^{de} ±0.06
7.4	50	25.4^{ab} ±1.8	2.61^{ab} ±0.28	2.74^{ab} ±0.05	1.78^{b} ±0.05
	52	27.6^{a-f} ±1.8	2.32^{a} ±0.11	2.65^{a-d} ±0.12	1.65^{cd} ±0.06
	54	32.4^{i-l} ±2.0	2.82^{bc} ±0.31	2.80^{a} ±0.16	1.80^{b} ±0.06
	56	32.0^{g-l} ±1.6	2.71^{b} ±0.18	2.67^{a-c} ±0.15	1.54^{c-e} ±0.06
	58	28.4^{b-h} ±2.4	3.17^{de} ±0.22	2.45^{c-g} ±0.14	1.45^{ef} ±0.12
	60	29.8^{d-j} ±1.4	3.44^{ef} ±0.23	2.46^{c-g} ±0.13	1.35^{fg} ±0.03
7.6	50	25.6^{a-c} ±2.6	3.27^{de} ±0.26	2.74^{ab} ±0.14	1.54^{c-e} ±0.04
	52	26.4^{a-d} ±1.7	2.59^{ab} ±0.02	2.80^{ab} ±0.14	1.79^{b} ±0.08
	54	27.6^{a-e} ±1.6	2.86^{bc} ±0.08	2.74^{ab} ±0.12	1.64^{cd} ±0.04
	56	33.4^{j-}l ±1.1	3.03^{cd} ±0.21	2.76^{ab} ±0.15	1.55^{c-e} ±0.06
	58	31.4^{e-k} ±1.1	3.86^{gh} ±0.21	2.74^{ab} ±0.06	1.55^{c-e} ±0.01
	60	31.4^{e-k} ±2.5	3.93^{h-j} ±0.25	2.61^{a-e} ±0.04	1.30^{gh} ±0.06
7.8	50	24.5^{a} ±2.4	3.88^{g-i} ±0.16	2.33^{f-I} ±0.06	1.54^{c-e} ±0.12
	52	28.4^{b-h} ±1.8	3.60^{fg} ±0.24	2.32^{f-I} ±0.16	1.46^{e}f ±0.06
	54	31.5^{f-k} ±1.5	4.37^{l-n} ±0.16	2.36^{e-i} ±0.08	1.13^{i-k} ±0.05

Table 5. continued

	56	28.6^{c-l} ±2.0	4.24^{j}-m±0.14	2.45^{c-g} ±0.13	1.10^{jk} ±0.09
	58	34.6^{k-l} ±1.4	4.07^{h}-l ±0.22	2.53^{b-f} ±0.13	1.24^{g-l} ±0.12
	60	30.0^{d-j} ±1.4	5.41^{pq} ±0.16	2.14^{i-m} ±0.14	1.08^{jk} ±0.09
8.0	50	28.6^{c-l} ±2.1	5.25^{p} ±0.17	2.06^{j-m} ±0.21	1.05^{jk} ±0.09
	52	31.5^{f-k} ±2.1	4.79^{o} ±0.21	2.31^{f-j} ±0.05	1.18^{h-j} ±0.06
	54	35.4^{l} ±1.9	5.67^{qr} ±0.14	1.90^{m} ±0.14	0.99^{k} ±0.12
	56	32.6^{jl} ±3.4	5.93^{r} ±0.11	1.94l^{m} ±0.13	0.87^{l} ±0.01
	58	32.0^{hl} ±1.5	5.50^{p-q} ±0.09	2.04^{k-m} ±0.02	1.01^{k} ±0.06
	60	34.0^{kl} ±2.2	3.94^{h-j} ±0.12	2.16^{h-l} ±0.16	1.24 ^{ig} ±0.07

Samples were treated with 2:3 A/U and hydrolyzed for 1 day.

Values within each column followed by a different letter are significantly different (P<0.05).

3.5. Effective sequence of treatments towards optimum efficacy in production of antioxidant extracts

Since there were three different effective treatments (heat treatment, pH control and enzymatic hydrolysis) to be combined/integrated in the most effective sequence, all the possible sequence combinations were investigated for production of antioxidant-rich extracts from Tot (Table 9). As we expected, the efficacy of different sequences were entirely different and conformed the importance of determination of the most effective sequence. Of the sequences investigated, the best processing sequence was in the order of heat treatment, enzymatic hydrolysis and pH control (alkaline hydrolysis).

Table 9. Determination of effective processing sequence of treatments for the production of Tot antioxidants

Processing Sequence	Extraction efficacy (g/100g dried sample)		Antioxidant activity SC_{50} (mg/ml)	
	Yield	Total polyphenols	DPPH	H_2O_2
E H	38.6^{a} ±1.12	7.21^{b} ±0.21	7.8^{a} ±0.04	1.26^{a}±0.08
E P	40.2^{ab} ±1.24	8.12^{c} ±0.09	1.3^{b} ±0.05	0.96^{b}±0.04
E P H	41.2^{abc} ±2.1	6.94^{b} ±0.16	0.74^{c}±0.12	0.42^{c}±0.05
E H P	44.4^{d} ±1.42	8.91^{c} ±0.08	0.63^{c}±0.04	0.16^{de}±0.07
H E P	42.6^{bcd}±1.36	10.01^{f}±0.14	0.42^{d}±0.04	0.14^{e}±0.06
H P E	43.2^{bcd}±1.43	6.29^{a} ±0.22	1.42^{b}±0.06	0.36^{c}±0.09
P E H	43.6^{cd} ±2.31	8.53^{d} ±0.21	0.76^{c}±0.09	0.34^{c}±0.01
P H E	42.4^{bcd}±1.22	9.16^{e} ±0.21	0.73^{c}±0.07	0.24^{d}±0.04
BHT			0.36^{e}±0.02	0.07^{e}±0.004
α-tocopherol			0.73^{f}±0.07	0.12^{e} ±0.04

E: enzymatic hydrolysis, H: heat treatment, P: alkaline pH treatment.

All the treatments were applied at their optimum conditions standardized in this experiment. Values within each column followed by a different letter are significantly different (P<0.05).

4. DISSUSSION

Alkaline hydrolysis could have contributed to this distinctly enhanced antioxidant extraction via a single or integrated effect of glycosidic bond splitting, ester breaking, ion exchange, desulfation and protein-polyphenol complex breaking abilities of alkaline medium. The complex matrix (a barrier for the extraction of interior compounds) of brown seaweeds is an association of both soluble (alginate, laminarin, fucoidin and polygalactose-sulphate esters) and insoluble (cellulose) fibers (Boney, 1969; Lahaye and Kaeffer, 1997; Ruperez and Saura-Calixto, 2001). Breakdown of such barriers can contribute to the liberation of interior compounds and further easiness in extraction. Specially, alginates at higher pH conditions (alkaline pH) can undergo alkaline β-elimination thus, occurring deploy-merization and further liberation of antioxidative materials (Haug et al., 1967; Holme et al., 2001). Moreover, the brown algal sulfated polysaccharides (fucoidan) are known to contain L-fucose residue as the main sugar constituent and sulfate ester. It is a well-known phenomenon that the esters can be easily hydrolysed with alkaline treatments. The strong alkaline medium can enhance the cation exchange in Ca^{+2} or Mg^{+2} alginates or the desulfation of sulfated polysaccharides, thus can alter/degrade/loose the stability of matrix. Likewise, alteration/breakdown of both extra and intracellular matrix of Tot can enhance the efficacy of antioxidative compound liberation.

In acidic conditions, polyphenols can cross-link with proteins resulting haze or precipitation. Thus, the tendency of proteins to complex with polyphenols under acidic conditions can be avoided by alkaline conditions. This clearly explains the comparatively lower

polyphenolic contents recorded under acidic conditions. There was a progressive increment of polyphenolic liberation in the ranges of pH 2.0-6.0 and again from pH 10.0-12.0. The significantly higher polyphenolic liberation at pH 12.0 (7.59 g/100g dried sample) compared to pH 2.0 can be obviously due to the breakdown of protein-polyphenol complex or inhibition of complex formation. There have been a number of research articles, which reported the correlation between polyphenols and antioxidant activity of both plants and seaweeds (Yan et al., 1996; Kim et al., 1998; Ruberto et al., 2001; Siriwardhana et al., 2003; Athukorala et al., 2003). Brown algae reported to have the highest polyhenolic content (5-15 % of the dried weight) compared to the green or red algae and we have already reported the possitive correlation between Tot antioxidant activity and its polyphenolic (phlorotannin) content (Siriwardhana et al., 2003).

In general, the thermal degradation reactions of alginic acid like high molecular weight polysaccharides are slow since it involves breaking of covalent –C-O- linkages. Thus, the heat treatment at 100 ^{o}C for 45 min could be compatible with the requirement for such a breakdown resulting liberation of antioxidative compounds from Tot. For the efficacy of extraction of antioxidative compounds, not only the breakdown of alginate may have contributed but also the breakdown of other polysaccharides (eg. fucoidan and laminarin) thus, loosing the ability of the matrix to be act as a consistent barrier.

In our first study, we reported the enhanced efficacy of extraction of antioxidative compounds in hydrolysates treated with Alcalase (alkaline endopeptidase) of proteases and Ultraflo (β-glucanese) of carbohydrases. Moreover, the combination of those two

enzymes could synergistically enhance the liberation of antioxidants than that of using those enzymes alone (Siriwardhana et al., 2004). Brown seaweeds contain soluble dietary fiber polysaccharides as alginates, fucans and laminarians together with the insoluble fibers made of cellulose (Lahaye and Kaeffer, 1997; Ruperez and Saura-Calixto, 2001). Alginic acid is the main structural component of the brown algal cell wall while laminarin (β-1,3-glucan) is the main storage polysaccharide (Moen et al., 1997; Kloareg and Quatrano, 1998). Furthermore, brown seaweeds reported to contain 5-15% proteins, which can easily combine with polyphenols/phlorotannins (one of potential antioxidative compounds). Likewise, both carbohydrates and proteins are barriers for the extraction of antioxidative compounds from seaweeds.

The higher antioxidant extraction efficacy of 2:3 A/U combination can be explained both qualitatively and quantitatively. The 2:3 A/U combination of endopeptidase and β-glucanase could effectively hydrolyse the endopeptides and laminarins (β-glucan) respectively. The content of carbohydrates (polysaccharides) is higher than that of proteins (peptides) thus, 3% of β-glucanase (carbohydrase) and 2% of alkaline endopeptidase (protease) could be compatible with the respective compositions of the substrates (polysaccharides and proteins).

Application of heat at the first step may involve with the random breakdown of –C-O- linkages in both structural and storage polysaccharides (specially in alginates, fucoidan and laminarin) thus, loosing the barriers against extraction via depolymerization. Application of the enzyme mixture (endopeptidase and β-glucanase) followed by the heat treatment can further hydrolyse proteins and

laminarin, which contain (β-1,3-glucan linkages. Application of alkaline treatment at the last stage of extraction process could significantly enhance the liberation of polyphenolic compounds. Under alkaline conditions, there is no possibility to form protein-polyphenol complexes, which can block the extraction of polyphenols (Vázquez and Antorrena, 1987; Sealy-Fisher and Pizzi, 1992). Theoretically, the antioxidative phenolic group is an excellent hydrogen donor that forms strong hydrogen bonds with the protein carboxyl group which facilitates the protein-polyphenol complex formation. This fact was clearly observed in the present results that both the EHP and HEP combinations (where alkaline hydrolysis was applied at the end of treatment combinations) have reported comparatively higher antioxidant extraction efficacies than those of other combinations tested. At higher pH conditions, phenolic hydroxyls are ionized and proteins have net negative charges thus, phenols become free at alkaline conditions. This fact suggests that essentially the alkaline hydrolysis should be applied as the final treatment in the extraction process.

In the case of heat and enzymatic hydrolysis, the initial nonspecific breakdown/depolymerization of structural and storage polysaccharides could enhance the easy access of endopeptidases and β-glucanase enzymes to their respective substrates (laminarin and proteins which are located inside the cells). Therefore, the initial nonspecific depolymerization by heat prior to enzymatic hydrolysis can effectively enhance not only the enzymatic hydrolysis of laminarin but also various proteins. Liberation of proteins or its breakdown products and interior compounds (specially polyphenols) can intern undergo the random aggregation/complex formation.

Beside the hydrolytic, ion exchange and desulfation properties of alkaline hydrolysis, its ability to prevent protein-polyphenol complex formation at the end of the extraction process is critical in effective extraction of Tot antioxidants.

Further studies to be performed towards isolation, purification, structural identification and characterization of active antioxidative compounds (phlorotannin) in the extracts produced with the novel methodology described above.

Part IV

Purification of antioxidant phlorotannin (HHpP8) from Tot (*H. fusiformis*) and characterization of its antioxidant activity

1. ABSTRACT

A potential hydrophilic phlorotannin antioxidant was purified from Tot extract prepared by integrating the treatments of enzymatic hydrolysis, pH control and heat. Initially, the extract was fractionated with methylene chloride and resultant aqueous fraction was further purified by gel filtration chromatography using Sephadex LH-20. The purity of the Hizikia hydrophilic phlorotannin 8 (HHpP8) was confirmed with a single spot in a thin-layer chromatography (TLC) plate. The antioxidative activity of the HHpP8 was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide scavenging in 2,2-Azino-bis (3-ethylbenzthiazolin)-6-sulfonic acid (ABTS) and in cell systems. The free radical (DPPH) scavenging at the concentrations of 0.05, 0.1, 0.25 and 0.5 mg/ml were 34.8, 59.2, 71.3 and 84.4 % respectively. The H_2O_2 scavenging activity determined in ABTS system at 0.05, 0.1, 0.25 and 0.5 HHpP8 mg/ml were 18.2, 52.2, 64.4 and 74.2% respectively. The H_2O_2 scavenging activity measured in cell system was 26.9, 40.8, 58.4 and 68.9% at 0.05, 0.1, 0.25 and 0.5 HHpP8 mg/ml respectively. The present study introduces HHpP8 as a potential free radical and H_2O_2 scavenging natural antioxidant. Therefore, it can be useful not only as an antioxidant, which can be imparted health benefits by scavenging free radicals but also scavenging H_2O_2 (pro oxidant), which can produce a variety of harmful radicals.

2. MATERIALS AND METHODS

2.1. Material

Tot was collected from the Jeju Island cost of Korea in March and June 2004. Epiphytes, salt and sand were removed using tap water and the samples were rinsed with deionised water before freeze-drying.

Ultraflo L (β-glucanases) and Alcalase 2.4 L FG (alkaline endopeptidase) enzymes were purchased from Novo Co. (Novozyme Nordisk, Bagsvaerd Denmark). 1,1-diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO) and low-melting agarose (LMA) was purchased from Sigma Co. (St. Louis, USA). 2,2-Azino-bis (3-ethylbenzthiazolin)-6-sulfonic acid (ABTS) and peroxidase were purchased from Fluka Co. (Buchs, Switzerland). All the other chemicals used were analytical grade supplied by Fluka or Sigma Co.

2.2. Enzymatic hydrolysis and preparation of crude extract of hydrophilic phlorotannins

The crude extract of hydrophilic phlorotannis was prepared according to the method developed by us (Siriwardhana et al. 2005). Freeze dried Tot was ground into a fine powder and 1 g was mixed with 100 ml of distilled water in a conical flask. Initially, the mixture was heated at 100 ^{o}C for 45 min and allowed to cool up to ambient temperature (25 ^{o}C). The pH and temperature of the resultant mixture was adjusted as 8.0 and 56 ^{o}C respectively. Alcalase (an alkaline endopeptidase/protease) and Ultraflo (a β–glucanase/carbohydrase) enzymes were then added as 2% and 3% respectively. After incubating for 1 day, the enzyme activity of hydrolysates was

inactivated by heat (100 ^{o}C for 10 min) and the pH of the mixture was increased until 12 (by using 1M NaOH) and placed in the shaking incubator for 12h. The resultant mixture was centrifuged and the supernatant was filtered in vacuum. The pH of the mixtures was adjusted to 7.0 by using 1M HCl. Finally, the extract was fractionated with a 100 ml of organic solvent mixture (methylene chloride and methanol at 1:1 ratio) and the respective aqueous fraction (upper dark brown layer) was separated and its total phenol content was measured. The aqueous layer containing significantly (P<0.05) higher amount of phenolics (crude hydrophilic phlorotanin extract) was then placed in the vacuum drier for 2 hr (in order to evaporate methylene chloride and methanol). The crude hydrophilic phlorotannin extract was then lyophilized and stored at -20 ^{o}C until use for the experiments.

2.3. Purification of phlorotannins

The phlorotannins were separated by column chromatography on a Sephadex LH-20 column (1.5 X 150 cm,) using mixture of H_2O and MeOH with increasing amounts of H_2O (H_2O:MeOH = 8:2 →10:0). As a preliminarily test to determine the suitable starting eluent (H_2O:MeOH = 8:2), different H_2O:MeOH mixtures such as 9:1, 8:2, 7.3, 6:4 and 5:5 were tested and compared for the separation efficacy. Separated phlorotannin fractions were tested for their antioxidant activities (DPPH radical and H_2O_2 scavenging activities) and consecutive active fractions that showed a single spot on the thin-layer chromatography (TLC) plates were pooled (8-11 fractions). Pooled and lyophilized active fraction hereafter referred as Hizikia hydrophilic phlorotannin 8 (HHpP8).

2.4. Hydrogen peroxide (H_2O_2) scavenging assay

The assay was based on the ability of the antioxidant to scavenge the hydrogen peroxide in ABTS-peroxidase medium according to the method of Muller. Hundred micro liter of Tot hydrolysate and 20 μl of 10 mM hydrogen peroxide were mixed with 100 μl of phosphate buffer (0.1 M, pH 5.0) in a 96-microwell plate and incubated at 37 ^{o}C for 5 min. Finally, 30 μl of freshly prepared 1.25 mM ABTS and 30 μl of peroxidase (1 U/ml) was mixed and incubated at 37 ^{o}C for 10 min before recording the absorbance in ELISA reader (Tecan, Sunrise Co. Ltd. Sweden) at 405 nm.

2.5. Cell cultures

Cells of the Chinese hamster lung fibroblast line (V79-4) were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 unit/ml).

2.5.1. Hydrogen peroxide scavenging in cells

For detection of intracellular H_2O_2, V79-4 cells were seeded in 96-well plates at the concentration of 1.0×105 cells/ml. After 16 hr, the cells were treated with diffrent concentrations of HHpP8 (100 ug/ml) and fraction sample (1, 10, 50 and 100 ug/ml) and 30 min. later H_2O_2 was added to the concentration of 1 mM. The cells were then incubated for an additional 30 min at 37 °C, followed by the addition of 2-7-dichlorofluorescin diacetate (DCF-DA; 5 μg/ml). The 2' 7'-dichlorodihydrofluorescein fluorescence was detected at 485

nm excitation and at 535 nm emission using a PerkinElmer LS-5B spectrofluorometer.

2.6. DPPH radical scavenging assay

The assay was based on the scavenging of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by the radical scavenging antioxidant. Modified method of Brand Williams was used to determine the free radical scavenging activity. DPPH solution in dimethyl sulfoxide (DMSO) was prepared at the concentration of 3 $x10^{-5}$ M. A 2 ml fraction of Tot extract and 2 ml of freshly prepared DPPH solution were thoroughly mixed. The reaction mixture was incubated for 1 hr and absorbance was recorded at 517 nm using UV-VIS spectrophotometer.

2.7. Statistical analysis

All data were the means of three determinations and the data was analyzed using the SPSS package for Windows (Version 11.5). In the data analysis, variance analyses were performed using Students t test and the differences between means of treatment were determined. *P*-value of less than 0.05 was considered significant.

3. RESULTS

3.1. Purification of phlorotannins

The crude hydrophilic phlorotannin fraction of Tot was separated in gel filteration chromatography by using Sephadex LH-20 (Fig. 15). Resultant active fractions were pooled and the pooled fraction with highest activity (fraction 8-11) was lypophilized. Lyophilized phlorotannin fraction was named as Hizikia hydrophilic phlorotannin 8 (HHpP8) and it was 13 mg in weight and a 1.3% yield form the 1 g of dried Tot sample used for the preparation of crude hydrophilic phlorotannin extract (104 mg). The purity of the compound was confirmed with a single spot in TLC plate.

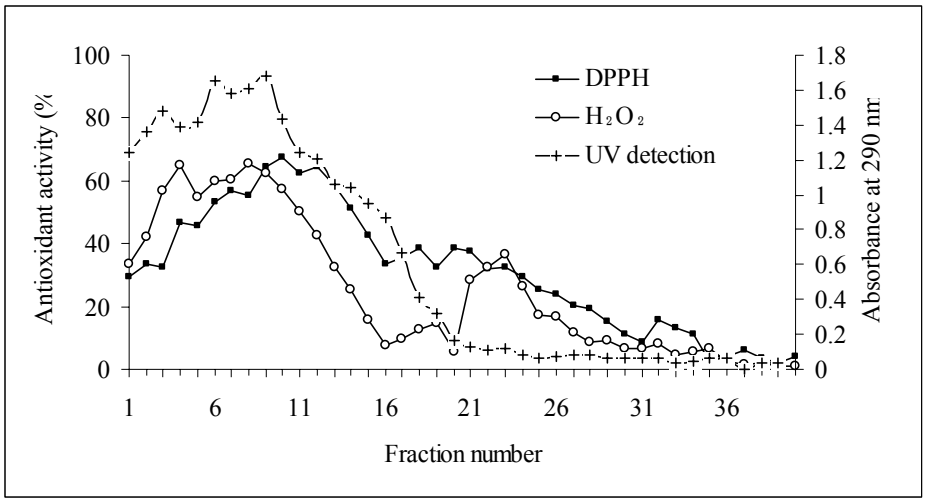

Fig. 16. Antioxidant (H_2O_2 and DPPH radical scavenging) activity detected for fractions (0.25 mg/ml) obtained by Sephadex LH-20 column chromatography.

3.2. DPPH radical scavenging activity

Free radical scavenging activity of the HHpP8 was determined by using DPPH radical. The absorbance (measured at 517 nm) of the purple colored DPPH radical mixture was decreased (turned to yellow) with the increasing HHpP8 concentration (Fig 17). The percentage free radical scavenging activities at the concentrations of 0.05, 0.1, 0.25 and 0.5 HHpP8 mg/ml were 34.8, 59.2, 71.3 and 84.4 % respectively.

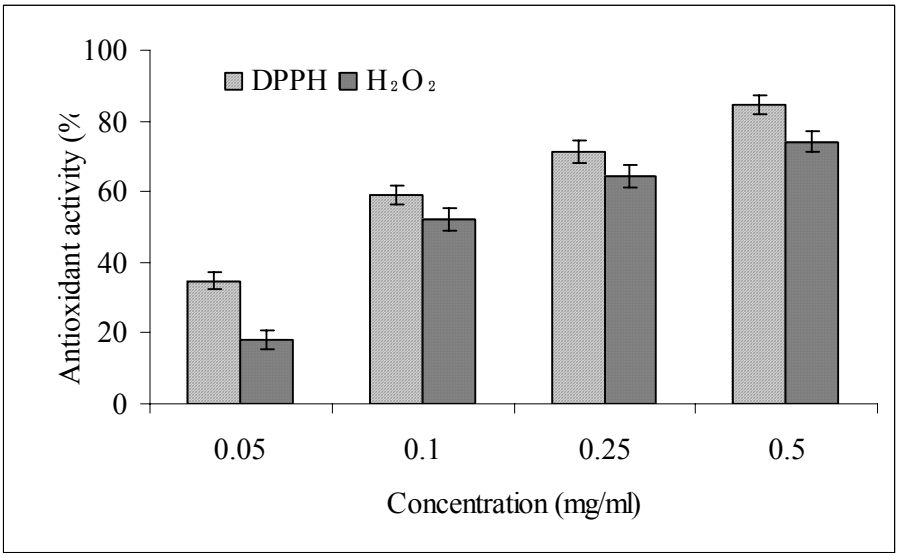

Fig. 17. Dose-dependant antioxidant activity of HHpP8. Antioxidant activity was determined in DPPH and H_2O_2 assays.

3.3. H_2O_2 scavenging activity

The H_2O_2 scavenging activity was determined by using ABTS oxidation reaction. The absorbance of blue green ABTS cation chromophore (405 nm) of was decreased with the increasing HHpP8 concentration (Fig 16). The percentage H_2O_2 scavenging activities at the concentrations of 0.05, 0.1, 0.25 and 0.5 HHpP8 mg/ml were 18.2, 52.2, 64.4 and 74.2% respectively.

3.4. H_2O_2 scavenging in cell system

The H_2O_2 scavenging potential of the HHpP8 was determined in Chinese hamster lung fibroblast cell line (V79-4). The cell damage was decreased with the increasing HHpP8 concentration. The per-centage H_2O_2 scavenging activities were of 26.9, 40.8, 58.4 and 68.9% at 0.05, 0.1, 0.25 and 0.5 HHpP8 mg/ml concentrations respect-tively (Fig. 18).

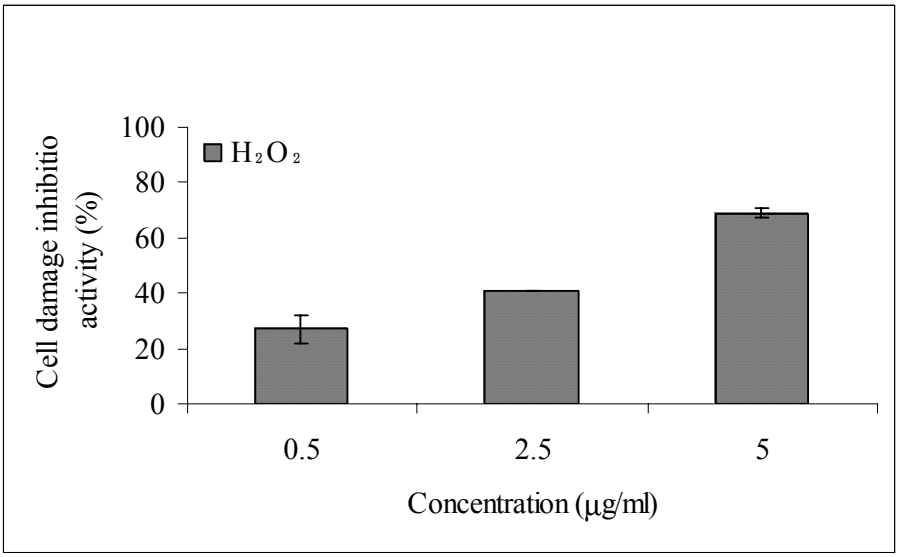

Fig. 18. H_2O_2 induce cell (investigated on V79-4 cells) damage inhibitory activity of various concentrations of HHpP8.

4. DISSCUSSION

Solvent fractionation (using methylene chloride and methanol) of original extract (OE) obtained after integrated extraction treatments (enzymatic hydrolysis, heat and pH control) could yield the hydrophilic aqueous fraction rich in polyphenols (hydrophilic phlorotannins). Polyphenols in marine brown algae are called as phlorotannins, which are formed by the polymerization of phloro-glucinol (1,3,5 trihydroxybenzene) monomer units (Ragan and Glom-bitza, 1986).

The unpaired/odd electrons (free radicals) are continuously formed in our body as a result of normal metabolism, immunological reactions or various unfavorable extracelluler conditions such as pollution, radiation, smoke and pesticides. The organic compound 1,1-diphenyl-2-picrylhydrazyl (DPPH) is a radical, in which an unpaired/odd electron located on one of the nitrogen atoms of the molecule. The DPPH radical scavenging potential reported in this study suggests that HHpP8 may have beneficial health effects by protecting biological systems from harmful damages of free radicals.

Hydrogen peroxide (H_2O_2) is an oxidant belongs to reactive oxygen species (ROS) but not a radical. H2O2 as a molecule is weakly reactive, but the single bond between the two oxygen atoms is easily broken, so that it readily fragments into a hydrogen and a hydroperoxyl radical or into two hydroxyl radicals. It's a long-lived molecule, which has a great potential to diffuse hence capable of long distance diffusion (freely miscible). In general, H_2O_2 levels over 50 mM considered as cytotoxic. It can produce other ROS like hypochlorous acid (HOCl) by enzymatic (myeleperoxidase) oxidation

of chloride ion. HOCl can leads to produce highly reactive singlet oxygen $^1O_2$ or even hydroxyl radicals. Furthermore, in the presence of abundant hydrogen carbonate (HCO_3^-), H_2O_2 reacts to produce hydrogen peroxycarbonate that reacts with bimolecular substances in vitro (Jensen, 2003). Therefore the elevated H_2O_2 levels must be minimized by the action of antioxidants. The potential H_2O_2 scavenging activity of HHpP8 could be beneficial in minimizing or retarding the adverse effects of H_2O_2. Moreover, H_2O_2 is a pro oxidant which serves as a starting material for the production of harmful hydroxyl radical ($HO^{\cdot}$) and non free-radical species like singlet oxygen ($^1O_2$). Hydroxyl radical is the most reactive among reactive oxygen species and the cell-damaging action of $HO^{\cdot}$ is the strongest among free radicals and its approximate half-life is 10^{-9} S (Kitada et al., 1979; Cheeseman and Slater, 1993; Liu and Ng, 2000). Therefore, the H_2O_2 activity of HHpP8 is important as a potential antioxidant that can maintain good health by overcoming complications possible with harmful oxidants. When the HHpP8 was passed through 5 and 30 kDa molecular weight cut-off filters, the solution was only filtered through the 30 kDa filter but not through the 5 kDa filter. This suggests that either the phlorotannin molecule is a high molecular weight complex compound (such as flavonoid glycoside) or it has strongly conjugated with polysaccharide of Tot. The preliminary data obtained by ^{1}HNMR and ^{13}CNMR also indicated the precence of suger groups in HHpP8 fraction. Therefore, further investigations on the structure and related bioactivities would be informative to the knowledge pool of marine biotechnology based pharmaceuticals.

Part V

Lipid peroxidation inhibitory effect of Tot (*H. fusiformis*) methanolic extract on fish oil and linoleic acid

1. ABSTRACT

The lipid peroxidation inhibitory effects of Tot *is* methanolic extracts (HME) on fish oil and linoleic acid were studied using peroxide value (PV), thiobabituric acid reactive substances (TBARS), conjugated diene hydroperoxides (CDH) and weight gaining assays. Heat and UV light stability was studied using DPPH assay. HME significantly (p<0.05) reduced the lipid peroxidation at different steps of the pathway in a dose-dependent manner. Increasing the level of HME from 0.01% to 0.1% caused the antioxidative effect increment over the effect of BHT (BHT reported the best effect compared to α-tocopherol and BHA). HME could reduce the formation of primary oxidation products form lipid radicals indicating lower CDH values compared to its control counterpart. Also, HME could reduce the addition of oxygen to form lipid peroxyl radicals indicating low weight gaining in HME-treated oils. Moreover, it could reduce the formation of lipid peroxide and indicated low PV. Furthermore, it could reduce the total lipid peroxidation resulting low TBARS values. The heat and UV light study showed that Tot contains heat and UV light resistant antioxidants. Total results indicated that the Tot could be useful for the food oils successfully.

2. Materials and Methods

2.1. Materials

Fish oil, linoleic acid, potassium iodide (KI), potassium dichromate ($K_2Cr_2O_7$), sodium thiosulfate ($Na_2S_2O_3$), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), trichloro acetic acid (TCA), butylated hydroxytoluene (BHT), butylated hydroxyl-anisole (BHA) and α-tocopherol were purchased from Sigma Co. (St. Louis, USA). All the other chemicals used were analytical grade supplied by Fluka (USA) or Sigma Co.

2.2. Sampling and extraction

Tot was collected from the Jeju Island costal of S. Korea in April 2003. Epiphytes, salt and sand were removed using tap water and the samples were rinsed with deionised water before freeze-drying. Ten grams of the ground powder were mixed with 200 ml of methanol and placed in a shaking incubator for 24 hr at 25 ^{o}C. The macerated mixture was filtered and centrifuged at 400 x g for 5 min. Finally, methanol was evaporated and the remained material was dissolved in absolute ethanol.

All the commercial antioxidants (BHA, BHT and α-tocopherol) were dissolved in absolute ethanol at the level of 0.01%.

2.3. Oxidation of oils

Oils (fish oil and linoleic acid) were exposed to accelerate oxidation similar to the method used by Abdalla and Roozen (1999). Oil samples (60 g) containing 0.01%, 0.05% and 0.1% of HME were

mixed in screw-capped glass bottles covered externally with aluminum foil and incubated at 60 ^{o}C in dark for 12 days. Initial 6 hr incubation was done without closing the cap of the bottles in order to remove the ethanol added with Tot extract.

2.4. Peroxide value (PV)

PV of oils stored under accelerated oxidation conditions was determined by the iodometric determination method according to the AOAC (1995) guidelines.

2.5. Thiobarbituric acid-reactive substances assay (TBARS)

The assay was conducted according to the method of Madsen et al. (1998). One gram of the oil samples was dissolved in 3.5 ml of cyclohexane and 4.5 ml of TCA-TBA mixture (7.5% TCA and 0.34% TBA) subsequently. Resultant mixture was shaken for 5 min and centrifuged at 2780 x g for 15 min. The TCA-TBA phase was removed and heated in a boiling water bath for 10 min. Absorbance was recorded at 532 nm (Opron 3000 Hanson Tech. Co. Ltd., Korea) and the antioxidative ability was expressed as equivalent μmol of malonaldehyde per kg oil using a standard curve (concentration ranges from 1 mM to 20 mM) based on tetraethoxypropane.

2.6. Conjugated diene hydroperoxides (CDH)

Conjugated diene hydroperoxide content was measured in every two days as described by Roozen et al. (1994). Fifty milligrams of each oil sample (stored under accelerated oxidation conditions) were mixed with 5 ml of cyclohexane and the conjugated diene

hydroperoxide absorbance was recorded at 234 nm using spectrophotometer.

2.7. Weight gaining

This experiment was conducted according to the modified method described by Wanasundara and Shahidi (1998). Two grams from each oil sample prepared for lipid peroxidation were separated into aluminum petri dishes and traces of water in the samples were removed in vacuum oven at 35 ^{o}C for 12 hr. Oxidation conditions of the samples were accelerated in a forced air oven at 65±1 ^{o}C. Percentage weight gaining was recorded for 12 days as similar to the Yan et al. (1996).

2.8. Heat and UV light stability of HME

This assay was based on the scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by the HME incubated at different temperatures (25 ^{o}C, 50 ^{o}C, 75 ^{o}C and 100 ^{o}C) for 6 hr and under UV light for 12 days. A modified method of Brand-Williams (1995) was used to investigate the free radical scavenging ability. DPPH solution was prepared at the concentration of $3x10^{-5}$ M using methanol as the solvent. A 2 ml fraction of HME and 2 ml of freshly prepared DPPH solution were thoroughly mixed. Absorbance was measured at 517 nm using UV-VIS spectrophotometer and used the following equation in % activity calculation.

$[1-(A_i - A_j)/A_c] \times 100$

A_i= the absorbance of HME mixed with DPPH solution;

A_j = the absorbance of same extract mixed with 2 mL methanol, and

A_c = the absorbance of DPPH solution adding 2 mL methanol.

2.9. Statistical analysis

All experiments were conducted in triplicate and Student's *t*-test was used to determine significant difference between average values of oxidation produced in oil with Tot extracts.

3. RESULTS

Initially, the PV of fish oil and linoleic acid was checked and confirmed the quality as the PV was below 6 meq/Kg and 5 meq/Kg in fish oil and linoleic acid respectively.

3.1. Peroxide Value (PV)

Addition of commercial antioxidants (BHT, BHA and α-tocopherol) and HME caused significant (p<0.05) reduction of PV compared to their control counterparts (Fig. 19 and 20). Delaying effect of PV increment of HME-added oils was dose-dependent and the effect of its highest concentration (0.1%) was slightly higher than the effect of BHT, which recorded the maximum inhibitory effect among the three commercial antioxidants. There was no marked synergistic activity recorded between HME and α-tocopherol. Also, the effect of α-tocopherol was comparatively lower than the effect of BHT or 0.1% HME. There was a significant (P<0.05) difference between PV reduction effects of 0.01% HME level and 0.1% HME level. Also, it was similar to the significant (P<0.05) difference occurred between α-tocopherol and BHT. The obvious effect of HME on PV reduction in fish oil and linoleic acid indicated the potential of Tot antioxidants to inhibit the lipid peroxidation in its early stages.

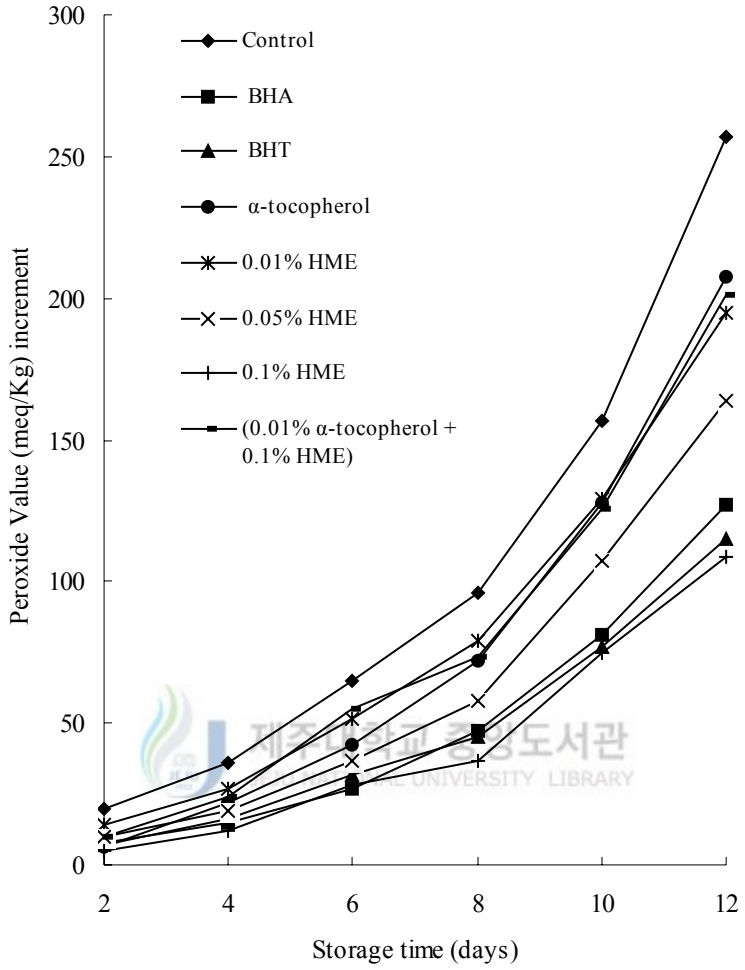

Fig. 19. Peroxide value (meq/Kg) increment (PV difference in respective days compared to its initial PV) of fish oil with anti-oxidants stored at 60±1 °C for 12 days.

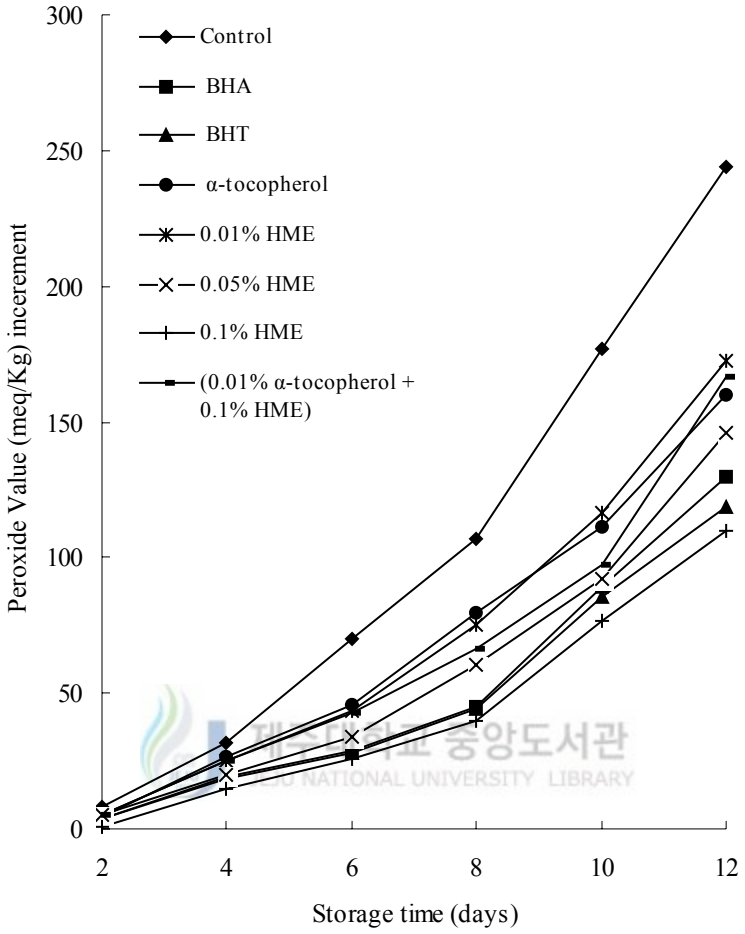

Fig. 20. Peroxide value (meq/Kg) increment (PV difference in respective days compared to its initial PV) of linoleic acid with anti-oxidants stored at 60±1 ^{o}C for 12 days.

3.2. Thiobarbituric acid reactive substances (TBARS)

The TBARS formation inhibitory effects of the commercial antioxidants and HME were significantly (P<0.05) higher than their control counterparts (Fig 21 and 22). Inhibition effect of HME on TBARS formation was also dose-dependent and its 0.1% level effect was compatible to the inhibition effect of BHA and BHT. The inhibitory effects of 0.01% and 0.05% HME were lower than the BHT and BHA but similar to the inhibition effect of α-tocopherol in fish oil. The 0.05 % HME in linoleic acid showed a significant (P<0.05) effect compared to α-tocopherol but such significance was not occurred in fish oil with the same HME level. The effect of 0.1 % HME was initially higher than the BHT but at the end it was slightly lower (but not significantly lower at (P<0.05) than the BHT. There was no synergistic effect recorded between the HME and α-tocopherol. Also, the inhibitory effect of TBARS formation in the α-tocopherol-added oils were not higher compared to the other anti-oxidants and its activity was suddenly decreased after the 8^{th} day at 60±1 ^{o}C.

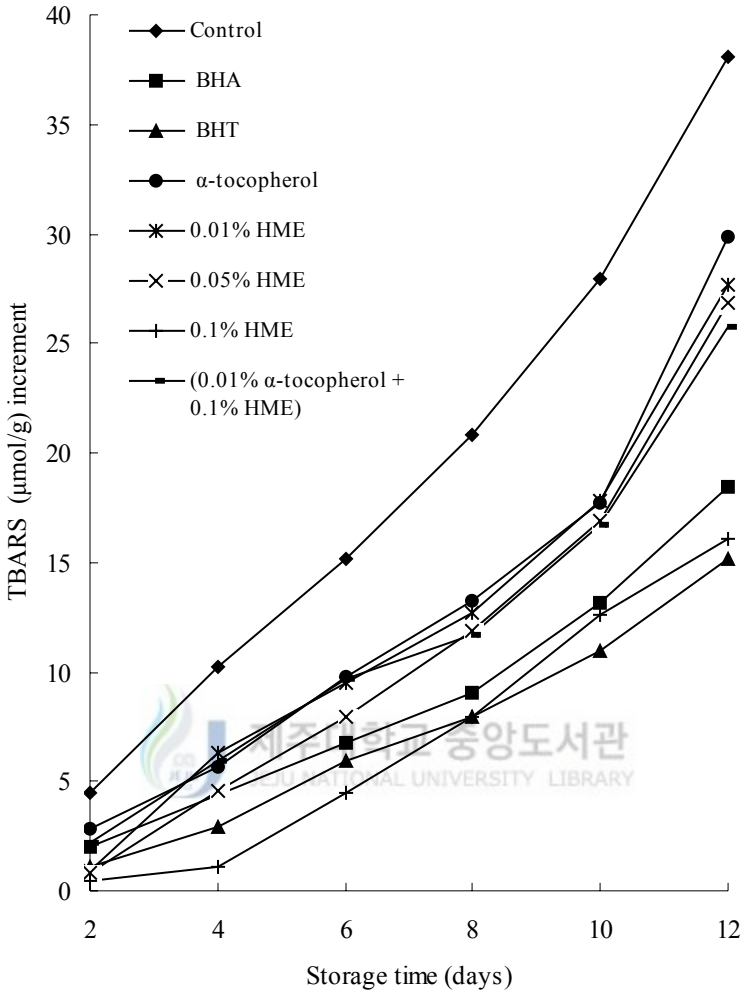

Fig. 21. TBARS value (μmol/g) increment (TBARS difference in respective days compared to its initial TBARS content) of fish oil with antioxidants stored at 60±1 ^{o}C for 12 days.

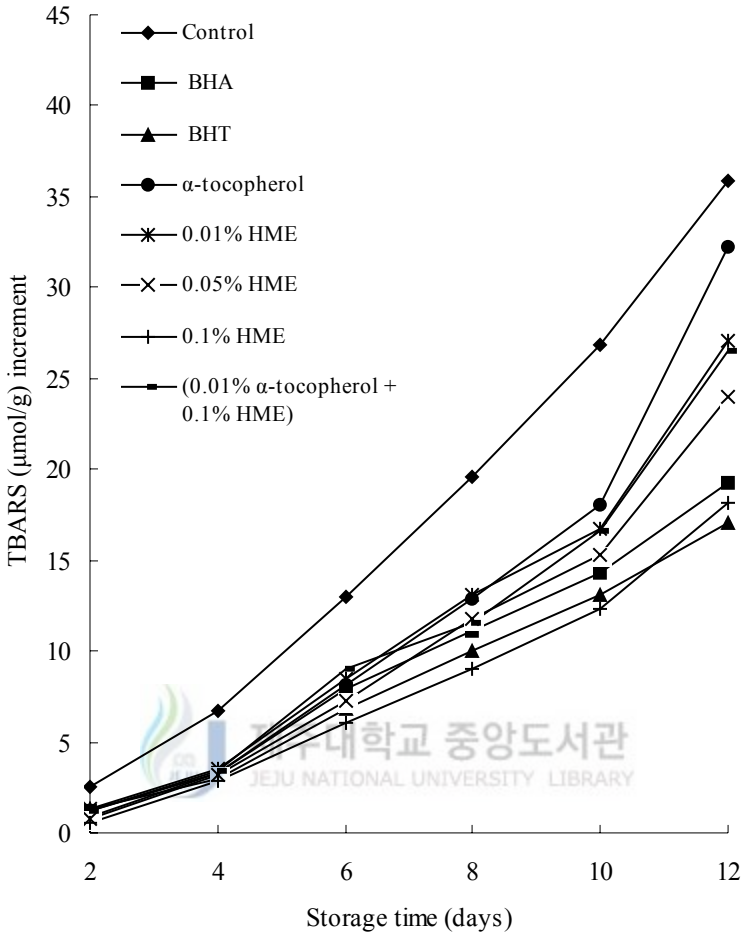

Fig. 22. TBARS value (μmol/g) increment (TBARS difference in respective days compared to its initial TBARS content) of linoleic acid with antioxidants stored at 60±1 ^{o}C for 12 days.

2.3. Conjugated diene hydroperoxides (CDH)

The rate of CDH formation decreased significantly ($P<0.05$) in the fish oil and linoleic acid treated with commercial antioxidants and HME (Fig. 23 and 24). Results showed that the CDH formation inhibitory effect of HME was dose-dependent and the inhibitory effect of its 0.1% level was higher than the effect of BHA and BHT. The inhibition effect of 0.05% level HME on CDH formation was similar to the inhibition effect of BHA. By contrast, the 0.05% HME in other parameters (PV, TBARS and weight gaining) remained less effective than BHA.

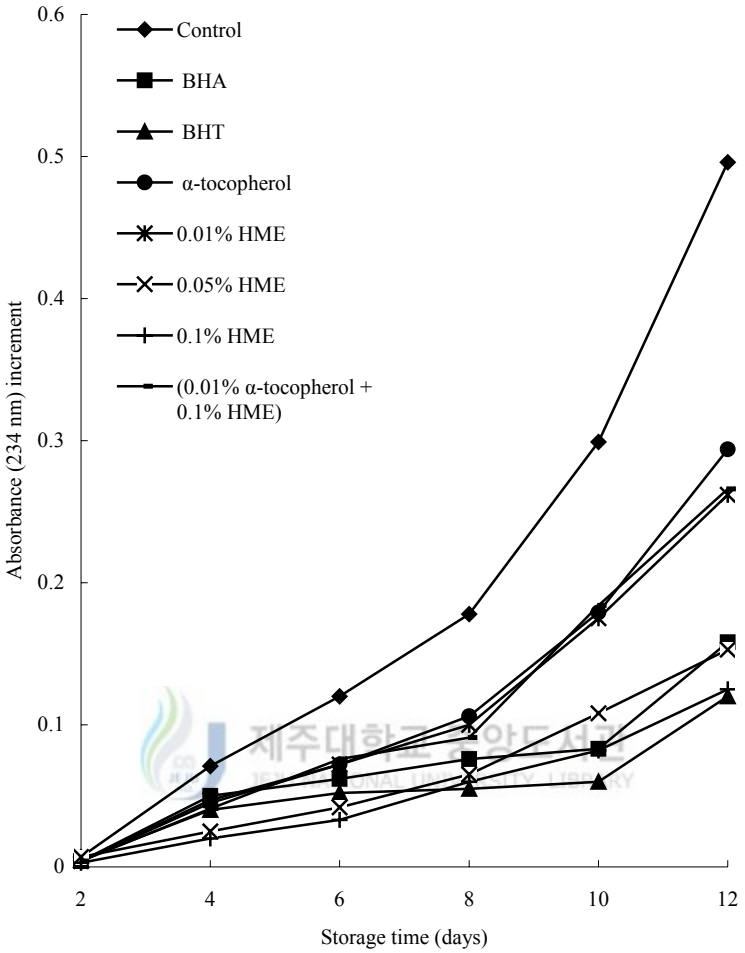

Fig. 23. Conjugated diene hydroperoxide absorbance (234 nm) increment (absorbance difference in respective days compared to its initial absorbance) of fish oil with antioxidants stored at 60±1 ^{o}C for 12 days.

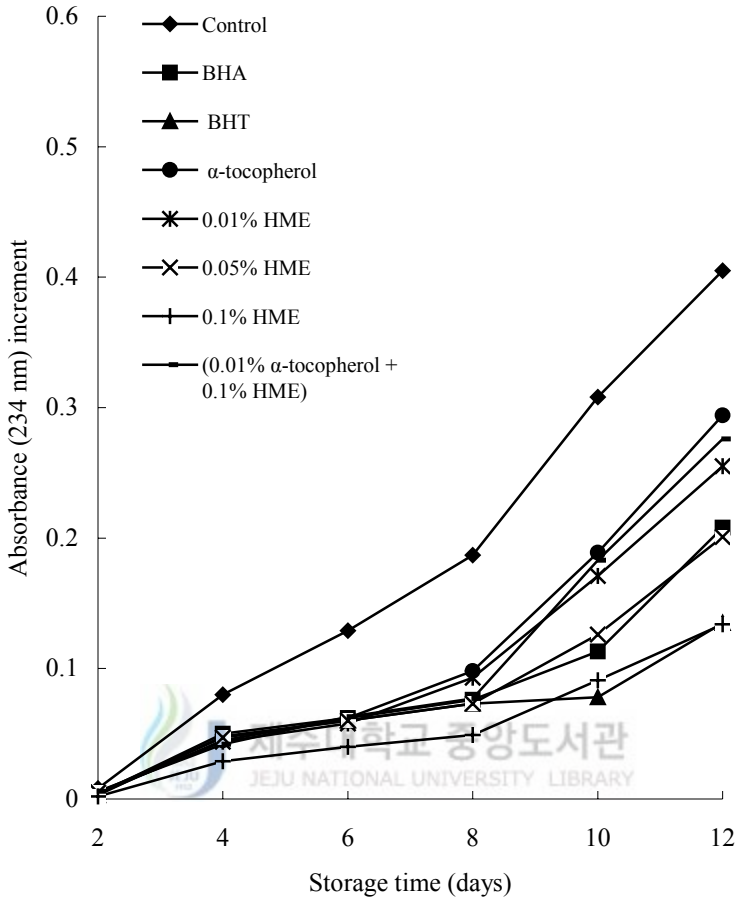

Fig. 24. Conjugated diene hydroperoxide absorbance (234 nm) increment (absorbance difference in respective days compared to its initial absorbance) of linoleic acid with antioxidants stored at 60±1 ^{o}C for 12 days.

3.4. Weight gaining

The inhibition of weight gaining in commercial antioxidants and HME-added oils were shown in Fig. 25 and 26. All the antioxidants were able to inhibit weight gaining significantly ($P<0.05$). Increment of HME level up to 0.1% improved the weight gain inhibitory effect similar to the inhibitory effect of BHA and BHT but 0.01% level showed inhibitory effect similar to α-tocopherol (α-tocopherol showed the lowest inhibitory activity among all the antioxidants). The inhibitory effect of the 0.05% level HME was slightly lower than the effect of BHA. Weight gaining inhibitory pattern of HME was different from the pattern of commercial antioxidants and it could be clearly observed in linoleic acid. HME-treated oils showed gradual weight gaining increment over 12 days while the synthetic antioxidant-treated oils showed a rapid weight gaining increment after the 8^{th} day.

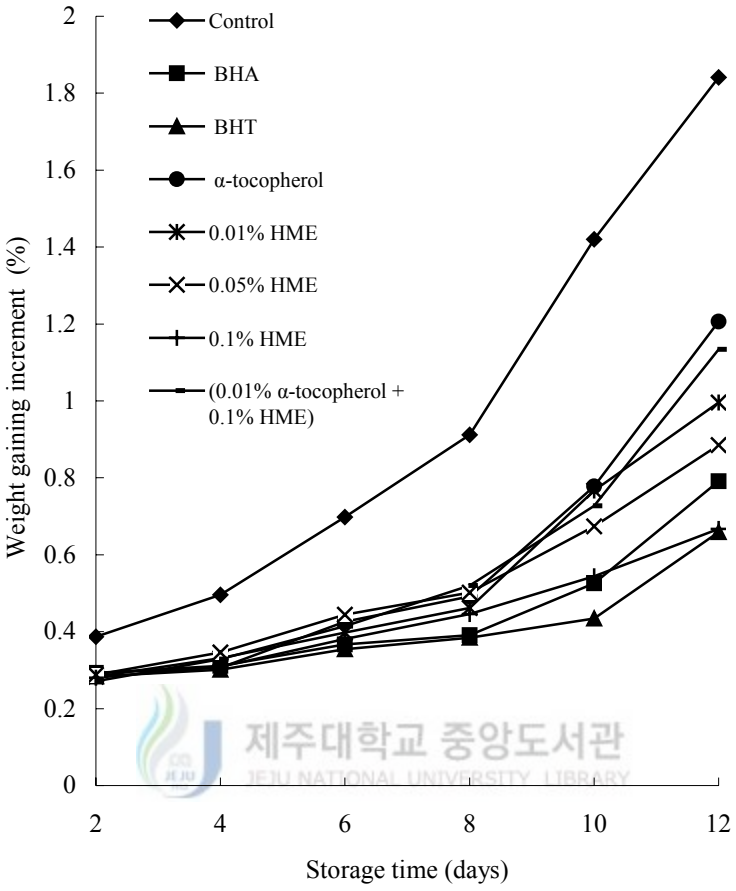

Fig. 25. Weight gaining increment (% weight difference in respective days compared to its initial weight) of fish oil with antioxidants stored under forced air at 65±1 ^{o}C for 12 days.

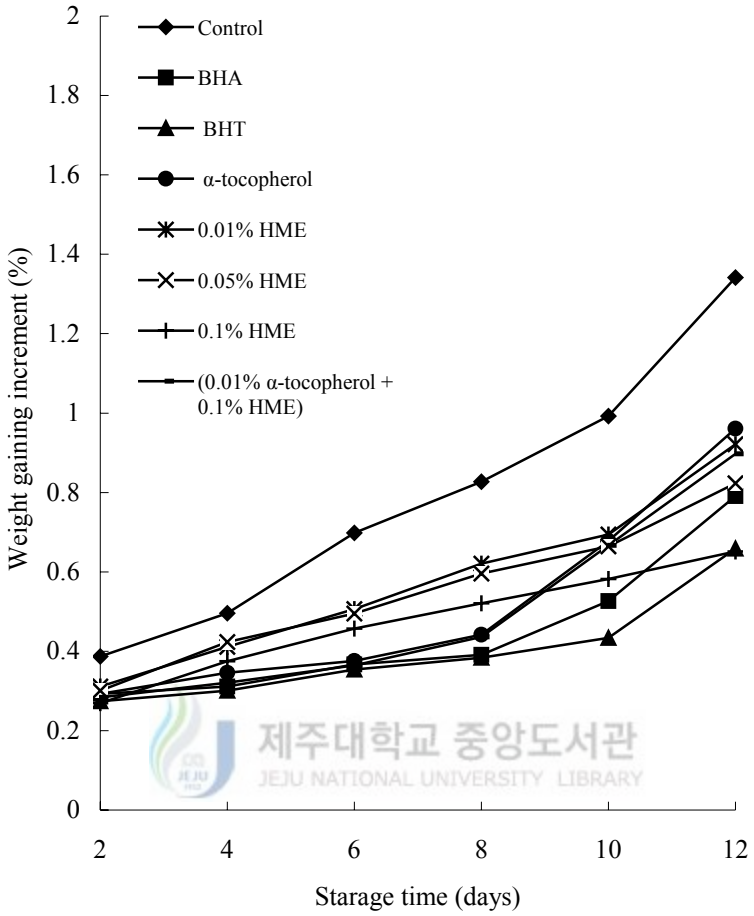

Fig. 26. Weight gaining increment (% weight difference in respective days compared to its initial weight) of linoleic acid with antioxidants stored under forced air at 65±1 ^{o}C for 12 days.

3.5. Heat and UV light stability of HME.

HME heated at 50 ^{o}C and 75 ^{o}C (for 90 min) showed no significant DPPH radical scavenging effect reduction compared to HME incubated in room temperature (Fig. 27). Also, the HME heated at 100 ^{o}C for 30 min, remained its activity over 55%. HME anti-oxidants showed no marked effect reduction over 12 days incubated under UV light but the α-tocopherol exposed to same conditions started to reduce its effect after the 8th day and reduced its effect by 10% within next 4 days (Fig 28).

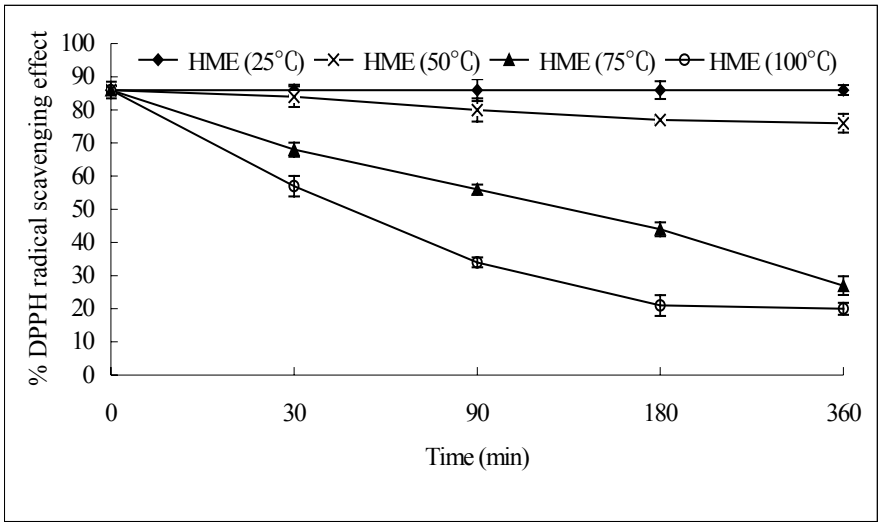

Fig. 27. Heat-stability of HME (0.1% HME) exposed to different heat treatments.

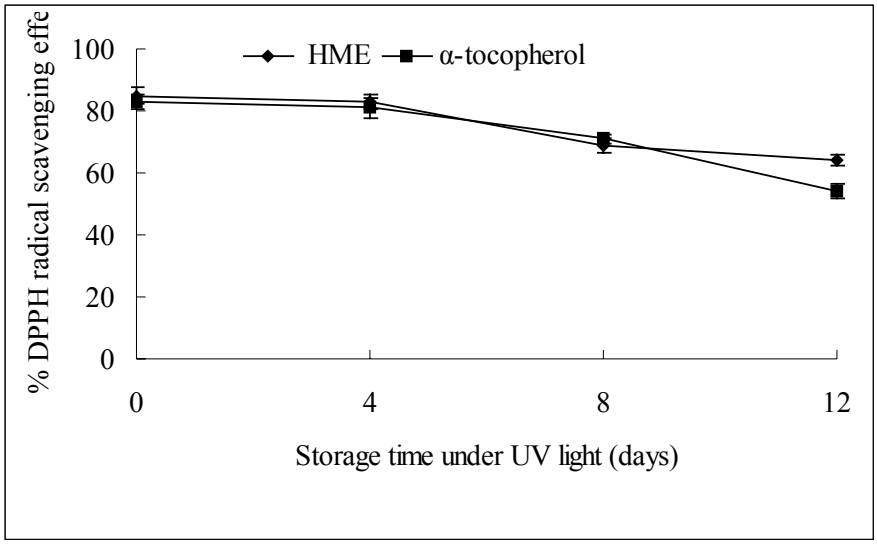

Fig. 28. UV light-stability of HME (0.1% HME and 0.01% α-tocopherol) exposed to UV light (40W x 2EA) for 12 days.

4. DISSCUSSION

PV is the chemical indication of how much of the oil is in the early stages of oxidation and it reflects the degree of oxidation. A study on oregano (a characteristic ingredient of Mediterranean cookery) antioxidant activity in mackeral oil (Tsimidou et al., 1995) showed that its 0.5% oregano level effect was compatible to that of 200 ppm BHA or 0.5% dry rosmery. Also, Wanasundara and Shahidi (1998) compared the antioxidant activity of dechlorophylized green tea extracts (DGTE) in marine oil with commercial antioxidants and reported that the PV reduction of DGTE at ≥200 ppm was comparatively higher than that of BHT, BHA and α-tocopherol at 200 ppm level. PV of fish oil was increased more rapidly compared to the linoleic acid and it was common in almost all the LOP assays investigated (PV, TBARS, CDH and weight gaining assays). This was mainly due to the more unsaturation of fish oil compared to linoleic acid where there are only two double bonds present in the molecule. Moreover, Hamilton et al. (1997) reported that fish oil is unstable even more than sunflower and maize oil.

Aldehydes especially the malonaldehydes, the breakdown products of oxidized fatty acids (lipid peroxyl radical), results off-flavors (rancid flavor) in oxidized oils that can quantified through their reaction with TBA. A number of studies have been conducted on different natural antioxidants (rosmery, green tea and spices extracts) and recorded similar kinds of results (Tsimidou et al., 1995; Wanasundara and Shahidi 1998; Madsen et al., 1998). TBARS values estimate the secondary lipid oxidation products (specially malonaldehyde) formation during lipid peroxidation and indicate the extent

of total lipid oxidation. Hence, the marked TBARS inhibition effect of HME-treated oils indicated its ability to retard the oxidation of fish oil and linoleic acid.

CDH are formed by the rearrangement of the lipid radicals and undergo further radical formation such as lipid peroxyl radical, lipid hydroperoxide, lipid alkoxyl radical and lipid aldehydes. The ability of HME antioxidants to inhibit the CDH is important in prevention of lipid peroxidation at its early stages by blocking further formation of reactive radicals. Similar kind of CDH formation inhibitory activity has reported by Abdalla and Roozen (1999) for the plant extracts applied to evaluate the oxidative stability of sunflower oil and emulsion.

The addition of oxygen to the lipid radicals results lipid peroxyl radicals and weight gaining. The time required to achieve a 0.5% weight increment in all the oils treated with HME was more than 8 days and it was more than 12 days for the 0.1% HME level. Evens et al. (1973) suggested that each storage day under Schaal oven condition at 65 ^{o}C is equivalent to one month of storage at ambient temperature. Therefore it was obvious that HME antioxidants posses potential to extend the shelf life of oils.

Heat stability of a natural antioxidant is important in order to use it efficiently in commercial level.

In general, the recorded lipid peroxidation inhibitory effect of BHA was lower than the BHT. Also, it is known that for oils like fish oil, BHA is not very effective (Ke et al., 1977). The recorded α-tocopherol lipid peroxidatory inhibition effect was slightly compatible with BHT in first few days but after the 8th day its effect

was not at a satisfactory level. This reduction may be due to the consumption of α-tocopherol to suppress the lipid oxidation under accelerated oxidation condition (Khan and Shahidi, 2001). Recorded lipid peroxidation inhibitory effect of HME was dose-dependent and its 0.1% level effect was compatible with the 0.01% BHT level. Unlike synthetic antioxidants, Tot antioxidative compounds were from natural edible seaweed and its antioxidants can be added in larger quantities to magnify the effect (synthetic antioxidants addition is thoroughly limited under food laws and regulations). Therefore the lipid peroxidation can be under control by using enough dosages of Tot antioxidative compounds and addition of Tot antioxidants in oils can considered as a supplementation of natural antioxidants via food oils. In our previous study, Tot reported to have a notable anti-oxidative effect and its polyphenolic content was clearly correlated with the antioxidative effect (Siriwardhana et al., 2003). The lipid peroxidation inhibition recorded in fish oil and linoleic acid could be due to the antioxidative polyphenolic compounds of Tot. Also, the effect of seaweed polyphenolic (specially brown seaweed phloro-tannins) compounds on lipid oxidation and human health has been described in numerous studies (Yan et al., 1996; Yan et al., 1999; Duval et al., 2000; Heo et al., 2003; Atukorala et al., 2003). Recorded heat and UV light stability of the HME antioxidants showed that Tot is a potential alternative natural antioxidant source over less stable natural antioxidants. The lipid peroxidation inhibition effect recorded in this study would be an innovative direction of the development of natural heat stable antioxidative compounds for oil stabilization from brown seaweeds like Tot.

Part VI

Isolation and purification of colon cancer cell (CT26) growth inhibitory phlorotannin (HHbP20) from Tot (*H. fusiformis*)

1. ABSTRACT

The cancer cell growth inhibitory potential of the crude phlorotannin fraction of Tot was initially investigated in 4 cell lines namely B16, CT26, U937 and Hela cells representing melanoma, colon, promonocytic and cervical cancer cells respectively. Except B16 cells, all the other cancer cell growth was successfully inhibited by the crude phlorotannin fraction. The cell growth inhibitory activities of the crude phlorotannin at 50 μg/ml were 82.4, 73.2 77.4 and 50.1% on B16, CT26, U937 and Hella cancer cells respectively. After further fractionation of crude phlorotannin fraction by gel filteration chromatography (Wakogel C-300HG), the resultant fractions showed a notable growth inhibition against CT26 cells than that of the other cancer cells. Therefore, the most active fraction (20^{th} fraction) against CT26 cell line was selected and named as Hizikia hydrophobic phlorotannin 20 (HHbP20). The purity of HHbP20 was confirmed by a single spot in thin-layer chromatography (TLC) and used for further studies on the mechanism of cytotoxicity. The cytotoxicity investigation performed by nuclear staining with Hoechst 33342 (Investigation of apoptotic body formation) method showed that the anticancer activity of the HHbP20 was obviously via apoptosis. These results suggest that Tot contains potential anticancer drug candidates specially the hydrophobic phlorotannis like HHbP20.

2. Materials and Methods

2.1. Materials

Tot was collected from the Jeju Island cost of Korea in March and June 2004. Epiphytes, salt and sand were removed using tap water and the samples were rinsed with deionised water before freeze-drying.

RPMI-1640, fetal bovine serum (FBS) and phosphate buffer saline (PBS) were purchased form Gibco BRL (Gaithersburg, MD, USA). Dimethyl sulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) were purchased form Sigma (St. Louis, MO, USA).

2.2. Preparation of crude extract of phlorotannins

One hundred grams of fresh Tot was mixed with 100 g of ice and 100 ml of cold water (4 ^{o}C). Then the mixture was thoroughly agitated in a blender for 20 min. The resultant mixture was then centrifuged at 3000 rpm for 15 min. The precipitate was extracted twice with ethanol (100 ml) by shaking at 4 ^{o}C for 1 day.

One hundred milliliter of ethanolic extract was first fractionated with 100 ml of chloroform and the upper layer (non-chloroform fraction) was separated. The upper layer was then again fractionated twice with ethylacetate and (200 ml). The ethylacetate fraction was evaporated under vaccume.

2.3. Purification of phlorotannins

The phlorotannins were separated by column chromatography on a column (1.5 X 150 cm, Wakogel C-300HG; Wako Pure Chem-

ical Industries, Osaka, Japan) using mixture of chloroform: methanol: water (80:20:2, v/v) with increasing amounts of methanol (chloroform: methanol: water 80:20:2 → 0:100:0 v/v). Separated phlorotannin fractions were tested for their anticancer activities against cancer cells and active fractions were pooled.

2.4. Total phenolic content

Content of the total phenolic compounds was determined according to the protocol similar to Chandler and Dodds (1993). One ml of the extract was mixed in a test tube containing 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 ml of 5% Na_2CO_3 was added. Thoroughly mixed mixture was placed in a dark room for 1 hr and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea). A gallic acid standard curve was used for the extrapolation of total phenolic content.

2.5. Cell culture

Cancer cells such as U937 (human promonocytic cell), B16(mouse melanoma cell), Hela (woman cervical carcinoma cell) and CT26 (mouse colon carcinoma) were grown in RPMI 1640 medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37 ^{o}C in 5% CO_2 incubator.

2.5.1. Cell growth inhibition assay

The cytotoxicity effect of phlorotannins was determined by a colorimetric MTT assay. Attach cells (Hela and CT26 cells) were seeded in a 96-well plate at a concentration of 2×10^4 cells/ml. Initially, the cells were incubated for 12 hr before the phlorotannin treatment. The suspention cells (B16 and U937) were treated (with phlorotannins) soon after the seeding. Then the cells were treated with phlorotannin (100 ug/ml). The cells were then incubated for an additional 72 h at 37 ^{o}C. MTT stock solution (50 μl; 2 mg/ml in PBS) was then added to each well for a total reaction volume of 200 ml. After incubating for 4 hr, the plate was centrifuged at 800 × g for 5 min and the supernatant was carefully aspirated. The formazan crystals in each well were dissolved in 150 μl of DMSO. The amount of purple formazan was spectrometricaly determined by measuring the absorbance at 540 nm.

2.6. Nuclear staining with Hoechst 33342 (Investigation of apoptotic body formation)

The nuclear morphology of CT26 cells was studied using the cell-permeable DNA dye Hoecht 33342. Cells with homogeneously stained nuclei were considered to be viable, whereas the presence of chromatin condensation/fragmentation was indicative of apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). The CT26 cells were placed in 24-well plates at a concentration of 1.0×105 cells/ml. After 16 hr of plating, the cells were treated with various concentrations of fraction sample, and after afuther incubation of 1h, H_2O_2 was added to 1 mM. After 24 hr, 1.5 μl of 10 mg/ml stock of Hoechst 33342, a DNA-specific fluorescent dye, was added into the 1.5 ml solution in each well, and the plates were incubated for 10 min at 37

°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

2.7. Statistical analysis

All the data were means of three determinations and the data was analyzed using the SPSS package for Windows (Version 11.5). In the data analysis, variance analyses were performed using Students t- test and the differences between means of treatment were determined. *P*-value of less than 0.05 was considered significant.

3. RESULTS

The growth inhibitory potential of crude phlorotannins of Tot against 4 cancer cells is shown in Fig. 29. Except in B16 cancer cells, all the other cell growth was progressively decreased with the increasing crude phlorotannin concentration. The growth inhibition in B16 cells were started when the crude phlorotannin concentration was increased over 10 μg/ml. The CT26 and U937 cell growth inhibition was initiated even at the 5μg/ml crude phlorotannin concentration. The crude phlorotannin growth inhibition activity of the Hela cells was comparatively less than that of the other cells. However, Hela cell growth inhibitory activity also reached to 50% at the concentration slightly over 50 μg/ml. At the crude phlorotannin concentration of 50 μg/ml, the growth inhibitory activities on B16, CT26 and U937 cancer cells were 82.4, 73.2 and 77.4% respectively.

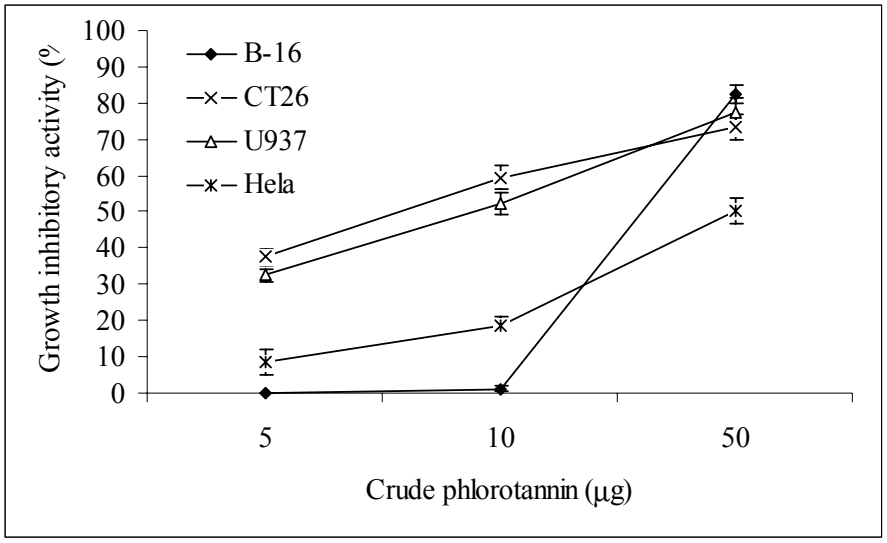

Fig. 29. Percentage of cancer cell inhibitory activity of crude phlorotannins from Tot.

3.1. Purification of HHbP20

From the crude phlorotannin fraction of Tot, the HHbP was isolated by gel filtration chromatography (Wakogel C-30). The chromatogram is shown in the Fig. 30. Of the 4 cancer cell lines tested, only the CT26 cells showed considerable activity after the chromatography. Therefore, CT26 cell line was selected for the further experiments. With increasing methanol concentration of the mobile phase, the anticancer activity of the eluting compounds was increased specially from fraction number 18. The highest anticancer activity was observed in the fraction number 20 and thereafter the activity was gradually decreased. Therefore, elute in fraction number 20 was evaporated under vacuum and the resultant compound was dissolved in water. The purified compound was named as Hizikia hydrophobic phlorotannin 20 (HHpP20).

The purity of the HHbP20 was confirmed with a single spot in TLC. HHbP20 was obtained as a light brown amorphous powder, soluble in both methanol and water.

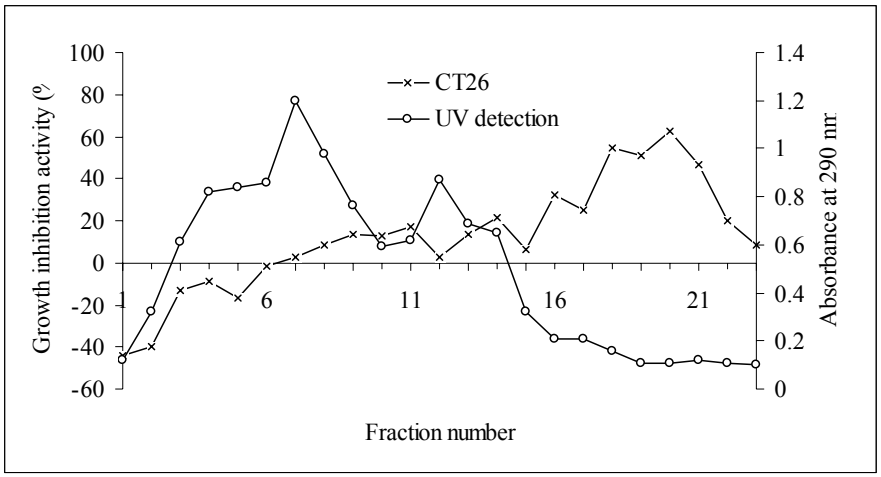

Fig. 30. Anticancer (growth inhibition of CT26 cancer cells) activity detected for fractions obtained by Wakogel C-300 column chromatography.

3.2. Anticancer activity of HHbP20

The IC_{50} value of the CT26 cancer cell growth inhibition by the purified HHbP20 was 1.96 μg/ml. The growth inhibitory activity was increased with the increasing HHbP20 concentration (Fig. 31). Further, the growth inhibitory activity was gradually increased with the increasing incubation time. At the concentration of 1 μg/ml of HHbP20, the growth inhibitory activities measured at 12, 24, 48 and 72 hr incubation periods were 24.3, 34.3, 42.5 and 59.1% respectively. At lower HHbP20 concentration (0.1 μg/ml), the growth inhibitory potential of the compound was not significant and almost no activity increment was observed after 24 hr of incubation.

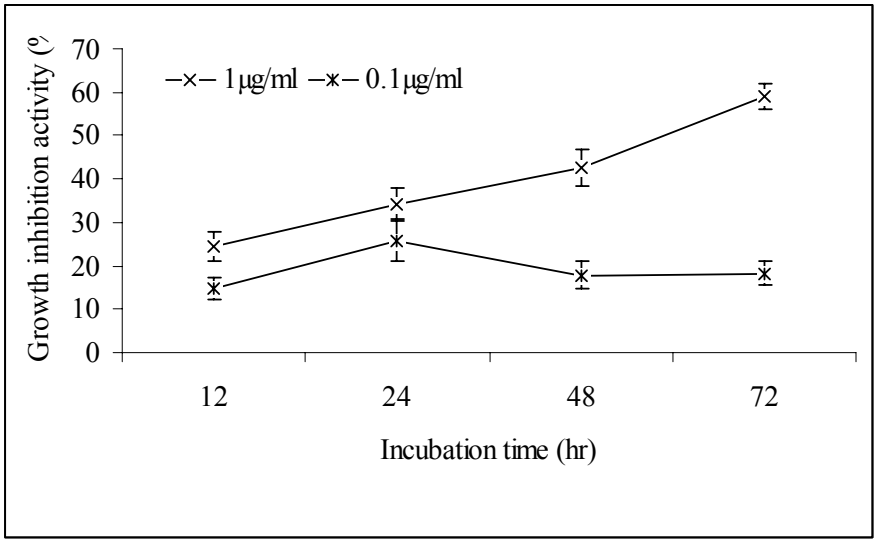

Fig. 31. Growth inhibition of CT26 colon cancer cells by HHbP20 investigated under different incubation periods.

3.3. Induction of apoptosis by HHbP20

The possibility of induction of apoptosis by HHbP20 was investigated by observing the apoptotic body formation upon HHbP20 treatments at different concentrations. The morphological observation suggested that the CT26 colon cancer cell cytotoxicity was an out come of HHbP20 induce apoptosis (Fig 32). HHbP20 dose-dependently increased the formation of apoptotic bodies.

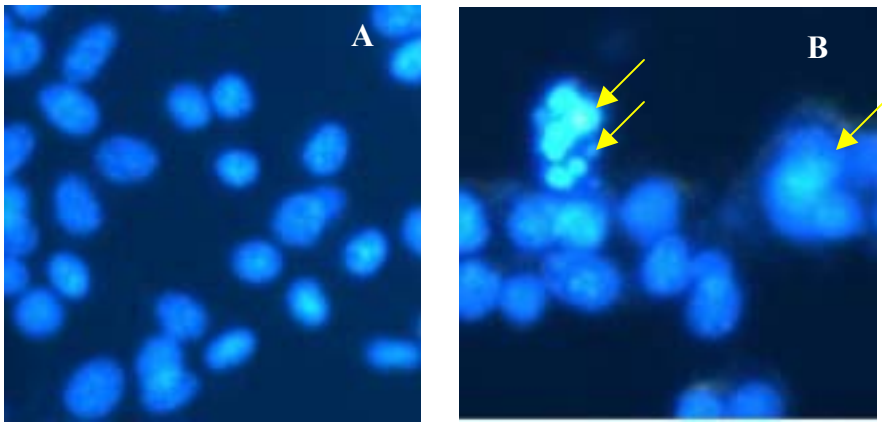

Fig. 32. Formation of apoptotic body during CT26 cell growth inhibition by HHbP20. Yellow colored arrows indicates the apoptotic body.

4. DISCUSSION

It was obsearved that the CT26 cancer cell growth inhibitory compound (HHbP20) is not strongly hydrophobic as it was eluted when the methanol concentration was increased over the chloroform concentration. Also, the HHbP20 was soluble in both water and methanol facilitating easy screening of its anticancer activity in cell culture systems.

The growth of any tissue, whether normal or malignant, is determined by the quantitative relationship between the rate of cell proliferation and the rate of cell death. For every cell, there is a time to live and a time to die. Apoptosis is an ordered and characteristic sequence of structural changes resulting in the programmed death of the cell. It leads to a cell death through a tightly regulated process resulting in the removal of damaged or unwanted tissues. Apoptosis plays an important role in the development of various diseases including cancer (Fisher, 1994 and McConkey et al., 1994). Cancer therapy targets the quantitative cell kinetic relationship which sets the cell death exceeds cancer cell proliferation. In chemotherapy, anticancer agents are being used to cause apoptosis in chemo-sensitive cancer cells. These results demonstrate that HHbP20 can inhibit/retard the growth of colon cancer cells by inducing apoptosis.

Colon cancer is a lengthy process, which takes about 5-10 years for a benign polyp to transform into cancer. HHbP20 demonstrate an anticancer activity (cancer cell growth inhibition) that increases with the increasing cell number. This suggests that HHbP20 may possess a strong ability to suppress or control the growth of colon cancer. Even though, the frequent medical investigations for

the early detection of the colon cancer have been recommended, the increased number of its occurrence suggests that it has been not properly practiced. Therefore, the frequent consumption of Tot could be beneficial in preventing the occurrence or progression of colon cancer. According to the results shown in Fig. 1, Tot is not only effective against colon cancer but also against skin, cervical and breast cancers. Moreover, researchers have reported that Tot contains several other promising health benefits such as antioxidative, antihypertensive and anticoa-gulative (Kim et al., 1998; Komori et al., 1993; Siriwardhana et al,. 2003; Karawita et al 2005).

Part VII

Isolation and partial purification of Angiotensin-1-converting enzyme inhibitory (antihypertensive) peptides from Tot *(H. fusiformis)*

1. ABSTRACT

The antihypertensive efficacy of different peptic digests of Tot protein extract was investigated by the angiotensin-1 converting enzyme inhibitory activity. Five proteolytic enzymes (Protamax, Kojizyme, Neutrase, Flavourzyme and Alcalase) were initially used in the preparation of peptic digests. Of the five peptic digests, Flavourzyme treated peptic digest was significantly (P<0.05) effective in ACE inhibition (IC_{50} : 83.2 μg/ml). Thereafter, Flavourzyme treated peptic digest was further hydrolysed according to the order of gastrointestinal proteases percent along the human digestive tract (Pepsin and Pepsin → Trypsin + Chymotripsin). Though the ACE inhibitory activities of the secondary peptic digests were not significantly increased, the peptide content of the digests was increased sharply (Pepsin; 0.89 mg/ml and Pepsin → Trypsin + Chymotripsin; 1.22 mg/ml). Followed by the treatment with gastrointestinal proteases, the Pepsin → Trypsin + Chymotripsin treated digest was fractionated with 3 ultrafilteration membranes (5, 10 and 30 kDa cut-off membranes). Of the resultant fractions, the 5 kDa fraction showed the highest significant (P<0.05) ACE inhibitory activity (IC_{50} : 53.2 μg/ml). In general, the activity was progressively increased with the decreasing molecular weight of the fraction. The 5 kDa fraction was further fractionated by cation exchange

chromatography using SP-Sephadex C-25. Desalting of the peptide fractions were performed with Sephadex G-10. The partially purified small moleculer peptides showed a good ACE inhibitory activity. This suggests that frequent consumption of Tot can be beneficial in maintaining blood pressure at a healthy level. Moreover, those peptides can be further purified and can be used as a safe drug for hypertention.

2. Materials and Methods

2.1. Materials

Tot was collected from the Jeju Island cost of Korea in March and June 2004. Epiphytes, salt and sand were removed using tap water and the samples were rinsed with deionised water before freeze-drying.

Protamex (endo-proteases), Kojizyme 500 MG (endo/exo-peptidase), Neutrase 0.8 L (neutral B. amyloliquefaciens proteases), Flavourzyme 500 MG (endo/exopeptidase) and Alcalase 2.4L FG (alcalase) were purchased form Novo Co. (Novozyme Nordisk, Bagsvaerd Denmark). Hippuryl-L-histydyl-L-leucine (HHL), angio-tensin 1-converting enzyme (ACE) Pepsin, Tripsin and Chymotripsin were purchased from Sigma chemical Co (St Louis, Mo. USA). SP Sephadex C-25 was purchased from Amersham Biosciences (Uppsala, Sweden).

2.2. Preparation of water extract from fresh Tot

All preparation and subsequent purification procedures were carried out at 3-4 ^{o}C. One hundred grams of fresh Tot was mixed with 100 g of ice and 100 ml of cold water (4 ^{o}C). Then the mixture was thoroughly agitated in blender for 20 min. The resultant mixture was then centrifuged at 3000 rpm for 15 min. Supernatant (water extract) was carefully decaned in to a beaker.

2.3. ACE inhibitory activity

ACE inhibitory activity was assayed by the method of Cushman and Chung (1971) with slight modifications. HHL was dissolved in 100 mM sodium borate buffer, pH 8.3, containing 300 mM NaCl. A 200 μL of a 5 mM HHL solution was mixed with 80 μL of a captopril solution, and then pre-incubated for 3 min at 37 ^{o}C. The reaction was initiated by adding 20 μL of ACE solution in distilled water (100 mU/mL), and the mixture was incubated for 30 min at 37 ^{o}C. The reaction was stopped by addition of 250 μL of 1M HCl. The hippuric acid liberated by the ACE reaction was extracted with 1.7 mL ethyl acetate, and the solvent was removed in an oven (120 ^{o}C). The content was dissolved by addition of 1 mL distilled water and its UV spectra density was measured at 228 nm.

The percentage ACE inhibitory activity was calculated as follows:

Inhibition % = Ac- (As-Ab)/Ac

Ac = Absorbance of control sample

As = Absorbance of test solution

Ab = Absorbance of blank solution

The concentration of the sample (μg/ml) that were shown 50% of ACE inhibitory activity under above conditions calculated by using scavenging activities of five different concent-rations.

2.4. Preparation of crude protein (CP) extract

Ten milliliter fractions of the above water extract was mixed with 2.91 g of ammonium sulfate (NH_4SO_4) in two steps and gently shaked for 2 min. The mixture was placed in ice for 15 min and

centrifuged at 10,000 x g for 10 min. The supernatant was then decaned into a 50 ml conical tube and the precipitate was mixed with 2 ml of water (50% NH_4SO_4 extract of proteins). The supernatant was again mixed with 1.25 g of NH_4SO_4 and repeated the same precipitation procedure (70% NH_4SO_4 extract of proteins). The resultant supernatant was again mixed with 2.09 g of NH_4SO_4 and performed same precipitation procedure (100% NH_4SO_4 extract of proteins). All the protein extracts were finally dialyzed against distilled water and stored at -20 ^oC.

2.5. Enzymatic hydrolysis of proteins with different proteases

Each 5 g of crude protein was mixed with 500 ml of distilled water in a conical flask. The pH and temperature of the protein solutions was adjusted according to the optimum conditions mentioned in Table 10. Enzymes were then added as a ratio of protein substrate to enzyme (100 to 1, w/w or w/v). After incubating for 1 day, the enzyme activity of hydrolysates was inactivated by heat (90 ^oC for 15 min). Each fraction was centrifuged at 4000 x g for 20 min and the supernatants were lyophilized. The ACE inhibitory potential of the hydrolysates was investigated for the determination of the best enzyme to be used in further experiments.

The lyophilized peptide fraction (1 g) was further hydrolyzed with (1) Pepsin only and (2) Pepsin and Tripsin + Chymotripsin consecutively. The Tripsin + Chymotrpsin hydrolysis was performed at pH 7.7 and 25^oC for 12 hr.

Table 10. Optimum conditions of proteases*

Enzyme	Optimum		Enzyme composition
	pH	temperature (^{o}C)	
Protamex	6.0	40	endo-proteases
Kojizyme	6.0	40	endo/exopeptidase
Neutrase	6.0	50	β amyloliquefaciens roteases
Flavourzyme	7.0	50	endo/exopeptidase
Alcalase	8.0	50	alcalase
Pepsin	2.0	37	endopeptidase
Tripsin	7.6	25	endopeptidase
Chymotrypsin	7.8	25	protease

Information provided in the table was obtained from the guidelines of the enzyme* supplier (Novozyme Nordisk, Bagsvaerd Denmark). Pepsin, Tripsin and Chymotripsin were from Sigma chemical Co. (St. Louis, Mo. USA).

2.6. Molecular weight distribution of hydrolysates

The active hydrolysate obtained with Flavourzyme was passed through a 30 kDa molecular weight cut-off membrane (Millipore Biomax 30, (50 cm^2) Billerica, USA). A portion of the solution was then passed through a 10 kDa molecular weight cut-off membrane. The same procedure was again followed with a 5 kDa membrane. Totally four molecular weight fractions were obtained as <5, 5-10, 10-30 and >30 kDa. The active peptidic fraction was selected by measuring the ACE inhibitory activity.

2.7. Purification of ACE inhibitory peptides using chromatography

The <5kDa fraction was initially fractionated using SP-Sephadex column C-25 (2.5 x 90 cm) previously equilibrated with 20 mM sodium acetate-citric acid buffer (pH 4.0). The elution was performed with a linear gradient from 0 to 1 M NaCl in the same buffer (sodium acetate-citric acid buffer) solution. Active fractions (potential ACE inhibitory peptides) were pooled and lyophilized. Afterwards, the pooled active fraction was dialysed with Sephadex G-10 column (2.5 x 45).

The peptide content of each fraction was measured according to the Lowery method (Lowery et al., 1951).

2.8. Statistic analysis

All data were the means of three determinations and the data was analyzed using the SPSS package for Windows (Version 11.5). In the data analysis, variance analyses were performed using Students

t-test and the differences between means of treatments were determined. *P*-value of less than 0.05 was considered significant.

3. RESULTS

3.1. ACE inhibitory potential of Tot.

The ACE inhibitory potential of water extract, crude protein extracts and different peptic fractions were investigated (Table 11). The crude protein fraction showed a significantly higher ACE inhibitory activity than that of its parent water extract. Of the hydrolysates treated with 5 proteases (Protamax, Kojizyme, Neutrase, Flavourzyme and Alcalase), ACE inhibitory activity of the Flavourzyme treated digest (IC_{50} : 83.2 μg/ml) was significantly (P<0.05) higher than that of the other digests. Therefore, the Flavourzyme treated fraction was selected for further digestion with gastrointestinal proteases.

Even though, the ACE inhibitory activity remained unchanged upon further digestion, the peptide content has notably increased. The increment of the peptide content was significantly (P<0.05) higher in Pepsin → Tripsin+ Chymotripsin treatment (0.89 mg/ml) than that of Pepsin digestion alone (1.22 mg/ml).

Table 11. ACE-1 inhibitory activity of proteins and protein hydrolysates of Tot

Extract/Hydrolysate	ACE-1 inhibitory activity IC_{50} (μg/ml)	Yield (%)	Peptide content (mg/ml)
Water	312.1 [e] ±0.08	100	
Proteins	246.3 [d] ±0.07	11.61	
Protamex	106.4 [b] ±0.05	1.34	0.54
Kojizyme	099.3 [b] ±0.07	1.13	0.58
Neutrase	167.6 [c] ±0.1	1.62	0.62
Flavourzyme	083.2 [a] ±0.06	1.46	0.53
Alcalase	102.0 [b] ±0.11	1.21	0.36

Water ; Fresh Tot extracted with water

Yield of the extracts and digests were calculated as a percentage to the water extract used in the initial step. Values within each column followed by a different letter are significantly different (P<0.05). Peptide content of the digests was measured in the supernatant after centrifugation at 4000 x g for 20 min.

Table 12. ACE-1 inhibitory activity of further hydrolyzed peptides after the initial Flavourzyme treatment

Extract/Hydrolysate	ACE-1 inhibitory activity IC_{50} (μg/ml)	Yield (%)	Peptide content (mg/ml)
Pepsin	76.3 [a] ±0.07	0.86	0.89
Pepsin ⟶ T+ C	76.6 [a] ±0.07	0.64	1.22

◀

The Tripsin + Chymotrpsin hydrolysis was performed at pH 7.7 and 25 °C for 12 h. Values within each column followed by a different letter are significantly different (P<0.05). Peptide content of the hydrolysates was measured in the supernatant after centrifugation at 4000 x g for 20 min. T+C; Tripsin+ Chymotripsin.

3.2. ACE inhibitory potential of different molecular weight fractions of Flavourzyme hydrolysate

The ACE inhibitory potential of four different (<5, 5-10, 10-30 and >30 kDa) molecular weight fractions were determined (Table 13). The <5 kDa fraction showed the highest ACE inhibitory activity (IC_{50} : 53.2 μg/ml) which was significantly (P<0.05) higher than that of all the other fractions. Moreover, the ACE inhibitory activity of the fractions was decreased with the increasing molecular weight.

Table 13. ACE-1 inhibitory activity of the diffrent moleculer weight fractions of Tot hydrolysates obtained with Flavourzyme→Pepsin→Tripsin + Chymotripsin

Sample	ACE-1 inhibitory activity IC_{50} (μg/ml)	Yield (%)
<5kDa	053.2±0.03[a]	0.49
5-10kDa	381.4±0.04[b]	0.47
10-30kDa	474.1±0.06[c]	0.31
>30kDa	834.6±0.09[d]	0.23

Yield of the extracts and hydrolysattes were calculated as a percentage to the water extract used in the initial step. Values within each column followed by a different letter are significantly different (P<0.05).

3.3. Purification of active peptides

The <5 kDa fraction was further purified by cation exchange chromatography using SP-Sephadex C-25. According to the chromatogram (Fig. 33), five distinct peptide peaks were detected and those were pooled (A; 36-41, B; 57-62, C; 72-77, D; 96-102 and E; 115-119) and ACE inhibitory activity was determined (Table 14). Although five distinct peptide peaks were detected, the peak responsible for A fraction was significantly (P<0.05) active in ACE inhibition (IC_{50} : 43.4 μg/ml). The active fraction was then subjected to dialysis in Sephadex G-10 coulm. Two distinct peptide peaks were obsearved (Fig. 34). The active peptide fration showed a good ACE inhibitory potential (IC_{50} : 36.4 μg/ml).

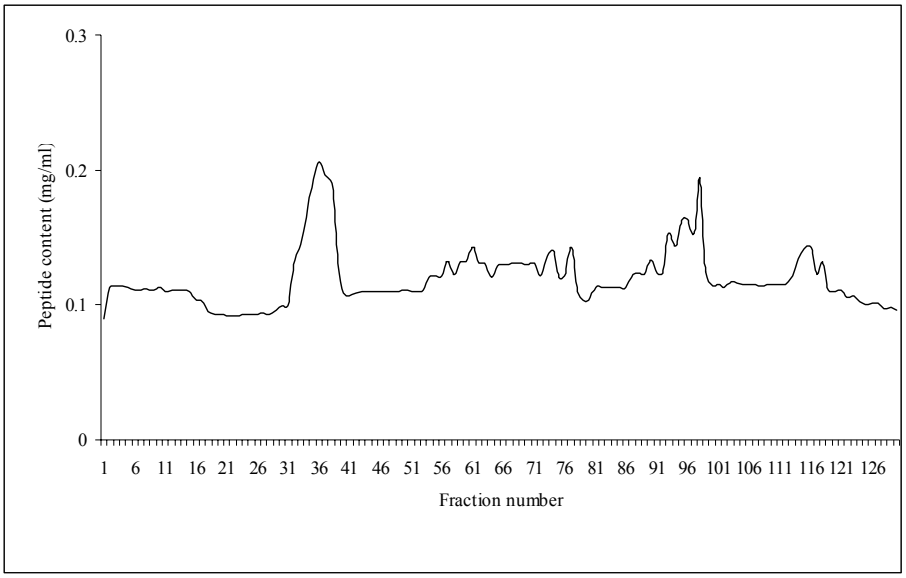

Fig. 33. Fractionation of ACE inhibitory peptides in cation exchange chromatography (SP-Sephadex C-25).

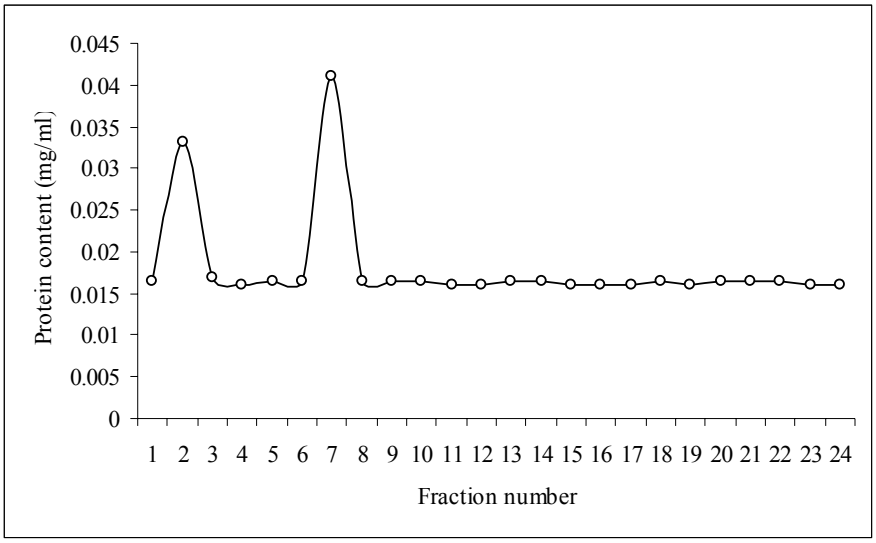

Fig. 34. Dialysis of ACE inhibitory peptides in cation exchange chromatography (Sephadex G-10).

Table 14. ACE-1 inhibitory activity of the chromatographic steps involved in purification

Sample	ACE-1 inhibitory activity IC_{50} (μg/ml)	Yield (%)	Peptide content (mg/ml)
SP-Sephadex C-25	43.4 [a] ±1.1	0.084	0.42
Spephadex G-10	36.4 [b] ±1.6	0.044	0.14

Yield of the extracts and hydrolysattes were calculated as a percentage to the water extract used in the initial step. Values within each column followed by a different letter are significantly different (P<0.05).

4. DISCUSSION

In this study, the protein fraction of Tot water extract was initially precipitated and digested with five commercially available food grade enzymes. Different ACE inhibitory potentials were reported in hydrolysates obtained with different proteases. The hydrolysate prepared with Flavourzyme reported the highest ACE inhibitoy activity than that of the all the other enzyme treated. Flavouezyme is a mixture of enzymes containing both endo and exo-peptidases. Endo-peptidases can hydrolyze peptide bonds in the interior of a polypeptide chain or protein molecule while exo-peptidases can hydrolyze single amino acids from the end of a polypeptide chain. Therefore, this would be a right combination of enzymes that can yield a variety of peptides including potential ACE inhibitory peptides.

In the digestive tract, the proteins are first hydrolysed with the Pepsin and then further hydrolyze with Tripsin and Chymotripsin. When the Flavourzyme treated hydrolysates were further hydrolyzed with Pepsin and Pepsin → Tripsin+ Chymotripsin the peptide content of the resultant hydrolysates were markedly increased compared to the peptide content reported alone with Flavourzyme or any other enzymes used in the initial hydrolysis. Although, the ACE inhibitory activity was increased with the Pepsin treatment, it was not significant (P<0.05) compared to the activity reported with Flavo-urzyme. This suggests that both Pepsin and Pepsin → Tripsin+ Chymotripsin treatment may involve in the further hydrolysis of existing proteins or high molecular peptides. This observation is in agreement with the research conducted by Sato et al 2002. In

particular, they have reported elevated ACE inhibitory activities upon further treatment of Wakame hydrolysates by Pepsin → Tripsin+ Chymotripsin.

When the hydrolysates were fractionated into different molecular weight fractions, the highest ACE inhibitory activity was reported with the lowest molecular weight fraction (<5 kDa). In a similar type of study with ultrafilteration membrane fractionation of corn glutan hydrolysate, approximately a four fold higher ACE inhibitory activity was recorded for the <5 kDa fraction than that of the original and 5-10 kDa fractions (Kim et al 2004). Moreover, the ACE inhibitory activity increment with the decreasing of molecular weight suggests that potential ACE inhibitors are small peptides. The ACE inhibitory activities of small molecular weight peptides have been previously reported by a number of researches (Miyoshi et al., 1991; Kinoshita et al., 1993; Maeno et al., 1996; Abubakar et al., 1998; Kohama et al, 1998; Byun and Kim, 2001 and Suetsuna 2001). Specially, in two investigations on Wakame (a popular brown seaweed in Korea and Japan) peptidic digest and hot water extract, four ACE inhibitory tetrapeptides (*Ala-Ile-Tyr-Lys*, *Tyr-Lys-Tyr-Tyr, Lys-Phe-Tyr-Gly and Tyr-Asn-Lys-Leu*) and four dipeptides (*Tyr-His, Lys-Tyr*, *Phe-Tyr*, *and Ile-Tyr*) were isolated by Suetsuna and Nakano (2000) and Suetsuna et al. (2004). In particuler, they have investigated the antihypertensive effect of those potential peptides on blood pressure in spontaneously hypertensive rats. In the human digestive system, small peptides such as dipeptides and tripeptides can be absorbed intact through the intestine by paracellular and transcellular routes (Adibi, 1971; Hara et al., 1984 and Hagihira et al., 1990). Masuda et al., 1996 have demonstrated that ACE inhibitory peptides

(dipeptides and tripeptides) are cable of absorbing in the intestine of rats. Moreover, such small peptides are not easily subjected to the enzymatic digestion of digestive enzymes.

The ACE inhibitory potential of low molecular peptides of Tot is an agreement with those studies and our study suggests that frequent intake of Tot may impart beneficial health effects by maintaining blood pressure at a healthy level.

Part VIII

Anticoagulant activity of enzymatic digests of marine brown alga Tot (*H. fusiformis*)

1. ABSTRACT

The anticoagulant activity of hot water fraction of Tot and its five enzymatic digests was investigated by activated thromboplastin time (APTT) assay. Anticoagulant activity was increased when the hot water fraction was digested with five carbohydrases. All five digests have reported significantly (P<0.05) higher anticoagulant activities than that of the control where the coagulation process was performed alone with water instead Tot constituents. Of the digests, the highest anticoagulant activity was recorded in the AMG (exo1, 4-alpha-d-glucosidase enzyme) treated digest. The activity was signify-cantly (P<0.05) higher than all the other digests. Even though, the anticoagulant activities were increased with enzymatic digestion, it was not satisfactory when compared to the commercial anticoa-gulant compound (Heparin).

2. MATERIAL AND METHODS

2.1. Materials

Tot was collected from the Jeju Island cost of Korea in March and June 2004. Epiphytes, salt and sand were removed using tap water and the samples were rinsed with deionised water before freeze-drying.

APPT (ellagic + bovine pospolipid) and $CaCl_2$ solutions were obtained from International Reagents Corporation (Japan), PT (rabbit thromboplastion) and TT reagents were purchased from Fisher scientific company (USA). Enzymes such as Viscozyme L (a multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, beta-glucanase, hemicellulase and xylanase), Celluclast 1.5L FG (catalyzing the breakdown of cellulose in to glucose, cellobiose and higher glucose polymers), AMG 300L (an exo1, 4-alpha-d-glucosidase), Termamyl 120L (a heat stable alpha–amylases), Ultraflo L (a heat stable multi-active beta-glucanase) were obtained from Navo Co. (Novozyme Nordisk, bagsvaed, Denmark).

Table 15. Optimum hydrolysation conditions and compositions of enzymes*

Enzyme	Optimum		Enzyme composition
	pH	temperature (^{o}C)	
Viscozyme	4.5	50	arabanase, beta-glucanase, hemicellulase 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

*Enzymes were purchased from Novozyme Nordisk, Bagsvaerd Denmark.

2.2. Preparation of hot water extract from fresh Tot

One hundred grams of fresh Tot was mixed with 100 ml of hot water (90 ^{o}C). Then the mixture was placed in a shaking hot water bath at 90 ^{o}C for 20 min. The resultant mixture was then centrifuged at 3000 rpm for 15 min. Supernatant (hot water extract) was carefully decaned in to a beaker.

2.3. Enzymatic digestion and preparation of crude polysaccharide fractions

Each 10 ml of the hot water extract was mixed with five carbohydrases namely Viscozyme L, Celluclast 1.5L FG, AMG 300L, Termamyl 120L and Ultraflo L. The pH and temperatre conditions were adjusted according to the guidelines of the enzyme manufacturer (Novozyme Nordisk, bagsvaed, Denmark). Digestion was performed for 12 h and the supernatant was obtained after centrifugation (3000 rpm for 15 min).

2.4. Measurement of extraction yield

Yields of the hydrolysates obtained by enzymatic hydrolysis of Tot were calculated by dry weight of hydrolyzed filtrate over the dry weight of the seaweed sample used.

2.5. Anticoagulant assays

2.5.1. Plasma sample

Normal pooled plasma was made from 10 individual healthy donors, without history of bleeding or thrombosis. Nine parts of blood collected by venipuncture ware drawn into one part of 3.8%

sodium citrate. Blood was centrifuged for 20 min at 2400 x g, and the plasma was stored at -60 ^{o}C until use.

2.6. Activated thromboplastin time (APTT)

Citrated normal human plasma (90 µl) was mixed with a solution of algal extract (10 µl) and incubated for 1min at 37 ^{o}C, then APTT reagent (100 µl) was added to the mixture and incubated for 5 min at 37 ^{o}C. Thereafter clotting was induced by adding 0,025 mol/l of $CaCl_2$ (100 µl). Finally, the clotting time was recorded.

3. RESULTS

3.1. Anticoagulant activity of Tot digests treated with carbohydrases

The anticoagulant activity of hot water extract of Tot and its digests prepared by five carbohydrases was determined by the Activated Thromboplastin Time (APTT) assay. The time (S) required for the coagulation after $CaCl_2$ addition was determined and shown in (Table 16). Higher the time taken for coagulation represents the higher anticoagulant activity. All the digests have demonstrated significantly higher activities than that of the control where the reaction was performed alone with water as a treatment. The highest anticoagulant activity was recoded for the digest prepared with AMG enzyme (104 S) while the lowest activity was recorded for the Celluclast (55 S). Only AMG digest showed the significantly higher anticoagulant activity than that of the other enzyme digests. Even though the activities of digests were higher than the control, all the activities were significantly lower compared to the commercial anticoagulant (Heparin) used in the experiment.

Table 16. Anticoagulant activity of enzymatic digests of Tot treated carbohydrases

Extract/digest	APTT (S)	Yield (%)
Control	32 [e] ±1	
Heparin	800 [a] ±3	
Hot water	84 [c] ±2	100
Viscozyme	86 [b] ±1	36
Celluclast	55 [d] ±3	29
AMG	104 [b] ±2	38
Termamyl	81 [c] ±2	39
Ultraflo	87 [c] ±3	46

Sample (hot water extract and enzyme digests) concentration used in the assay was 80 μg/ml. Heparin concentration was 0.03 μg/ml. All the data are the means of 3 diterminations. Values withing each colomn followed by a different letter are significantly different (P<0.05). Yield was calculated as a percentage weight (%) to the hot water extract used in the initial step.

4. DISCUSSION

It was observed that the anticoagulant activity was increased when the water extracts were digests with carbohydrases. This suggests that the carbohydrate derivatives (polysaccharides) may have attributed to the higher anticoagulant activities. The enhanced anticoagulant activity of the AMG digest can be attributed to the products formed by the action of exo1, 4-alpha-d-glucosidase enzyme activities. AMG contains exo1, 4-alpha-d-glucosidase enzyme which removes terminal alpha-1, 4-linked d-glucose residues from non-reducing ends of chains, with release of beta-d-glucose. Brown seaweeds contain soluble dietary fiber polysaccharides as alginates, fucans and laminarians together with the insoluble fibers made of cellulose (Lahaye et al., 1997; Reperez et al., 2001). Alginic acid is the main structural component of the brown algal cell wall, which is a linear copolymer of β-1,4-D-mannuronic acid and α-1,4-L-guluronic acid, with the residues organized in blocks of polymannuronic acid and polyguluronic acid, as well as heteropolymeric sequences of guluronic and mannuronic acid (Moen et al., 1997; Kloareg and Quatrano, 1998).

Even though, the anticoagulant activities were increased with enzymatic digestion, the activities of the digests were not satisfactory when compared to the commercial anticoagulant compound (Heparin). Therefore, no attempt was taken on further purification of anticoagulant polysaccharides.

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