Enzymatic Hydrolysis for Effective Extraction of Antioxidative Compounds from *Hizikia fusiformis*

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*Hizikia fusiformis* hydrolysates prepared by five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamex, 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 proteases were selected by 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 as 5%. Simultaneously, Ultraflo of the two carbohydrases and Alcalase 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 *H. fusiformis*. The hydrolysate obtained by combining the enzymes was separated into four different molecular weight fractions (< 1kD, 1-10 kD, 10-30 kD and > 30 kD) and recorded the polyphenolic content distribution and respective antioxidative ability. The fraction < 1kD was identified as less effective and those fractions > 1kD indicated comparatively higher antioxidative activities related to their polyphenolic content.

**Key Words:** antioxidative, carbohydrate, enzymatic hydrolysis, fractionation, *Hizikia fusiformis*, protease

INTRODUCTION

Numerous phycotechnological studies on bioactive materials revealed that seaweeds are potential sources of pharmacological compounds and food additives, which potentially exert beneficial health effects like antioxidative and anti-carcinogenic (Ireland et al. 1993; Okai et al. 1996; Duval et al. 2000; Rupérez and Calixto 2001; Lim et al. 2002; Athukorala et al. 2003). *Hizikia fusiformis* (Class Pheophyceae, Order Fucales, Family Sargassaceae) is edible brown seaweed being widely consumed in Korea, Japan and some European countries. During last two decades, a number of researches on *H. fusiformis* 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). Our previous studies on *H. fusiformis* antioxidative compounds reported notable activities in reactive oxygen species (ROS) scavenging and lipid peroxidation inhibition (Siriwardhana et al. 2003 a, b). In the first investigation of antioxidative activity variation among different extracts, we observed that the free radical and hydrogen peroxide scavenging activities of aqueous extracts were almost compatible with commercial antioxidants tested. Moreover, there was a tendency of resulting higher activities towards high polar solvents (ethanol, methanol and water) than that of non-polar solvents (Diethyl ether and chloroform).

Seaweeds originally contain large amount of highly viscous polysaccharides in large quantities (Mabeau and Kloareg 1987). Brown seaweeds contain soluble dietary fiber polysaccharides as alginates, fucans and laminarians together with the insoluble fibers made of cellulose (Lahaye et al. 1997, Rupérez et al. 2001). Alginate 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
hydrolysis for efficient extraction of antioxidative compounds from brown seaweeds like H. fusiformis. The investigation was continued to the fractionation of constituents according to the molecular weight followed by the hydrolysis of H. fusiformis with effective and commercially available proteases and carbohydrases in order to provide the information for optimized extraction of desired bioactive materials (especially antioxidants).

MATERIALS AND METHODS

Materials

Hizikia fusiformis 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, betaglucanase, 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. amyloliquefaciens proteases), Flavourzyme 500 MG (endo/exopeptidase) and Alcalase 2.4L FG (alcalase) were purchased form Novo Co. (Novozyme Nordisk, Bagsvaerd Denmark). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), butylated hydroxytoluene (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.

Enzymatic hydrolysis procedure

Freeze dried H. fusiformis 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 those reported by Heo et al. (2003). Enzymes were then added at the dosage (enzyme/substrate ratio) of 5% (except in the optimum concentration investigation experiment). The mixtures were placed in the 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 Whatmann No. 1 (Whatmann Ltd. England) filter paper and the enzymes activity of hydrolysates was inactivated by heat (100°C for 10 min). Finally, the pH of the each hydrolysate was adjusted to pH 7 with 1M HCl/NaOH.

**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.

**Measurement of extraction yield**

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

**Total polyphenolic compounds**

Total polyphenolic compounds were determined according to the protocol similar to Chandler and Dodds (1993). One ml of *H. fusiformis* 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% Na₂CO₃ was added. Thoroughly mixed mixture was placed in a dark room for 1 h and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer (Optron 3000 Hanson Tech. Co. Ltd., Korea). A gallic acid standard curve was obtained for the calculation of polyphenolic content.

**DPPH radical scavenging assay**

This assay was based on the scavenging of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by the radical scavenging components in *H. fusiformis* 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 x 10⁻³ M. A 2 ml fraction of *H. fusiformis* hydrolysate and 2 ml of freshly prepared DPPH solution were thoroughly mixed. The reaction mixture was incubated for 1 h and absorbance was recorded at 517 nm using UV-VIS spectrophotometer.

**Hydrogen peroxide scavenging assay**

This assay was based on the ability of *H. fusiformis* hydrolysates to scavenge the hydrogen peroxide in ABTS-peroxidase medium according to the method of Müller (1995). Twenty µl of *H. fusiformis* 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°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°C for 10 min before recording the absorbance in ELISA reader (ELX tech Instruments Inc) at 405 nm.

**Scavenging activity calculation**

Scavenging activity was calculated as

\[ 1 - (A_t - A_s)/A_c \times 100 \]

Where in the DPPH method: \( A_t \) = the absorbance of enzymatic extract mixed with DPPH solution; \( A_s \) = 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: \( A_t \) = the absorbance measured with enzymatic extract \( A_s \) = the absorbance measured with same extract but without ABTS; \( A_c \) = the absorbance of control with particular solvent (without enzymatic extract).

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

Two most effective enzymes of each carbohydrases and proteases 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 h (every day) and the effects on yield, total polyphenolic content, DPPH radical and hydrogen peroxide scavenging activity were investigated.

**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 polyphenolic content, DPPH radical and hydrogen peroxide scavenging activity were investigated.
Fractionation of hydrolysates

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

RESULTS AND DISCUSSION

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

Extraction efficacy of *H. fusiformis* 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. 2). Total polyphenolics of the hydrolysates extracted by Ultraflo of carbohydrases and Neutrase, Flavourzyme and Alcalase of proteases were comparatively higher than the hydrolysates by the other enzymes (Fig. 3). These correlations between extracted yield and total polyphenolic content of the hydrolysates were not significant and all the correlation coefficient values were less than 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. 4). Hydrolysates obtained by AMG, Termamyl and Ultraflo of carbohydrases and Flavourzyme and Alcalase of proteases were comparatively stronger in hydrogen peroxide scavenging (Fig. 5). The correlation coefficient of hydrogen peroxide scavenging activity and total polyphenolic content was comparatively higher than
Fig. 7. Total polyphenolic content of *H. fusiformis* hydrolysates obtained using four selected enzymes at their optimum pHs and temperatures for 3 days. Total polyphenolic content was calculated as the gallic acid equivalents (mg) extracted from 100g *H. fusiformis* dried samples. Enzymes were applied at different dosages as 1%, 2.5% and 5%. Experiments were performed in triplicates.

Fig. 8. DPPH radical scavenging activity (%) of *H. fusiformis* hydrolysates obtained using four selected enzymes at their optimum pHs and temperatures for 3 days. Enzymes were applied at different dosages as 1%, 2.5% and 5%. Samples were tested at the original level of hydrolysates resulted (without adjusting their concentrations). DPPH concentration: 3 x 10^{-5} M. Experiments were performed in triplicates.

Fig. 9. Hydrogen peroxide scavenging activity (%) of *H. fusiformis* hydrolysates obtained using four selected enzymes at their optimum pHs and temperatures for 3 days. Enzymes were applied at different dosages as 1%, 2.5% and 5%. Samples were tested at the original level of hydrolysates resulted (without adjusting their concentrations).

suggested 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 *H. fusiformis* 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 laminarin by β-glucanase of Ultraflo effectively contributes to the enhanced antioxidative activity. Flavourzyme contains both endo and exopeptidase 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, Celliclast, 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 carbohydrates are more suitable than Termamyl and Flavourzyme.
Fig. 4. DPPH radical scavenging activity (%) of H. fusiformis hydrolysates obtained using different enzymes at their optimum pHs and temperatures for 3 days. Enzymes were added at the dosage of 5% relative to the dried weight of samples. Samples were tested at the original level of hydrolysates resulted (without adjusting their concentrations). DPPH concentration; 3 x 10⁻² M. Commercial antioxidants were tested at the level of 2 mg/ml. Experiments were performed in triplicates. (bar ± S.D.)

Fig. 5. Hydrogen peroxide scavenging activity (%) of H. fusiformis hydrolysates obtained using different hydrolytic enzymes at their optimum pHs and temperatures for 3 days. Enzymes were added at the dosage of 5% relative to the dried weight of samples. Samples were tested at the original level of hydrolysates resulted (without adjusting their concentrations). Commercial antioxidants were tested at the level of 2 mg/ml. Experiments were performed in triplicates. (bar ± S.D.)

those of other comparisons of DPPH and total polyphenolic content, yield and DPPH radical scavenging activity or hydrogen peroxide scavenging activity. Considering the notable results recorded in this primary investigation, Termamyl and Ultraflo of carbohydrases and Flavourzyme and Alcalase of proteases 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 alcalse (alkaline endopeptidase) containing Alcalase of proteases were more effective than other types of enzymes used. This can be mainly due to the breakdown of laminarin (β-1,3-glucans) by β-glucanase and amylose α-amylase and peptide bonds endo/exopeptidase and alcalse.

Effect of enzymatic reaction time and enzyme dosage

The yield of each hydrolysate treated with all the selected enzymes was increased gradually with the time (Fig. 6). Effect of the enzyme dosage (enzyme/substrate ratio) on yield was dose-dependent. As 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. 7). Hydrolysates treated with proteases were relatively higher in total polyphenolics compared to the hydrolysates treated with carbohydrases. Of the two carbohydrases 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 dependent manner and also increased with the increment of the time (Fig. 8). DPPH radical scavenging activity of proteases treated hydrolysates was comparatively higher than the hydrolysates treated with carbohydrases. In the third day after the enzymatic
Table 1. *Hizikia fusiformis* hydrolysates obtained by mixed enzyme (Alcalase 2.5% and 2.5% Ultraflo) treatment for 3 days. Samples were at the original level after hydrolysis (without adjusting their concentration). DPPH concentration: $3 \times 10^{-5}$ M

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield</td>
<td>41.3 (g/100 g, dried sample)</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>26.2 (g/100 g, dried sample)</td>
</tr>
<tr>
<td>Protein</td>
<td>11.3 (g/100 g, dried sample)</td>
</tr>
<tr>
<td>Total polyphenols</td>
<td>820 (mg/100 g, dried sample)</td>
</tr>
<tr>
<td>DPPH radical scavenging activity</td>
<td>84.5%</td>
</tr>
<tr>
<td>Hydrogen peroxide scavenging activity</td>
<td>71%</td>
</tr>
</tbody>
</table>

Effect of combination of protease and carbohydrases

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 antimutagenic effects (Beress et al. 1993; Nardella et al. 1996; Durig et al. 1997; Rupérez et al. 2002). Breakdown of proteins disrupts protein-polyphenol complex formation and Ramos and Xiong (2002) reported notable antioxidative activities of enzymatically hydrolyzed protein hydrolysates. Therefore, the combination of proteases and carbohydrases to hydrolyze both proteins and carbohydrates can be 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 1). Investigated antioxidative activities of DPPH radical and hydrogen peroxide scavenging activity also increased due to the combination of enzymes.

**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 kD was significantly higher than the other three fraction of < 30 kD (Fig. 10). Of the < 30 kD fractions, 1-10 kD fraction contains the highest content and < 1 kD was the lowest. Distribution pattern of total polyphenolic content was slightly similar to the yield distribution except the net total polyphenolic content of < 30 kD fractions was higher than total polyphenolic content of > 30 kD fraction (Fig. 11). The DPPH radical scavenging activity of < 1 kD fraction was comparatively lower than the other three fractions (Fig. 12). Of the fractions investigated at the concentration of 2mg/ml, 1-10 kD fraction showed the highest DPPH radical scavenging activity but which was not significantly
different form the 10-30 kD and >30 kD 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 kD fraction (Fig. 13). The concentration (2mg/ml) used in fractionated samples (<1 kD, 1-10 kD, 10-30 kD and >30 kD) 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 confirms the promising antioxidative activity of the constituents > 1 kD molecular weight. Moreover, the comparatively higher polyphenolic content of 1-10 kD 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 (Sirirawadhan et al. 2003a).

Antioxidative activity of H. fusiformis 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 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 Glombitz 1996). Generally, a major part of brown seaweed polyphenols is high molecular weight (> 10 kDa) and potentially bind with proteins (Moen et al. 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 of > 1 kD suggests the further research work to identify the specific beneficial effects, not only the antioxidant activity but also a variety of bioactivities including antimutagenic, anticoagulant and antihypertensive activities. Moreover, the different molecular weight polyphenolic compounds involved with antioxidative activities to be further investigated related to their structure and functional properties.

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