The antioxidative activities of hydrolysates isolated from the freshwater rotifer using enzymes Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin were identified and evaluated using direct free radical scavenging activity. Among the six hydrolysates, Neutrase hydrolysate had the highest antioxidative activity compared to the other hydrolysates. The free radical scavenging activity of Neutrase hydrolysate was 45.26% at 1.0 mg/mL. The peptide demonstrating the strongest antioxidative activity was isolated from the hydrolysate using consecutive chromatographic methods including Sephadex G-25 Gel chromatography and high performance liquid chromatography on an ODS column. The IC$_{50}$ value of purified antioxidant peptide was 100.8 μM. The antioxidant peptide was identified as a sequence of 10 amino acids, Gly-His-Asp-Gly-Tyr-Glu-Pro-Leu-Ser-Ser (1091 Da) by N-terminal amino acid sequence analysis. The purified peptide exhibited an inhibitory effect against induced DNA oxidation. Our results suggested that antioxidative hydrolysates freshwater rotifer may be useful ingredients in food and nutraceutical applications.

Key words: antioxidant, DPPH radical scavenging, enzymatic hydrolysis, freshwater rotifer, peptide

The onset of many human diseases such as cancers [Leanderson et al., 1997] gastric ulcers [Debashis et al., 1997], Alzheimer’s, arthritis [Vajragupta et al., 2000] can be attributed to the presence of free radicals, while natural antioxidants can act as free radical scavengers and prevent mentioned diseases [Chang et al., 2007]. Antioxidants primarily serve to protect against diseases by eliminating excessive free radicals and reactive oxygen species (ROS) in the body [Fridorich, 1978]. Formation of free radicals including superoxide (O$_2^-$), nitric oxide (NO•), nitrogen dioxide (NO•$_2$), hydroxyl (*OH), peroxyl (ROO•), alkoxyl (RO•), and hydroperoxyl (HO•$_2$) is a natural consequence of cell respiration in aerobic organisms [Je et al., 2007]. In human cells and tissue, free radicals and ROS are constantly removed by an enzyme mediated system involving catalase, superoxide dismutase (SOD), peroxidase, glutathione peroxidase, ascorbic acid and α-tocopherol [Halliwell and Gutteridge, 1989].

Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are used as food additives and preservatives. Although, these synthetic antioxidants show stronger activity than natural compounds such as α-tocopherol and ascorbic acid. However, the use of synthetic antioxidants is under strict regulation because of the potential health hazards, but natural compounds were concern about their safety [Ito et al., 1986]. Therefore, the development and use of natural antioxidants as an alternative to synthetic ones is of great interest. Efficiently dietary antioxidant was needed in modern complex lifecycle exposure to inordinate stress. Vitamin C, α-tocopherol and phenolic compounds, which are present naturally in vegetables, fruits and seeds, possess the ability to reduce oxidative damage associated with many diseases.

Recently, hydrolyzed proteins from many animal and plant sources have been found to possess antioxidant activity, aquatic products and by-products have proven to be good sources of antioxidant peptides, such as grass carp [Ren et al., 2008], jumbo squid skin [Mendis et al., 2005a], yellow stripe trevally (Selaroides leptolepis) [Klompong et al., 2007], Pacific hake (Merluccius productus) [Samaranayaka et al., 2008], algae protein waste [Sheih et al., 2009]. Protein hydrolysates or peptides have health-related functions such as blood pressure reduction and antioxidant function.
The rotifer, *Brachionus calyciflorus*, is most commonly used as live feed for freshwater fish larvae cultures. The *B. calyciflorus* has been studied as food source for freshwater fish because of its small size and rich protein and lipid [Rønnestad *et al.*, 2003]. The protein of rotifer widely could be used in bioactivity materials and functional resources. However, bioactivity material had not been reported from freshwater rotifer, and even antioxidative activities have not studied. Therefore, freshwater rotifer be a good source of antioxidants, which make it a good source for large quantity cultivation.

Therefore, the purpose of this study was purification and characterization of an antioxidative peptide derived from enzymatic hydrolysates of freshwater rotifer. Finally, separated peptide analyze of amino acid sequence and molecular weight. Then, identify novel bioactivity peptide by DNA oxidation *in-vitro* test.

**Materials and Methods**

**Materials.** The freshwater rotifer, *B. calyciflorus* was large quantity cultured under controlled condition of water temperature, feed frequency, pH, and dissolved oxygen according to Park [1998]. Fully-grown rotifers were harvested and lyophilized at −80°C using a freeze dryer. Alcalase and Neutrase were purchased from Novozyme Co. (Bagsvaerd, Denmark), α-chymotrypsin, trypsin, papain, BHA, BHT and Sephadex G-25 were purchased from the Sigma Chemical Co. (St. Louis, MO). Pepsin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were of the highest grade commercially available.

**Preparation of hydrolysates.** To produce antioxidant peptide from freshwater rotifer, enzymatic hydrolysis was performed using six different enzymes: Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin with their optimal conditions for pH, temperature, and buffer (Table 1). At enzyme-substrate ratio of 1/100 (w/w), 1% substrate and enzyme were mixed in a shaking water bath and sample mixture was immediately broken down using an ultrasonicator (Sonic and Materials, Inc., CT, USA). The sample mixture was incubated for 12 h at each optimal temperature with shaking at 120~130 rpm, and then heated in a boiling water bath for 5 min to inactivate the enzyme. Degree of hydrolysis was determined by measuring the soluble nitrogen content in 10% trichloroacetic acid as followed by Kim *et al.* [1990], and lyophilized hydrolysates were stored at −80°C until use.

**DPPH radical scavenging assay.** DPPH radical scavenging activity of hydrolysates was modified as described by Bersuder *et al.* [1998]. A volume of 40 μL of each sample at different concentrations was mixed with 120 μL of 100% methanol and 40 μL of 0.15 mM DPPH in 100% methanol. The mixture was then kept at room temperature in the dark for 30 min, and the reduction of DPPH radicals were measured at 517 nm using a UV-Visible spectrophotometer (JASCO, Tokyo, Japan). The DPPH radical scavenging activity was calculated as follows: Radical scavenging activity (％)=Absorbance of control−Absorbance of sample/Absorbance control×100. The control was conducted in the same method, except that distilled water was used instead of sample. Standard was using artificial antioxidant, BHA and BHT. The test was carried out in triplicate.

**Purification of antioxidative peptide.** The potent fraction as determined from DPPH radical scavenger activity assay was further purified by size exclusion chromatography on a Sephadex G-25 gel filtration column (25×750 mm) equilibrated with distilled water. Separated fractions were monitored at 280 nm, collected at a volume of 7.5 mL and measured for antioxidative activities. The highest active fraction was then injected into a preparative reverse phase HPLC column (Grom-sil 120 ODS-5 ST, 10.0×250 mm, 5 μm) and separated using a linear gradient of acetonitrile (0-40% v/v) containing 0.1% trifluoroacetic acid on an HPLC system (Agilent Technologies, Santa Clara, CA). The potent peak was finally purified into a single peptide on a reverse phase HPLC analytical C8 column (4.6×250 mm, 5 μm) using a linear gradient of acetonitrile (0-20%, v/v) in 0.1% TFA.

**Identification of antioxidative peptide.** The antioxidative activity of purified peptide from freshwater rotifer was determined through amino acid sequence analysis by N-terminal amino acid sequencing at Korea Basic Science Institute. The active section of the purified peptide was excised from the 2-DE gels and subjected to in-gel digestion [Shevchenko *et al.*, 1996]. The trypsin- or pepsin-digested peptide mixtures were analyzed with a 4700 Proteomics Discovery System (PerSeptive Biosystems, PerkinElmer, USA). The potent peptide was identified using a BenchMark II software (2002). The peptide purity was confirmed by HPLC, and finally purified into a single peptide on a reverse phase HPLC column (4.6×250 mm, 5 μm) using a linear gradient of acetonitrile (0-20%, v/v) in 0.1% TFA.

**Table 1. Optimum conditions of enzymatic hydrolysis for various enzymes**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Buffer</th>
<th>pH</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcalase</td>
<td>50 mM sodium phosphate</td>
<td>7.0</td>
<td>50</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>50 mM sodium phosphate</td>
<td>8.0</td>
<td>37</td>
</tr>
<tr>
<td>Neutrase</td>
<td>50 mM sodium phosphate</td>
<td>8.0</td>
<td>50</td>
</tr>
<tr>
<td>Papain</td>
<td>50 mM sodium phosphate</td>
<td>6.0</td>
<td>37</td>
</tr>
<tr>
<td>Pepsin</td>
<td>20 mM glycine-HCl</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>50 mM sodium phosphate</td>
<td>8.0</td>
<td>37</td>
</tr>
</tbody>
</table>
Framingham, MA) and with Ultraflex MALDITOF/TOF MS (BrukerDaltonics GmbH, Bremen, Germany). Peptide mass fingerprintings were acquired by summing 750 laser shots. Monoisotopic peptide masses were selected in the range of 800-3000 Da and were identified using the Matrix Science Mascot and National Center for Biotechnology Information protein sequences database [Na et al., 2006].

Protective effect of antioxidative peptide on oxidation-induced DNA damage. Genomic DNA, which was extracted from RAW cell 264.7 (American Type Culture Collection, ATCC, Rockville, MD) using standard phenol/proteinase K procedure with modification [Sambrook and Kussell, 2001] was pre-treated with different concentrations of hydrolysates and exposed •OH group using Fenton assay as described by Milne et al. [1993]. Briefly, 40 μL of DNA reaction mixture was prepared by adding different concentrations of test sample, final concentrations of test samples were 100 mM FeSO₄, 0.1 mM H₂O₂ and 5 μL genomic DNA. The mixture was subsequently incubated for 10 min at 25°C and the reaction was terminated by the addition of ethylenediaminetetraacetic acid, final concentration of 10 mM. 20 μL of the reaction mixture containing about 1 μg of DNA was electrophoresed on a 1% agarose gel for 40 min at 100 V. Agarose gel were stained with 1 mg/mL ethidium bromide and than visualized by a UV lamp.

Results

Enzymatic hydrolysates of freshwater rotifer. The freshwater rotifer protein was separately hydrolyzed by Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin, to product of antioxidant hydrolysates. The extent of protein degradation by enzymatic hydrolysates was estimated by evaluating the degree of hydrolysis. Analysis revealed that DH of trypsin, α-chymotrypsin and pepsin were 70.05%, 65.41%, 60.51%, respectively. The remaining enzymatic hydrolysates had DH values greater than 50% (Fig. 1).

Antioxidative activity of hydrolysates. Peptides from six hydrolysates were evaluated for their antioxidant activities using a DPPH radical scavenging assay. As shown in Fig. 2, DPPH radical scavenging activity of extracts produced by various enzymes, Neutrase, Alcalase and trypsin were 45.26%, 28.02%, 5.44% at 1.0 mg/mL, respectively. All hydrolysat extract showed dose-dependent DPPH radical scavenging activity, and interestingly Neutrase hydrolysat exhibited the highest antioxidative activity.

Purification of antioxidative peptide. Purifying antioxidative peptide from Neutrase hydrolysat of freshwater rotifer required the use of different chromatographic techniques. Figure 3 demonstrates the chromatographic profiles obtained during different purification steps of antioxidative peptide from freshwater rotifer and their scavenging effects on DPPH radical. Initially, Neutrase hydrolysat was separated into five fractions (F1-F5) on a Sephadex G-25 chromatography column (Fig. 3A). Among the separated fractions, F2 had the highest scavenging activity on DPPH radical at 67.45% (Fig. 3B). Further separation of F2 was carried out using reverse phase HPLC on a C₁₈ HPLC column, where five different scavenging activities were obtained (Fig. 4A). A clear scavenging difference was observed among the activities of five fractions and F2-1 was the more potent antioxidant (Fig. 4B). F2-1 was further purified using reverse phase HPLC on analytical C₁₈ column with liner gradients. Single peptide fraction that exhibited antioxidant activity, were finally purified on an analytical HPLC column and their amino acid sequences were
Antioxidant peptide isolated from freshwater rotifer

Amino acid sequence of purified antioxidative peptide. F2-I was analyzed for molecular weight with amino acid sequence using N-terminal sequencing analysis. F2-I was identified as Gly-His-Asp-Gly-Tyr-Glu-Pro-Leu-Ser-Ser with a molecular weight of 1091 Da (Fig. 5). The purified peptide F2-I was effectively quenched in DPPH radical, and IC$_{50}$ value was 100.8 μM.

Protective effect of the purified peptide on oxidation-induced DNA damage. In this study, genomic DNA was isolated from RAW 264.7 cells to investigate the protective effects of purified peptide from freshwater rotifer against DNA oxidative damage (Fig. 6). The results of this study strongly suggest that purified peptide can prevent oxidative damage to DNA when DNA is exposed to •OH generated by Fenton reaction.

Discussion

In this study we purified antioxidant peptide from freshwater rotifer. Antioxidant activity peptide was produced from freshwater rotifer protein using enzymatic hydrolysis. Enzymatic hydrolysis is one of the approaches for the effective release of bioactive peptides from protein sources. It is widely applied to improve and upgrade the functional and nutritional properties of proteins. In the present study, various hydrolysates were produced by different proteases, and their antioxidant activities were evaluated. Among six enzymes, trypsin was highest DH (Fig. 1). The remaining enzymatic hydrolysates had DH values greater than 50%. Similar to our study, Tang et al. [2009] reported that hemp (Cannabis sativa L.) protein was separately hydrolyzed by six enzymes (Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin) for production of antioxidative peptides. The peptic and tryptic hydrolysates from hemp (Cannabis sativa L.) [Tang et al., 2009] had DH of 48.5% and 11.2%. Qian et al. [2008] reported that an antioxidant peptide was isolated from bullfrog protein hydrolysates, DH of bullfrog hydrolysates were 73.9% and 65.4%, pepsin and trypsin, respectively. As compared to our result, enzymatic hydrolysate DH from freshwater rotifer protein was higher than DH values of hemp and less than bullfrog hydrolysate. Hydrolysis was necessary to liberate antioxidative peptides from an inactive form in freshwater rotifer protein, and the presence of antioxidative activity in the hydrolysates was probably due to the presence of a variety of peptide sequences.

Purification of antioxidative peptide Sephadex G-25 column chromatography was fractionated according to molecular size, where F1 and F2 represented large molecular size peptides, and fractions F3-F5 contained small molecular size peptides. According to Pihlanto-Leppala’s [2001] report most bioactive peptides contain
According to reverse phase-HPLC methods, we purified the most potent antioxidant activity peptide with the sequence of Gly-His-Asp-Gly-Tyr-Glu-Pro-Leu-Ser-Ser with a molecular weight of 1091 Da (Fig. 5). The C-terminals of purified peptides were represented by proline, leucine and serine residues, while glycine and histidine residues were located at the N-terminals. Similar to our data, an antioxidative activity peptide was observed in the hoki skin gelatin hydrolysate at IC₅₀ value of His-Gly-Pro-Leu-Gly-Pro-Leu was 156.8 μM [Mendis et al., 2005b]. The C-terminal of purified peptide from freshwater rotifer was located between leucine, and proline in the C-terminal. Interestingly, we found a study that reported [Suetsuna et al., 2000] purified peptide from casein hydrolysate contained leucine and proline at the C-terminal end of the sequence. We therefore suggest that leucine and proline are also important for antioxidant activity peptide sequence at the C-terminal. Hydroxyl radicals have been known to cause oxidative damage in DNA structure by relaxing the DNA or denaturing it. The results of this study strongly suggest that purified peptide can prevent oxidative damage to DNA when DNA is exposed to •OH generated by Fenton reaction. The purified peptide of protective activity against DNA oxidation, a clear dose-dependent effect was observed. In this result, the effect of purified peptide to protect hydroxyl radical induced DNA damage.

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References

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