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Chemical Compositions and Antioxidant Properties of Enzymatic Hydrolysates from Velvet Antler in Elk (*Cervus elaphus canadensis*).

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ABSTRACT

In this study, velvet antler of elk was hydrolyzed by various enzyme and measured chemical compositions and 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, and alkyl radical scavenging activities by electron spin resonance (ESR) spectroscopy. Velvet antler of elk was divided into 3 sections including upper, middle and base section. In addition, the velvet antler was hydrolyzed by two carbohydrases such as celluclast, termamyl and two proteases such as pepsin and flavourzyme. In the present study, the content of uronic acid was the highest in the middle section, but it was the lowest in the base section. The flavourzyme extract of the upper section and the pepsin extract of the middle section showed the highest sialic acid and glycosaminoglycan (GAG) contents. The content of TC was the highest in the middle section of the termamyl and pepsin extracts. On the other hand, the collagen contents showed the highest content in the base section in all enzymatic hydrolysates. Especially, the collagen content was mostly higher in carbohydrase (celluclast and termamyl) extracts compared to those of protease (pepsin and flavourzyme) extracts. In addition, all enzymatic hydrolysates from velvet antler effectively reduced intensities of free radicals. The flavourzyme extracts of all section particularly showed the highest DPPH scavenging activity. On the other hand, flavourzyme and pepsin hydrolysates of the upper sections showed the highest scavenging activity against hydroxyl (IC_{50}=0.89 mg/ml) and alkyl radicals (IC_{50}=0.42 mg/ml), respectively. These results suggested that the contents of chemical and biological substances of velvet antler are strongly influenced by the kind of enzymes and portion of velvet antler.

Keywords: Antioxidant properties; Chemical composition; Enzymatic hydrolysates; Elk (*Cervus elaphus canadensis*); Velvet antler

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INTRODUCTION

Velvet antlers have been used in medical stuff for the treatment of diseases in Eastern countries for a hundred years. Every year, over 4000 tons of velvet antler international markets of supplement local production in Eastern countries [1]. Especially, South Korea is the world's largest market for velvet antler; it imports about 1,000 tons, or 80 percent of total world production annually. Deer antler has been used in the East as a traditional animal-based medicine for over 2000 years to prevent or treat various diseases, including bone-resorption diseases [2], rheumatoid arthritis [3], and vascular necrosis [4]. More recently, it has been reported that the hypoglycemic, hypolipidemic and antioxidant properties of peptides from red deer antler in vivo, as well as in islet cells [5].

The difference between horns and antlers was that horns, like hooves, were composed of keratin, whereas antlers were composed of bone. Growing antlers were 80 % protein and 20 % ash. Hardened antlers were roughly 63 % ash including 22 % calcium [6]. Velvet antlers, morphological appendage comprised primarily of minerals, protein, carbohydrates and fatty acids are bonelike branches that growth from base of the skulls of deer [1, 6]. The chemical composition of velvet antlers have been studied in caribou [7], Reindeer [8], red deer [9], Wapiti [10] and sika deer [11]. Although some studies on the chemical components and bioavailability of entire velvet antler have been carried out, there has been little research on the chemical content and antioxidant activities of different enzymatic hydrolysates of each section from velvet antler in elk. Therefore, the aim of the present study was to provide basic information to allow the improved assessment of velvet antler quality by investigating the antioxidant properties as well as chemical and biological composition of enzymatic hydrolysates from velvet antler in elk (*Cervus elaphus canadensis*).

MATERIALS AND METHODS

Sample collection

Velvet antler harvested on 80 days after casting were collected from randomly-selected deer farms in Korea, 2014. Antlers were harvested from elk (*Cervus elaphus canadensis*) stags, 4-5 years old, and one of the antler pair from each stag was used as a sample for analysis. All stags were healthy, with no clinical signs of disease. Each antler sample was divided into three equal sections (upper, middle and base) along the main beam. Samples of each section were sliced with a bone slicer, freeze-dried and ground by a sample mill (KNIFETEC 1095 Sample Mill) to pass a 0.1 mm screen. Ground samples were stored in a freezer (–40°C) until preparation of velvet antler extracts.

Preparation of velvet antler extracts

3 g samples were put into 300 ml of distilled water. After they were extracted by water bath at 10 °C for 5 h, the extractions were filtered 3 times and lyophilized in a freeze dryer. 1L of distilled water was added to 30 g of each lyophilized velvet antler sample and then a 300 µl (or mg) of the enzymes were mixed. The enzymatic hydrolysis reactions were performed for 24 h to achieve an optimum degree of the hydrolysis as shown in Table 1. After each sample was clarified by centrifugation at 3000 rpm for 10 min, they were inactively enzymatic hydrolysis by water bath at 100 °C for 10 min. Enzymatic extracts of velvet antler was filtered 3 times and lyophilized in a freeze dryer. The prepared extracts were stored at 4 °C until used.

Table 1: Specification of enzymes used for hydrolysis of velvet antler

Enzyme	Type	Enzyme characterization	Optimum conditions	
			pH	Temp (°C)
Celluclast	Mono	Cellulase	4.5	50
Termamyl	Mono	α-amylase	6	60
Pepsin	Mono	Protease	7	37
Flavourzyme	Mono	Exopeptidase	7	50

Chemical analysis

The velvet antler samples were analyzed for crude protein, fat (ether extracts), crude fiber, total ash and mineral contents by the method of the Association of Official Chemists (AOAC, 1990). The biological substances from velvet antler extracts were analyzed for total carbohydrate (TC), collagen, glycosaminoglycan (GAG), uronic acid, sialic acid. TC content was determined by a phenol-sulfuric acid method [12]. Collagen content was evaluated by spectrophotometric determination of hydroxyproline [13]. For GAG, uronic acid and sialic acid analysis, 50 mg of velvet antler sample was decalcified in 1 ml 0.05 M Na_2EDTA (pH 7.4, including 0.5 M Tris) for 2 days at 4 °C. After decalcification, samples were centrifuged at 12,000 rpm for 10 min at 4 °C to obtain a precipitate. A 3 ml sample of 0.1 M phosphate buffer (pH 6.5, containing 0.05 M cystein hydrochloride and 0.005 M Na_2EDTA) was mixed with 20 mg crude papain, and incubated for 30 min at 65 °C to activate the enzyme. The activated enzyme was then mixed with the decalcified samples. The reaction was carried out for 16 h at 65 °C, after which the upper liquid layer was removed. GAG content was determined by a microfilter plate adaptation of a dimethylmethylene blue assay of Farndale et al. [14]. Uronic acid content of the antler was determined by the method of Scott [15] and Kosakaki and Yosizawa [16]. Sialic acid content was determined by the method of Warren [17]. Reagent A (40 µl) was added to the samples, standards and controls (80 µl) and mixed well. The mixture was left at room temperature for 20 min. Reagent B (400 µl) was then added and the tubes were shaken vigorously to remove the yellow-colored iodine. The tubes were left for a further 5 min at room temperature. Reagent C (1.2 ml) was then added before the tubes were shaken and heated at 100 °C for 15 min. Samples were cooled rapidly to room temperature, and two liquid layers formed: one was red in color and the other was transparent. The red-colored solution was extracted and centrifuged for a few minutes to properly separate the two layers. The upper cyclohexanone layer was determined at the absorbance level of 549 nm.

DPPH radical scavenging activity

Velvet antler enzymatic hydrolysate samples were homogenized in nine volumes of ice cold homogenizing buffer containing 50% KCl, 1 M TRIS-HCl, and 0.5 M EDTA (pH 7.0). The homogenate was centrifuged (55,000 g at 4 °C) for 30 min, and the supernatant was used for the electron spin resonance (ESR) assays. DPPH radical scavenging activity was measured using the method described by Nanjo et al. [18]. Briefly, 60 ml of each sample was added to 60 ml of DPPH (60 mM) in methanol solution. After mixing vigorously for 10 s, the solution was transferred into a 100 ml Teflon capillary tube, and the scavenging activity of each sample for the DPPH radical was measured using an ESR spectrometer. A spin adduct was measured on an ESR spectrometer exactly 2 min later. Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5 and temperature, 298 K.

Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by iron-catalysed Haber–Weiss reaction (Fenton-driven Haber–Weiss reaction), and the generated hydroxyl radicals rapidly reacted with nitrone spin-trap DMPO. The resultant DMPO-OH adduct was detectable with an ESR spectrometer. Briefly, 0.2 ml of each sample was mixed with 0.2 ml of DMPO (0.3 M), 0.2 ml of $FeSO_4$ (10 mM) and 0.2 ml of H_2O_2 (10 mM) in a phosphate buffer solution (pH 7.2), and then transferred to a 100 ml Teflon capillary tube. After 2.5 min, an ESR spectrum was recorded using a JES-FA ESR spectrometer (JEOL Ltd, Tokyo, Japan). Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5 and temperature, 298 K.

Alkyl radical scavenging activity

Alkyl radicals were generated by AAPH. The phosphate buffered saline (pH 7.4) reaction mixtures containing 10 mM AAPH, 10 mM (4-pyridyl-1-oxide)-N-tert-butylnitrone, and the indicated concentrations of tested samples were incubated at 37 °C in a water bath for 30 min and then transferred to a 100-mL quartz capillary tube. The spin adduct was recorded on an ESR spectrometer. Measurement conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5; and temperature, 298 K.

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RESULTS AND DISCUSSION

Chemical composition of enzymatic hydrolysates from velvet antler in elk

In this study, we investigated the composition of chemical and biological substances of enzymatic hydrolysates from velvet antler in elk (*Cervus elaphus canadensis*) in order to develop value-added food ingredients and apply to the food and pharmaceutical industries as new materials using velvet antler. The contents of crude protein, crude fat, ash, Ca and P in velvet antlers are shown in Table 2. Dry matter of velvet antler in each section are appeared in the range from 27 to 38%. They were decreased downward, significant differences in contents of crude protein and crude fat. In contrast, the contents of ash, Ca and P were the highest in the base section. Similar results were reported in Iberian red deer where the base section had greater ash, Ca and P contents compared to those of upper and middle sections [9]. In addition, it was also reported that the mineral content of antlers was higher in the base section than in the tip [19].

Table 2: Contents of crude protein, crude fat, crude ash, Ca, and P by each section of velvet antler from elk

	Dry matter	Crude protein	Crude fat	Ash	Ca (µl/ml)	P (µl/ml)
Upper	27.7±1.6	51.37±7.8	2.82±0.4	26.09±4.4	13.1±3.8	2.9±1.6
Middle	32.8±2.3	33.27±3.4	2.41±0.4	38.55±3.4	14.6±2.5	6.4±1.2
Base	37.9±4.3	25.99±2.8	2.45±0.4	42.06±3.4	15.9±1.4	9.4±1.0

Data are mean ± SD values (n=3).

Table 3: Uronic acid, sialic acid and GAG contents of enzymatic hydrolysates from velvet antler in elk

		Enzymatic hydrolysates			
		Celluclast	Termamyl	Pepsin	Flavourzyme
Uronic acid	Upper	0.037±0.001	0.050±0.005	0.017±0.002	0.029±0.001
(mg/mL)	Middle	0.082±0.002	0.163±0.022	0.037±0.015	0.057±0.007
	Base	0.023±0.006	0.038±0.002	0.005±0.001	0.010±0.002
Sialic acid	Upper	0.332±0.009	0.399±0.011	0.343±0.007	0.622±0.009
(mg/mL)	Middle	0.377±0.011	0.157±0.008	0.699±0.014	0.478±0.015
	Base	0.366±0.009	0.028±0.002	0.475±0.067	0.456±0.012
GAG	Upper	0.193±0.067	1.252±0.102	1.064±0.178	4.260±0.271
(mg/mL)	Middle	0.273±0.067	0.853±0.193	8.907±0.292	2.834±0.114
	Base	1.577±0.056	0.697±0.102	2.866±0.564	2.505±0.138

Data are mean ± SD values (n=3).

Uronic acid, sialic acid and GAG contents of enzymatic hydrolysates from velvet antler in elk

The contents of uronic acid, sialic acid and GAG of enzymatic hydrolysates from elk velvet antler are shown in Table 3. As four kinds of enzymatic hydrolysates, the content of uronic acid was the highest in the middle section, but it was the lowest in the base section. The flavourzyme extract of the upper section and the pepsin extract of the middle section showed the highest sialic acid contents. Similarly, the flavourzyme extract of the upper section and the pepsin extract of the middle section showed the highest GAG contents, but mostly higher in protease (pepsin and flavourzyme) extracts compared to those of carbohydrase (celluclast and termamyl) extracts. It has been reported that sialic acids are a family of 9-carbon α-keto acids found predominantly at distal positions of oligosaccharide chains of glycoproteins and glycolipids and are considered key determinants in a variety of complex biological regulatory and signalling events [20]. Similar to our results, numerous studies have reported that uronic acid and sialic acid contents decreased from the top to the base

section [1, 21] however, the biological components of enzymatic hydrolysates of velvet antler have not been elucidataed.

Total carbohydrate (TC) and collagen contents of enzymatic hydrolysates from velvet antler in elk

In the present study, total carbohydrate (TC) and collagen contents of enzymatic hydrolysates from velvet antler in elk are shown in Table 4. The content of TC was the highest in the middle section of the termamyl and pepsin extracts in contrast, it was the highest in the base section of the flavourzyme extract. In general, collagen is considered the major protein involved in reinforcing the mineralized tissue structure and providing mechanical strength to the tissue [10, 22]. In our study, the collagen contents showed the highest content in the base section compared to those of upper and middle sections in all enzymatic hydrolysates. Especially, the collagen content was mostly higher in carbohydrase (celluclast and termamyl) extracts compared to those of protease (pepsin and flavourzyme) extracts. These results indicate that the bio-active substances of elk velvet antler might be related to different parts of velvet antler as well as enzymatic hydrolysates.

Table 4: Total carbohydrate (TC) and collagen contents of enzymatic hydrolysates from velvet antler in elk

		Enzymatic hydrolysates			
		Celluclast	Termamyl	Pepsin	Flavourzyme
TC	Upper	2.588±0.001	3.115±0.024	3.889±0.018	1.956±0.154
	Middle	2.750±0.007	5.851±0.191	8.975±0.050	5.918±0.051
	Base	2.542±0.019	1.751±0.007	4.545±0.005	9.461±0.117
Collagen	Upper	0.538±0.013	0.510±0.001	0.359±0.036	0.393±0.002
	Middle	0.711±0.023	0.652±0.012	0.165±0.016	0.761±0.014
	Base	1.238±0.034	1.001±0.021	0.823±0.008	0.894±0.001

Data are mean ± SD values (n=3).

Free radical scavenging activities of enzymatic hydrolysates from velvet antler

Enzymatic hydrolysates containing peptides are attractive drug candidates because of their potent biological activities as well as high target specificities. Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and the biological system's ability to detoxify the reactive intermediates [23]. Excessive oxidative stress can cause direct cell damage and induce several harmful effects in the human body such as cancer, liver injury, skin damage and aging [24]. A number of extracts from nature sources have exhibited antioxidant effects however, the free radical scavenging effect of enzymatic hydrolysates of elk velvet antler have not been elucidataed. In the present study, therefore, the radical scavenging activities of velvet antler enzymatic hydrolysates were investigated using DPPH, hydroxyl, and alkyl radical scavenging properties measured using an ESR spectrometer. The decrease of IC_{50} value in all groups was observed with the dose increment of the velvet antler enzymatic hydrolysates (Fig. 1). DPPH is a stable radical that is used to screen the free radical scavenging ability of compounds or antioxidant activity of plant or animal extracts. In the present study, flavourzyme extract exhibited stronger DPPH radical scavenging activity (IC_{50} value, upper 1.28 mg/ml, middle 2.34 mg/ml, and base 2.65 mg/ml, respectively) than celluclast, termamyl and pepsin extract as shown in Fig. 1A. On the other hand, flavourzyme and pepsin extracts showed the strongest hydroxyl and alkyl radical scavenging activity in the upper section, respectively (Fig. 1B and C). In a previous study, it was reported that the stronger reactive oxygen species scavenging activities were found with the high content of uronic acids in polysaccharide conjugates [25]. These results indicate that the velvet antler enzymatic hydrolysates effectively scavenged various reactive radicals, including DPPH, hydroxyl, and alkyl radicals.

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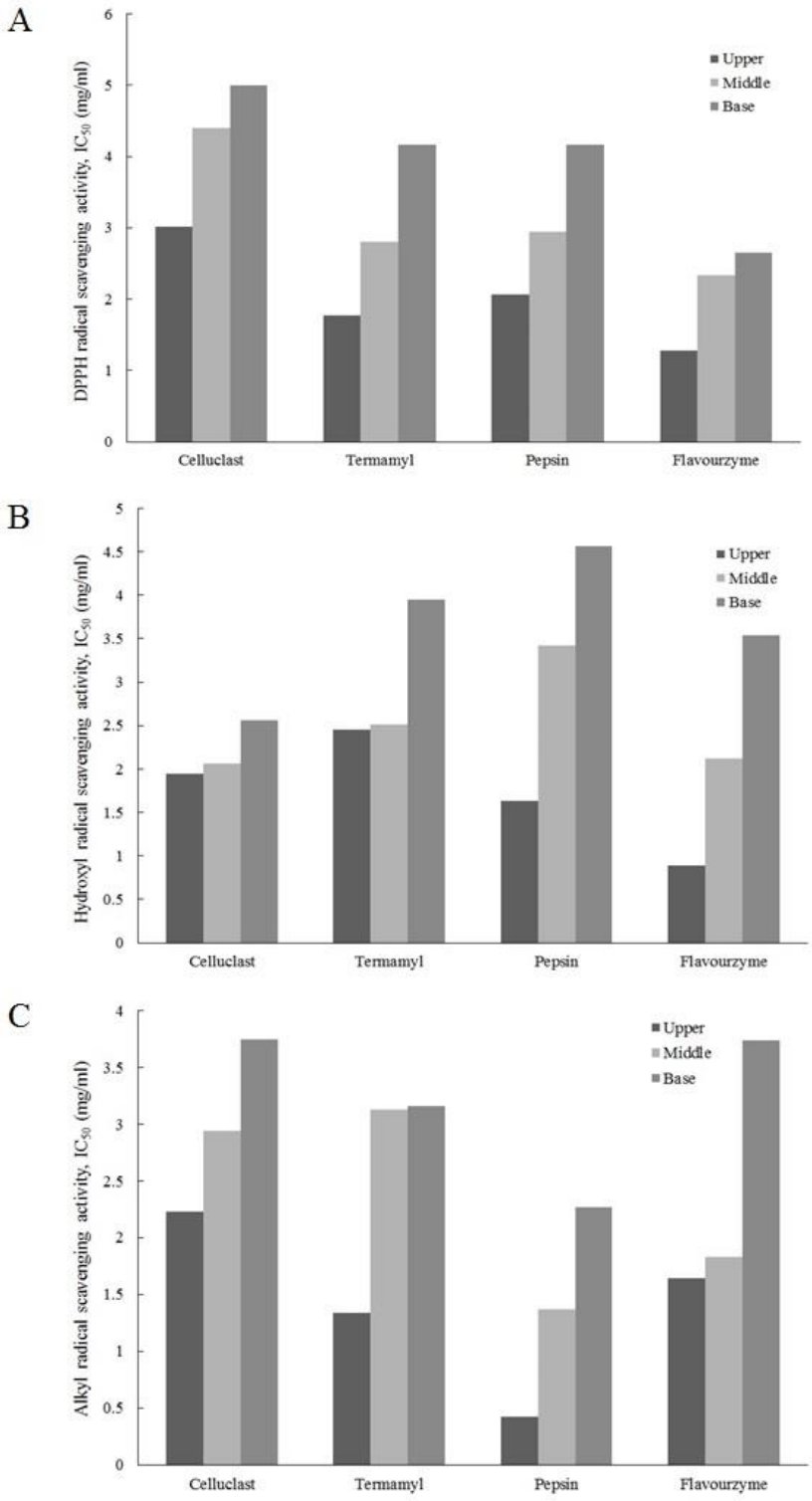

Figure 1: DPPH (A), hydroxyl (B) and alkyl radical (C) scavenging activities expressed by the value at the concentration inhibiting 50% of free radical generation (IC_{50}) of enzymatic hydrolysates from velvet antler in elk.

CONCLUSIONS

In conclusion, our results indicated that enzymatic hydrolysates of elk velvet antler containing biological substances such as uronic acid, sialic acid, GAG and collagen resulted in beneficial effects on the scavenging activities against several free radicals suggesting that it may be a useful natural radical scavenger

and a potential supplement for developing value-added food ingredients, pharmaceutical, and cosmetic industries as well as cattle feed and/or antibiotic because of its potent antioxidant capacities. However, further study will be required to identify the components in the enzymatic hydrolysates of velvet antler for the several biological activities by in vitro and in vivo studies.

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