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Original Article

Antioxidant Activity of Gelatins from Sika Deer (*Cervus nippon*)

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ABSTRACT

Background: In Japan, the rapid increase in the number of sika deer (*Cervus nippon*) has become a social issue and measures for the resource utilization of extensively captured sika deer have not kept pace. In contrast, various body parts of deer have been used in traditional Chinese medicine since ancient times. **Objectives:** In the present study, the *in vitro* antioxidant activity of gelatins from sika deer was evaluated in order to increase their usefulness as health promoting resources. **Materials and Methods:** Three kinds of deer gelatins were prepared through hot water extraction of antlers, skins, or bones obtained from sika deer. The antioxidant activity was evaluated along with commercially available gelatins and collagen peptides from donkey, bovine, pig and fish using three kinds of *in vitro* antioxidant assays including DPPH free radical scavenging activity (DPPH) assay, Superoxide anion scavenging activity (SOD) assay and Oxygen Radical Absorption Capacity (ORAC) assay. **Results:** All kinds of gelatins from deer exhibited more than 50% inhibition of DPPH free radical and superoxide anion scavenging activities, as well as H-ORAC values exceeding 8,000 µmol TE/L. The gelatins from deer were further separated into high molecular-weight and low molecular-weight fractions by ethanol precipitation method and the low molecular-weight fractions showed stronger antioxidant activity than corresponding high molecular-weight fractions in all measurements. **Conclusion:** The result of present study provided rudimentary evidence for the antioxidant activity of gelatins from sika deer, which need further research for the use as health promoting resources.

Keywords: Sika deer, Gelatin, Collagen peptide, Antioxidant activity.

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INTRODUCTION

In recent decades, various factors such as the decline in hunting, the lack of natural predators, habitat modification due to agriculture and forestry and climate change have led to a significant increase in the population and expansion of habitats for wild deer in countries of Japan, the United States and Europe.[1] The irruption of sika deer (*Cervus nippon*) has become a societal issue in Japan, causing damage to agriculture and forestry, an increase in traffic accidents and biodiversity loss due to the extinction of rare plants and a landslide disaster in national parks and world heritage site.[2,3] Forest damage caused by sika deer is particularly serious in Japan, accounting for about 70% of all forest damage caused by wild birds and mammals. For address these issues, efforts to capture and manage the population of sika deer are underway nationwide. The number of sika deer captured has increased by 51.9-fold over 45 years, from 1975 to 2020.[4] Despite the large number of sika deer being captured, measures related to the utilization of captured sika deer resources have not kept pace. The primary use of sika deer is for consumption as meat, while other parts are mostly discarded and the methods of utilization are still in the consideration stage.

In contrast, various body parts of deer, such as antlers, bones, tails and sinews have been used in traditional Chinese medicine since ancient times.[5] Deer antlers are highly valued for their nourishing and strengthening effects, leading to extensive research on their biological activities, such as antioxidant, anti-inflammatory, neuroprotective and antitumor activities.[6,7] Moreover, gelatins from deer antler is well-known gelatinous animal-derived traditional Chinese medicines for the treatment of dizziness due to blood deficiency, soreness and coldness of the waist and knees and emaciation due to fatigue. The major constituents have been reported peptides and proteins produced by collagens.[6,7] Gelatins and collagens are widely used in manufacturing industries such as foods, pharmaceuticals and cosmetics due to their excellent functional and technological properties.[8] Most of the gelatins available in the market are obtained from donkey, bovine, pig, or fish.[9,10] Hence, to increase the usefulness of sika deer as health

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promoting resources, the present study evaluated the *in vitro* antioxidant activity of the gelatins from antlers, skins and bones of sika deer and compared with those from donkey, bovine, pig and fish.

MATERIALS AND METHODS

Materials

Three kinds of deer gelatins (deer antler gelatin, DAG; deer skin gelatin, DSG; and deer bone gelatin, DBG) were prepared through hot water extraction of the antlers, skins, or bones from sika deer (*Cervus nippon*) inhabiting Hokkaido, Japan. The Donkey skin Gelatin (DG) was provided from Wakan SINCA Co., Ltd., (Tokyo, Japan). Bovine Gelatin (BG), Pig Gelatin (PG), Fish Gelatin (FG), Bovine collagen Peptide (BP), Pig collagen Peptide (PP) and Fish collagen Peptide (FP) were purchased from Nippi, Inc. (Tokyo, Japan).

Chemicals

Ethanol (purity≥99.5%) and 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) (purity>97%) were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). Fluorescein sodium salt, potassium phosphate dibasic (purity≥98%) and potassium phosphate monobasic (purity≥99%) were purchased from Sigma-Aldrich (St Louis, MO, USA). Chlorogenic acid hydrate (purity>98%) and *trans*-ferulic acid (purity>98%) were purchased from Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan). Trolox standard (purity>98%) was purchased from Dojindo Laboratories (Kumamoto, Japan)

In vitro antioxidant activity

Sample preparations

The gelatins and collagen peptides were dissolved in purified water at a concentration of 100 mg/mL for DPPH and ORAC assays and 120 mg/mL for SOD assay and extracted by sonication at 37°C for 1 hr. Each mixture was then centrifuged at 13,200 rpm for 15 min and the supernatant or supernatant diluent by purified water was used as the sample solution for *in vitro* antioxidant assays. Ethanol precipitation for deer and donkey gelatins were performed by adding ethanol to each water dissolved gelatin solutions to a final concentration of 70 vol%. The mixture was centrifuged at 1,4800 rpm for 15 min to separate the supernatant and precipitate. Then, the supernatant was vacuum-concentrated and the precipitate was freeze-dried. The resulting supernatant and precipitate fractions were dissolved in purified water at a concentration of 100 mg/mL or 120 mg/mL for *in vitro* antioxidant assays.

DPPH free radical scavenging activity (DPPH) assay

The DPPH assay was performed using a DPPH antioxidant assay kit (Dojindo Laboratories, Kumamoto, Japan) based on the manufacturer's manual. Concisely, 20 µL of sample solutions and 80 µL assay buffer were added to each well of a 96-well microplate. Subsequently, 100 µL DPPH working solution was added to each well. Then, the plate was incubated at 25°C for 30 min in the dark. The absorbance at 517 nm was measured using an Infinite M Nano$^+$ microplate reader (Tecan Japan Co., Ltd., Kanagawa, Japan). The concentration-activity dependency was examined at multiple concentrations range from 0.63 mg/mL to 10 mgmL. XY curves fitted with non-linear model and the IC_{50} values were calculated by four-parameter logistic curve fitting method (GraphPad Prism 7).

Superoxide anion scavenging activity (SOD) assay

The SOD assay was performed using a SOD assay kit-WST (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's manual. Briefly, 20 µL of sample solutions were added to each well of a 96-well microplate, followed by 200 µL WST working solution and 20 µL enzyme working solution. Then, the plate was incubated at 37°C for 20 min in the dark. The absorbance at 450 nm was measured using an Infinite M Nano$^+$ microplate reader. The concentration-activity dependence was measured at the concentrations range from 0.63 mg/mL to 10 mg/mL. XY curves fitted with non-linear model and the IC_{50} values were calculated by four-parameter logistic curve fitting method (GraphPad Prism 7).

Oxygen Radical Absorption Capacity (ORAC) assay

The ORAC assay was performed according to a literature method with modification.[11,12] Namely, 35 µL of the assay buffer and trolox standard solutions (50.0, 25.0, 12.5 and 6.25 µM) or the sample solutions in 75 mM potassium phosphate buffer (pH 7.4) were added to each well of a 96-well microplate, followed by 115 µL of fluorescein working solution (110.7 mM). After the plate was incubated at 37°C for 10 min in the dark, the fluorescence (Ex. 485 nm, Em. 530 nm) was measured using an Infinite M Nano$^+$ microplate reader. Then, 50 µL of AAPH working solution (31.7 mM AAPH solution) was added to each well of the plate and the fluorescence (Ex. 485 nm, Em. 530 nm) was immediately measured every 2 min for 45 times (0-90 min) at 37°C. The H-ORAC values were calculated from net AUC of trolox standard solutions and sample solutions and expressed as µmol trolox equivalent/L (µmol TE/L).

Statistical analysis

All results are expressed as the mean±Standard Deviation (SD) of a triplicate measurement. The data were analyzed using one-way Analysis of Variance (ANOVA) followed by Holm-Sidak test. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc. San Diego, CA, USA).

RESULTS

In vitro antioxidant activity of deer gelatins and commercially available gelatins and collagen peptides

Deer Gelatins (DAG, DSG and DBG) from the antlers, skins and bones of sika deer were evaluated for their antioxidant activity, along with commercially available gelatins (DG, BG, PG and FG) and collagen peptides (BP, PP and FP) from donkey, bovine, pig and fish. The antioxidant activity was measured by three kinds of *in vitro* assay methods: DPPH free radical scavenging activity (DPPH) assay, superoxide anion scavenging activity (SOD) assay and Oxygen Radical Absorption Capacity (ORAC) assay.

In the DPPH assay, all samples were firstly measured their DPPH free radical scavenging activity at a final concentration of 10 mg/mL. As a result, Gelatins from Donkey (DG) inhibited the production of DPPH free radical at 81.5%, followed by those from deer at 67.7% for DAG, 69.9% for DSG and 65.2% for DBG, but gelatins and collagen peptides from bovine, pig and fish showed weak activity (20.9-31.0%) (Figure 1A). Gelatins from Donkey (DG) and Deer (DAG, DSG and DBG) were further measured their DPPH free radical scavenging activity in the concentration range from 0.63 mg/mL to 10 mg/mL and all samples displayed concentration-dependent inhibitory activity (Figure 1B). Their IC_{50} values were determined as 3.76 mg/mL for DG, 6.33 mg/mL for DSG, 6.92 mg/mL for DAG and 8.00 mg/mL for DBG, using the non-linear regression formula (Table 1).

In the SOD assay, at a final concentration of 10 mg/mL, Gelatins from Donkey (DG) and deer (DAG, DSG and DBG) showed superoxide anion scavenging activity at 94.8% for DSG, 86.1%

Table 1: IC_{50} and H-ORAC values of gelatins and collagen peptides from deer, donkey, bovine, pig and fish.

Sample	DPPH assay	SOD assay	ORAC assay
	IC_{50} (mg/mL)	IC_{50} (mg/mL)	H-ORAC (μmol TE/L)
DAG	6.92±0.18	5.90±0.25	8689.6±118.7
DSG	6.33±0.17	2.09±0.05	8725.2±124.7
DBG	8.00±0.63	6.35±0.13	8124.0±279.8
DG	3.76±0.13	2.10±0.06	8664.6±160.4
BG	>10	>10	2581.0±44.3
PG	>10	>10	1793.1±29.0
FG	>10	>10	9673.8±270.3
BP	>10	>10	1499.9±67.9
PP	>10	>10	1329.4±23.4
FP	>10	>10	1457.8±34.4
trolox[a]	6.59±0.12	-	-
chlorogenic acid[a]	-	1.45±0.09	-
trans-ferulic acid	-	-	14627.4±216.4

[a]IC_{50} value at μg/mL.

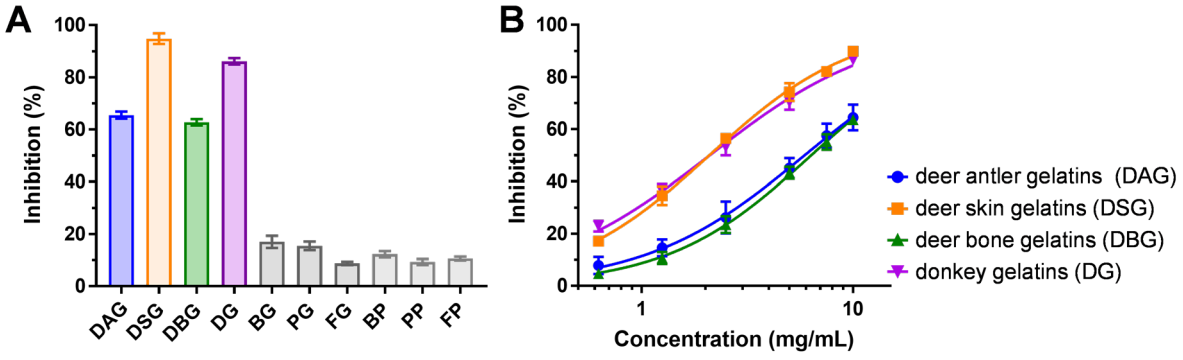

Figure 2: Superoxide anion scavenging activity of deer gelatins and commercially available gelatins and collagen peptides. Inhibition rates of gelatins and collagen peptides from deer, donkey, bovine, pig and fish (A) and inhibition curves of gelatins from deer and donkey (B). Each value represents a mean±SD (*n*=3).

for DG, 65.5% for DAG and 62.8% for DBG, while gelatins and collagen peptides from bovine, pig and fish showed weak activity (8.8-17.0%) (Figure 2A). For the samples with more than 50% inhibition, their inhibitory activities were further measured at different concentrations from 0.63 mg/mL to 10 mg/mL to obtain the IC_{50} values by regression analyses (Figure 2B). DSG (IC_{50}=2.09 mg/mL) and DG (IC_{50}=2.10 mg/mL) showed the most potent activity, followed by DAG (IC_{50}=5.90 mg/mL) and DBG (IC_{50}=6.35 mg/mL).

The ORAC assay was performed using the H-ORAC method, which focuses on hydrophilic antioxidants. As a result, gelatins from Donkey (DG), deer (DAG, DSG and DBG) and Fish (FG) exhibited antioxidant activity with H-ORAC values exceeding 8,000 µmol TE/L, which were much potent than other samples (Table 1).

Antioxidant activity of fractions of gelatins from deer and donkey by ethanol precipitation

Since the major constituents of the four antioxidant samples (DAG, DSG, DBG and DG) are gelatins from deer and donkey, these samples were further separated into high molecular-weight and low molecular-weight fractions by ethanol precipitation method. The method of precipitating gelatin with ethanol utilizes the insolubility of gelatin in ethanol, that gelatin becomes insoluble because electron donation from the ethyl group reduces the hydrogen bond donating ability of the hydroxy group.[13] In this study, 70% ethanol was used to fractionate gelatins from deer and donkey, separating them into supernatant (low molecular-weight fractions: DAG-L, DSG-L, DBG-L and DG-L) and precipitate (high molecular-weight fractions: DAG-H, DSG-H, DBG-H and DG-H). All fractions were evaluated their antioxidant activities by DPPH, SOD and ORAC assays. The results are shown in Figure 3.

When comparing the activity between the fractions from the same gelatin sample, the low molecular-weight fractions exhibited stronger antioxidant activity than corresponding high molecular-weight fractions. Further, the differences between antioxidant activities of two fractions were observed more significant in gelatins from deer than donkey. It is also notable that DSG-L fraction exhibited the most potent antioxidant activity in all measurements.

DISCUSSION

Three frequently used *in vitro* antioxidant activity assays, DPPH assay, SOD assay and ORAC assay, were used to elucidate the antioxidant activity of gelatins from sika deer (DAG, DSG and

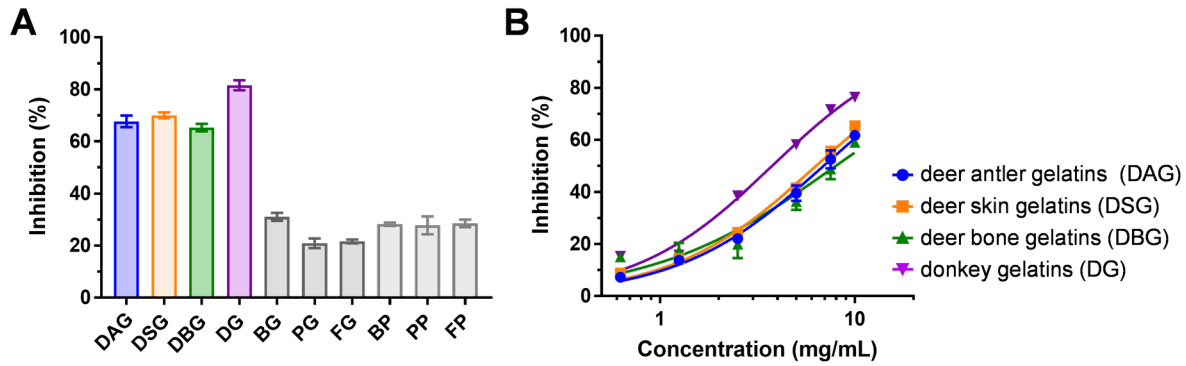

Figure 1: DPPH free radical scavenging activity of deer gelatins and commercially available gelatins and collagen peptides. Inhibition rates of gelatins and collagen peptides from deer, donkey, bovine, pig and fish (A) and inhibition curves of gelatins from deer and donkey (B). Each value represents a mean±SD (*n*=3).

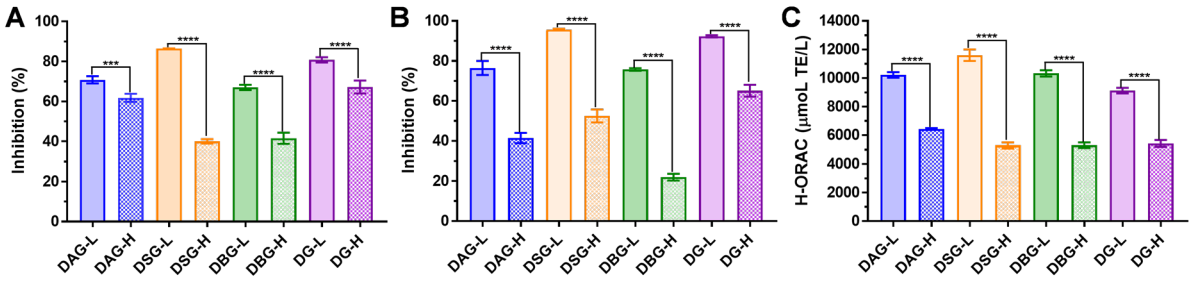

Figure 3: Antioxidant activity of low molecular-weight fractions (DAG-L, DSG-L, DBG-L and DG-L) and high molecular-weight fractions (DAG-H, DSG-H, DBG-H and DG-H) of gelatins from deer and donkey separated by ethanol precipitation. DPPH (A), SOD (B) and ORAC (C) assays. Each value represents a mean±SD (*n*=3). ***p<0.001, ****p<0.0001, supernatant fraction vs. precipitate fraction (ANOVA, Holm-Sidak test).

DBG), along with gelatins (DG, BG, PG and FG) and collagen peptides (BP, PP and FP) from donkey, bovine, pig and fish. The DPPH assay utilizes the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical reagent, which represents the ability of an antioxidant to scavenge stable free radical DPPH.[14] Since the DPPH radical is not present in the human body, the extent of biological relevance is unclear, but the DPPH assay remains one of the simplest and most commonly used antioxidant assays. Superoxide Dismutase (SOD) is one of the most important antioxidant enzymes present in the human body that plays a role in scavenging superoxide anion (O_2^-), one of the Reactive Oxygen Species (ROS). The SOD assay using xanthine/xanthine oxidase as a superoxide anion generating system was measured by a simple and rapid WST-1 method, which performed using the water-soluble tetrazolium salt, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1).[15] Oxygen Radical Absorbance Capacity (ORAC) is an antioxidant capacity evaluation method established by Cao *et al.* at the National Institute on Aging in the United States, widely used for assessing the antioxidant activity of the nutraceutical, pharmaceutical and food samples.[16] The ORAC assay utilizes AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) as a radical reagent and evaluates the relative antioxidant activity of the sample against the standard substance trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

The results suggested that gelatins from sika deer and donkey were found to exhibit stronger antioxidant activity than gelatins and collagen peptides from bovine, pig and fish in three *in vitro* methods. In particular, the Deer Skin Gelatin (DSG) exhibited more potent activity than the Deer Antler Gelatin (DAG), which is well known to demonstrate antioxidant activity.[7] Exceptionally, the Fish Gelatin (FG) exhibited highest antioxidant activity in the ORAC assay. Previous studies have reported that fish gelatin hydrolysates show antioxidant activity,[17-19] and the results of ORAC assay in this study suggested the presence of hydrophilic peptides with potent antioxidant activity in the fish gelatin sample.

Gelatins from Donkey (DG) have been used in traditional Chinese medicine as hemostatic and tonic agents from more than 2000 years ago.[20] DG has been revealed to exhibit various biological activities, including hemostasis, anticancer, antioxidant, immunomodulatory and anti-inflammatory effects.[9] In addition, DG was demonstrated to enhance the activities of superoxide dismutase, catalase and glutathione peroxidase, leading to scavenge free radicals such as malondialdehyde in a D-galactose induced aging mice.[21] These results suggested that DG exhibits an anti-aging effect through its antioxidant activity and a similar effect is expected in gelatins from sika deer.

The gelatins from animals are a water-soluble protein obtained by partial hydrolysis of collagen. It has been reported that the precipitation of gelatin by 70% ethanol at 37°C could precipitate most gelatin with a molecular weight>50 kDa.[13] Therefore, the supernatant fractions of gelatins from deer and donkey separated by ethanol precipitation method in this study showed stronger antioxidant activity than corresponding precipitate fractions, which suggested gelatin with a molecular weight of <50 kDa extracted from deer and donkey may contribute to the antioxidant activity.

CONCLUSION

The present study was conducted to elucidate the antioxidant activity of the gelatins from antlers, skins and bones of sika deer inhabiting Hokkaido, Japan, using three *in vitro* methods including DPPH, SOD and ORAC assays. As a result, gelatins from deer exhibited stronger antioxidant activity than gelatins and collagen peptides from bovine, pig and fish and showed concentration-dependent antioxidant activity at DPPH and SOD assays as well as gelatins from donkey. Furthermore, the gelatins from deer and donkey were separated into high molecular-weight and low molecular-weight fractions using 70% ethanol and found that low-molecular weight fractions exhibited stronger antioxidant activity than corresponding high molecular-weight fractions. Notably, the low-molecular weight fraction of deer skin gelatin showed the most potent antioxidant activity in all measurements. The result of present study provided rudimentary evidence for the antioxidant activity of gelatins from sika deer, which need further research for the use as health promoting resources.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DPPH: 2,2-diphenyl-1-picrylhydrazyl; **SOD:** Superoxide dismutase; **ORAC:** Oxygen radical absorbance capacity; **H-ORAC:** Hydroxyl radical antioxidant capacity; **DAG:** Deer antler gelatin; **DSG:** Deer skin gelatin; **DBG:** Deer bone gelatin; **DG:** Donkey gelatin; **BG:** Bovine gelatin; **PG:** Pig gelatin; **FG:** Fish gelatin; **BP:** Bovine collagen peptide; **PP:** Pig collagen peptide; **FP:** Fish collagen peptide.

SUMMARY

In this study, the *in vitro* antioxidant activity of gelatins from antlers, skins and bones of sika deer was evaluated to increase their usefulness as health promoting resources. As a result, gelatins from deer exhibited stronger antioxidant activity than commercially available gelatins and collagen peptides. Furthermore, the low-molecular weight fractions of gelatins from deer exhibited stronger antioxidant activity than corresponding high molecular-weight fractions. The present study provided rudimentary evidence for the antioxidant activity of gelatins from sika deer.

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