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Collagen hydrolysates derived from Yezo sika deer (*Cervus nippon yesoensis*) tendon have highly health-promoting potentials

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Abstract

Hydrolysis was applied to collagen of Yezo sika deer tendon using six proteases (pepsin, trypsin, papain, chymotrypsin, pronase E, and thermolysin). The yields of hydrolysates and its protein contents ranged from 69 to 96% (w/w) and 185.7 to 260.6 µg/mg lyophilized powder, respectively. Next, the functional properties of hydrolysates were demonstrated using a different four systems. Antioxidative activities were moderate; the hydrolysates prepared by pepsin, trypsin, chymotrypsin, and pronase E showed the same activities as 1 mM ascorbic acid. Reactive oxygen species scavenging activities were also measured. Highly scavenging activities were observed in pronase E, papain, and chymotrypsin hydrolysates. On the other hand, thermolysin hydrolysate strongly inhibited ACE activity. From the above investigation, Yezo sika deer tendon can be used as an easily accessible underutilized bioresource of natural antioxidants, ACE inhibitors, and as a possible food supplement or in biomedical fields and pharmaceutical industry.

Introduction

In all living organisms, antioxidant property is particularly important characteristics because oxidation is identified as reactive oxygen species-mediated process that has a lot of undesirable impacts on biological systems. Hydroxyl radical, superoxide anion radical, and hydrogen peroxide as free radicals are mutagens produced by radiation and unfavorable metabolic by-products of normal aerobic metabolism. Among them hydroxyl radical and superoxide anion radical are highly unstable and these react rapidly with other chemical groups and substances in the body, leading to damage against proteins, lipids, and nucleic acids (Tabaraki and Ghadiri, 2013). It is widely evidenced that these radicals are associated with the occurrence of several chronic diseases including carcinogenesis, inflammation, and atherosclerosis, and so on (Sarmadi and Ismail, 2010). Reactive oxygen species can remove and scavenge by enzymes such as catalase, glutathione peroxidase, peroxidase, and superoxide dismutase and by non-enzymatic compounds as vitamins (A, C and D), minerals (zinc and selenium), and proteins or oligopeptides. Thus, oxidative stress is produced by an imbalance between production of reactive oxygen species and antioxidant defences.

Angiotensin-converting enzyme (ACE) is an important enzyme and plays physiological role in the regulation of blood pressure on rennin-angiotensin system and kinin-kalicrein system and hypertension (Montecucco *et al.*, 2011). For patients suffering from hypertension that is one of risk factors for cardiovascular disease, ACE inhibitiors (antihypertensive drugs) such as alcacepril, captopril, enalapril, and lisinopril are beneficial. However these synthetic inhibitors have unfavorable effects such as allergic reactions, angioedema, coughing, skin rashes, and taste disturbances. It is desired, therefore, for the prevention and treatment of hypertension to find safety and economical ACE inhibitors. By the World Health Organization, it is estimated that heart disease and stroke will be the leading cause of death and disability worldwide till 2020 (Erdmann *et al*., 2008).

Dietary proteins are recognized as a source of energy (about 4 kcal/g) and essential amino acids. These are needed for growth and maintenance of physiological functions in the body. The functionalities of dietary proteins can be modified by enzymatic, physical, and chemical treatments. Particularly proteolytic enzymes are used as attractive tools or techniques to modify or change the protein structure under moderate conditions (neutral pH and temperature). Enzymatic hydrolysates or oligopeptides obtained can decrease the molecular

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weight and enhance the functional properties of proteins. A large quantity of proteins in animals, eggs, Aves, fishes, shellfishes, algae, and plants are sources of bioactive peptides. In fact, these protein hydrolysates and peptides have health-related functionalities such as antioxidant function (Park *et al*., 2009; Sheih *et al*., 2009; Lee *et al*., 2010; Liu *et al*., 2010; Jayakumar *et al.*, 2011; Kumar *et al.*, 2011; Lee *et al*., 2011; Pownall *et al*., 2011; Yang *et al*., 2011; Zhang *et al*., 2011; Chen *et al*., 2012; Chen *et al*., 2012; Fan *et al*., 2012; Kumar *et al.*, 2012; Zhang *et al.*, 2012; Amza *et al*., 2013; Chai *et al*., 2013; Chi *et al*., 2014; Edgar *et al.*, 2013; Lapsongphon and Yongsawatdigul, 2013; Wu *et al.*, 2013) and high blood pressure reduction (Nakajuma *et al.*, 2009; Liu *et al*., 2010; Jakubczyk and Baraniak, 2013; Ketnawa and Rawdkuen, 2013a, 2013b; Lau *et al*., 2013; Sila *et al*., 2013; Wu *et al.*, 2013; Zambrowicz *et al.*, 2013; Zeng *et al.*, 2013; Alashi *et al.*, 2014). Among enzymatic hydrolysates, it is also shown to possess combined antioxidative activity and antihypertensive activitiy (Samaranayaka *et al*., 2010; Alemán *et al*., 2011).

Many species of wild animals such as Yezo sika deer (*Cervus nippon yesoensis*), Hokkaido brown bear (*Vrsus arctos yesoensis*), Hokkaido squirrel (*Sciurus vulgaris orientis*), and Schrenks red fox (*Vulpes vulpes schrencki*) lives in the eastern part of Hokkaido, Japan. Particularly, Shiretoko, in the east Hokkaido, have been recorded as one of the World Heritage by United Nations Educational, Scientific and Cultural Organization (UNESCO) in July 15, 2005. While we have no active from the standpoint of nature conservation, the quantity of Yezo sika deer explosively increased and now it is known to live about six hundred of Yezo sika deer in this area. As a result, it is increasing not only any damage to the crops and forest, but also traffic accident to people. To solve the problem as quick as possible, it is necessary to use Yezo sika deer as one of the utilized bioresources to human life. At present, many of the skins, bone, and tendon are not used and disposed as waste materials, although some companies process and sell the meat of Yezo sika deer. Therefore, it is deeply desired to highly utilize these bioresources that have not been utilized that much in human health applications. The focus of this study was to investigate the antioxidative activities and antihypertensive activities of collagen hydrolysates from Yezo sika deer tendon as bioresource by proteolytic digestion model system using gastric and intestinal proteases: these results may establish their potential effects on health promotion and disease prevention against reactive oxygen species-mediated oxidative stress and hypertension.

Materials and Methods

Sample and chemicals

Tendon from Yezo sika deer was obtained from Hokusen Development Co. Ltd., Kushiro, Hokkaido, Japan. The tendon was stored at -30°C until used. Pepsin from porcine stomach mucosa (EC 3.4.23.1; 2 x crystallized; 3,085 U/mg protein) was from Sigma, USA. ACE from bovine lung (1U), 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH), bovine serum albumin (BSA), α-chymotrypsin from bovine pancreas (EC 3.4.21.1; 1,000 usp chymotrypsin units/mg), 2-deoxy-D-ribose, ethylenediaminetetraacetic acid disodium salt (EDTA), 2,6-di-*t*-butyl-4-methylphenol (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethyl acetate for spectrochemical analysis grade, hippuryl-L-histidyl-L-leucine as substrate peptide, linoleic acid, nitroblue tetrazolium salt (NBT), papain (EC 3.4.22.2; digestive powder; 1:350), thermolysin from *Bacillus thermoprotealyticus* Rokko (EC 3.4.24.4; 7,000 PU/mg), α-tocopherol, trypsin from porcine pancreas crystallized (EC 3.4.21.4; 4,500 USP trypsin U/mg protein), and xanthine were from Wako Chemicals Co. Ltd. (Osaka, Japan). *tert*-Butyl-4-hydroxyanisole (BHA) was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Pronase E from *Streptomyces griseus* (1,000,000 tyrosine U/g) was from Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan). Xanthine oxidase from butter milk (XOD; 0.33U/mg powder) was from Oriental Yeast Co. Ltd. (Tokyo, Japan). All other chemicals were of regent grade.

Preparation of collagen from Yezo sika deer tendon

All the preparative procedures were carried out at 4°C. The frozen tendon was thawed at half and cut into small pieces (0.5 x 1.0 cm) by a scalpel (D-4: As One Co. Ltd., Osaka, Japan). The pieces were extracted with 10 volumes of 10% ethanol to remove fat for 3 days by changing the solution once a day. The resultant matter was washed with distilled water for a day by changing the solution. The matter was extracted with 0.1 M NaOH to remove noncollagenous proteins for 2 days by changing the solution twice a day. The resultant matter was washed with distilled water for 2 days by changing the solution three time a day and lyophilized. The dried matter was extracted with 0.5 M acetic acid for 3 days. The extract was centrifuged at 50,000 x g for 1 h. The supernatants were pooled and salted out to purify the collagen by adding NaCl to a final concentration of 0.7 M and followed by precipitation of the collagen by the addition of NaCl

at a final concentration of 2.2 M at a neutral pH in 0.05 M Tris-HCl (pH 7.5). The resultant precipitate was obtained by centrifugation at 50,000 x g for 1 h, and dissolved in a minimum volume of 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, distilled water, and then lyophilized (acid-soluble collagen: ASC). The residue produced by the extraction of acid-soluble collagen was washed with distilled water for a day twice a day, suspended in 0.5 M acetic acid, and was digested with 1.0% (w/w) pepsin (EC 3.4.23.1; 2 x crystallized; 3,085 U/mg protein, Sigma-Aldrich Co., USA) by continuous stirring for 2 days. After centrifugation at 50,000 x g for 1 h, the supernatants were pooled and dialyzed against 0.02 M Na_2HPO_4 (pH 7.2) for 3 days with the change of solution twice a day to inactivate the pepsin. The precipitate collected by centrifugation in the same conditions was dissolved in 0.5 M acetic acid and salted out by selective salt precipitation at above conditions. The resultant precipitate was obtained by centrifugation at 50,000 x g for 1 h, dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid for a day, distilled water for 3 days by changing the solution twice a day, and then lyophilized (pepsin-solubilized collagen: PSC).

Preparation of PSC hydrolysates from Yezo sika deer tendon

Pepsin digestion

PSC was added and suspended in distilled water and the suspension was boiled for 10 min. After the pH of the suspension was adjusted at 2.0 using HCl, the digestion was started by the addition of 0.1% (w/w) pepsin at 37°C for 2 days. The hydrolysis stopped by boiling for 10 min to inactivate the enzyme. The hydrolysate was centrifuged at 50,000 x g at 20°C for 30 min, and then the supernatants were pooled, adjusted pH at 7.0 using NaOH, and lyophilized (pepsin peptide).

Trypsin digestion

PSC was added and suspended in distilled water and the suspension was boiled for 10 min. After the pH of the suspension was adjusted at 7.5 using NaOH, the digestion was started by adding 0.1% (w/w) trypsin at 37°C for 2 days. The hydrolysis stopped by boiling for 10 min, and then the hydrolysate was centrifuged at 50,000 x g at 20°C for 30 min to remove the residue. The supernatants were collected and then lyophilized (trypsin peptide).

Papain digestion

PSC was added and suspended in distilled water and the suspension was boiled for 10 min. The pH of the suspension was adjusted at 7.0 using NaOH and the digestion was started by addition of 0.1% (w/w) papain at 37°C for 2 days. The hydrolysis stopped by boiling for 10 min and the hydrolysate was centrifuged at 50,000 x g at 20°C for 30 min. The supernatants were collected and then lyophilized (papain peptide).

Chymotrypsin digestion

PSC was added and suspended in distilled water and then the suspension was boiled for 10 min. After the pH of the suspension was adjusted at 7.9 using NaOH, the digestion was started by the addition of 0.1% (w/w) chymotrypsin at 37°C for 2 days. The hydrolysis stopped by boiling for 10 min, and then the hydrolysate was centrifuged at 50,000 x g at 20°C for 30 min. The supernatants were pooled, and then lyophilized (chymotrypsin peptide).

Pronase E digestion

PSC was added and suspended in distilled water, and the pH of the suspension was adjusted at 7.0 using NaOH. After the digestion was done adding 0.1% (w/w) pronase E at 37°C for 2 days, the hydrolysis stopped by boiling for 10 min. The hydrolysate was centrifuged at 50,000 x g at 20°C for 30 min, and then the supernatants were collected, and lyophilized (pronase E peptide).

Thermolysin digestion

PSC was added and suspended in distilled water, and the pH of the suspension was adjusted at 7.5 using NaOH. The digestion was started by the addition of 0.1% (w/w) thermolysin at 37°C for 2 days, and then the hydrolysis stopped by boiling for 10 min. The supernatants were collected by the centrifugation at 50,000 x g at 20°C for 30 min, and then lyophilized (thermolysin peptide).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described previously (Nagai and Nagashima, 2006).

Ultraviolet absorption spectrum analysis of PSC

The ultraviolet absorption spectrum of PSC from Yezo sika deer tendon was recorded by a PerkinElmer model Lambda 11 (PerkinElmer, Tokyo, Japan) UV/VIS spectrometer. PSC was dissolved in 0.5 M acetic acid and the viscous solution was centrifuged at 50,000 x g at 4°C for 30 min to remove the undissolved collagen fibre. The supernatants were used in the analysis.

Functional properties of PSC hydrolysates from Yezo sika deer tendon

Antioxidative activity

The antioxidative activities of PSC hydrolysates were measured in a linoleic acid oxidation system described by Nagai and Nagashima (2006). BHA (0.01, 0.1, and 1.0 mM), BHT (0.01, 0.1, and 1.0 mM), trolox (0.01, 0.1, and 1.0 mM), ascorbic acid (1 and 5 mM), and α-tocopherol (1 mM) were used as positive control. Distilled water was used as negative control.

Radical scavenging activity

The superoxide anion radicals, hydroxyl radicals, and DPPH radicals scavenging activities of PSC hydrolysates were evaluated as described by Nagai and Nagashima (2006). Positive and negative controls were used as the same as above. The IC_{50} value was defined as the concentration of hydrolysates required to inhibit 50% of these activities.

ACE-inhibitory activity

The ACE inhibitory activities of PSC hydrolysates were performed as described by Nagai and Nagashima (2006). The IC_{50} value was defined as the concentration of hydrolysates required to inhibit 50% of the ACE activity.

Statistical analysis

Each assay was repeated 3 times independently and the results were reported as means ± standard deviation.

Results

Preparation and purification of collagen from Yezo sika deer tendon

The tendon was treated with cold ethanol to remove fat and continuously was with NaOH to remove noncollagenous proteins. The resultant matter was washed with cold distilled water and was extracted with cold 0.5 M acetic acid for 3 days to obtain acid-soluble collagen. The yield of the lyophilized ASC powder was very low about 0.08% (w/w) on a dry weight basis of lyophilized tendon. The residue was washed with cold distilled water and then was extracted with cold 0.5 M acetic acid containing 1.0% (w/w) pepsin for 2 days with gentle stirring. As a result, a viscous solution was obtained and it was salted out by selective salt precipitation to purify the collagen. PSC was effectively obtained and its yield was high about 35.7% (w/w) on a dry weight basis. The results suggested that most of the collagen was rigidly bound to the connective tissue and therefore was not easily solubilized with acetic acid.

Table 1. Yields and its protein contents of PSC hydrolysates derived from Yezo sika deer tendon

Enzyme	Yield	Protein (μg/mg powder)
Pepsin	96	245.9±4.3
Trypsin	76	229.7±3.7
Papain	81	185.7±2.1
Chymotrypsin	75	234.7±3.6
Pronase E	69	204.6±2.5
Thermolysin	82	260.6±4.4

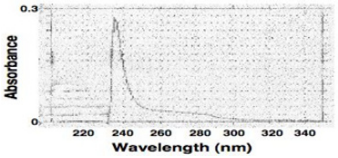

Figure 1. Ultraviolet absorption spectrum of PSC derived from Yezo sika deer tendon

To confirm the purity of ASC and PSC, SDS-PAGE was performed using 7.5% gel. Two distinct α-chains corresponding to α1 and α2 chain and a small amount of β-chain and γ-chain were detected (data not shown). In comparison with the collagen from porcine skins, these collagens from Yezo sika deer tendon may have a chain composition of $(\alpha1)_2\alpha2$ heterotrimer similar to that of the collagen such as porcine skins.

The ultraviolet absorption spectrum of PSC from Yezo sika deer tendon was measured. The distinct absorption was obtained near 235.2 nm and no obvious absorption was detected at near 280 nm because of not existence of tryptophan residue in PSC (Figure 1). The absorption bands between 250 nm and 290 nm were not shown for low contents of phenylalanine and tyrosine. These were in accordance with the characteristics absorption of collagen. Li *et al.* (2004) reported that the collagen from the skins of bullfrog has absorption near 236 nm. Moreover, other researchers reported the maximum absorptions of the collagens from the skins as follows: channel catfish; 232 nm (Liu *et al*., 2007) and largefin longbarbel catfish; 233 nm (Zhang *et al*., 2009). From the above investigation, it was suggests that the purified PSC was obtained from Yezo sika deer tendon.

Functional properties of PSC hydrolysates from Yezo sika deer tendon

Hydrolysates were prepared from PSC using six proteases such as pepsin, trypsin, papain, chymotrypsin, pronase E, and thermolysin. As a result, these hydrolysates were successfully prepared and these yields were very high, ranging from 69 to 96% (w/w) on the basis of lyophilized PSC (Table 1). The protein contents of these hydrolysates ranged from 185.7 to 260.6 μg/mg lyophilized peptide powder (Table 1).

The antioxidative activities of PSC hydrolysates from Yezo sika deer tendon were measured for 200

Table 2. Antioxidative activities of PSC hydrolysates derived from Yezo sika deer tendon

Sample species	Absorbance (500 nm) Time (min) 50	100	200
Pepsin (mg/ml)			
1.0	0.242±0.010	0.357±0.020	0.693±0.036
10	0.172±0.008	0.346±0.018	0.473±0.032
100	0.113±0.006	0.241±0.012	0.322±0.019
Trypsin (mg/ml)			
1.0	0.203±0.009	0.389±0.022	0.522±0.030
10	0.178±0.009	0.355±0.020	0.373±0.024
100	0.136±0.007	0.261±0.015	0.368±0.022
Papain (mg/ml)			
1.0	0.326±0.017	0.426±0.028	0.615±0.033
10	0.215±0.014	0.409±0.025	0.553±0.030
100	0.153±0.007	0.298±0.015	0.457±0.028
Chymotrypsin (mg/ml)			
1.0	0.271±0.009	0.382±0.020	0.518±0.027
10	0.186±0.010	0.343±0.018	0.512±0.028
100	0.099±0.008	0.199±0.013	0.335±0.017
Pronase E (mg/ml)			
1.0	0.166±0.011	0.395±0.024	0.483±0.035
10	0.165±0.010	0.327±0.015	0.481±0.033
100	0.116±0.007	0.216±0.011	0.346±0.019
Thermolysin (mg/ml)			
1.0	0.271±0.012	0.430±0.030	0.570±0.032
10	0.176±0.009	0.353±0.022	0.525±0.030
100	0.170±0.008	0.310±0.018	0.473±0.029
BHA (mM)			
0.01	0.084±0.005	0.120±0.008	0.245±0.012
0.1	0.056±0.003	0.090±0.006	0.165±0.010
1.0	0.054±0.002	0.057±0.003	0.100±0.006
BHT (mM)			
0.01	0.082±0.003	0.112±0.009	0.248±0.011
0.1	0.058±0.004	0.108±0.005	0.173±0.008
1.0	0.044±0.002	0.051±0.003	0.093±0.005
Trolox (mM)			
0.01	0.084±0.005	0.094±0.006	0.262±0.013
0.1	0.038±0.002	0.051±0.003	0.123±0.008
1.0	0.011±0.001	0.031±0.002	0.032±0.002
Ascorbic acid (mM)			
1.0	0.022±0.001	0.135±0.006	0.469±0.027
5.0	0.016±0.001	0.032±0.003	0.090±0.008
α-Tocopherol (mM)			
1.0	0.006	0.025±0.001	0.028±0.002
Control	0.379±0.008	0.715±0.025	1.406±0.041

Table 3. Superoxide anion radical, hydroxyl radical, and DPPH radical scavenging activities of PSC hydrolysates derived from Yezo sika deer tendon

Sample species	Scavenging activity (%) Superoxide anion radical	Hydroxyl radical	DPPH radical
Pepsin (mg/ml)			
1.0	28.3±1.67	3.3±0.03	0.0
10	43.7±3.12	5.1±0.07	0.0
100	65.0±3.89	51.6±2.78	1.0
Trypsin (mg/ml)			
1.0	41.3±2.91	0.0	0.0
10	63.5±3.50	12.9±0.15	0.0
100	89.9±4.67	69.2±3.44	35.6±1.78
Papain (mg/ml)			
1.0	79.7±4.11	1.4±0.02	0.0
10	93.8±4.64	9.9±0.09	1.6±0.02
100	>100.0	53.1±2.85	8.1±0.09
Chymotrypsin (mg/ml)			
1.0	41.4±2.95	0.0	0.0
10	59.7±3.83	10.6±0.12	8.9±0.10
100	83.4±4.24	68.7±3.50	40.2±1.95
Pronase E (mg/ml)			
1.0	71.3±3.78	9.3±0.11	1.4±0.03
10	90.2±4.04	16.4±0.14	4.8±0.07
100	>100.0	67.1±3.39	40.6±1.89
Thermolysin (mg/ml)			
1.0	66.1±3.79	0.0	0.0
10	86.4±4.10	15.6±0.12	3.1±0.04
100	>100.0	63.6±3.37	10.2±0.12
BHA (mM)			
0.01	29.3±0.52	59.1±0.78	5.5±0.04
0.1	36.4±0.91	93.3±1.39	17.5±0.36
1.0	51.9±1.36	95.2±1.44	72.7±3.58
BHT (mM)			
0.01	11.7±0.19	82.8±0.91	3.9±0.03
0.1	46.6±1.02	97.6±1.55	7.9±0.08
1.0	48.4±1.17	>100.0	31.7±0.76
Trolox (mM)			
0.01	46.4±0.98	81.5±0.63	0.1±0.01
0.1	58.1±1.12	91.8±1.17	17.9±0.20
1.0	76.1±1.89	>100.0	86.3±3.27
Ascorbic acid (mM)			
1.0	14.7±0.20	13.2±0.21	3.1±0.04*
5.0	89.9±5.31	17.6±0.71	34.1±2.01**
α-Tocopherol (mM)			
1.0	52.6±4.18	67.6±4.34	87.6±2.75

*0.1 mM ascorbic acid; **1.0 mM ascorbic acid

min to evaluate the inhibition effects at the initiation stage of linoleic acid peroxidation. The antioxidative activities of these hydrolysates increased with increasing the hydrolysate concentration, although the activity of each hydrolysate decreased with the passage of time (Table 2). The hydrolysates for 1 and 10 mg/ml showed low antioxidative activities: these activities were lower than those of 0.01 mM BHA, BHT, and trolox and 1 mM ascorbic acid. On the other hand, the hydrolysates for 100 mg/ml possessed the same or slightly higher activities than 1 mM ascorbic acid. Among them, hydrolysates prepared by pepsin, trypsin, chymotrypsin, and pronase E had higher activities than those by papain and thermolysin. Particularly, the activities after the reaction for 200 min were the following order from high to low; pepsin > chymotrypsin > pronase E > trypsin > papain > thermolysin. The antioxidative activities of enzymatic hydrolysates from protein sources are influenced by source of protein, enzyme specificity, the degree of hydrolysis, the structure and molecular weight, and amino acid composition. It is known, particularly, that amino acids such as cysteine, histidine, leucine, phenylalanine, tryptophan, and tyrosine enhance to the antioxidative activities and redical scavenging activities of food-derived peptides (Erdmann *et al*., 2008; Pownall *et al.*, 2010). Moreover, amino group and carboxylic group in branches of acidic amino acid as glutamic acid and aspartic acid and basic amino acids as arginine, histidine, and lysine contribute to increase metal chelating acticvity (Liu *et al*., 2010). It suggested that hydrolysates having higher antioxidative activities might be obtained by combination of some enzymes treatment.

Superoxide anion radical scavenging activities of PSC hydrolysates from Yezo sika deer tendon were investigated by xanthine/xanthine oxidase system. Each hydrolysate showed high scavenging activity. The activities of 1mg/ml pepsin, trypsin, and chymotrypsin hydrolysates were much higher than those of 0.01 mM BHA and BHT, and 1 mM ascorbic acid (Table 3). Papain, pronase E, and thermolysin hydrolysates exhibited remarkable higher activities than antioxidants except for 5 mM ascorbic acid and 1 mM α-tocopherol. For 10 mg/ml, the activities of hydrolysates by papain, pronase E, and thermolysin were the same or higher than that of 5 mM ascorbic acid. Moreover, these hydrolysates for 100 mg/ml perfectly scavenged superoxide anion radical (Table 3). The activities for 100 mg/ml hydrolysates were in the following order from high to low; papain = pronase E = thermolysin > trypsin > chymotrypsin > pepsin. Papain hydrolyzes the peptide bonds with basic amino acids such as arginine, glutamic acid, glycine, histidine, lysine, and tyrosine residues.

Table 4. The IC_{50} values of PSC hydrolysates derived from Yezo sika deer tendon against the activities of superoxide anion radical, hydroxyl radical, DPPH radical and ACE

Species	IC_{50} value (mg protein/ml)			
	Superoxide anion radical	Hydroxyl radical	DPPH radical	ACE
Pepsin	15.4	22.3	N.D.	3.49
Trypsin	6.87	16.3	31.4	5.02
Papain	0.45	17.4	N.D.	1.31
Chymotrypain	8.17	16.9	29.2	0.65
Pronase E	0.62	14.6	25.3	2.07
Thermolysin	0.92	20.0	49.3	0.99

Pronase E has a wide range of substrate specificities because of the mixture of many kinds of proteases. It suggests that large quantities of peptides are produced by papain and pronase E hydrolysis contributes to the superoxide anion radical scavenging activities. These tendencies are consistent with the reports by Liu *et al.* (2010) and Pownall *et al.* (2010). On the other hand, the IC_{50} values of PSC hydrolysates from Yezo sika deer tendon against superoxide anion radicals were calculated and ranged from 0.45 to 15.4 mg protein/ml (Table 4).

Hydroxyl radical scavenging activities of PSC hydrolysates from Yezo sika deer tendon were determined. The hydrolysates for 1 mg/ml hardly showed the scavenging activities (Table 3) except for pepsin and pronase E. The activities for 10 mg/ml except for pepsin hydrolysate were similar to that of ascorbic acid. For 100 mg/ml, the activities were similar or higher than that of 1 mM α-tocopherol. It would be inferred from the results that increasing the concentration of hydrolysates in the reaction mixture would ultimately raise the potency to scavenge hydroxyl radicals; hence an increase in the antioxidative properties. It was shown, however, the difference of these activities among enzyme species tested. The IC_{50} values of PSC hydrolysates against hydroxyl radicals were 14.6 to 22.3 mg protein/ml (Table 4).

The relatively stable DPPH radical is used to investigate the ability of compounds to act as free radical scavengers and to test the antioxidative activity. DPPH radical scavenging activities of PSC hydrolysates from Yezo sika deer tendon were measured and were compared with those of antioxidants. As a result, the hydrolysates for 1 and 10 mg/ml had no activities or lower ones; the activities were similar to those of 0.01 mM BHA and BHT and 1.0 mM ascorbic acid (Table 3). On the other hand, the activities for 100 mg/ml trypsin, chymotrypsin, and pronase E hydrolysates were higher than those of 1 mM ascorbic acid and BHT, but were lower than those of 1 mM BHA, α-tocopherol, and trolox. Chymotrypsin cleavages the peptide bonds of C terminus of hydrophobic amino acids such as alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, trypyophan, and valine. The peptides having these amino acid residues may strongly scavenge DPPH radical. In fact, Pownall *et al.* (2010) reported the increase of antioxidant activities by amino acid hydrophobicity. Nevertheless, it could not expect the activity higher than 1 mM α-tocopherol for 100 mg/ml hydrolysates. The IC_{50} values of PSC hydrolysates against DPPH radicals were as follows: 31.4 mg protein/ml (trypsin), 29.2 mg protein/ml (chymotrypsin), 25.3 mg protein/ml (pronase E), and 49.3 mg protein/ml (thermolysin), respectively (Table 4).

Table 5. ACE inhibitory activities of PSC hydrolysates derived from Yezo sika deer tendon

Species	Activity (%)
Pepsin (mg/ml)	
1.0	33.0±0.85
10	37.9±1.01
100	> 100
Trypsin (mg/ml)	
1.0	57.6±1.35
10	59.1±1.31
100	98.5±1.01
Papain (mg/ml)	
1.0	66.0±1.47
10	82.3±1.53
100	91.6±1.12
Chymotrypsin (mg/ml)	
1.0	77.8±1.02
10	80.8±1.18
100	83.7±1.21
Pronase E (mg/ml)	
1.0	71.4±0.99
10	73.4±1.10
100	83.7±1.17
Thermolysin (mg/ml)	
1.0	71.4±1.02
10	76.8±1.09
100	> 100

Hypertension is a significant public problem worldwide. Therefore, finding new sources of ACE inhibitors, especially in food resources, is of great interest. ACE inhibitory activities of PSC hydrolysates from Yezo sika deer tendon were investigated and are shown in Table 5. Each hydrolysate possessed high activity and the activities increased with increasing the hydrolysate concentration (Table 5). Except for pepsin hydrolysate, others had fairly higher activities about 58-78% for 1 mg/ml and about 59-82% for 10 mg/ml. For 100 mg/ml, hydrolysates by chymotrypsin and pronase E showed the activities about 84%. Besides, hydrolysates by trypsin and papain almost perfectly inhibited ACE activity. On the other hand, pepsin and thermolysin hydrolysates perfectly inhibited the activity (Table 5). That is, the inhibitory activities for 100 mg/ml were in the following order from high to low; pepsin = thermolysin > trypsin > papain > chymotrypsin = pronase E. It suggested that many species of active oligopeptides inhibited ACE activity were produced by pepsin and thermolysin having broad substrate specificities. In other words, pepsin and thermolysin were good enzymes that could prepare the hydrolysates having high ACE inhibitory activities from Yezo sika deer tendon PSC. On the

other hand, the IC_{50} values of PSC hydrolysates against ACE were calculated as the range of 0.65 to 5.02 mg protein/ml (Table 4).

Discussion

Li *et al.* (2007) prepared collagen hydrolysates using different proteases such as pepsin, papain, protease from bovine pancreas (PP), protease from *Streptomyces* (PS), and protease from *Bicillus polymyxa* (PB) and investigated these antioxidative activities in a linoleic acid peroxidation system and on DPPH radicals and metal chelating. They further hydrolyzed pepsin hydrolysate using papain, PP, and mixture of PP, PS, and PB to obtain smaller peptides than large peptides and protein. In a linoleic acid peroxidation tests for 4 days, the more the hydrolysis by enzymes and the degree of hydrolysis are high, the more the inhibitory activities against oxidation were high, although these activities were less than that of BHT. Next, DPPH radical scavenging activities were as follows: pepsin (13.4%), papain (20.5%), PP (27.0%), and mixture (87.2%), respectively. These results indicated the same pattern as those of linoleic acid peroxidation tests. The activities of porcine collagen hydrolysates were higher in comparison with those of Yezo sika deer tendon. On the other hand, ferrous ion chelating potential were also tested. These activities were the range of 9.5-37.4%.

Many researchers are progressing the studies on functionalities of food resources related to functional foods and nutraceuticals to improve the human health status. Among them, there are many reports about antioxidative activities and antihypertensive activities. Recently, large quantities of peptides and components as antioxidants were discovered in plants (Park *et al*., 2010; Jayakumar *et al*., 2011; Pownall *et al.*, 2011; Yang *et al*., 2011; Zhang *et al*., 2011; Edgar *et al.*, 2013; Lapsongphon and Yongsawatdigul, 2013), animal (Liu *et al.*, 2010), Aves (Lee *et al*., 2010), egg white (Chen *et al*., 2012; Chen *et al.*, 2012), fishes (Lee *et al.*, 2011; Kumar *et al*., 2011; Fan *et al*., 2012; Kumar *et al*., 2012; Chai *et al*., 2013; Chi *et al.*, 2014), shellfish (Wu *et al*., 2013), algae (Sheih *et al.*, 2009). Enzymatic hydrolysates and peptides derived from food resources also possesses ACE inhibitory activities or blood pressure lowering effects as follows: plants (Jakubczyk and Baraniak, 2013; Lau *et al.*, 2013; Zeng *et al*., 2013; Alashi *et al.*, 2014), egg white (Liu *et al.*, 2010; Zambrowicz *et al.*, 2013), fishes (Ketnawa and Rawdkuen, 2013a, 2013b; Nakajima *et al.*, 2009; Sila *et al*., 2013; Wu *et al*., 2013).

Collagen is converted into gelatin when heated above 40°C. The functional researches to fish and shellfish gelatin are aggressively performed. Zhang *et al*. (2012) prepared enzymatic hydrolysates of tilapia (*Oreochromis niloticus*) skin gelatin by properase E and multifect neutral and investigated the antioxidative activities against superoxide anion radical, hydroxyl radical, and DPPH radical. The activities against which radicals were dependent on the hydrolysate concentration used, although the activities in gelatin were higher than those in gelatin hydrolysates. At the same time, the degree of hydrolysis and hydroxyl radical scavenging activities of the hydrolysates from different enzyme treatments was determined. The degree of hydrolysis in any treatment was low, 12.6-22.1%. However, hydroxyl radical scavenging activity strongly increased to 99.2%, when it had changed in the order of enzyme treatment (it was hydrolyzed with multifect neutral and then properase E). The IC_{50} values of gelatin and its hydrolysate against radicals were as follows: 0.27 and 0.56 mg/ml (superoxide anion radical), 0.74 mg/ml (gelatin hydrolysate against hydroxyl radical), and 0.69 and 3.66 mg/ml (DPPH radical), respectively. You *et al.* (2010) reported the IC_{50} value of loach (*Misgurnus anguillicaudatus*) protein hydrolysate against DPPH radical was 2.64 mg/ml.

Jellyfish umbrella is rich in collagenous protein. Gelatin polypeptides of jellyfish (*Rhopilema esculentum*) umbrella were prepared by trypsin and properase E and the mixture of these enzymes, and the antioxidative activities were investigated *in vitro* (Zhuang *et al*., 2010). The degrees of hydrolysis of two enzymatic processes were 14.3 and 14.5%, respectively. Moreover, double-enzyme hydrolysis could improve the degree of hydrolysis to 20.2-27.8%. These hydrolysates exhibited high scavenging activities about 30.3-52.8% against superoxide anion radical and about 66.5-94.2% against hydroxyl radical.

Giménez *et al.* (2009) were evaluated the antioxidant properties of gelatin hydrolysates obtained from skin of sole and squid by alcalase treatment. The hydrolysis of these gelatins resulted in ~35% and ~50% degree of hydrolysis, respectively. The chelating ability of gelatins at 25 mg/ml assay concentration was low below 5%. High chelating activities were, however, detected in each hydrolysate at only 0.2 mg/ml above 80%. Hydrolysates were tested by ABTS assay. Each hydrolysate possessed the activity approximately 8- (sole) and 10-fold (squid) higher than the corresponding gelatins. Moreover, ferric iron reducing activities of hydrolysates increased 2-fold compared with gelatins. Alemán *et al.* (2011) reported antihypertensive activities of hydrolysates

from giant squid (*Dosidicu gigas*) gelatin produced by seven proteases (protamex, trypsin, neutrase, savinase, NS37005, esperase, and alcalase). As a result, it was obtained the most potential ACE inhibitor (IC_{50} = 0.34 mg/ml) by alcalase. It is expected the production of potential hydrolysates to act as antioxidants and/or as inhibitors of ACE from food resources by proteolytic enzyme treatments. Collagen is the most primary protein of animal origin and comprised approximately 30% of total animal protein. Gelatin is converted from collagen by heat treatment above 40°C is applied additive to enhance and change water-holding capacity, stability, texture in food and biomaterial industries. In the present study, hydrolysates derived from Yezo sika deer tendon collagen have shown potent antioxidative activities and antihypertensive activities in different oxidative systems and proven to act as potent antioxidants and ACE inhibitors. From the investigation by many researchers (Li *et al*., 2007; Giménez *et al.*, 2009; You *et al*., 2010; Zhuang *et al*., 2010; Zhang *et al*., 2012), it found use double-enzyme hydrolysis could improve and increase functionalities such as antioxidant and antihypertensive capacities. It needs to investigate the optimal conditions to obtain enzymatic hydrolysates and detailed functional properties to utilize Yezo sika deer tendon as an underutilized bioresource.

Conclusions

PSC hydrolysates derived from Yezo sika deer tendon have higher antioxidative activities, scavenging activities against active oxygen species, and antihypertensive activities by proteolytic digestion using some proteases in the body. Yezo sika deer tendon can be used as an easily accessible underutilized bioresource of natural antioxidants and as a possible food supplement or in biomedical fields and pharmaceutical industry.

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