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ARTICLE

Relationships between Antioxidants and Quality Characteristics from Velvet Antlers of Formosan Sambar Deer

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Abstract

The quality characteristics of velvet antlers obtained from Formosan sambar deer (*Cervus unicolor* Swinhoi) (SDVA), harvested from 63 to 81 d during the velvet antler growth period, were evaluated by investigating the relationships between antioxidant levels; including content, activity, and content/activity ratios, and physical properties; including shear force values, color, and Ca content. The hardness of samples from base velvet antler sections increased, and that the color of these samples tended to become reddish-yellow (redder and more yellow), suggesting that the Ca content in the base section of the sample was not ossified yet. Samples from the upper sections of velvet antler showed higher superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) content (3.91 to 1.50 mg/mL, 2.53 to 0.90 mg/mL, and 3.95 to 1.58 mg/mL, respectively) than did samples from the middle and base sections ($p<0.05$). The activity and content/activity ratios of GPX measured in the upper section were also found to be significantly greater than in the middle and base sections ($p<0.05$). We further observed that the content and activity of GPX was significantly and negatively correlated with Ca content, shear force values, and the content/activity ratio of this antioxidant ($p<0.01$). The study findings may serve as a reference index for quality evaluations of velvet antlers of Formosan sambar deer in future.

Keywords antioxidant, deer velvet antler, Formosan sambar deer, quality characteristics

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Introduction

Deer velvet antler (DVA) is an expensive material that has been used in traditional Chinese medicine for over two thousand years. DVA has also been recognized as one of the most important and effective functional ingredients in natural medicines that seek to improve nutrition and physical strength. Medical records from "Shengnong's herbal classic" (a traditional herbal medical compendium) as well as long-term clinical observations have confirmed that DVA contains many bioactive components. In human, these bioactive components may serve many functions, including reinforcing physical and mental strength, reducing the effects of aging, and extending life span. Many reports have also shown that DVA can scavenge free radicals and increase antioxidant activity (Kim *et al*., 2005; Sunwoo *et al*., 1997; Wang *et al*., 1988). Such evidence has convincingly demonstrated that

DVA contains many endogenous antioxidative enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). Indeed, these enzymes may be responsible for the anti-aging and curative properties of DVA.

Injuries can occur as a result of oxidative damage if an excess of oxygen free radicals are generated from in the human body and/or if deficiencies exist in the body's ability to scavenge free radicals. These are the major factors cause cancers, aging or senescent and apoplexy (Hai, 2006). Oxidative stress may be the cause and consequence with aging for each other, and also plays important roles in degenerative diseases (Orr and Sohal, 1994). Malondialdehyde (MDA) is an important degradation product of fat peroxides that contributes to oxidative stress and free radicals. In cells, the MDA content can change in response to the level of lipid peroxidation, which can measure in cells, the extent of damage to biological membranes, and the serious attacking extent of free radicals. Indeed, an increasing amount of *in vivo* evidence has implicated O_2^- • in oxidative damage. Numerous psychological and biochemical functional mechanisms allow antioxidants to be able to directly scavenge peroxides that are generated from inside the human body or derived from the external environment stimuli. SOD is the first line in the enzymatic antioxidant defense system against reactive oxygen spices (ROS), such as toxic O_2^- • (Scandalios, 1993). Specifically, SOD converts O_2^- • into O_2 and H_2O_2. CAT then catalyzes a reaction which converts H_2O_2 into O_2 and H_2O. GPX is able to eliminate peroxidases indirectly and catalyze reduction reactions that involve hydroperoxides under existence by supplied hydrogen-GPX and others (Matés and Sánchez-Jiménez, 1999). Natural SOD can only be extracted from humans or other organisms; therefore, it is difficult to obtain natural SOD in high yields. SOD is a macromolecule that cannot easily enter cells, shows poor cell permeability, and has a short half-life in blood. The stable period of SOD is even shorter *in vivo*, its half-life is only minutes (Hai, 2006). In addition, to ensure that the extraction process does not adversely affect function, SOD can only be obtained from water-soluble extracts. Extraction processes such as those that involve heating and/or cooking can render SOD inactive. For example, in one earlier study, that measured the antioxidants contained in the tissue of deep-sea fish species and the commercial DVA product, reported that the maximum and minimum averages for the AUR (activity /protein unit ratio) of SOD were 416.40 U/mg and 16.46 U/mg protein respectively, equivalent to 0.54 U/ mg protein (Janssens *et al.,* 2000). Furthermore, the above evidence also indicates that DVA contains functional ingredients that can provide health benefits to humans. For example, oxidative damage may be one of the major O_2^-• related factors that is responsible for aging / senescence, cardiovascular diseases, cancers, stroke, neurological illness, diabetes, mental disorders, rheumatoid arthritis, and other diseases (Hai, 2006; Valko *et al.,* 2007; Yuan, 2007).

Normally, the endogenous enzymatic antioxidants defense system could protect cells from injuries caused by oxidative stress and maintains the balance between oxides and antioxidative compounds, thereby preventing diseases. Therefore, in evaluating DVA, the protein content and activity of the antioxidants could serve as important quality indicators.

At present, sensory evaluation is still the primary method used to evaluate the quality of DVA for quality judgment methods assisted by various auxiliary apparatuses are also used to determine whole body integrity, moisture content, extent of ossification, color, aroma, and adulterated impurities of finished DVA products (Lin, 2007). However, many factors, such as deer breed, the part at the given section where DVA is harvested from, and time of harvest, can cause variations in moisture content, composition of organic and inorganic components, and levels of major antioxidants in DVA. Greater ossification tends to occur near the base section of DVA, where the inorganic compound content is higher and the moisture levels and organic compound content is lower. In this study, we investigated the changes in the content, activity and AUR of major antioxidants and their specific components concerned with physical quality in the velvet antlers obtained from Formosan sambar deer (*Cervus unicolor* Swinhoi) (SDVA) during DVA growth period. Results from this study can serve as a reference index to establish quality standards for future evaluations of SDVA.

Materials and Methods

Collection and preparation of DVA samples

Fresh SDVA tissue (weighed between 1,388 and 3,225 g) was collected from eight heads of 5 to 10 year-old Formosan sambar deer from a local deer farm in Taiwan during the SDVA growth period (63 to 81 d, during late spring). These deer had been fed a diet of 13% crude protein content in dry matter. The surfaces of SDVA tissues

were cleaned and shaved, and velvet that had adhered to the sample surface was removed. SVDA tissue was then dissected and rapidly divided into three parts (upper-, middle-, and base-section) using a trephine; wherein cutting and storage methods were the same as those used in the studies of Bubenik *et al*. (2005), Gu *et al*. (2007), and Jeon *et al*. (2006). Following this, each of the three SDVA sections were immediately sectioned into two to three thin slices (approximately 2.0 to 3.0 mm). Finally, samples were vacuum packaged and frozen at -80°C until analysis (Gu *et al*., 2007).

Preparation of SVD samples for chemical analysis

Homogenization

After being removed from storage, SDVA samples were thawed at ambient temperature. SDVA samples were then homogenized used an ice crush mixer at medium speed (Osterizer, USA). The homogenized samples were packed in No. 4 double-layer zip bags and stored at -80°C for later analysis (Gu *et al*., 2007).

Measuring the physical characteristics of SDVA

Quantification of shear force values

SDVA samples were cut into pieces (2.0 to 3.0 mm thick) for later analysis of physical characteristics. Shear force values (kg/cm^2) were measured according to a method modified from Purchas (2010). In brief, to determine the hardness of samples, a texture analyzer (TA.XT. plus, Texture Technologies Corp., USA) equipped with a craft knife adaptor and an A/CKB set probe (height of 6 mm) was used to measure shear force in cross the areas of central cores obtained from samples collected at the upper, middle, and base sections of SVDA. The samples were sheared crosswise (1.5 mm/s) using a 30-kg cell.

Determination of color

The color of samples obtained from the three different SVDA sections was measured using the CIE L*, a*, b* system on a colorimeter (Color Meter, Nippon Denshoku ZE 2000, Japan). Color measurements of each sample, including lightness, redness, and yellowness, were taken in triplicate (Purchas, 2010).

Analyzing the Ca content of SDVA samples

Analysis of Ca content (%) in SDVA samples was conducted according to methods provided by Environmental Protection Administration, Executive Yuan of Taiwan, R.O.C.: NIEA C303.01T (1994) and NIEA M105.00B (2004) (Environmental Protection Administration, 2004 and 1994). Ca content was quantified using an inductively coupled plasma optical emission spectrometer (VARIAN VISTA-MPX simultaneous ICP-OES) combined with a microwave accelerated reaction system (CEM MARS-5).

Samples Preparation and Determination of antioxidants in SDVA

SDVA tissue samples were cleaned by phosphate buffered saline (PBS, pH of 7.4) several times in order to remove blood clots. Per gram of the SDVA tissue sample was mixed with 5 mL of 20 mM pH 7.2 HEPES(4-(2-hydroxyethyl)-piperazine ethane sulfonic acid ($C_8H_{18}N_2O_4S$, Sigma, H4034-100G, USA) at 4°C, buffered by ultrasonic extract for 15 min, then centrifuged at 1,500 *g* for 5 min. Samples were then mixed with 50 mM pH 7.0 potassium phosphate (Sigma, USA), buffered by ultrasonic extract for 15 mins, and centrifuged at 10,000 *g* for 15 min. Following this, samples were mixed with 50 mM pH 7.0 Tris-HCl (Sigma, USA), buffered by ultrasonic extract for 15 min; and then centrifuged 10,000 *g* for 15 min. Finally, the supernatant was extracted for detected solution, which was stored at -80°C. Samples of detected solution have to be used up within one month.

Extraction of GPX from SDVA samples

SDVA tissue samples per gram were mixed with 5 mL of 50 mM Tris-HCl buffer (pH of 7.0) (Sigma, USA) by ultrasonic extraction for 15 min at a temperature of 4°C. Following this, SDVA samples were subjected to centrifugation at 10,000 *g* for 15 min in order to facilitate supernatant collection (from which GPX was detected). The supernatant was stored at -80°C. The supernatant is only viable for one month after collection.

Quantitative analysis of antioxidants

In performing quantitative analysis of SOD, CAT and GPX, we measured the protein content of each antioxidant using experimental procedures developed by Bradford (1976).

Determining the standard curves of SOD, CAT, and GPX proteins

The protein content of SOD, CAT and GPX contained in supernatant samples were measured using an Absorbance Microplate Reader (Epoch, BioTek) with the absor-

bance wavelength set at 595 nm. All measurements were taken in duplicate. Standard curve equations of SOD, CAT and GPX proteins were determined by linear regression analysis.

SOD, CAT and GPX activity in SDVA samples

After antioxidants were extracted from supernatant samples, they were analyzed using (1) a superoxide dismutase assay kit (catalog No. 706002, Cayman Chemical Company, USA) to quantify SOD activity, (2) a catalase assay kit (catalog No. 707002, Cayman Chemical Company, USA) to quantify CAT activity, or (3) a glutathione peroxidase assay kit (catalog No. 703102, Cayman Chemical Company, USA) to quantify GPX activity.

Data processing and statistical analysis

The antioxidants levels of SOD, CAT, and GPX extracted from supernatant samples were measured by absorbance using wavelengths at 450 nm, 540 nm and 340 nm, respectively. CV15% was adopted as the standard for adoption or abandonment of data (triplicate).

The activity levels of SOD and CAT in SDVA samples were calculated according to the following linear regression equation (determined using the standard curve): SOD activity (U/mL) = {[sample LR − (y-intercept)] / slope} × (0.23 mL / 0.01 mL) × sample dilution. The concentration of formaldehyde (used to determine CAT activity) was calculated by applying the following equation: Formaldehyde (μM) = (sample absorbance − y-intercept) / slope × (0.17 mL / 0.02 mL). The level of CAT activity in SDV samples was calculated according to the following equation (one unit was defined that enzyme contents was formed by 1.0 nmol formaldehyde concentrations per min at 25°C): CAT activity = (formaldehyde μM of sample / 20 min) ⁻ sample dilution = nmol/min/mL. To calculate the level of GPX activity, we first selected two points on the standard curve and determined the change in absorption that occurred between them, as follows:

$$\Delta A340/\text{min.} = \frac{A340(Time2) \times A340(Time1)}{Time2(\text{min.}) \times Time1(\text{min.})}$$

The GPX activity was then calculated using the following equation:

$$\text{GPX activity} = \frac{A340 / \text{min}}{0.00373\ \mu\text{M}^{-1}} \times \frac{0.19\ \text{mL}}{0.02\ \text{mL}} \times \text{sample dilution} = \text{nmol/min/mL}$$

Exchange methods for activity units of antioxidants were as follows: SOD activity (U/mL) / protein (mg/mL) = SOD activity (U/mg protein); CAT activity (nmol/min/mL) / protein (mg/mL) = CAT activity (nmol/min/mg protein); GPX activity (nmol/min/mL) / protein (mg/mL) = GPX activity (nmol/min/mg protein). However, antioxidant contents are reported from the averages from antioxidant content of upper, middle, and base sections of SDVA samples x the weights from each SDVA samples (g).

All of the experimental data were analyzed using general linear modeling (GLM) and one way analysis of variance (ANOVA) procedures applied in SAS (Statistical Analysis System) software (SAS 9.1, 2011). Duncan's new multiple range tests were also used to determine the significance of differences. Finally, correlation coefficients (r) were calculated to investigate the relationships among each of the data metrics we measured.

Results

Comparison of the levels of major antioxidants and the physical properties and Ca content in the three different SDVA sections

The levels of the three major antioxidants (SOD, CAT, and GPX) contained in the upper, middle and base sections of SDVA samples were individually measured. Specifically, we determined protein content (contents mg/mL), activity (U/mL), and activity / per mg unit ratio (AUR) (nmol/mL) of SOD, CAT, and GPX (Table 1). The SOD and CAT content in the various SDVA sections were significantly different ($p<0.05$), and the content of all three major antioxidants were highest in the upper section. Furthermore, the content and activity of GPX were markedly higher in the upper section compared to the other two sections ($p<0.05$). In contrast, the GPX-AUR was the highest in the base section ($p<0.05$).

Relationships between the major antioxidants, physical characteristics, and Ca content obtained from various sections of SDVA samples

The shear force values (SFV), Ca content, and L* values in the upper section of SDVA samples (upper-SDVA) were significantly lower than those in the other two sections ($p<0.05$). This suggests that the upper-SDVA section had the smallest ossification extent, a tender texture, and a color that was lighter and whiter. However, a* values in the middle section of SDVA samples (middle-SDVA) were markedly higher than that of those in the

Table 1. Comparisons for the major antioxidants, physical characteristics and Ca content at various sections obtained from of deer velvet of Formosan sambar deer (*Cervus unicolor* Swinhoi)

Item	Various sections of deer velvet antler		
	Upper	Middle	Base
SOD_pro (mg/mL)	3.91 ± 0.54[a]	2.15 ± 0.28[b]	1.50 ± 0.19[b]
SOD_act (U/mL)	60.20 ± 11.17[a]	21.11 ± 4.06[b]	16.97 ± 4.30[b]
SOD_act/pro (U/mg protein)	14.97 ± 1.33[a]	9.45 ± 1.03[b]	9.94 ± 1.88[b]
CAT_pro (mg/mL)	2.53 ± 0.31[a]	1.40 ± 0.26[b]	0.90 ± 0.06[b]
CAT_act (nmol/mL)	153.27 ± 25.50[a]	77.91 ± 19.44[a]	98.34 ± 33.05[a]
CAT_act/pro (nmol/mg protein)	66.02 ± 11.96[a]	59.99 ± 14.02[a]	101.66 ± 29.32[a]
GPX_pro (mg/mL)	3.95 ± 0.41[a]	2.58 ± 0.29[b]	1.58 ± 0.18[c]
GPX_act (nmol/mL)	259.49 ± 3.49[a]	237.71 ± 9.66[ab]	221.79 ± 11.14[b]
GPX_act/pro (nmol/mg protein)	68.93 ± 7.34[b]	98.50 ± 8.94[b]	156.53 ± 22.79[a]
Shear force value (kg/cm^2)	16.50 ± 1.75[c]	29.72 ± 2.76[b]	40.00 ± 1.87[a]
L*	40.91 ± 1.43[a]	36.37 ± 1.03[b]	40.49 ± 1.46[ab]
a*	23.40 ± 1.05[b]	29.16 ± 1.09[a]	25.37 ± 1.37[b]
b*	17.50 ± 2.89[a]	17.64 ± 1.60[a]	15.83 ± 0.41[a]
Ca^{2+}(%)	2.63 ± 1.01[b]	8.17 ± 0.45[a]	8.83 ± 0.78[a]

[a,b]Means in the same row with no common superscript differ significantly (p<0.05).
pro, protein; act, activity; act/pro, activity/protein.

other two sections. In other words, we found that the color in the middle section of SDVA samples are the reddest among the three sections (p<0.05). The b* values of SDVA samples were not significantly differed among the three sections. Generally speaking, upper-SDVA had a tender texture, a lighter - whiter color, and the highest antioxidant (SOD, CAT, and GPX) content, antioxidant activity and AUR (Table 1), all of which suggests higher quality SDVA.

The content and activity of GPX were much lower in the base section of SDVA samples (base-SDVA) than in the other two sections. In addition, we found that there was a very significant and positive correlation between the activities of GPX and SFV (p<0.01). Finally, we determined that, when GPX activity was higher, the extent of ossification was lower and samples were much more tender. However, no significant correlations were found between GPX activity and color (L*, a* and b* values) (Table 3).

In all sections (upper, middle, and base), there were significant and very insignificant negative correlations between SFV and the protein content (r=-0.816, p<0.01), activity (r=-0.736, p<0.01) and AUR (r=-0.432, p<0.05) of SOD, respectively. Both the content and activity of SOD showed negative correlations with Ca content (r=-0.806, p<0.01). This finding revealed that the extent of ossification was fairly low in SDVA samples, however; SOD content and SOD activity were correlated with higher L* values and lower Ca content and SFV also showed a very significant positive correlation (r=0.773, p<0.01). These combined results demonstrate that SDVA samples with a lower extent of ossification tend to be more tender, more lighter and whiter in color, and show higher SOD content and SOD activity (Table 3).

With respect to CAT, we found that the protein content was higher in the upper-SDVA than in the middle and base sections (p<0.05); however, differences among these three sections were not significant (Table 1). Nonetheless, we found a significantly positive correlation between the activity of CAT and the AUR of CAT (r=0.741, p<0.01). This reveals that the AUR of CAT significantly increases when the CAT content is higher (Table 2).

However, no significant differences were found in the activity or AUR of CAT. Results of this study also showed that the CAT content in SDVA samples is significantly negatively correlated to the SFV from those samples (r=-0.780, p<0.01). However, we did not find significant correlations between SFV and the activity or AUR of CAT. Nonetheless, both the content and activity of CAT were negatively correlated to the Ca content of those. Conversely, SFV showed a very significantly positive correlation with Ca content (r=0.773, p<0.01) (Table 3). However, the content and activity of CAT were negatively correlated with Ca content and SFV. These findings suggest that SVDA samples with a lower calcification extent were more tender and redder.

Results of this study also showed that the GPX content in SDVA samples had a significantly positive correlation with GPX activity (r=0.452, p<0.05) and a significantly

Table 2. Relationships between the contents, activity and AUR of the major antioxidants in SDVA[1] samples

Items	SOD_pro (mg/mL)	SOD_act (U/mL)	SOD-act/pro (U/mg protein)	CAT_pro (mg/mL)	CAT_act (nmol/mL)	CAT-act/pro (nmol/mg protein)	GPX_pro (mg/mL)	GPX_act (nmol/mL)	GPX-act/pro (nmol/mg protein)
Content	2.52± 1.34[B]	32.76± 26.30[B]	11.46± 4.40[B]	1.61± 0.88[B]	109.84± 75.67[B]	75.89± 57.29[B]	2.70± 1.26[B]	239.66± 28.22[B]	107.98± 53.95[B]
SOD_pro (mg/mL)	1.000	0.967	0.686	0.818	0.153	-0.336	0.911	0.514	-0.700
SOD_act (U/mL)	0.001[ΔΔ]	1.000	0.786	0.798	0.160	-0.286	0.890	0.419	-0.657
SOD-act/pro (U/mg protein)	0.001[ΔΔ]	0.001[ΔΔ]	1.000	0.646	0.361	-0.008	0.640	0.104	-0.664
CAT_pro (mg/mL)	0.001[ΔΔ]	0.001[ΔΔ]	0.646	1.000	0.366	-0.255	0.801	0.494	-0.620
CAT_act (nmol/mL)	NS	NS	NS	NS	1.000	0.741	0.052	0.288	-0.019
CAT-act/pro (nmol/mg protein)	NS	NS	NS	NS	0.001[ΔΔ]	1.000	-0.442	-0.054	0.436
GPX_pro (mg/mL)	0.001[ΔΔ]	0.001[ΔΔ]	0.001[ΔΔ]	0.001[ΔΔ]	NS	0.031[Δ]	1.000	0.452	-0.805
GPX_act (nmol/mL)	0.010[Δ]	0.042[Δ]	NS	0.014[Δ]	NS	NS	0.027[Δ]	1.000	-0.114
GPX-act/pro (nmol/mg protein)	0.001[ΔΔ]	0.001[ΔΔ]	0.001[ΔΔ]	0.001[ΔΔ]	NS	0.033[Δ]	0.001[ΔΔ]	NS	1.000

[1]SDVA: deer velvet antler of the deer velvet of Formosan sambar (*Cervus unicolor* Swinhoi).
[a]the data at top-right of an oblique angle represents contrast correlation (r); the data at bottom-left of an oblique angle represents probability level (p).
[B]The averages of 8 groups from DVA at the upper, middle and base sections, mean±SEM.
[Δ], [ΔΔ]Significantly related at the 5% ([Δ]) or 1% ([ΔΔ]) probability level, n=24 (8 × 3 × 3).
NS, no significant difference (p>0.05).
p, protein; a, activity; a/p, activity/protein; SOD – a/p = SOD – activity/protein; CAT – a/p = CAT – activity/protein; GPX – a/p = GPX – activity/protein.

negative correlation with the AUR of GPX (r=-0.805, p< 0.01). Nonetheless, the content, activity, and AUR of GPX were not significantly different from their SFV, and both the content and activity of GPX showed a very significantly negative correlation with Ca content, as well as the AUR of GPX from those were adverse to Ca contents of those (Table 2 and 3). In summary, the combined results shown in Table 2 and 3 revealed that SDVA samples with lower ossification extent contained higher levels of GPX.

According to data collected from SDVA samples, the results found that except for CAT activity had the higher correlations with the contents and AUR of CAT and GPX activity. That also had the lower correlations with other measured items. Except for GPX-AUR and CAT- AUR, CAT-AUR and their activity had positive correlations; GPX-AUR and CAT-AUR had negative correlation with the rest of measured items, except for the contents of CAT and GPX were positively correlated with SFV. Conversely, the contents and activities of the others in these major SDVA antioxidants showed negative correlations with SFV and with Ca contents.

Relationships between SDVA quality characteristics and growth days or SDVA yields

Considering the eight groups of SDVA samples, the average growth days was 73.75±5.50 d, and the average SDVA yield was 2,189±557.07 g. These results reveal that SDVA yield increases with a greater number of growing days (r=0.807, p<0.001) (details not shown). The determined data of the major antioxidants were different among them, and there were significant differences between the levels of antioxidants found in different sections of SDVA samples (p<0.05). The activity of CAT was highest in the upper-SDVA, followed by the base and middle section. Ca content as well as the AUR of GPX and SFV showed a different trend, wherein values were highest in the upper

Table 3. Relationships for the major antioxidants, physical characteristics, Ca content, growth characteristics obtained from SDVA[1] samples

Items	Physical characteristics				Calcium content	Growth characteristics	
	Shear force value (kg/cm^2)	L*	a*	b*	Ca^{2+} (%)	Growth days (d)	SDVA[2] yields (pair, g)
Content	28.74± 11.05[B]	39.36± 4.06[B]	25.97± 4.01[B]	16.99± 4.72[B]	6.54± 3.34[B]	73.75± 5.50	2189± 557.07
SOD_pro (mg/mL)	-0.816	0.124	-0.242	-0.111	-0.806	-0.189	-0.089
SOD_act (U/mL)	-0.736	0.215	-0.316	-0.068	-0.791	-0.170	-0.092
SOD_act/pro (U/mg protein)	-0.432	0.344	-0.349	0.092	-0.579	-0.157	-0.208
CAT_pro (mg/mL)	-0.780	0.344	-0.349	0.092	-0.692	-0.034	-0.084
CAT_act (nmol/mL)	0.299	0.050	-0.181	-0.073	-0.243	-0.090	-0.243
CAT _act/pro (U/mg protein)	0.639	0.040	-0.053	-0.098	0.234	-0.001	-0.148
GPX_pro (mg/mL)	-0.432	0.344	-0.349	0.092	-0.785	-0.048	0.023
GPX_act (nmol/mL)	0.299	0.050	-0.181	-0.073	-0.642	-0.049	0.029
GPX _act/pro (U/mg protein)	0.639	0.040	-0.053	-0.098	0.602	0.073	-0.010

[1]SDVA: deer velvet antler of the deer velvet of Formosan sambar (*Cervus unicolor* Swinhoi).
[a]the data at top-right of an oblique angle represents contrast correlation (r); the data at bottom-left of an oblique angle represents probability level (p), mean±SEM.
$^{\Delta}$, $^{\Delta\Delta}$Significantly related at the 5% ($^{\Delta}$) or 1% ($^{\Delta\Delta}$) probability level, n=24 (8 × 3 × 3).
NS, no significant difference (p>0.05); pro, protein; act, activity; act/pro, activity/protein.

section, secondly highest in the middle section, and the lowest in the base section. All other metrics related to antioxidant levels showed a similar tendency. In addition, L* and a* values of upper sections were different than those of middle sections. In other words, the color of upper-, middle-, and base-SDVA samples was lighter-whiter, darker and much redder, and lighter and reddish-yellow (redder and more yellow), respectively (Table 1). Nevertheless, no significant relationships between growth days or SDVA yields and the content, activity, and AUR of major antioxidants were identified in any section (i.e., upper, middle, or base) of SDVA samples (Table 3).

Our results also showed that a simultaneous increase in Ca content and SFV caused the SDVA samples to gradually calcify, whereupon their texture hardened. As calcification occurred, the content and activity of major antioxidants also decreased and showed positive and negative correlations with both L* and a* values, individually. However, Ca content and SFV showed slightly positive correlations with CAT-AUR (p=0.234) and GPX-AUR (p =0.602) (Table 3). It can therefore be concluded that, while GPX-AUR remained high, Ca content also remained high and SDVA samples had already started to harden. Conversely, when CAT-AUR was high, Ca content decreased to lower levels and SDVA samples were tended to be completely hardened.

Taken together, the results of this study indicate that CAT-AUR, GPX-AUR and GPX activity should be able to serve as a calcification / hardening index for SDVA; combing the measuring data of SOD had highly negative correlation with SFV and Ca content could also serve as reference index of the relative final goal to determine the extent to which SDVA samples have undergone calcification and hardening. However, the activity of GPX showed a strongly negative and weak positive correlation with Ca content (-0.642) and SFV (0.299) (Table 3), which suggests that it would be the better time for the reference index of optimal harvest periods while Ca contents may achieve the maximum levels, but SDVA samples had not yet hardened, and the sample color tended to show the highest ratio of red / yellow reach (around at the base section). Therefore, SDVA color should be useful for establishing a reference index to determine optimal harvest period.

SDVA may have calcified and hardened when CAT-AUR and GPX-AUR reached values of 101.66 and 156.63 nmol/mg protein, respectively. The contents, activity and AUR of SOD respectively declined to 1.50 mg/mL, 16.97 nmol/mL and 9.94 nmol/mg protein (Table 1). Nonetheless, the various metrics that we suggest could be useful indicators in determining the optimal SDVA harvest period will need to be further confirmed by advanced researches.

Discussion

In general, characteristics of the major antioxidants

showed the highest values in the upper-SDVA in the present study. This section also exhibited a more tender texture, a lighter-whiter color, and the lowest extent of ossification. Conversely, antioxidant characteristics tended to be lowest in base-SDVA, and this section exhibited the hardest texture, a lighter and more yellow color, and the greatest extent of ossification. The antioxidant levels in the middle-SDVA tended to have moderate values. This section exhibited a color that was reddish-yellow (the reddest and much more yellow) and a moderate extent of calcification (neither soft nor hard). We can therefore conclude that the base-SDVA reached the maximum extent of calcification (thereby becoming harder) but still did not complete calcification. This suggests that the SDVA samples had calcified gradually. Furthermore, data from antioxidant characteristics indicate that the levels of SOD, CAT, and GPX also gradually decreased from upper- to base-section. As this happened, the color of SDVA samples gradually became lighter and changed from red to yellow (or reddish-yellow). Previous studies reported that the SOD activity in fresh DVA is 380 times greater than that of swine blood (Long *et al.,* 1991; Yuan *et al.,* 1987). Furthermore, while the upper of DVA from red deer contained SOD, CAT, and GPX, the distribution of these antioxidants were not significantly different in various DVA sections. Nonetheless, the top of the DVA showed the highest GPX content, and GPX levels gradually decreased from the upper section to the base section of antlers (Suttie and Fennessy, 1992). The data in this study also revealed that GPX had the lowest activity and highest AUR in base-SDVA samples, likely due to the fact that the base section had a lower GPX content than the other two sections.

It can therefore be concluded that Ca content underwent the greatest degree of calcification in base section, although calcification was nonetheless gradual and remained incomplete. When the extent of calcification was greater, SDVA texture also became harder, which present that SDVA has gradually been calcifying, and the determining data of the aforementioned levels of the three major antioxidants are gradually decreased and the color of SDVA changed from lighter- whiter become to reddish-yellow.

As the price of DVA is generally determined according to weight. A higher calcification extent can increase DVA weight i.e. harvesting SDVA at the optimal when the extent of calcification is greater, and thereby increase profit. Therefore, the optimal harvest time of SDVA is coincides with the optimal extent of calcification, which occurs when base-SDVA shows a high color ratio of red and yellow reach to the highest (around location at the base section), of SDVA with much higher antioxidant levels, that SDVA may also be evident from higher antioxidant levels before SDVA is harvested. Furthermore, when the activity of GPX reached the minimal level of 221.79 nmol/mL, SFV was as high as 40.00 kg/cm^2, Ca content was around 8.83%, and L*, a* and b* values were 40.49, 25.37, and 15.83, respectively, as showed in Table 1. These levels can help determine the optimal time to harvest SDVA so that it contains much higher antioxidant levels. Findings from this study can be used to establish a reference index for the evaluation of SDVA quality characteristics and the improvement of SDVA yields. The most important indicator of DVA yield is weight. DVA size is also largely determined by genetics (Kruuk *et al.,* 2002; Ma and Yang, 1996). The SDVA samples in this study were collected during the optimal harvest period (Lin, 2007) when the top SDVA (in the upper section) changes from a round shape to a keen edge.

In this study, upper-SDVA had significantly lower SFV and Ca content than did the middle and base sections. The tissues in the upper-SDVA were more tender and slightly whiter, and that the lower extent of ossification could be used as a reference index in determining the quality of SDVA. At present, the SFV of SDVA samples can be directly determined at deer farms (*in situ*). A lower SFV indicates that the SDVA has a more tender texture. The average SFV from SDVA samples obtained from the upper, middle, and base sections ranged between 16.50 to 40.00 kg/cm^2. Consequently, the base-SDVA were sectioned into slices that measured approximately 2 to 3 mm, which was determined to be sufficient to evaluate SDVA quality. In this study, Ca content of SDVA samples from the middle and base sections were 8.17% and 8.83%, respectively. These findings are similar to those of Kuo *et al.* (2009), who measured the Ca content in bone pieces from the base-SDVA and reported that the highest values were between 6.09 and 7.89%. Therefore, results of the current study can potentially be used to help set reference values to define the ossification extent of SDVA.

SOD is very important in the human body. Consequently, the quality of SDVA samples which showed a higher SOD activity, can earn value-added effects. In another previous study, the content of SOD, CAT, and GPX in healthy human blood was 90, 0.5 and 9 mg/L, respectively. Moreover, animal experiments confirmed that the differences in SOD activity levels between individual animals were

quite low with a standard error of only around 10%. Conversely, the difference in GPX activity levels among different species can be 50 times greater than the difference in GPX level among individuals of the same species, and differences in CAT activity levels among different species can be up to 100 times greater than differences among individuals of the same species. These previous findings revealed the important roles that SOD plays in the prevention systems of multiple species could develop the more important functions (Hai, 2006).

In another earlier study, administered oral DVA extracts prepared from sika deer (*Cervus Nippon* Temminck) in elderly (12-mon-old) Kunming mice. That study found that oral administered of DVA extracts (1) strengthened the activity of SOD, CAT and GPX in the liver and (2) strengthened the activity of CAT and GPX in the brain and kidney. In addition, the administration of DVA extracts led to a greater reduction of MDA content in elderly mice than in juvenile mice (1-mon-old), which indicates that DVA oral liquids provide antioxidation functions (Wan *et al.,* 2004). Furthermore, Yuan *et al.* (2007) employed DVA extracts to rats with myocardial tissues during a later stage of acute myocardial ischemia and found that DVA extracts performed preventative functions by enhancing the SOD activity in ischemic myocardial tissues and reducing the MDA content associated with secondary injuries of ischemia myocardial tissues (Yuan *et al.*, 2007). Results from another study indicated that DVA ethanol extracts from sika deer can enhance SOD activity in mice, thereby increasing their ability to scavenge free radicals and potentially delaying senility (Chen and Nie, 2000). Other research reported that rats that received oral DVA exhibited anti-fatigue effects. Specifically, the exhaust periods of swinging were greatly increased compared to mice that did not receive DVA (Wu, 2006). Another study indicated that the diabetes mice oral administered DVA ethanol extract can reduce blood sugar levels and MDA concentrations; enhance the activity of SOD, CAT, and GPX; and strengthen antioxidative abilities (Liu *et al.,* 2010). Orally administering freshly extracted DVA liquid (with PBS or ethanol) to male ICR mice have also been found to increase overall sperm numbers, increase the number of live sperm, and reduce the number of abnormal sperm (Chou, 2008). Furthermore, extracted DVA liquid was also found to helpful in increasing the gene expression of antioxidants when incubating mice embryos were exposed to peroxide and hydroxide, in relaxing and reducing the harmful effects of oxidative attacks, and in maintaining the ability of the embryo development (Cheng *et al.,* 2014).

Long *et al.* (1991) demonstrated that SOD activity is much stronger in DVA than in cattle or pig blood (more than 380 times greater in the case of pig blood). In our current study, the AURs of antioxidants in SDVA samples were more than numerous times greater than that which is found in most of the famous commercial health supplement. In addition, Niwa (1996) reported that the rate of SOD drainage from the kidney is very fast following injection. Injection amount of six min can be drained out of body by urine after 0.5 min due to cells (nucleus) produced SOD did not send out automatic control command, which is the command to existence of residual SOD, this situation make exterior injection of SOD did not achieve the function that it originally owned. DVA can be obtained from natural food, and results from several studies have confirmed that the components of DVA, which can provide antioxidative and anti-aging functions regardless of the method of administration (e.g., orally or through the abdominal cavity), are probably related to molecular weight sizes of antioxidants, make it has the less efficiency absorption and utilization. However, the effects of molecular weight on the stimulation and activation of factors requires further investigation.

The results from both the current study and previous researches indicate that agents derived from DVA have many health benefits, including scavenging free radicals, reducing fatigue, and promoting anti-aging effects. Results of this study which pertain to (1) the content and activity of antioxidants in the upper, middle and base sections and (2) the physical quality and Ca content of SVDA may be useful in establishing a reference index to evaluate the quality of SDVA. Nonetheless, SOD activity in SDVA samples still showed differences (details not shown). This implies that future studies which consider how genetics and metabolic functions influence the content and activity of antioxidants in DVA is worthy of investigation and may help to fill an important research gap.

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