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Antioxidant Potential of Enzymatic Extracts from Blueberry (*Vaccinium corymbosum* L.)

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Enzymatic extracts were prepared from the blueberry (*Vaccinium corymbosum* L.) collected in Jeju, Korea. Five carbohydrases namely AMG, Celluclast, Termamyl, Ultraflo and Viscozyme, and five proteases namely Alcalase, Flavourzyme, Kojizyme, Neutrase and Protamex were used to prepare the enzymatic extracts. Antioxidant properties of each extracts were studied using stable 1,1-diphenyl 2-picrylhydrazyl (DPPH), reactive oxygen species (ROS), nitric oxide (NO·) scavenging, metal chelating assays and lipid peroxidation inhibitory activity in hemoglobin-induced linoleic acid system. The phenolic content of all enzymatic extracts was in the range of 517.85 - 597.96 mg/100 g dried sample. DPPH and NO· scavenging, and metal chelating assays exhibited prominent activities. Viscozyme showed the highest DPPH activity (0.046±0.002 mg/mL) while AMG showed the highest activity in NO· scavenging (0.339±0.011 mg/mL). All the extracts exhibited strong metal chelating activities. Blueberry enzymatic extracts also showed relatively good activity in hydrogen peroxide scavenging. AMG showed the highest lipid peroxidation inhibitory activity (0.28±0.01 mg/mL) in hemoglobin-induced linoleic acid system. In this results, the blueberry, which has potential antioxidant components, may be a good candidate as a natural antioxidant source.

Key words – Highbush blueberry (*Vaccinium corymbosum* L.), enzymatic extraction, reactive oxygen species, antioxidant activity

Free radicals are molecules that have an odd number of electrons. They can occur both organic (i.e., quinones) and inorganic molecules (i.e., $O_2\cdot^-$). Various types of free radicals are generated *in vivo* as by-products of normal metabolism and these radicals are highly reactive once they were formed and therefore, transient. They are also produced when an organism is exposed to ionizing radiation, to drugs capable of redox cycling or to xenobiotics that can form free radical metabolites *in situ*.

According to modern theory of free radical biology and medicine[13], free radical species (ROS, NO·) are involved in several disorders. However, the harmful effect of the free radicals can be blocked by antioxidant substances, which scavenge the free radicals and consequently detoxify the organism. Most research about free radicals have confirmed that foods rich in antioxidants play an essential role in prevention of cardiovascular diseases and cancers[21], neurodegenerative diseases, including Parkinson's and Alzheimer's diseases [11] as well as inflammation and problems caused by cell and cutaneous aging [3]. Antioxidants that can neutralize free radicals can be used to protect the human body from diseases and retard lipid rancidity in foods [28].

Many synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-Butylhydroquinone (TBHQ) are widely used in the food industry. However, animal models have proved that BHA and BHT accumulate in the body and cause liver damage and carcinogenesis[16]. Therefore, development and utilization of more effective and non-toxic antioxidants of natural origin are desired.

Highbush blueberry (*Vaccinium corymbosum* L.) is abundantly grown in Canada and the United States and more recently has become a popular commercial crop in Korea and Japan. A higher antioxidant capacity has been reported in blueberries compared to other fruits and vegetables[27]. Lowbush blueberry has higher *in vitro* antioxidant capacity than the cultivated highbush blueberry[18]. Further, blueberry contains chlorogenic acid, which is an important antioxidant compound, and they are also rich in phytochemicals such as anthocyanin (secondary plant metabolite). Numerous *in vitro* experiments have been indicated that anthocyanins and other phenolics in berries

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have a wide range of potential anti-cancer and heart disease properties including antioxidant, anti-inflammatory, and cell regulatory effects[19].

The enzymatic hydrolysis has reported excellent yields of desired compounds from the raw material tissues and cells and it present an easily accessible extraction and purification process in the industry[17]. Furthermore, enzymatic extraction process possesses the number of advantages and characteristic features compared to conventional extraction procedures. Water solubility, higher extraction efficacy, greater variation of constituents, minimized environmental pollution and relatively less expensiveness are some of those advantages in the enzymatic extraction process. The enzymes can work primarily by macerating the tissues and breaking down the cell walls and complex interior storage materials to release the bioactive compounds. Furthermore, high molecular weight polysaccharides and protein will be resulted in the extraction process, which can contribute to enhance the antioxidant potential[2].

The objective of the present work was to evaluate the potential of the antioxidant compounds present in enzymatic extract from highbush blueberry using variety of *in vitro* methods such as stable DPPH, HO·, NO·, $O_2 \cdot^-$ radicals scavenging, H_2O_2 scavenging and metal chelating. Furthermore, the lipid peroxidation activity in hemoglobin induced linoleic acid system was also evaluated in this work.

Materials and Methods

Materials

Blueberry samples were collected from the farm of Jeju Nong San Co., Ltd, Jeju, in Korea. Carbohydrases such as Viscozyme L (Ability to liberate bound materials and to degrade non-sratch polysaccharides), Celluclast 1.5 L FG (Catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose polymers), AMG 300 L (Hydrolyzes 1,4- and 1,6-α-linkages in liquefied starch), Termamyl 120 L Hydrolyses 1,4-α-glucosidic linkages in amylose and amylopectin), Ultraflo L (Breakdown of β-glucans, pentosans and other gums) and Protease such as Protamex (Production of non-bitter protein hydrolysis), Kojizyme 500 MG (Amino and carboxy peptidase activities), Neutrase 0.8 L (Endopeptidase activities), Flavourzyme 500 MG (Endoprotease and exopeptidase activities), Alcalase 2.4 L (Endopeptidase activity) were purchased from Novo Co.(Novozyme Nordisk, Bagsvaed, Denmark). Butylated hydroxytoluene (BHT), α-tocopherol, dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitro blue tetrazolium salt (NBT), xanthine, xanthine oxidase, thiobarbituric acid (TBA), trichloro acetic acid (TCA), Folin Ciocalteu reagent, sodium nitroprusside, sulfanilic acid were purchased from Sigma Co. (St. Louis, USA) and N-1-naphthylethylene diamine dihydrochloride was purchased from Hayashi pure chemical Industries Ltd. (Osaka, Japan). Ethylinediamine tetra-acetic acid (EDTA), peroxidase, 2, 3-Azino-bis (3-ethyl-benzthiazolin)-6-sulfonic acid (ABTS), and deoxyribose were purchased from Fluka Co. (Buchs, Switzerland). All other chemicals used were analytical grade supplied by Fluka or Sigma Co.

Preparation of enzymatic extracts

Method described by Heo *et al.*[15] was used with slight modifications to perform the enzymatic extracts from blueberry. One gram of ground blueberry was mixed with 100 mL of buffer solution and then, 100 μL (or 100 mg) of enzyme was mixed. Then the relevant pH and temperature was adjusted to optimize the digestion process in each sample. Later, enzymatic extraction was performed for 12 hrs to reach an optimum degree of extraction Fig 1. Afterwards, the samples were kept in a boiling water bath (100℃) for 10 min to inactivate the enzyme. Enzymatic extracts were obtained after filtering with Whatman filter paper and pH was adjusted to 7. Each enzymatic extract was tested for ROS, NO, metal chelating and lipid peroxidation assays together with measurement of phenolic content. In each assay, all activities were compared with the values of commercial antioxidants (BHT and α-tocopherol) dissolved in methanol.

DPPH radical scavenging assay

DPPH scavenging potential of the blueberry extracts were measured based on the scavenging ability of stable 1, 1-diphenyl-2-picrylhdrazyl (DPPH) radicals. The method modified by Brand-Williams *et al.*[5] was employed to investigate the free radical scavenging activity. Freshly prepared 2 mL DPPH ($3x10^{-5}$ M in DMSO) solution was thoroughly mixed with 2 mL of blueberry extracts. The reaction mixture was incubated for 1 hr at room temperature. The absorbance of resultant mixture was recorded at 517 nm using UV-VIS spectrophotometer (Opron

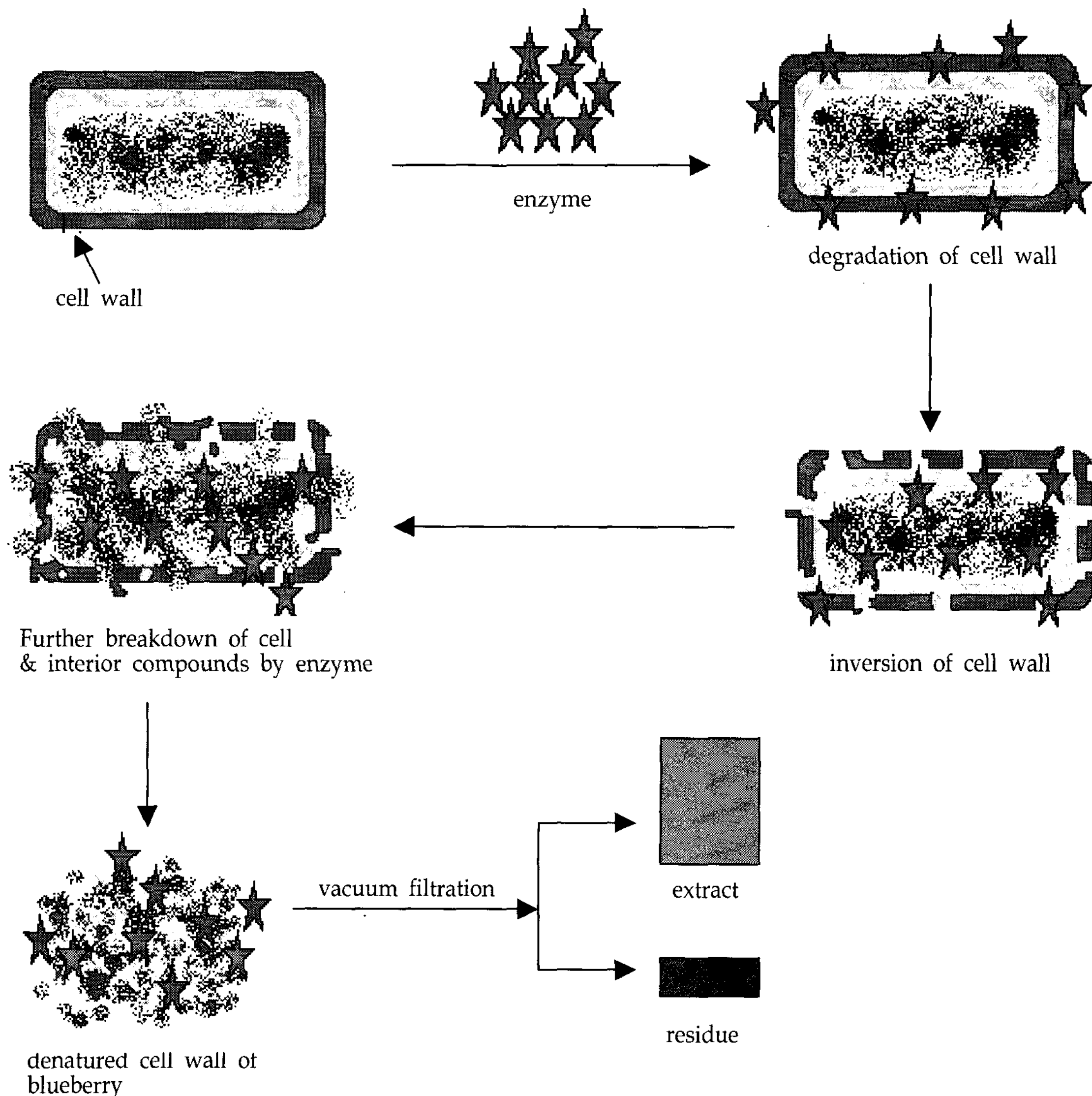

Fig. 1. Schematic diagram to explain the enzymatic extraction of blueberry.

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Superoxide anion ($O_2 \cdot^-$) scavenging assay

The superoxide scavenging ability of the blueberry extracts were assessed by the method described by Nagai *et al.*[25]. The reaction mixture contained 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, and 0.02 mL of 0.15% bovine serum albumin, 0.02 mL of 0.75 mM NBT and 0.02 mL of blueberry extracts. After incubation at 25℃ for 20 min, 6mU XOD was added to the mixture to initiate the reaction, which was carried out at 25℃ for 20 min. Reaction was terminated, by adding 0.02 mL of 6 mM CuCl. The absorbance of the mixture was recorded at 560 nm.

Hydrogen peroxide (H_2O_2) scavenging assay

The hydrogen peroxide scavenging ability of the blueberry extracts was investigated based on the scavenging of the hydrogen peroxide in ABTS-peroxidase system described by Muller[24]. Eighty microliter of each blueberry extracts and 20 μL of 10 mM hydrogen peroxide was mixed with 100 μL of phosphate buffer (pH 5.0, 0.1 M) in a 96-microwell plate and the samples were incubated at 37℃ for 5 min. Subsequently, 30 μL of freshly prepared ABTS (1.25 mM) and 30 μL of peroxidase were added and incubated at 37℃ for another 10 min. The absorbance of the resulting mixture was recorded using ELISA reader

(Sunrise; Tecan Co. Ltd., Austria) at 405 nm.

Hydroxyl radical (HO ·) scavenging assay

Ability of the blueberry extracts to scavenge the HO · generated by Fenton reaction was measured according to the modified method of Chung *et al.*[7]. The Fenton reaction mixture containing of 200 μL of 10 mM $FeSO_4 \cdot 7H_2O$, 200 μL of 10 mM EDTA and 200 μL of 10 mM 2-deoxyribose was mixed with 1.2 mL of 0.1M phosphate buffer (pH 7.4) containing 200 μL of blueberry extracts. Thereafter, 200 μL of 10 mM H_2O_2 was added to the mixture before incubation at 37℃ for 4 h. After incubation, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added and placed in the boiling water bath for *10 min. Then, the resultant mixture was allowed to cool to room temperature and centrifuged at 395xg for 5 min. The absorbance was recorded at 532 nm.

Nitric Oxide radical (NO ·) scavenging assay

Sodium nitroprusside in aqueous solution at physiological pH (7.4) spontaneously produce nitric oxide, which reacts with oxygen to produce nitrite ions and it can be determined by the use of Griess Illosvoy reaction[12]. Griess Illosvoy reagent was slightly modified using naphthylethylenediamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). The reaction mixture (3 mL) containing 2 mL of 10 mM sodium nitroprusside, 0.5 mL of phosphate buffer saline (pH 7.4, 0.01 M) and 0.5 mL of extract was incubated at 25℃ for 150 min. Thereafter, 0.5mL of the reaction mixture containing nitrite was taken out and mixed with 1 mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 mL of naphthylethylenediamine dihydrochloride (0.1%) was added, and allowed to stand for 30 min in diffused light. The absorbance of the pink colored chromophore was measured at 540 nm.

Ferrous ion chelating ability

A method described by Decker and Welch[10] was used to investigate the ferrous ion chelating ability of blueberry extracts. Five milliliters of each blueberry extracts was mixed with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine solutions. The absorbance at 562 nm was determined after 10 min. A complex of Fe^{2+}/ferrozine showed strong absorbance at 562 nm.

Lipid peroxidation inhibitory activity in a hemoglobin-induced linoleic acid system

The lipid peroxidation inhibitory activity of blueberry extracts was determined according to the method described by Kuo *et al.*[22] with slight modifications. Each extract (0.1 mL) was thoroughly mixed with 0.025 mL of 0.1 M linoleic acid/ethanol and 0.075 mL of 0.2 M phosphate buffer (pH 7.2). Afterwards, 0.08% hemoglobin (0.05 mL) was added to start the autoauxidation and mixture was incubated at 37℃ for 60 min. The lipid peroxidation was stopped by adding 5 mL of 0.6% HCl/ethanol. The peroxidation value of the mixture (0.2 mL) was measured after reaction with 0.02 mL of 20 mM $FeCl_2$ and 0.01 mL of 30% ammonium thiocyanate. The absorbance was recorded at 490 nm with an ELISA reader (Sunrise; Tecan Co., Austria)

Total phenolic content assay

Total phenolic content was determined according to the method described by Chandler and Dodds[6]. One millilitre of blueberry extract was mixed in a test tube containing 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Foiln-Ciocalteu reagent. The resultant mixture was allowed to react for 5 min and 1 mL of 5% Na_2CO_3 was added. It was mixed thoroughly and placed in dark for 1 hr and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer. A gallic acid standard curve was obtained for the calculation of phenolic content.

Calculation of 50% inhibition concentration (IC_{50})

The concentration of the extract (mg/mL) that required scavenging 50% of radicals was calculated by using the percent scavenging activities of four different extract concentrations. Percent scavenging activity was calculated as $[1-(A_i-A_j)/Ac]$ X 100%.

Where; A_i is the absorbance measured with different blueberry extracts in the particular assay with ROS source; A_j is the absorbance measured with different blueberry extracts in the particular assay but without ROS source; A_c is the absorbance of control with particular solvent (without blueberry extracts).

Statistical analysis

All experiments were conducted in triplicate (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 parameters were determined by using Duncan test ($P<0.05$).

Results and Discussion

DPPH radical scavenging activity

Proton-radical scavenging activity is an important attribute of antioxidants, which can be measured using DPPH radical scavenging assay. DPPH, a protonated radical, has characteristic maximum absorbance peak at 517 nm, which decreases with the scavenging of the proton radical[30]. Further, 2,2-diphenyl-2-picrylhydrazyl radical has been widely used to evaluate the free radical scavenging capacity of antioxidants[5].

DPPH radical scavenging activity is shown in Table 1 and 2. Viscozyme and Flavourzyme extracts showed significantly higher ($P<0.05$) scavenging activities (0.046±0.002 and 0.045±0.001 mg/mL respectively) when compared with the values of BHT (0.374±0.003 mg/mL) but significantly lower scavenging activities ($P<0.05$) than that of α-tocopherol (0.018±0.0 mg/mL). All the enzyme extracts showed higher values than that of BHT but lower values than that of α-tocopherol. Furthermore, DPPH scavenging activity of carbohydrases treated extracts was relatively higher than the extracts treated with proteases. Oki *et al.*[26] have reported that the radical scavenging ability may increase with the increase of the phenolic content. In this study, however, some enzymatic extracts showed high activities, even though they contained low amount of phenolics. This may be due to the presence of other bioactive materials such as low molecular weight polysaccharides, pigments, proteins or peptides (due to enzymatic extraction). The results reveal that enzymatic extracts from blueberry might contain radical scavenging compounds.

Superoxide anion ($O_2 \cdot^-$) scavenging activity

Superoxide radicals are generated during the normal physiological process mainly in mitochondria. Although superoxide anion is by itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to the oxida-

Table 1. Antioxidative effect of enzymatic extracts (carbohydrases) from blueberry.

Extracts	Total phenolic content (mg/100g dried sample)	IC_{50} (mg/mL)					
		DPPH	$O_2 \cdot^-$	H_2O_2	HO ·	NO ·	Metal chelating
AMG	589.38±9.4	0.057^c±0.005	3.77^f±0.13	2.10^a±0.12	3.84^c±0.09	0.339^a±0.011	0.120^a±0.007
Celluclast	540.74±8.4	0.104^e±0.003	2.23^d±0.13	2.11^a±0.12	3.94^c±0.15	0.486^{bc}±0.013	0.108^a±0.002
Termamyl	546.63±7.6	0.057^c±0.004	1.83^c±0.14	2.50^b±0.14	3.25^b±0.14	0.442^b±0.014	0.089^a±0.003
Ultaraflo	563.63±8.7	0.064^d±0.001	2.56^e±0.17	2.83^c±0.09	3.86^c±0.17	0.405^b±0.015	0.117^a±0.009
Viscozyme	532.12±7.3	0.046^b±0.002	3.71^f±0.03	2.72^{bc}±0.19	3.11^b±0.15	0.675^d±0.012	0.103^a±0.005
BHT		0.374^f±0.003	0.18^a±0.01	2.20^a±0.11	0.03^a±0.00	1.630^e±0.060	3.300^b±0.140
α-Tocopherol		0.018^a±0.000	1.60^b±0.08	3.20^d±0.14	0.05^a±0.00	2.340^f±0.060	4.300^c±0.170

All data are means of three determinations. (mean ± SD, n=3)
Significant differences at $P < 0.05$ indicated with different letters.

Table 2. Antioxidative effect of enzymatic extracts (proteases) from blueberry.

Extracts	Total phenolic content (mg/100g dried sample)	IC_{50} (mg/mL)					
		DPPH	$O_2 \cdot^-$	H_2O_2	HO ·	NO ·	Metal chelating
Alcalase	597.96±3.1	0.062^c±0.004	0.89^b±0.12	2.10^a±0.14	3.98^c±0.08	0.349^a±0.011	0.149^a±0.006
Flavourzyme	577.93±5.1	0.045^b±0.001	2.84^e±0.12	2.35^{bc}±0.13	3.72^b±0.10	0.697^c±0.013	0.117^a±0.001
Kojizyme	532.16±4.1	0.113^d±0.003	3.28^f±0.15	2.75^d±0.12	3.90^c±0.04	0.429^b±0.015	0.129^a±0.003
Neutrase	517.85±3.2	0.061^c±0.001	2.46^d±0.11	2.41^c±0.11	3.97^c±0.05	0.482^b±0.015	0.117^a±0.001
Protamex	549.32±6.2	0.152^e±0.002	2.83^e±0.13	2.16^{ab}±0.11	3.97^c±0.05	0.420^b±0.011	0.115^a±0.006
BHT		0.374^f±0.003	0.18^a±0.01	2.20^{ab}±0.11	0.03^a±0.00	1.630^d±0.060	3.300^b±0.140
α-Tocopherol		0.018^a±0.000	1.60^c±0.08	3.20^e±0.14	0.05^a±0.00	2.340^e±0.060	4.300^c±0.170

All data are means of three determinations. (mean±SD, n=3)
Significant differences at $P < 0.05$ indicated with different letters.

tive stress[9]. Therefore, superoxide radical scavenging by antioxidants has physiological implications.

Superoxide anion scavenging activity of blueberry extracts is illustrated in Tables 1 and 2. Alcalase extract showed significantly higher (*P<0.05*) activity (0.89±0.12 mg/mL) than that of α-tocopherol (1.60±0.08 mg/mL) but significantly lower (*P<0.05*) activity than that of BHT (0.18±0.01 mg/mL) while Termamyl extract showed higher activity (1.83±0.14 mg/mL) among carbohydrases extracts but significantly lower (*P<0.05*) than those of commercial antioxidants tested. Furthermore, all the other extracts showed relatively moderate values in superoxide scavenging activity when compared with the commercial antioxidants. Cotelle *et al.*[8] have reported that flavones, a class of flavonoids, bearing hydroxyl groups at positions 3′, 4′ or 3′, 4′, 5′ scavenge $O_2\cdot^-$. These authors also have reported that flavonoids containing one hydroxyl group in the C-7 position of its ring A could inhibit generation of $O_2\cdot^-$ in the xanthine/xanthine oxidase system by inhibiting the enzyme. Therefore, $O_2\cdot^-$ scavenging properties of blueberry extracts may also be attributed to both neutralization of superoxide radicals via hydrogen donation and inhibition of xanthine oxidase by various phenolic components present in the extracts. Further, the differences in $O_2\cdot^-$ scavenging potentials of blueberry extracts could be attributed to the structural features of the bioactive compounds.

Hydrogen peroxide (H_2O_2) scavenging activity

Hydrogen peroxide converts into singlet oxygen ($^1O_2$) and hydroxyl radical, and thereby become a very powerful oxidizing agent. Further, it can cross membranes and may oxidize number of compounds. Thus, a removing of H_2O_2 is very important for antioxidant defense in cell or food systems.

In the results of hydrogen peroxide scavenging activity, AMG extract exhibited the highest activity (2.10±0.12 mg/mL) followed by Celluclast extract (2.11±0.12 mg/mL) among carbohydrase extracts (Table1). Further, Alcalase extract showed the highest activity (2.10±0.14 mg/mL) among protease extracts (Table2). Both activities were significantly higher (*P<0.05*) than that of α-tocopherol (3.20±0.14 mg/mL). All extracts showed higher activities than that of α-tocopherol. Scavenging of H_2O_2 by blueberry extracts may be attributed to the phenolic compounds in each enzymatic extracts which could donate electrons to H_2O_2, thus neutralizing it to water[14]. Further, the other materials in blueberry extracts, such as small molecular weight polysaccharides, pigments, proteins or peptides, may also influence the inhibitory activity.

Hydroxyl radical (HO·) scavenging activity

An antioxidant ability to scavenge hydroxyl radicals is an important antioxidant action because of the high reactivity of hydroxyl radicals that enables the radical to react with a wide range of molecules found in living cells, such as amino acids, lipids, nucleotides and sugars. Hydroxyl radicals can be generated *in situ* by decomposition of hydrogen peroxide by high redox potential EDTA - Fe^{2+} complex (non-site specific) and, in the presence of 2-deoxy-D-ribose substrate, it forms thiobarbituric acid reactive substances which can be measured photometrically[4].

Viscozyme showed the highest activity (3.11±0.15 mg/mL) among the carbohydrase extracts while Flavourzyme showed the highest activity (3.72±0.10 mg/mL) among the protease extracts. However, both activities were significantly lower (*P<0.05*) than those of commercial antioxidants, BHT and α-tocopherol (0.03±0.0 and 0.05±0.0 mg/mL respectively). Furthermore, all the enzymatic extracts showed significantly lower (*P<0.05*) activities in hydroxyl radical scavenging when compared with the commercial antioxidants. Hydroxyl radicals are capable of abstracting hydrogen atoms from cellular membranes and bring about peroxidic reactions in lipids[20]. It is therefore thought that blueberry extracts demonstrate antioxidant effects against lipid peroxidation by scavenging the hydroxyl radicals and superoxide anions at the stage of initiation and termination of peroxy radicals.

Nitric oxide radical (NO·) scavenging activity

In addition to reactive oxygen species, nitric oxide is also implicated in cancer, inflammation and other pathological conditions[23]. The plant or plant products may have the property to counteract the effect of NO· and in turn may be of considerable interest in preventing the bad effects of excessive generation of NO· in the human body. Therefore, the scavenging ability of NO· may also help to disrupt the chain reactions initiated by excessive generation of NO· that are detrimental to human health. AMG extract exhibited significantly higher activity (*P<0.05*) than other carbohydrase extracts (0.339±0.011 mg/mL) and

Alcalase extract showed significantly higher activity ($P<0.05$) than other protease extracts (0.349±0.011 mg/mL). Further, all values were significantly higher ($P<0.05$) than those of commercial antioxidants; BHT and α-tocopherol (1.630±0.06 and 2.340±0.06 mg/mL respectively). Nitric oxide has both beneficial as well as deleterious effects. NO · is a mediator of inflammation, which plays an essential role in the defense mechanism of macrophages against micro-organisms[23]. NO · produced by inflammatory cells are toxic as they give rise to peroxynitrite ($ONOO^-$) reacting with $O_2 \cdot ^-$. Therefore NO · scavenging is an alternative to Nitric oxide synthase (NOS) inhibition and may be an interesting approach to reduce toxicity of NO · .

Ferrous ion chelating activity

Iron is known as the most important lipid oxidizing pro-oxidant among the transition metals due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxidase to reactive free radicals via the Fenton type reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO \cdot$). Ferrozine was used to indicate the presence of chelator and it forms a complex with free Fe^{2+}. In the presence of chelating agent, the complex formation of ferrous and ferrozine is disrupted, resulting in a decrease in red color of the complex.

Tables 1 and 2 showed the metal chelating activity of enzymatic extracts from blueberry. Both, the protease and carbohydrase extracts from blueberry showed significantly higher ($P<0.05$) chelating activities when compared with the commercial antioxidants. Termamyl exhibited the highest activity among carbohydrases and Protomax showed the highest activity among proteases. The iron chelation properties of a plant extract may be attributed to their endogenous chelating agents, mainly phenolics. Some phenolic compounds show high activity due to their properly oriented functional groups, which chelate metal irons[29]. These authors have also reported that six-membered ring complexes show higher stability in metal-antioxidant complexes than that of five-membered ring complexes. Therefore the strong iron chelating activities of blueberry obtained by enzymatic extracts could be attributed to the formation of six-membered complexes with iron.

The inhibitory activity of lipid peroxidation

Lipid peroxidation is a critical problem, which affects the quality of food leading to rancidity, toxicity and destruction of bioactive components. As shown in Table 3, the highest inhibitory activity of lipid peroxidation in a hemoglobin-induced linoleic acid system was found in AMG (0.28±0.01 mg/mL) among carbohydrases extracts and Alcalase (0.65±0.05 mg/mL) among proteases extracts. Those activities were compared with the values of BHT and α-tocopherol. All the enzymatic extracts showed moderate activities in this assay. There have been a number of researches describing that oxidative damage of important biological molecules can be mediated by hemoglobin, myoglobin and hemin[1]. It is proposed that heme group may either act as a Fenton reagent which can produce hydroxyl radicals or oxidizing species such as ferryl intermediates and radical species in amino acid residues of hemoproteins. Our results showed that blueberry enzymatic extracts can inhibit the hemoglobin-induced lipid peroxidation. Strong inhibitory activities could be attributed to the chain braking or metal chelating antioxidants present in the blueberry extract. However, this method could assess the results with only 1 hr of oxidation time.

In conclusion, the results obtained in the present study clearly demonstrate that the enzymatic extract of blueberry may contain a number of antioxidant compounds, which can effectively scavenge various reactive oxygen species and chelating ferrous ions under *in vitro* conditions. Additionally, higher phenolic content also dispersed in the extracts giving different antioxidant activities. Thus, highbush bluberry may be a good candidate as a natural antioxidant source and as a food supplement or in pharma

Table 3. Lipid peroxidation inhibitory activity of enzymatic extracts from blueberry in a hemoglobin induced linoleic acid system.

	Sample	IC_{50} (mg/mL)
Carbohydrases	AMG	0.28±0.01
	Celluclast	0.97±0.04
	Termamyl	0.38±0.05
	Ultaraflo	1.06±0.08
	Viscozyme	0.66±0.04
Proteases	Alcalase	0.65±0.05
	Flavourzyme	0.95±0.06
	Kojizyme	1.98±0.04
	Neutrase	0.96±0.03
	Protamex	2.14±0.09
	BHT	0.13±0.02
	α-Tocopherol	0.16±0.03

All data are means of three determinations. (mean ± SD, n=3)

ceutical industry. Further investigations are needed to find-out the bioactive compounds present in the blueberry.

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초록 : 블루베리 (*Vaccinium corymbosum* L.) 유래 효소 추출물의 항산화성

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제주지역에 서식하고 있는 블루베리 (*Vaccinium corymbosum* L.)로부터 수용성 추출물을 효과적으로 제조하기 위하여 5가지 종류의 탄수화물 분해효소 (AMG, Celluclast, Termamyl, Ultraflo, Viscozyme)와 또 다른 5종의 단백질 분해효소 (Alcalase, Flavourzyme, Kojizyme, Neutrase, Protamex)를 이용하여 효소적 추출을 시도하였다. 이들 효소를 이용하여 제조된 여러 가지 블루베리 효소 추출물에 대하여 1,1-diphenyl 2-picrylhydrazyl (DPPH), 활성산소종 (ROS), 일산화질소 (NO ·) 등의 소거활성, 금속 킬레이트결합능, 그리고 지질 과산화 저해능과 같은 항산화 효과를 검토하였다. 모든 효소 추출물의 페놀함량은 517.85~597.96 mg/100 g 건조시료이었으며, 항산화 효과에 대한 평가에서 특히 DPPH 및 NO · 소거활성, 그리고 금속 킬레이트 결합능 등이 우수한 것으로 나타났다. Viscozyme 추출물은 DPPH 소거활성 (0.046±0.002 mg/mL)이, 그리고 AMG 추출물은 NO · 소거활성 (0.339±0.011 mg/mL) 및 지질 과산화 억제활성 (0.28±0.01 mg/mL)이 각각 우수하였다. 과산화 수소 소거활성에 있어서는 블루베리 효소 추출물들이 천연 항산화제인 α-토코페롤보다 상대적으로 높았다. 이러한 결과로 볼 때 블루베리는 항산화 효과가 우수한 화합물들을 함유하고 있을 것으로 판단되며, 향후 천연 항산화 자원으로서 이용이 가능할 것으로 사료된다.