Food Sci. Biotechnol. Vol. 18, No. 4, pp. 917 ~ 922 (2009)

Food Science and Biotechnology

Nutritional Compositions of Ray and Effects of Steaming on Antioxidative Activities of Ray Hydrolysates

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Abstract This study investigated the effects of steaming on the antioxidative activities of ray hydrolysates *in vitro*. Based on the results of 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, steamed ray hydrolysate possessed significantly higher antioxidative activities than raw ray hydrolysates ($p<0.05$). The 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity of steamed ray hydrolysate by pancreatin was slightly lower than raw ray hydrolysate at all concentrations. Raw ray hydrolysate displayed moderate hydroxyl radical scavenging activity, while steamed ray hydrolysate showed a greater increase in hydroxyl radical scavenging activity than raw ray hydrolysate at concentrations above 21.33 mg/mL, and it reached 58.21% at 42.67 mg/mL. The results of this study show that ray hydrolysates have potent free radical scavenging activities and reducing power, and the steaming has a partial impact on the antioxidative activity of ray hydrolysates.

Keywords: ray, hydrolysate, steaming, antioxidative activity

Introduction

The interest in fish hydrolysates dates back to the 1960s, with most work being directed towards the use of fish protein for animal feed and non-dietary purposes, rather than for human food (1,2). In 1970, Bertullo and Pereira (3) provided the first investigation into fish hydrolysates for human consumption. And in recent years, the interest in their use for human consumption has been increasing (4). In several studies, it was reported that fish hydrolysates from black tilapia (5), hoki (6), and tuna (7) acted as physiological modulators of antioxidant activity. Therefore, fish hydrolysates derived from ray are possible regulatory antioxidants as well; however, there are few reports on their effects or mechanisms.

The health-promoting capacities are dependent on processing history. Food processing not only improves the flavor and palatability of foods but also increases the bioavailability of nutrients, by inactivating antinutritional factors. It is recognized that the cooking process can affect the concentration and biological activities of different compounds present in foods (8). This aspect would seem very important considering that few ray are consumed in the raw state and most are processed before consumption. Ray is usually steamed before eating and steaming could cause some detrimental effects to its quality.

Steaming may cause the complex physical and chemical reactions of compositions, including the leaching of water soluble phenolics, freeing phenolics from bonded forms of phenolics, the degradation of polyphenols, the breakdown and transformation of phenolics, and the formation of Maillard reaction products, such as formation of complex products from phenolics and proteins (9,10). It is important to elucidate the effect of steaming on the components and bioactivities of ray or ray hydrolysate. Thus, we evaluated the antioxidative properties of raw and steamed ray hydrolysates by performing reducing power assays and free radical scavenging activity, using methods for detection of the oxidation end-point. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and hydroxyl radical scavenging methods are common spectrophotometric procedures for determining the antioxidative capacities of components (11). And the nutritional characteristics of raw and steamed rays, including amino acid and fatty acid compositions, were also analyzed.

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Received December 14, 2008; Revised February 28, 2009;
Accepted February 28, 2009

Materials and Methods

Ray The ray (*Raja kenojei*) was obtained live from sea near Korea. The ray was gutted and deheaded immediately upon arrival at the laboratory and deboned using a double-drum fish deboner. The flesh was then washed 3-4 times to remove water soluble nitrogenous compounds, minerals, naturally occurring proteolytic enzymes, and pigments. Subsequently, excess water was drained and the flesh was packed in polyvinyl chloride (PVC) bags (raw ray). After fish meat was steamed for 30 min using boiling water, it was cooled and packed in PVC bags (steamed ray). The raw and steamed rays were homogenized and freeze-dried, then the homogenate was stored at −70°C until the analysis.

Hydrolysate The enzymatic hydrolysate was performed

using pepsin and pancreatin with their optimal conditions by the method of Akeson and Stahman (12). The pepsin hydrolysate was prepared as fellows; The 400 mg ray was incubated with 1.5 mg of 3,280 U/mg pepsin (EC 3.4.4.1; Sigma-Aldrich, St. Louis, MO, USA) in 15 mL of 0.1 N HCl at 37°C for 3 hr. It was heated in boiling water for 5 min to inactivate pepsin. Then it was centrifuged at 3,000×g for 10 min, and the supernatants were freeze-dried, then homogenate was stored at −70°C until the analysis.

The pancreatic hydrolysate was prepared as follows; The 400 mg ray was incubated with 4 mg pancreatin (EC 3.4.21.4; Sigma-Aldrich) in 7.5 mL of 5 mM phosphate buffer (pH 8.0). The 1 mL toluene was added to prevent microbial growth and the solution was incubated at 37°C for 24 hr (13). It was then heated in boiling water for 5 min to inactivate pancreatin. Next, hydrolysate was centrifuged at 5,000×*g* for 10 min, and the resultant supernatants were freeze-dried. Homogenate was stored at −70°C until analysis.

Assay of chemical components The contents of moisture, crude fat, crude protein, crude ash, and crude carbohydrate in raw and steamed rays were determined according to AOAC Methods (14). Protein (Method 920.39) was determined by multiplying the nitrogen content with a factor of 6.25. Moisture (Method 934.01), crude fat (Method 920.39), and total ash (Method 942.05) were estimated by the AOAC Official Methods (14). Carbohydrate was defined as the residue excluding moisture, crude protein, crude fat, and crude ash.

The amino acids were determined by high performance liquid chromatography (HPLC, Varian 230; Varian Inc., Palo Alto, CA, USA) using the modified method of Hodgin *et al.* (15). The analysis was performed with an ODS-μ-Bondapak C_{18} column (3.9 mm×30 cm, Waters, Milford, MA, USA) and an HPLC equipped with a fluorescence detector (Varian L-2485; Varian Inc.). The fluorescence intensity of *O*-phthalaldehyde (OPA)-amino acid derivatives was detected at 360 nm of excitation and 450 nm of emission. This standard mixture and sample was diluted to 1:10 v/v with iodoacetic acid solution. Fifty μL of OPA reagent (16) in an Eppendorf tube at room temperature were vortex-mixed. After 120 sec (optimun derivatization time for the reaction) a 10 μL aliquot was injected. The OPA amino acid adduct eluted from the column by a gradient elution performed in 45 mins, from 15 to 70% of eluent B in 4 linear steps 1) at a flow rate of 1.5 mL/min. The gradient elution program was followed by a 10 min washing step, programmed to 100% B, so that any residual sample components would be cleaned from the column, before and after every run. The column was equilibrated with 10% eluent B for 10 min. Eluent A was a 50 mM sodium acetate buffer, pH 6.8, and eluent B was methanol. All quantitative analyses were performed by relating the peak areas of individual amino acids to those of external standard amino acids (Wako Chemical Co., Osaka, Japan).

The fatty acid compositions were determined using gas chromatography (GC, Varian 3900; Varian Inc.) from fatty acid methyl esters (FAME) that were prepared according to the modified method of Surh and Kwon (17). The FAME were extracted with 3 mL *n*-hexane, dried over sodium sulfate, and concentrated under nitrogen. A gas chromatograph equipped with a Innowax capillary column (30 m×0.32 mm i.d.; film thickness 0.5 μm, Hewlett-Packard Scientific Co., Folsom, CA, USA). Initially, the column was held at 180°C for 1 min and programmed to rise to 230°C at a rate of 2.0°C/min. It was then held at 230°C for 20 min. The carrier gas was helium, and the total gas flow rate was 50 mL/min. The fatty acid methyl esters were identified by comparing the retention times with standards.

DPPH radical scavenging activity The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was measured according to the method of Cheung *et al.* (18) with some modifications. Here, the 0.8 mL of 0.2 mM DPPH ethanolic solution was mixed with 0.2 mL of ray hydrolysate. The mixture was vigorously shaken and left to stand for 10 min under subdued light. The absorbance was measured at 520 nm.

ABTS radical scavenging activity The 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity was measured according to the method of Re *et al.* (19) with some modifications. The ABTS radical cations were generated by adding 7 mM ABTS to a 2.45 mM potassium persulfate solution and allowing the mixture to stand overnight in the dark at room temperature. The ABTS radical cation solution was then diluted with distilled water to obtain the absorbance of 1.4-1.5 at 414 nm (molar extinction coefficient, $\varepsilon=3.6\times10^4$ mol/cm) (20). The 1 mL diluted ABTS radical cation solution was added to 50 μL ray hydrolysate. After 90 min, the absorbance was measured at 414 nm.

Hydroxyl radical scavenging activity The hydroxyl radical scavenging activity was measured by the modified deoxyribose method of Halliwell *et al.* (21). The reaction was performed in a 10 mM phosphate buffer (pH 7.4) containing 2.8 mM deoxyribose, 2.8 mM H_2O_2, 25 μM $FeCl_3$, 100 μM ethylene-diamine tetraaceticacid (EDTA), and 250 μg/mL ray hydrolysate. The reaction was started by adding ascorbic acid to a final concentration of 100 μM. The reaction mixture was incubated in a water bath at 37°C for 1 hr. After incubation, the color was developed by adding 1% thiobarbituric acid (TBA) followed by ice-cold 2.8% trichloroacetic acid (TCA) and then heating in a water bath at 95-100°C for 20 min. After the solution was cooled, the absorbance was measured at 532 nm.

The free radical scavenging activity (%) was calculated by the following equation:

$$\text{Radical scavenging activity (\%)} = (1 - \text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}) \times 100$$

where $\text{Abs}_{\text{sample}}$ is the absorbance in the presence of ray hydrolysate, and $\text{Abs}_{\text{control}}$ is the absorbance in the absence of ray hydrolysate, respectively.

Reducing power The reducing power was determined by the method of Yen and Chen (22). The ray hydrolysate in 2.5 mL of 0.2 M phosphate buffer (pH 6.6) was added to 2.5 mL of 1% potassium ferricyanide, and the mixture was

incubated at 50°C for 20 min. After 2.5 mL of 1% TCA was added, the mixture was centrifuged at 3,000×g for 10 min and then 2.5 mL supernatant was mixed with 2.5 mL distilled water and 0.5 mL of 0.1% ferric chloride. The absorbance then was measured at 700 nm.

Statistical analysis All expressed values are the means of triplicate determinations. All statistical analyses were performed using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., Chicago, IL, USA). The *t*-test was used to assess the differences between raw and steamed ray hydrolysates. All data were two-sided at the 5% significance level and reported as mean±standard deviation (SD).

Results and Discussion

Chemical components The nutrient contents of raw and steamed ray hydrolysates are shown in Table 1. The analysis revealed that raw and steamed rays consisted of 50.88-56.25% moisture, 11.42-12.38% crude fat, 31.80-32.91% crude protein, 4.01-5.02% crude ash, and 0.41-0.94% crude carbohydrate. The changes of chemical components were seen with steaming, however, the protein contents of both 2 type rays were found still to be high. Cha *et al*. (23) reported that crude protein in sun-dried ray was 71.3%. It is thought that ray might be a good source for protein regardless of processing. The significant differences of moisture ($p<0.05$) and crude carbohydrate ($p<0.01$) contents were observed between raw and steamed rays. The steaming caused the increase of moisture and the reduction of crude carbohydrate. The crude fat, protein, and ash contents, however, were not significantly affected by steaming.

As shown in Table 2, ray contained 335.52-340.52 mg/100 g calcium and could be a good candidate as a nutraceutical. There were no significant differences on calcium, phosphorus, and iron contents between raw and steamed rays. The mineral components were not affected by steaming.

Amino acid analysis The amino acid profiles of raw and steamed rays are summarized in Table 3. Differences were observed between the amino acid contents of the 2 type rays, and this may be attributed to the destruction of certain amino acids by the steaming. Valine was not detected in raw ray, and cysteine was not detected in both raw and steamed rays. Steaming significantly decreased serine, histidine, methionine, phenylalanine, and lysine contents ($p<0.05$, 0.01, or 0.001). On the other hand, steaming significantly increased aspartate, glutamate, arginine, isoleucine, and leucine ($p<0.01$ or 0.001). There were no significant differences between the glycine, threonine, alanine, and tyrosine contents in raw and steamed rays. Lysine, which is generally accepted as being an antioxidant despite its occasional pro-oxidative effect, was the most abundant amino acid in both 2 type rays. In a study by Cha *et al*. (23), the content of total amino acids in sun-dried ray was 1,773.3 mg% on dry basis and the abundant amino acids were taurine, lysine, leucine, alanine, glycine, glutamate, proline, and valine in order.

Fatty acid analysis The fatty acid compositions of raw and steamed rays are shown in Table 4. Their fatty acid compositions were examined by evaluating the percentages of individual fatty acids and correlating the percentages of the main saturated and unsaturated residues, along with the sums of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), and an indicative ratio (PUFA/SFA).

$C_{14:0}$ was not detected in raw ray, and $C_{18:3}$ was not detected in steamed ray. According to the results, steaming affected severely the fatty acids in ray. With the exceptions of $C_{18:1}$ and $C_{18:2}$, steaming significantly increased levels of SFA, MUFA, and PUFA ($p<0.01$ or 0.001). Steamed ray (SFA, 53.72%; MUFA, 23.52%; PUFA, 22.52%) exhibited a significantly higher total SFA ratio and lower total UFA (MUFA and PUFA) ratio as compared to the raw ray (SFA, 17.42%; MUFA, 34.32%; PUFA, 48.22%), because

Table 1. Chemical components of raw and steamed rays

Chemical components (%)	Raw ray	Steamed ray	*p*-value
Moisture	50.88±4.33[1)]	56.25±3.35	0.014
Crude fat	12.38±1.02	11.42±1.41	0.356
Crude protein	31.80±2.15	32.91±2.23	0.249
Crude ash	4.01±0.68	5.02±0.79	0.185
Crude carbohydrate	0.94±0.03	0.41±0.08	0.002

[1)]Data are mean±SD.

Table 2. Major mineral compositions of raw and steamed rays

Minerals (mg/100 g)	Raw ray	Steamed ray	*p*-value
Calcium	335.52±18.89[1)]	340.52±17.45	0.405
Phosphorus	235.08±9.69	243.84±10.23	0.344
Iron	1.42±0.05	1.23±0.06	0.081

[1)]Data are mean±SD.

Table 3. Amino acid compositions of raw and steamed rays

Amino acids (mg/g)	Raw ray	Steamed ray	*p*-value
Aspartate	19.53±1.45[1)]	25.23±1.69	0.002
Glutamate	20.26±1.68	36.40±2.04	<0.001
Cysteine (hydrochloride)	ND	ND	-
Serine	18.74±1.25	9.17±0.78	<0.001
Histidine	4.21±0.05	1.52±0.09	0.011
Glycine	38.58±2.23	40.67±2.47	0.063
Threonine	6.27±0.08	8.24±0.19	0.073
Arginine	44.66±3.28	85.11±2.34	<0.001
Alanine	50.24±2.47	52.55±2.63	0.061
Tyrosine	3.51±0.25	2.67±0.17	0.062
Valine	ND	32.09±2.04	-
Methionine	52.91±3.44	21.03±1.29	<0.001
Phenylalanine	6.92±0.06	5.92±0.03	0.042
Isoleucine	4.08±0.06	6.56±0.07	0.003
Leucine	8.86±0.56	16.21±1.21	0.002
Lysine	319.78±14.45	254.81±16.23	0.007

[1)]Data are mean±SD; ND, not detected.

Table 4. Fatty acid compositions of raw and steamed rays

Fatty acids[1] (mol%)	Raw ray	Steamed ray	*p*-value
$C_{14:0}$	ND	2.02±0.08[2]	-
$C_{16:0}$	9.92±0.08	23.73±1.29	<0.001
$C_{16:1}$	0.32±0.08	1.82±0.06	<0.001
$C_{18:0}$	6.94±0.09	26.72±2.13	<0.001
$C_{18:1}$	33.92±2.03	20.3±0.68	0.003
$C_{18:2}$	43.22±2.78	5.87±0.26	<0.001
$C_{18:3}$	3.81±0.08	ND	-
$C_{20:0}$	0.62±0.09	1.62±0.07	0.002
$C_{20:4}$	0.21±0.01	1.54±0.02	<0.001
$C_{20:5}$	0.62±0.07	10.82±1.23	<0.001
$C_{22:1}$	0.21±0.03	1.41±0.08	<0.001
$C_{22:6}$	0.22±0.09	4.41±1.09	<0.001
SFA (%)	17.42±1.12	53.72±3.68	<0.001
MUFA (%)	34.32±2.89	23.52±2.23	0.003
PUFA (%)	48.22±2.35	22.52±2.63	<0.001
PUFA/SFA	22.71±0.03	0.42±0.02	<0.001

[1]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[2]Data are mean±SD; ND, not detected.

heating process by steaming might breakdown double bond of PUFA. Steaming increased the total SFA ratio and reduced the total PUFA ratio of steamed ray, so its PUFA/SFA ratio was significantly lower than that of raw ray ($p<0.001$). It has long been known that the type of fat in the diet influences the development of atherosclerosis, in which the consumption of MUFA or PUFA, as compared to SFA, decreases the risk (24). Therefore, steaming could present adverse effects to the quality of ray by changing its fatty acid composition.

Antioxidative activities The assessment of DPPH radical scavenging activity was one method used to evaluate the antioxidative activity of ray hydrolysates. The results are presented in Fig. 1. In DPPH assay, the steamed ray hydrolysate by pepsin (26.11%) possessed significantly higher antioxidative activity than raw ray hydrolysate (11.16%, $p<0.05$). Also, the steamed ray hydrolysate by pancreatin (26.49%) exhibited more effective DPPH radical scavenging as compared to raw ray hydrolysate (9.33%, $p<0.05$).

The ABTS radical scavenging activity of pancreatic hydrolysate ray is shown in Fig. 2. In this study, only pancreatic ray hydrolysate was examined in ABTS and hydroxyl assays, because in DPPH assay, they were more effective free radical scavengers than pepsin ray hydrolysate. ABTS radical scavenging activity increased with increasing concentrations. The ABTS radical scavenging activity of the steamed ray hydrolysate was slightly lower than that of raw ray hydrolysate at all concentrations; however, there was no significant difference between the 2 ray hydrolysates. When comparing the ABTS radical and DPPH radical scavenging effects of ray hydrolysates, ABTS radical scavenging activity seems to have been less affected by steaming.

As shown in Fig. 3, both 2 ray hydrolysates exhibited

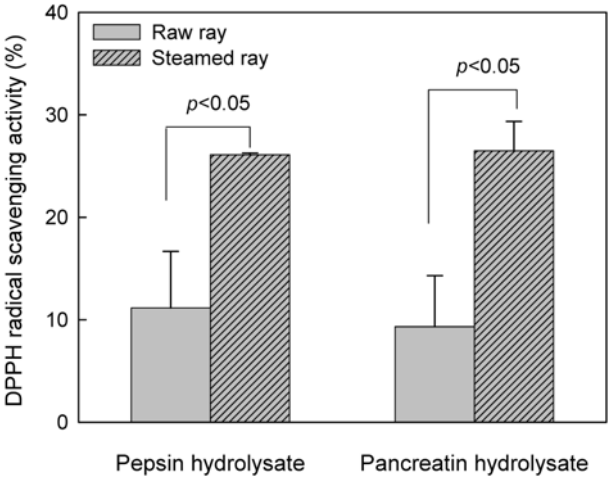

Fig. 1. DPPH radical scavenging activity of raw and steamed ray hydrolysates by pepsin and pancreatin. Values are mean±SD of triple determinations.

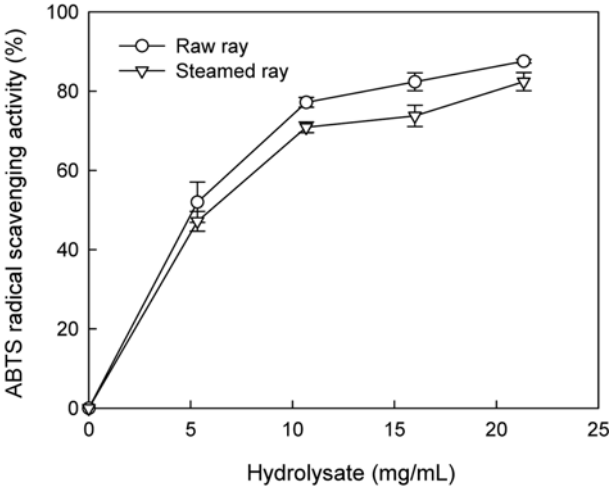

Fig. 2. ABTS radical scavenging activity of raw and steamed ray hydrolysates by pancreatin. Values are mean±SD of triple determinations.

concentrate dependent hydroxyl radical scavenging activities, similar to their other antioxidative effects. Above 32.00 mg/mL, steamed ray hydrolysate (32.00 mg/mL, 41.79%; 42.67 mg/mL, 58.21%) showed significantly greater antioxidative activity than raw ray hydrolysate (32.00 mg/mL, 29.85%; 42.67 mg/mL, 35.82%) in hydroxyl assay ($p<0.05$ or 0.01).

The results of reducing power assay for pancreatic ray hydrolysate at the absorbance of 700 nm are displayed in Fig. 4. The reductive potentials of both 2 ray hydrolysates were concentration dependent within the concentration range of 0-53.33 mg/mL. Below 21.33 mg/mL, the absorbance of steamed ray hydrolysate was significantly lower than that of raw ray hydrolysate ($p<0.01$ or 0.05); however, above 21.33 mg/mL, steamed ray hydrolysate showed a similar reductive potential to that of raw ray hydrolysate.

It is widely believed that many food antioxidative components can be significantly lost as a consequence of industrial sterilization, pasteurization, and dehydration, as

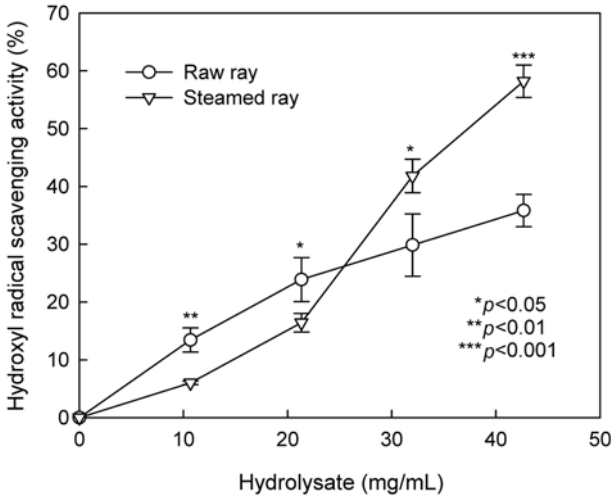

Fig. 3. Hydroxyl radical scavenging activity of raw and steamed ray hydrolysates by pancreatin. Values are mean±SD of triple determinations.

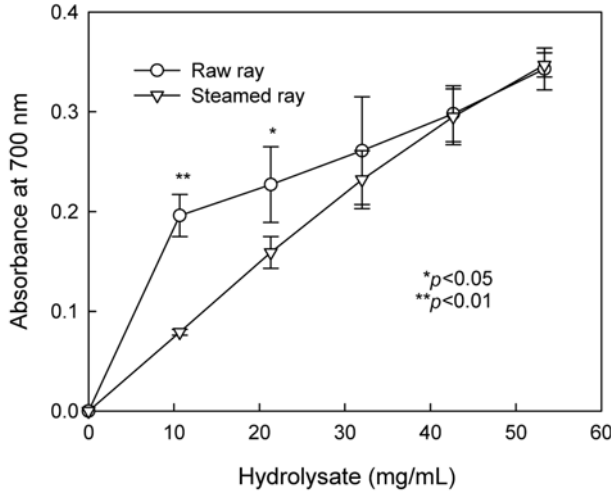

Fig. 4. Reducing power of raw and steamed ray hydrolysates by pancreatin. Values are mean±SD of triple determinations.

well as home cooking (25,26). However, processing does not always result in the destruction of the antioxidant components. In some cases, processing can induce the formation of compounds (27). In a study by Gliszczydska-Swiglo *et al.* (8), steaming broccoli had an approximate 2.2 fold increase in antioxidative activity when compared to raw broccoli. A similar effect of steaming was reported by Turkmen *et al.* (28) for the antioxidative activity of green beans. Steaming generally leads to increases in levels of polyphenols, flavonoids, phenolic acids, and carotenoids (29-31). Their increased concentrations by steaming are most likely the result of improved extraction, partly due to the disruption of complexes between these compounds and proteins, and the inactivation of oxidizing enzymes (32). A similar pattern had been reported for ginseng steamed at 100 and 120°C which showed better radical scavenging activity than air-dried ginseng (33). The increased activities in steaming ginseng may be contributed by the improvement of antioxidative properties or formation of novel compounds having antioxidative activity upon steaming treatments. This hypothesis had been verified by the fact that the content of maltol in ginsengs was remarkably increased in a temperature-dependent manner by steaming (33). In our study, steaming also improved the DPPH and hydroxyl radical scavenging activities of ray hydrolysates. Steaming induces biological activity as well as changes chemical constituents. The results obtained from the DPPH, ABTS, and hydroxyl radical scavenging and reducing power assays varied for ray hydrolysates. This suggests that the same type and level of antioxidant in ray could not give the same response with respect to its DPPH, ABTS, and hydroxyl radical scavenging, and reducing power effects.

The results of this study show that ray hydrolysates have potent free radical scavenging activities and reducing power, and they suggest that the physiological functions of ray hydrolysates are due to their protective effects against oxidation. Additionally, one can conclude that steaming has a partial impact on the antioxidative activities of ray hydrolysates. Therefore, steaming is recommended for ray hydrolysate in domestic and industrial processes to preserve antioxidative components. The changes in the overall antioxidative properties of processed ray can be attributed to the synergistic combinations or counteracting of several types of chemical reactions, leaching of water soluble antioxidant compositions, and formation or breakdown of antioxidative compositions. To better understand the role and fate of steamed ray or ray hydrolysate on stability and human health, the following chemical composition research was performed to investigate the molecular mechanisms responsible for loss or formation of antioxidants.

Acknowledgments

This research was financially supported by the Ministry of Knowledge Economy (MKE) through the Center for Traditional Microorganism Resources (TMR) at Keimyung University.

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