Korean J. Food Sci. An.
Vol. 34, No. 3, pp. 325~332(2014)
DOI http://dx.doi.org/10.5851/kosfa.2014.34.3.325

ARTICLE

Porcine Splenic Hydrolysate has Antioxidant Activity *in vivo* and *in vitro*

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Abstract

The antioxidant capacity of porcine splenic hydrolysate (PSH) was studied *in vitro* and *in vivo*. Peptide hydrolysates were prepared, using the proteolytic enzyme Alcalase®. The molecular weights of PSH were 37,666, 10,673, 6,029, and 2,918 g/mol. Rats were fed a 5% (w/v) PSH diet, instead of a casein diet, for 4 wk. The food intake, body weight gain, and liver weight of rats in the PSH group were similar to those in the control (CONT) group. There were no differences in the serum total cholesterol, triglyceride, total protein, or albumin levels between PSH and CONT groups. However, the level of *in vivo* hepatic lipid peroxidation in PSH group was significantly lower than that in CONT. *In vivo* hepatic catalase and glutathione peroxidase activities in the PSH group were significantly higher than those in the control group. The *in vitro* protein digestibility of PSH was lower than that of casein. The *in vitro* trolox equivalent antioxidant capacity of PSH was significantly higher than that of the peptide hydrolysate from casein. The *in vitro* radical scavenging activities of PSH were significantly higher than those of the peptide hydrolysate from casein. The present findings suggest that porcine splenic peptides improve the antioxidant status in rats by enhancing hepatic catalase and GSH-Px activities, and indicate a potential mechanism of radical scavenging activity during gastrointestinal passage.

Key words: porcine splenic hydrolysate, antioxidant, radical scavenging capacity, protein digestibility

Introduction

Dietary protein is one of the main energy sources and becomes a physiologically active component via the gastrointestinal tract. However, the antihypertensive, antioxidant, anticarcinogenic, and hypolipidemic properties of some proteins and peptides from plant and marine sources have been also studied (Di Bernardini *et al*., 2012; Latham 1999). Indeed, a few research investigations have focused on some peptides from milk casein, egg proteins, and animal muscle due to their antioxidant activities (Dávalos *et al*., 2004; Rival *et al*., 2001; Saiga *et al*., 2003). The antioxidant mechanism seems to be related to metal chelation, free radical scavenging, hydroperoxide reduction, and aldehyde adduction (Wang and Kurtz 2000). For example, casein-derived peptides have been shown to be capable of inhibiting enzymatic and non-enzymatic lipid peroxidation (Rival *et al*., 2001). An egg white hydrolysate has been also demonstrated to inhibit low density lipoprotein oxidation-induced copper ions and to quench radicals (Dávalos *et al*., 2004). Furthermore, Di Bernardini *et al*. (2012) reported that peptide hydrolysates of beef brisket sarcoplasmic proteins produced to scavenge DPPH radicals and chelate metal ions.

Recently, there is need for disposal of undesirable animal byproducts associated with the carbon footprint worldwide. The hydrolytic processing of animal proteins to produce new bioactive peptides might enable key question to be resolved effectively. The production might promote environmental sustainability as well as human health (Udenigwe and Howard 2012). In fact, an oligopeptide purified from porcine spleen (Polyerga®) was has been used for up-regulating immune responses in human subjects (Borghardt *et al*., 2000). Moreover, preparation of enzymatic hydrolysates of proteins from animal resources has been introduced into the food industry to develop antioxidant peptides (Elias *et al*., 2008; Udenigwe and Howard, 2012). It is suggested that success in obtaining new antioxidant peptides from animal byproducts could positively affect meat-based food preservation (Udenigwe and Howard, 2012) because the operating environment of

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meat processing is easily affected by prooxidative factors such as light exposure, thermal treatment, metal contamination, and lipids (Elias *et al*., 2008). To the best of our knowledge, however, the antioxidant activity of porcine splenic byproduct has not been investigated.

The objective of the present investigation was to examine the antioxidant activity of a hydrolysate from a porcine spleen compared to hydrolysate of casein using an *in vitro* system to examine radical scavenging activities. Furthermore, the antioxidant effect of the porcine splenic hydrolysate was evaluated in normal rats.

Materials and Methods

Preparation of porcine splenic hydrolysate

The hydrolysate was prepared using the proteolytic enzyme Alcalase® (Sigma-Aldrich, USA) (Dong *et al*., 2010), with slight modification. After slaughter, the fresh pig spleen was weighed, minced, and mixed with same volume of deionized water. The solution was adjusted to pH 8.0 and hydrolyzed using a protease at a 100 to 0.5 ratio (substrate/enzyme, v/v). The pH of the solution was constantly maintained during hydrolysis using 5 M NaOH at 55°C in a water bath for 18 h. After hydroxylation, the mixture was boiled at 90°C for 10 min to inactivate the protease, followed by filtration to remove insoluble materials. Then the porcine splenic extract was spray-dried to a powder and kept at 4°C until analysis. For comparison of the antioxidant activities *in vitro*, hydrated peptide from casein (from bovine milk, Sigma-Aldrich) was also prepared under similar experimental conditions except for being freeze-dried.

Determination of micronutrient and amino acid contents

Protein, fat, carbohydrate, moisture, and ash contents in the splenic hydrolysate were measured by AOAC methods (1990). Amino acid analyses were conducted using the method of Fujiwara *et al.* (1987). In brief, the hydrolysates was hydrolyzed in 6 M HCl at 110°C for 24 h, vacuum dried, reconstituted with 0.2 M HCl, and filtered with a 0.45 μm diameter filter (W-13-5; Tosoh, Japan). The composition of the hydrolyzed amino acids in the splenic hydrolysate was determined using an amino acid analyzer (Hitachi-8700, Hitachi, Japan).

Determination of the molecular weight (MW) of the hydrolysate

The molecular weights of splenic hydrolysates were determined by gel size exclusion chromatography. The hydrolysate at a concentration of 2 mg/mL in acetonitrile/water/trifluoroacetic acid (30/70/0.1, v/v/v) was filtered with a syringe filter (0.45 μm, Millipore) prior to injection. First, 50 μL of the filtrate was loaded into a Shimadzu gel permeation chromatography (GPC) system (Japan) equipped with an LC-20AD pump (Shimadzu, Japan), and a diode array UV detector (SPD-M20A, Shimadzu, Japan). The analytical column (Superdex Peptide 10/300 GL, 10×300 mm, 13 mm; GE Healthcare, USA) was eluted with acetonitrile/water/trifluoroacetic acid (30/70/0.1, v/v/v) at a flow rate of 0.5 mL/min under the maximum operating pressure of 18 kgf/cm^2 and monitored at 214 nm. The calibration curve of MW was obtained using the following standards: bovine serum albumin (MW 69,000, Sigma-Aldrich), aprotinin (MW 6,512, Sigma-Aldrich), insulin chain A (MW 2,531, Sigma-Aldrich), angiotensin 2 (MW 1,046, Sigma-Aldrich), tuftsin (MW 500, Peptide Institute, Japan), and triglycine (MW189, Peptide Institute). The MW of the peptide from the splenic hydrolysate was determined using GPC Software (LCsolution GPC Ver. 1.21, Shimadzu).

Trolox equivalent antioxidant capacity (TEAC)

The total antioxidant status of the hydrated peptides from casein and porcine spleen was determined using a commercial kit (NX 2332, Randox Laboratories Ltd., UK), based on the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) radical cation decolorization assay. Results were expressed as micromoles of Trolox per g of each sample.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging test

For stock solutions, each sample (100 mg) was dissolved in 10 mL of deionized water. Then 300 mL of the solution, which contained different concentrations of stock solution samples (0-3 mg/assay), was added to 900 mL of a mixture consisting of 400 mM DPPH (Wako, Japan) and 20% (v/v) ethanol in 0.2 M 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.0). The optical density (OD) of the solution was then measured at 520 nm using a spectrophotometer (UV-1600, Shimadzu) (Brand-Williams *et al*., 1995). Trolox (0.2 mM, Sigma-Aldrich) was used as the standard.

Superoxide dismutase (SOD)-like activity

The SOD-like activities of samples were investigated using a commercial kit (AB-2970 CLETA-S, ATTO, Japan). This method is based on the luminescence (LC)

generated by MPEC (ATTO) with the superoxide anions generated by the xanthine-xanthine oxidase system. LC was measured using a luminometer (AB-2350, ATTO).

$$\text{SOD-like activity (\%)} = [1 - (LC_{\text{sample positive}} - LC_{\text{sample negative}})/(LC_{\text{control positive}} - LC_{\text{control negative}})] \times 100,$$

where $LC_{\text{sample positive}}$ represents the total LC count of the sample solution and $LC_{\text{sample negative}}$ is that for the sample solution not containing xanthine oxidase. LC_{control} represents the total LC count of the control solution without any sample or enzyme.

Ferric thiocyanate test

Peroxyl radical scavenging activity of each sample was measured by the method of López-Alarcón and Lissi (2005). In brief, 100 mL of a 1% (w/v) hydrolysate sample was added to a solution of 15 mL of 600 mM 2,2'-azobis-(2-amidinopropane)dihydrochloride (AAPH) and 1 mL of 60 mM pyrogallol red (both from Sigma-Aldrich) in phosphate-buffered saline (PBS) buffer (pH 7.0) with 30% (v/v) ethanol. Then the mixture was incubated in a water bath at 37°C for 2 h, and the absorbance was measured at 540 nm. The radical scavenging activity was calculated as follows:

$$\text{Reduction (\%)} = 100 - [(OD_{\text{control}} - OD_{\text{control blank}})/(OD_{\text{sample}} - OD_{\text{sample blank}})] \times 100,$$

OD_{sample} represents the absorption of the sample solution and OD_{control} that for the control solution not containing the sample. Both OD_{blank} are for the blank solution not containing AAPH.

Chelation of ferrous ions

The chelation of ferrous ions by each sample was assayed according to the method of Dinis *et al*. (1994). In brief, a 1% (w/v) sample was mixed with 2 mM $FeCl_2$. The reaction was initiated by the addition of 5 mM ferrizine (Sigma-Aldrich). The mixture was shaken vigorously and left to stand for 10 min. The absorbance of the solution was then measured at 562 nm using a spectrophotometer.

$$\text{Inhibition (\%)} = [OD_{\text{control}} - (OD_{\text{sample}} - OD_{\text{blank}})]/OD_{\text{control}} \times 100,$$

where OD_{sample} represents the absorption of the sample solution, OD_{control} that for the control solution not containing the sample, and OD_{blank} is that for the blank solution not containing ferrizine.

Protein digestibility

The *in vitro* protein digestibility of the porcine splenic hydrolysate and the casein was evaluated by the pH-shift method using the multi-enzyme technique (AOAC, 1990). Based on Kjeldahl nitrogen at a concentration of 1 mg/mL in distilled water each sample was adjusted to pH 8.0 at 37°C in a water bath. Then a multi-enzyme mixture, freshly prepared using trypsin, α-chymotrypsin, and peptidase (all from Sigma-Aldrich), was added to the solution for 10 min at 37°C followed by bacterial protease (Sigma-Aldrich, USA) at 55°C for 9 min. Following this the temperature was changed back to 37°C for 1 min. Finally the reduction in pH of the sample from pH 8.0 was monitored after 20 min of incubation. The protein digestibility was calculated as follows:

$$\text{\% Digestibility} = 234.84 - 22.56\ (X),$$

where X = pH reduction at 20 min.

Animals and diets

Male F344/DuCrj rats (13 wk of age) were obtained from Charles River Japan (Japan). The animals were kept in plastic cages at a temperature of 23±1°C and a relative humidity of 60±5% with a 12 h light-dark cycle. All animal studies conformed to the principles of the NIH Guide for the Care and Use of Laboratory Animals. This experimental animal procedure was approved by the Animal Experiment Committee of Obihiro University of Agriculture and Veterinary Medicine. Rats were acclimated for 1 wk and given access to food and water *ad libitum*. Then the animals were randomly divided into two diet groups (n=5) with no significant difference in body weight at the start of the experiment. The control group (CONT) was fed a diet based on the AIN-93G semi-purified rodent diet containing 200 g/kg of casein, 100 g/kg of sucrose, 3.5 g/kg of mineral mixture, 1.0 g/kg of vitamin mixture, 549.5 g/kg of α-corn starch, 50 g/kg of cellulose, 3 g/kg of L-cystine, 70 g/kg of soybean oil, 2.5 g/kg of choline bitartrate, and 0.014 g/kg of *tert*-butyl hydroquinone for 4 wk. The treatment group was fed a diet containing 50 g/kg of porcine splenic hydrolysate (PSH) instead of casein for 4 wk. Body weight and food consumption were recorded weekly and daily, respectively. At the end of the experimental period, the rats were anesthetized with Nembutal

(sodium pentobarbital, 40 mg/kg of body weight; Abbott Laboratories, USA) and killed without fasting. The blood was drawn quickly and the biochemical parameters were determined using a Toshiba TBA-120FR autoanalyzer (Toshiba Medical Systems Corp., Japan). The livers were frozen (-80°C) in tubes before further analysis.

Hepatic malondialdehyde (MDA) and glutathione (GSH) levels, and antioxidant enzyme activities

The degree of oxidation was immediately measured by thiobarbituric acid reactive substances assay (Ohkawa *et al*., 1979). Liver samples were homogenized in 10 volumes of PBS (pH 7.4). Protein concentrations were determined by Lowry assay (Bio-Rad, CA). The hepatic GSH level was analyzed by the method of Cohn and Lye (1966). Hepatic catalase activity was analyzed by the method of Aebi (1984) based on measuring the rate of H_2O_2 depletion using a spectrophotometer at 240 nm. Glutathione peroxidase (GSH-Px) activity was analyzed by the method of Lawrence and Burk (1976). Glutathione reductase (GSH-R) and glutathione-*S*-transferase (GST) activities were analyzed by the methods of Worthington and Rosemeyer (1976) and Habig *et al*. (1974), respectively.

Statistics

The results reported here are the means of at least three measurements (*in vitro*) and the standard deviations (SD). Significant differences between two groups (*in vivo* and *in vitro*) were determined by Student's *t*-test. Analyses were performed using PASW Statistics 17.0 software (SPSS Institute, USA).

Results

Micronutrient and amino acid contents and MW

Protein, fat, carbohydrate, moisture, and ash contents in the splenic hydrolysate were as follows (g/100 g): protein (calculated by multiplying the nitrogen contents by 6.25), 79.9; fat, 0.1; carbohydrate, 4.1; moisture, 8.3; ash, 7.6. The amino acid composition in the splenic hydrolysate was similar to that of casein (Table 1), the data for which were obtained from the Food Composition Database in Japan (http://fooddb.jp/). However, Tyr, Pro and Glu levels in the splenic hydrolysate were lower than those in casein, whereas those of Ala, Gly, and Cys in PSH were higher than in casein. Fig. 1 shows the molecular weight distribution from high to intermediate molecular weights of the splenic hydrolysate, which indicated that PSH was successfully treated by enzymatic hydrolysis. The main

Table 1. Amino acid compositions of casein and porcine splenic hydrolysate

Amino acid	MW	Casein*		Splenic hydrolysate	
		g 100 g^{-1}	mol%	g 100 g^{-1}	mol%
Arg	174.2	3.30	2.67	3.64	3.62
Lys	146.2	7.10	6.85	5.89	6.99
His	155.2	2.70	2.45	2.23	2.49
Phe	165.2	4.50	3.84	3.23	3.39
Tyr	181.2	5.00	3.89	2.32	2.22
Leu	131.2	8.40	9.03	6.46	8.54
Ile	131.2	4.90	5.27	3.06	4.04
Met	131.2	2.60	2.79	1.57	2.07
Val	117.1	6.00	7.23	4.62	6.84
Ala	89.1	2.70	4.27	4.82	9.38
Gly	75.1	1.60	3.00	4.99	11.5
Pro	115.1	10.0	12.3	3.72	5.60
Glu	147.1	19.0	18.2	10.2	12.0
Ser	105.1	4.60	6.17	3.49	5.76
Thr	119.1	3.70	4.38	3.31	4.82
Asp	133.1	6.30	6.67	7.10	9.25
Trp	204.1	1.10	0.76	0.87	0.74
Cys	240.3	0.43	0.25	0.97	0.70
Total		93.9	100	72.5	100

*Data from Food Composition Database in Japan (see http://fooddb.jp/)

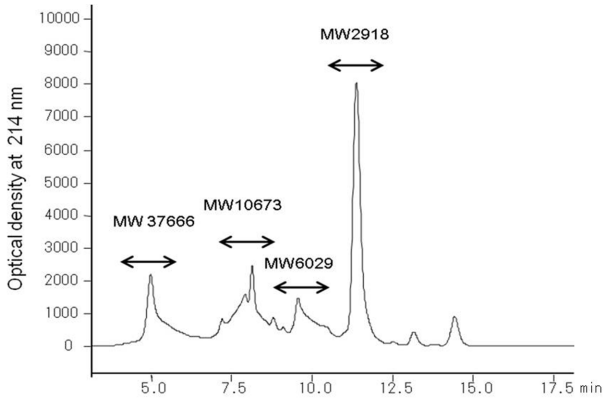

Fig. 1. The molecular weights of splenic hydrolysates. They were determined by gel size exclusion chromatography.

MWs obtained from the splenic hydrolysate were 37,666, 10,673, 6,029, and 2,918.

In vitro study

TEAC, radical scavenging activity, ferrous ion chelating activity and protein digestibility

The total antioxidant potential in each sample measured by Randox-TEAC is shown in Table 2. The antioxidant potential in splenic hydrolysate was higher than in casein hydrolysate. The Trolox equivalent of the splenic hydrolysate was approximately 3 μmol per g of powder, which

Table 2. Trolox equivalent antioxidant capacity (TEAC), radical scavenging activities, ferrous ion chelating activity and protein digestibility of casein and porcine splenic hydrolysates *in vitro*

	Group		*P* value
	Casein hydrolysate	Spleen hydrolysate	
TEAC (μmol TE/g)	1.20 ± 1.13	3.18 ± 0.50	0.05
Peroxyl radical scavenging activity (%)*	ND	41.0 ± 2.1	-
O_2^- radical scavenging activity (%)*	18.3 ± 4.4	80.3 ± 1.1	< 0.001
Ferrous ion chelating activity (%)*	98.5 ± 0.5	70.1 ± 1.0	< 0.001
Protein digestibility (g/100 g)	89.9 ± 5.5	69.6 ± 2.4	0.004

ND, not detected. *1% hydrolysate sample was added to each assay.

was three-fold that of the casein hydrolysate. DPPH radical scavenging activities of the casein hydrolysate and splenic hydrolysate were expressed as the OD of 0.4 mM DPPH solution (Fig. 2). The splenic hydrolysate had a significantly (p<0.05) lower effective dose (EC_{50}) for scavenging 50% (w/v) of DPPH radicals (EC_{50}=1.16 mg/assay) than the casein hydrolysate (EC_{50}=1.67 mg/assay). The Trolox equivalents (pmol/assay) of the casein hydrolysate and splenic hydrolysate for the DPPH radical scavenging activity were 1.69 and 16.00, respectively. O_2^- radical scavenging activity (SOD-like activity) of the hydrolysates was significantly (p<0.05) higher in the order splenic hydrolysate > casein hydrolysate (Table 2). The antioxidant capacity of the splenic hydrolysate against peroxyl radicals was 40%. However, in 1% (w/v) casein hydrolysate, the peroxyl radical scavenging activity was not detected (Table 2). Table 2 also shows the Fe^{2+} ion chelating activities (%) of the casein hydrolysate and splenic hydrolysate at the 1% concentration level. The activity was significantly (p<0.05) higher in the order casein hydrolysate > splenic hydrolysate. The *in vitro* protein digestibilities of the casein and the splenic hydrolysate by gastrointestinal digestive enzymes were 89.9 and 69.6%, respectively (Table 2).

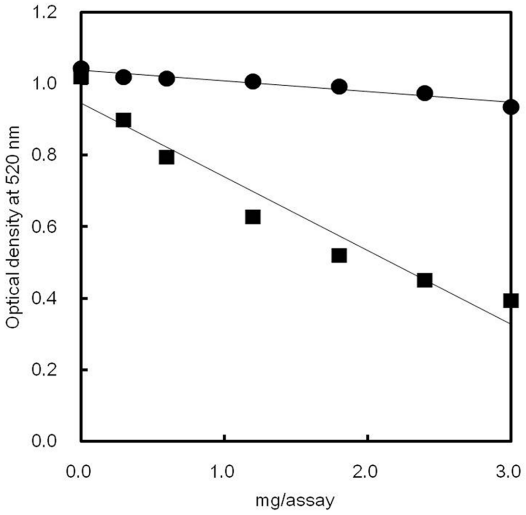

Fig. 2. Antioxidant activity in *in vitro* 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging test. The data are expressed as the means of at least three measurements and the standard deviations (SD). Significant differences were determined using Student's *t*-test (n=3). All concentration were significantly different at p<0.05 except 0 mg/assay. (●) and (■) are for casein hydrolysate and PSH hydrolysate, respectively.

In vivo study

Food intake, body weight, blood biochemical parameters

The food intake, body weight gain and liver weight of rats in the PSH group were not significantly different from those in the CONT group (Table 3). Furthermore, the serum total cholesterol, triglyceride, total protein, and albumin levels, and blood leukocyte concentration in the PSH group were similar to those in the CONT group.

Hepatic MDA and GSH levels, and antioxidant enzyme activities

The liver MDA level in the SPH group was significantly (p<0.05) lower than that in the CONT group, though there was no significant difference in the hepatic GSH level between the two groups (Table 3). Hepatic catalase and GSH-Px activities in the PSH group were significantly (p<0.05) higher than those in the CONT group. However, hepatic GST and GR activities in the PSH group were not significantly different between the CONT group and PSH group (Table 3).

Discussion

The oxidative stability of biological tissue is related to the balance between antioxidant and prooxidative factors. The production of reactive oxygen species (ROS) such as superoxide anions, singlet oxygen, hydrogen peroxide and free radicals in living species during enzymatic reactions and xenobiotic metabolism leads to an increase in

Table 3. Body weight, food intake, liver weight, blood biological parameters, hepatic TBARS and glutathione concentrations, and hepatic antioxidant enzyme activities

	Dietary group		*P* value
	CONT	PSH	
Body weight gain (g/4 wk)	32.1 ± 3.2	34.2 ± 4.2	0.392
Food intake (g/4 wk)	507 ± 34	495 ± 29	0.567
Liver weight (g)	7.08 ± 0.61	6.98 ± 0.33	0.231
Serum total cholesterol (mmol/L)	2.07 ± 0.18	2.00 ± 0.07	0.449
Serum triglyceride (mmol/L)	1.08 ± 0.23	1.06 ± 0.12	0.906
Serum total protein (g/L)	80.5 ± 1.6	80.5 ± 0.8	0.941
Serum albumin (g/L)	50.0 ± 1.2	50.0 ± 0.4	0.918
Blood leukocytes (10^3/mL)	3.00 ± 0.19	2.65 ± 0.34	0.076
Liver TBARS (nmol/mg protein)	0.483 ± 0.074	0.392 ± 0.016	0.026
Liver GSH (μmol/g liver)	4.15 ± 0.32	4.49 ± 0.48	0.223
Liver catalase (U/mg protein)	49.2 ± 3.2	58.2 ± 4.8	0.008
Liver GSH-Px (mU/g protein)	292 ± 13	353 ± 53	0.038
Liver GST (mU/mg protein)	208 ± 15	205 ± 16	0.770
Liver GSH-R (mU/mg protein)	57.7 ± 2.0	60.5 ± 5.7	0.329

Values are expressed as means±standard deviations for five rats. Significant differences were determined using Student's *t*-test.

lipid oxidation and subsequent cell injury (Cadenas and Davies, 2000). Biological tissue, especially liver cells, guards against ROS via a protective mechanism in which the potential toxicity of the partial products of oxygen is minimized by catalytic actions of SOD, catalase, GSH-Px, and other enzymes (Cadenas and Davies, 2000). However, the oxidative stress in living species gradually increases dependant on aging (Cadenas and Davies, 2000). For these reasons, constant intake of antioxidant agents from a proper diet is required to reduce or delay this aging-dependent loss. To date, there is little information on evaluation of the efficacy and detailed mechanisms of physiological functions of meat-based antioxidant peptides, especially against oxidative stress, in animal studies. However, Nazeer *et al*. (2012) recently examined the antioxidant effect of a peptide purified from a fish muscle hydrolysate in rats with ethanol-induced oxidative stress, showing increases in catalase and SOD activities in an erythrocyte lysate by orally injecting the peptide. Similarly, in this study, the hepatic MDA level in the PSH group was lower than in the CONT group, which might have been due to the increases in the hepatic catalase and GSH-Px activities. Both enzymes deactivate hydroperoxide into water, and GSH-Px also reduces organic peroxides into their corresponding alcohols (Cadenas and Davies, 2000). Thus it is considered that providing dietary PSH to rats might achieve antioxidant activity enhancing those systems.

The underlying mechanism for the health-promoting activity of the peptide mixture of porcine spleen in the animal might be free radical scavenging and hydroperoxide reduction. In this study, various free radical scavenging capacities of the splenic hydrolysate were comparable to those of casein when hydrolyzed with Alcalase®. Those activities were in accord with the results of TEAC assays, in which the Trolox equivalent value of the splenic hydrolysate was approximately three times higher than those of the hydrolysate of casein. However, the ferrous metal chelation activity in the casein hydrolysate was stronger than in the porcin splenic hydrolysate. The biological functions of the parent proteins or free amino acids seem to be more limited than after hydrolysis (Gill *et al*., 1996), suggesting roles for specific peptide sequences. The porcine splenic hydrolysate had a higher Cys residue content than casein. This amino acid residue exhibits substantial radical generation *in vitro* (Hernandez-Ledesma *et al*., 2005). However, most peptides are normally degraded into dipeptide or amino acid during gastrointestinal digestion. Thus, it is profoundly difficult for a bioactive peptide, especially a high-molecular-weight peptide, to be directly linked to the antioxidant system of hepatic cells as *in vitro* free radical scavenging activity. In this study, the *in vitro* protein digestibility of the porcine splenic hydrolysate was lower than that of casein. Excessive intakes of carbohydrates raise the susceptibility to oxidative stress, affecting the biological tissue antioxidant network (Bae *et al*., 2001). Some antioxidants such as phytochemicals may play important roles in limiting potential postprandial oxidative stress by reducing radical generation during gastrointestinal passage (Halliwell *et al*., 2000; Sies *et al*., 2005; Stahl *et al*., 2002). Halliwell *et al*. (2000) suggested that the gastrointestinal tract is a major location

of antioxidant action. Owing to the limited absorption of the splenic hydrolysate, therefore, the antioxidant properties of PSH might be exerted mainly in the gastrointestinal tract.

Although peptides and hydrolysates are available as antioxidants in food additives, the potential safety issue of allergenicity still remains. This is because that, when high-molecular-weight polypeptides are absorbed via tight junctions, they might become specific allergy antigens (Gardner, 1998). However, it has been reported that some peptides might reduce allergic reactions in atopic subjects and improve mucosal immunity in the gastrointestinal tract (Korhonen and Pihlanto, 2003). Furthermore, Polyerga®, which is an oligopeptide purified from porcine spleen, was reported to be effective in up-regulating immune responses in human subjects (Borghardt *et al*., 2000). The immunomodulatory actions of some peptides have been evaluated by lymphocyte proliferation, natural killer (NK) cell activity, antibody synthesis, and cytokine regulation (Fitzgerald and Murray, 2006; Horiguchi *et al*., 2005). However, the splenic NK cell activity did not significantly differ between the CONT and PSH groups in this study, which suggested that PSH did not induce an immune response in the animals (data not shown). This observation might be attributable to the differences between purified hydrolysates (Polyerga®) and crude hydrolysates, or the lowered digestibility of the splenic hydrolysate. If the latter is the case, it might consolidate our hypothesis on the antioxidant action of the porcine splenic hydrolysate in the gastrointestinal tract.

In conclusions, the present study demonstrated that the porcine splenic hydrolysate improved antioxidant activity both *in vivo* and *in vitro* via a potential mechanism that might involve radical scavenging activity. In future, the safety of porcine splenic peptides with regard to toxicity and allergenicity remains to be determined.

Acknowledgements

The authors wish to thank Mr. Koudai Semba and Mr. Kyouichi Machida (Department of Food Science, Obihiro University of Agriculture and Veterinary Medicine) for technical assistance and Mr. Yoshihisa Takahata (Nipponham R&D Center, Nippon Meat Packers) for supporting materials. This research was carried out with the support of a grant from the Regional Innovation Cluster Program (City Area Type) of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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(Received 2014.3.13/Revised 2014.4.28/Accepted 2014.4.29)