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ORIGINAL PAPER

Antioxidant Activities of Hydrolysates from Abalone Viscera Using Subcritical Water-Assisted Enzymatic Hydrolysis

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Abstract

Antioxidant hydrolysates were prepared from abalone viscera using subcritical water (AVS)-assisted by enzymatic hydrolysis with papain (AVSE-P), bromelain (AVSE-B), neutral protease (AVSE-N), and flavourzyme (AVSE-F). The protein and carbohydrate contents reached 38.33% and 24.36%, respectively. When AVS was digested by any of the proteases, the protein content increased, but carbohydrate content decreased. The main amino acids of AVSEs included alanine, glycine, and aspartic acid. The IC_{50} values of ferric reducing antioxidant power, and 2,2-diphenyl-1-picrylhydrazyl $(DPPH)^{\bullet}$, 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) $(ABTS)^{\bullet+}$, N,N-Dimethyl-p-phenylenediamine dihydrochloride $(DMPD)^{\bullet+}$, and hydroxyl radical ($^{\bullet}OH$) scavenging abilities of AVS were 2.93, 1.48, 1.61, 3.72, and 5.51 mg/mL, respectively, which decreased after enzymatic hydrolysis by any of the proteases. The $DMPD^{\bullet+}$ and $^{\bullet}OH$ scavenging abilities of AVSE-P and AVSE-B were higher than those of others, whereas the opposite was observed in lipid peroxidation inhibition efficiency, $DPPH^{\bullet}$, and $ABTS^{\bullet+}$ scavenging abilities. Hence, antioxidant activities of AVS could be enhanced by enzymatic hydrolysis, but the influence depends on the type of protease. At the same time, results also suggest that the proposed approach can be used for treating abalone viscera, and the obtained antioxidant hydrolysates could be used in nutraceutical and pharmaceutical industries.

Keywords Abalone viscera · Subcritical water extraction · Enzymatic hydrolysis · Proximate composition · Antioxidant activities

Introduction

Abalone (*Haliotis discus hannai* Ino) is a high-nutrition shellfish, which is widely cultured in China and Southeast Asian countries. The production of abalone in China has increased considerably in recent years, and the yield was about 148,000 tons in 2017. Abalone viscera, which are considered as by-products of abalone processing, account for 15–25% of the total abalone weight, are rich in protein, and usually processed into low-value products, such as silage (Zhou et al. 2012). These proteins from aquatic by-products are reported to be good raw material sources of functional foods (Abdollahi and Undeland 2018). Therefore, the use of abalone viscera to produce bioactive compounds can effectively expand precious abalone resources.

Enzymatic hydrolysis is a specific, simple, and safe method, widely used to recover proteins from aquatic by-products and convert them into marketable and bioactive materials (Sila and Bougatef 2016; Ovissipour et al. 2012). Furthermore, considerable bioactive hydrolysates were prepared by enzymatic hydrolysis from various sources, such as tuna backbone protein (Je et al. 2007), tuna liver (Ahn et al. 2010), and bluefin leatherjacket head (Chi et al. 2015a). Recently, antioxidant activities, and antithrombotic and anticoagulant properties were also observed in the abalone viscera hydrolysates prepared by enzymatic hydrolysis (Zhou et al. 2012; Je et al. 2015; Suleria et al. 2017). Abalone viscera contain not only proteins but also polysaccharides, of which a portion might exist in the form of glycoproteins that negatively affect enzymatic hydrolysis. Moreover, simple enzymatic hydrolysis is limited by low conversion rate, hydrolysis rate, and efficiency, whereas proteases cannot readily bind with an undenatured protein substrate (Qu et al. 2013).

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To expose the enzyme cutting site, considerable pretreatment methods, including heat, hydrostatic pressure, ultrasound, microwave, and pulsed electric field, are often used to treat materials (Uluko et al. 2015). Application of subcritical water in preparation of by-products has recently attracted considerable attention, due to its green, convenient, nontoxic, and low-cost qualities. Subcritical water, also called pressurized hot water, is liquid at temperatures above the atmospheric boiling point (100 °C, 0.1 MPa) but below the critical point (374 °C, 22.1 MPa) of water (Plaza and Turner 2015). Under subcritical conditions, glycosidic bonds are less stable than peptide bonds of proteins, resulting in rapid decomposition of carbohydrates (Toor et al. 2011). Subcritical water could be used to hydrolyze deoiled rice bran in the preparation of proteins and amino acids (Sereewatthanawut et al. 2008). Polyphenols and flavonoids from carrot leaves are extracted using subcritical water, and the optimum temperature and time are reported at 120 °C and 10 min, respectively (Song et al. 2018). The antioxidant capabilities of subcritical water extracts from golden oyster mushroom increase with increasing temperature and time (Jo et al. 2013). Antioxidant and antimicrobial hydrolysates are also extracted from tuna skin collagen using subcritical water extraction (Ahmed and Chun 2018). Considerable studies have been conducted on recovery proteins, amino acids, phenolic compounds, and bioactive compounds from plants and by-products using subcritical water extraction, whereas no studies have been performed on the preparation of antioxidant hydrolysates from aquatic by-products using subcritical water-assisted enzymatic hydrolysis.

This study aimed to prepare antioxidant hydrolysates from abalone viscera using subcritical water (AVS) assisted by enzymatic hydrolysis with papain (AVSE-P), bromelain (AVSE-B), neutral protease (AVSE-N), and flavourzyme (AVSE-F). The proximate composition and antioxidant activities including ferric reducing antioxidant power (FRAP) assay, scavenging abilities of 2,2-diphenyl-1-picrylhydrazyl (DPPH)$^{\bullet}$, 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)$^{\bullet+}$, N,N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD)$^{\bullet+}$, and hydroxyl radical ($^{\bullet}$OH), and lipid peroxidation inhibition assay, were investigated. This study will aid in the development of new approaches for extracting bioactive compounds from abalone viscera.

Material and Methods

Material and Chemicals

Abalone (*Haliotis discus hannai* Ino) viscera were obtained from Xiamen Daozhiyuan Biological Technology Co., Ltd (Xiamen, Fujian, China). Papain, bromelain, neutral protease, and flavourzyme were purchased from Pangbo Biological Engineering Co. (Nanning, Guangxi, China). Linoleic acid, ABTS, and DMPD were purchased from Sigma-Aldrich (St. Louis, MO, USA). DPPH was purchased from Wako Pure Chemical Industries (Japan). All other chemicals used were of analytical grade.

Sample Preparation

Abalone viscera (25 kg) and tap water (100 L) were added into a high-pressure autoclave (Zhucheng Jinding Food Machinery Co., Ltd, Shandong, China), and the AVS was obtained at 130 °C for 30 min. In the heating step, the time of temperature increase from room temperature to 130 °C was approximately 30 min. After being cooled below 50 °C, AVS was hydrolyzed using papain, bromelain, neutral protease, and flavourzyme respectively, at 50 °C for 3 h with an enzyme/solution ratio of 1:2000 (*w*/*v*). After hydrolysis, the abalone viscera subcritical water-assisted enzymatic extracts (AVSEs) were boiled for 10 min to inactivate the enzyme, and then cooled with ice water. The AVS and AVSEs were then fractionated successively by ultrafiltration (UF) and nanofiltration membranes with molecular weight cutoff (MWCO) of 10 and 0.2 kDa, respectively. The fraction of 0.2 kDa < MW < 10 kDa was collected and lyophilized at − 40 °C. The obtained samples were stored at − 20 °C for further analyses.

Determination of Proximate Composition

The protein, ash, and moisture contents in AVS and AVSEs were determined according to the methods of AOAC 992.15, AOAC 920.153, and AOAC 930.15 (AOAC 2005). Carbohydrate content was measured by phenolsulfuric acid method using D-glucose as reference (Dubois et al. 1956). All analyses were performed in triplicate.

Determination of Amino Acid Composition

The lyophilized samples were hydrolyzed in 6 mol/L HCl at 110 °C for 22 h containing 0.1% phenol and HCl was eliminated by evaporation (Chen et al. 2015). The amino acid composition of an aliquot derived with o-phthalaldehyde was analyzed by Agilent 1200 high-performance liquid chromatography (HPLC) System (Agilent Technologies, Palo Alto, USA) with Zorbax Eclipse AAA C18 (4.6 mm × 150 mm, 5 μm) column (Agilent Technologies, Palo Alto, USA).

Determination of Molecular Weight Distribution

The samples dissolved in ultrapure water (10 mg/mL) were filtered with a 0.22-μm syringe filter. Molecular weight distribution was determined by HPLC using a TSK gel G2000 SWXL column (7.8 mm × 300 mm, TOSOH, Tokyo, Japan) at a wavelength of 214 nm (Weng et al. 2014). The mobile

phase was methyl alcohol: acetonitrile : ultrapure water (45:55:10 ratio) at a flow rate of 0.50 mL/min. A molecular weight calibration curve was established according to standards of cytochrome C (12,327 Da), trasylol (6533 Da), oxidized glutathione (613 Da), triglycine (Gly-Gly-Gly, 189 Da), and glycine (Gly, 75 Da).

Determination of Antioxidant Activity

FRAP Assay

FRAP assays of AVS and AVSEs were determined according to the process established by He et al. (2013). One milliliter of sample was mixed with 1.0 mL phosphate buffer (0.2 mol/L, pH 6.6) and 1.0 mL 1% [$K_3Fe(CN)_6$] and incubated at 50 °C for 20 min. The obtained solution was mixed with 1 mL 10% trichloroacetic acid and centrifuged (4000*g*, 10 min). The supernatant (2 mL) was mixed with distilled water (2 mL), followed by addition of 0.4 mL 0.1% $FeCl_3$. After incubation at room temperature for 10 min, absorbance was measured at 700 nm using a UV-8000A spectrophotometer (Shanghai Yuanxi Instrument Co., China).

DPPH$^{\bullet}$ Scavenging Ability

DPPH$^{\bullet}$ scavenging abilities of AVS and AVSEs were measured according to the method established by Weng et al. (2014). One milliliter of sample were added to 1 mL DPPH$^{\bullet}$ solution (0.2 mmol/L in ethanol). After incubation in the dark at room temperature for 30 min, the mixture was centrifuged (10,000*g*, 10 min) and absorbance was measured at 517 nm using a spectrophotometer. DPPH$^{\bullet}$ scavenging ability was calculated using the following equation:

$$\text{Scavenging ability}(\%) = \left(1 - \frac{A_S - A_C}{A_0}\right) \times 100 \quad (1)$$

where A_S refers to the absorbance in the presence of samples, A_C denotes the absorbance when ethanol replaces the DPPH solution, and A_0 corresponds to the absorbance of control reaction.

ABTS$^{\bullet+}$ Scavenging Ability

The ABTS$^{\bullet+}$ scavenging abilities of AVS and AVSEs were measured according to the method established by Li et al. (2012). ABTS$^{\bullet+}$ was produced by mixing the ethanol solution of ABTS (2 mmol/L) with potassium persulfate (2.45 mmol/L) at equal volumes. The mixture was stored in the dark at room temperature for 12 h to ensure complete radical generation. For analysis, the ABTS$^{\bullet+}$ stock was diluted using 95% ethanol to obtain an absorbance of 0.80–1.00 at 734 nm. Antioxidant ability was determined by mixing 1.2 mL ABTS$^{\bullet+}$ stock solution and 0.3 mL sample. After incubation for 6 min, absorbance was measured at 734 nm and ABTS$^{\bullet+}$ scavenging ability was calculated using Eq. (1), where A_0 represents the initial concentration of the ABTS$^{\bullet+}$, A_S specifies absorbance of the remaining concentration of ABTS$^{\bullet+}$ in the presence of the sample, and A_C denotes the absorbance when distilled water replaces the ABTS$^{\bullet+}$ reagent.

DMPD$^{\bullet+}$ Scavenging Ability

The DMPD$^{\bullet+}$ scavenging abilities of AVS and AVSEs were measured using the method established by Rodríguez-Nogales et al. (2011). DMPD$^{\bullet+}$ was generated from 1 mL DMPD (100 mmol/L), 100 mL acetate buffer (100 mmol/L, pH 5.3), and 0.2 mL $FeCl_3$ (50 mmol/L). The mixture was kept in the dark at room temperature until its absorbance reached values between 0.80 and 1.00 at 505 nm. One milliliter of DMPD$^{\bullet+}$ reagent was mixed with 0.5 mL sample solution. After incubation for 10 min, absorbance was measured at 505 nm, and DMPD$^{\bullet+}$ scavenging ability was calculated using Eq. (1), where A_0 corresponds the initial concentration of DMPD$^{\bullet+}$, A_S indicates the absorbance of DMPD$^{\bullet+}$ in the presence of sample, and A_C signifies the absorbance when distilled water replaces the DMPD$^{\bullet+}$ reagent.

$^{\bullet}$OH Scavenging Ability

The $^{\bullet}$OH scavenging abilities of AVS and AVSEs were measured according to the method established by Weng et al. (2014). One milliliter of sample solution was mixed with 0.3 mL $FeSO_4$ (8 mmol/L), 0.25 mL H_2O_2 (20 mmol/L), and 1 mL salicylic acid (3 mmol/L). After incubation at 37 °C for 30 min, the mixture was cooled rapidly in ice water and centrifuged at 4000×*g* for 10 min. Absorbance was measured at 510 nm, and $^{\bullet}$OH scavenging ability was calculated using Eq. (1), where A_S denotes the absorbance in the presence of sample, A_C refers the absorbance when distilled water replaces the salicylic acid solution, and A_0 stands for the absorbance of control reaction.

Inhibition of Lipid Peroxidation

The sample dissolved in 0.5 mL distilled water (2.0 mg/mL) was mixed with 2.0 mL sodium phosphate buffer (200 mmol/L, pH 7.0), and then added to 2.5 mL linoleic acid emulsion in sodium phosphate buffer (40 mmol/L, pH 7.0), which was prepared using the method established by Gülçin (2010). The reaction mixture was incubated at 37 °C in glass vials under dark condition. At intervals during incubation, the resultant mixture (0.1 mL) was extracted and mixed with 4.7 mL 75% ethanol, 0.1 mL 30% ammonium thiocyanate, and 0.1 mL ferric chloride dissolved in 3.5% HCl. After 3 min

incubation at room temperature, the absorbance was measured using a spectrophotometer at 500 nm.

Statistical Analysis

The obtained data were subjected to variance (one- and two-way ANOVA) analysis (Minitab Inc., State College, PA). The probability value of $P < 0.05$ was used as criterion for significant differences.

Results and Discussions

Proximate Composition

Table 1 shows the proximate compositions of AVS and AVSEs. The contents of protein and carbohydrate in AVS reached 38.33% and 24.36%, respectively. After AVS was hydrolyzed by protease, the protein contents in the obtained AVSEs increased significantly ($P < 0.05$), whereas carbohydrate contents decreased markedly ($P < 0.05$) regardless of the proteases used. High levels of H^+ and OH^- were reported in subcritical water, wherein macromolecules, including proteins and carbohydrates, could be decomposed into small molecules (Toor et al. 2011). Therefore, portions of proteins and carbohydrates were dissolved out from abalone viscera after subcritical water treatment. When AVS was enzymatically hydrolyzed, the protein percentage in AVSEs increased as denatured protein dissolved easily by degrading into small mass peptide fractions, which could pass through UF membrane with MWCO of 10 kDa and be collected. On the other hand, the ash content of AVS reached 16.37%, which further increased significantly ($P < 0.05$) after hydrolyzation by any of the proteases (Table 1). The hydrolysates derived from herring (*Clupea harengus*) by-product and squid viscera were also reported at rates of 15.30% and 17.38%, respectively (Sathivel et al. 2003; Kondo et al. 2017).

Amino Acid Composition

Table 2 summarizes the amino acid compositions of AVS and AVSE. AVS and AVSEs are rich in aspartic and glutamic acids, which are types of umami flavor amino acids. These results are consistent with the abalone viscera hydrolysates in previous studies (Zhou et al. 2012; Viana et al. 2007). Although amino acid compositions vary in marine protein hydrolysates, the major amino acids include aspartic and glutamic acids in fish and shellfish protein hydrolysates (Je et al. 2015). In this study, high contents of alanine and glycine were found in AVS and various AVSEs, different from the reported enzymatic hydrolysates of abalone viscera (Zhou et al. 2012; Viana et al. 2007; Je et al. 2015), but similar to subcritical water hydrolysates of bovine serum albumin (Rogalinski et al. 2005), enabling the decomposition of abalone viscera protein into simple amino acids (alanine and glycine) that are stable under hot pressurized water.

When AVS was hydrolyzed by papain, bromelain, neutral protease, or flavourzyme, the contents of glycine, arginine, tyrosine, methionine, and phenylalanine increased significantly ($P < 0.05$). Among these amino acids, tyrosine, methionine, and phenylalanine enhanced the potency of antioxidant peptides (Dávalos et al. 2004). Thus, the results of Table 2 also suggest that AVS expectedly increased the antioxidant ability by digesting with these enzymes. Furthermore, differences in amino acid compositions of AVSEs produced by the four proteases were observed probably due to the different specificities of the enzymes (Table 2). Alanine content of AVSE-F was higher than that of other AVSEs, consistent with the other findings observed in brown stripe red snapper (Khantaphant et al. 2011) and yellow stripe trevally (*Selaroides leptolepis*) muscle (Klompong et al. 2009).

Molecular Weight Distribution

The molecular weight distribution of AVS and AVSEs were determined by HPLC (Fig. 1). It was reported that subcritical liquid water could diminish electrostatic interactions between molecules and rendered a less polar (Plaza and Turner 2015), leading to organic decomposition. The macromolecules in the organic compound were degraded into smaller molecules during subcritical water extraction (Toor et al. 2011). Therefore, abalone viscera could be decomposed by subcritical water, and the obtained decompositions of AVS mainly consisted of peptide fractions with 180–1000 Da (Fig. 1). When papain was used to hydrolyze AVS, the relative proportion of molecular weight above 1000 Da in the obtained AVSE-P increased

Table 1 Proximate composition of abalone viscera subcritical water (AVS)-assisted enzymatic hydrolysis with papain (AVSE-P), bromelain (AVSE-B), neutral protease (AVSE-N), and flavourzyme (AVSE-F)

	Protein (%)	Carbohydrate (%)	Ash (%)	Moisture (%)
AVS	38.33 ± 0.59^{a}	24.36 ± 0.25^{e}	16.37 ± 0.51^{a}	7.79 ± 0.20^{b}
AVSE-P	51.95 ± 0.56^{c}	10.43 ± 0.04^{c}	17.47 ± 0.37^{b}	8.10 ± 0.32^{b}
AVSE-B	48.26 ± 2.60^{b}	12.86 ± 0.09^{d}	22.56 ± 0.61^{e}	7.99 ± 0.34^{b}
AVSE-N	59.17 ± 1.16^{d}	8.61 ± 0.03^{a}	18.82 ± 0.12^{c}	9.50 ± 0.20^{c}
AVSE-F	59.83 ± 2.88^{d}	9.33 ± 0.02^{b}	19.63 ± 0.33^{d}	7.04 ± 0.38^{a}

Any two means in the same column followed by the same letter are not significantly different ($P > 0.05$)

Table 2 Amino acid compositions of abalone viscera subcritical water (AVS)-assisted enzymatic hydrolysis with papain (AVSE-P), bromelain (AVSE-B), neutral protease (AVSE-N), and flavourzyme (AVSE-F) (AA/1000 AA)

Amino acid	AVS	AVSE-P	AVSE-B	AVSE-N	AVSE-F
Aspartic acid	101.0 ± 1.4^{d}	76.5 ± 0.7^{c}	72.5 ± 2.1^{b}	75.5 ± 2.1^{bc}	57.5 ± 0.7^{a}
Glutamic acid	151.0 ± 2.8^{c}	118.0 ± 2.8^{a}	135 ± 5.7^{b}	112.5 ± 5.0^{a}	113.5 ± 0.7^{a}
Serine	52.5 ± 4.7^{a}	47.5 ± 2.1^{a}	50.0 ± 7.1^{a}	44.5 ± 3.5^{a}	46.0 ± 4.2^{a}
Histidine	14.0 ± 0.0^{a}	0	14.5 ± 3.5^{a}	0	11.5 ± 5.0^{a}
Glycine	119.0 ± 1.4^{a}	183.0 ± 3.4^{bc}	176.0 ± 5.7^{b}	186.5 ± 0.7^{c}	175.0 ± 2.8^{b}
Threonine	42.0 ± 4.2^{c}	35.5 ± 2.1^{b}	31.5 ± 1.8^{ab}	34.5 ± 1.7^{b}	28.5 ± 1.7^{a}
Arginine	30.0 ± 2.8^{a}	39.0 ± 1.4^{b}	43.5 ± 3.5^{b}	38.5 ± 2.1^{b}	40.0 ± 5.7^{b}
*Alanine	238.0 ± 1.4^{bc}	244.0 ± 4.2^{c}	200.5 ± 2.7^{a}	234.0 ± 5.6^{b}	323.5 ± 3.5^{d}
*Tyrosine	15.0 ± 1.4^{a}	17.0 ± 1.4^{ab}	20.5 ± 1.7^{b}	16.5 ± 2.1^{ab}	18.0 ± 2.8^{ab}
Cysteine	0	0	0	0	0
*Valine	36.0 ± 1.4^{a}	33.0 ± 0.0^{a}	32.5 ± 3.5^{a}	32.0 ± 2.4^{a}	33.0 ± 1.4^{a}
*Methionine	9.0 ± 0.3^{a}	12.5 ± 0.7^{b}	12.5 ± 2.1^{b}	11.5 ± 0.7^{ab}	12.5 ± 0.7^{b}
*Phenylalanine	16.0 ± 2.8^{a}	19.5 ± 0.7^{a}	22.5 ± 6.3^{a}	21.0 ± 2.8^{a}	20.0 ± 0.7^{a}
*Isoleucine	33.0 ± 4.2^{b}	21.5 ± 0.7^{a}	25.5 ± 3.5^{a}	21.5 ± 2.1^{a}	21.5 ± 0.7^{a}
*Leucine	37.5 ± 3.5^{a}	38.0 ± 1.4^{a}	40.0 ± 1.4^{ab}	44.5 ± 2.1^{b}	39.5 ± 2.1^{ab}
Lysine	31.5 ± 3.5^{b}	23.0 ± 1.4^{a}	25.0 ± 0.0^{a}	24.5 ± 2.1^{a}	23.0 ± 2.8^{a}
*Proline	48.0 ± 4.2^{b}	48.5 ± 2.1^{b}	50.0 ± 5.7^{b}	55.0 ± 4.2^{b}	18.0 ± 5.7^{a}
Hydroxyproline	26.5 ± 3.7^{a}	43.5 ± 3.5^{b}	50.0 ± 2.8^{b}	46.5 ± 2.1^{b}	19.0 ± 5.7^{a}
Total	1000	1000	1000	1000	1000

*Hydrophobic amino acids

Any two means in the same row followed by the same letter are not significantly different ($P > 0.05$)

markedly ($P < 0.05$) from 13.23 to 27.01% (Table 3). A proportion of small peptide fractions usually increased when proteins were hydrolyzed by enzymes. However, the opposite was observed in AVSE-P, possibly due to the high molecular weight of AVS fractions, which were intercepted by UF membrane with MWCO of 10 kDa but allowed to pass through when hydrolyzed by papain. Similar phenomena were also noted in AVSE-B and AVSE-F. No remarkable change was

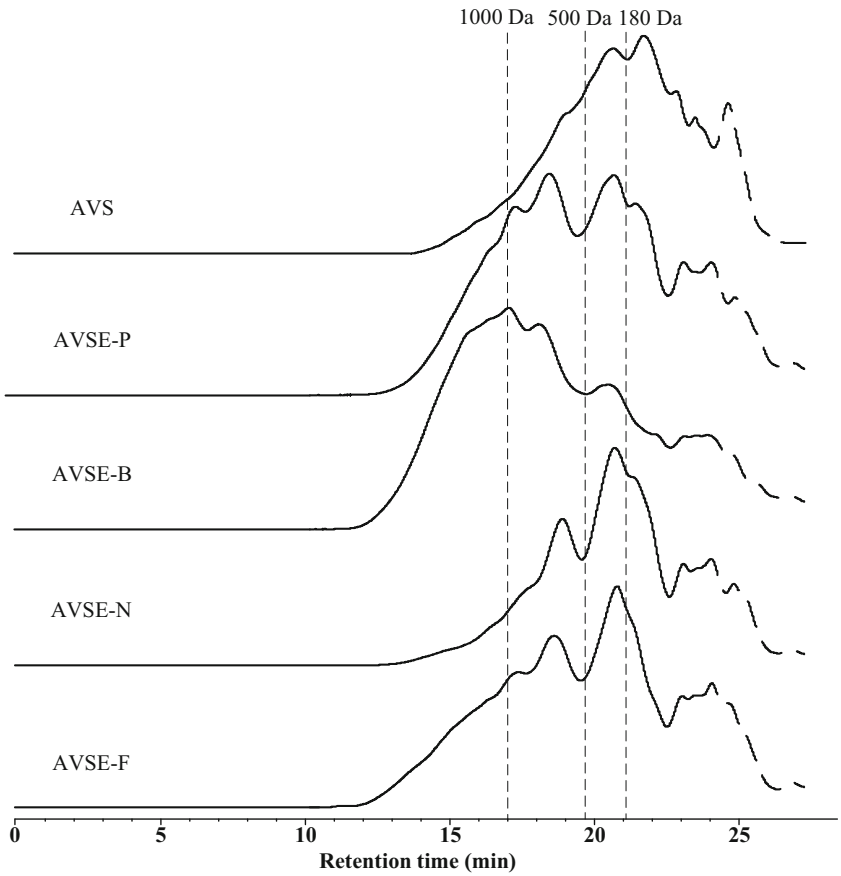

Fig. 1 HPLC profiles of molecular weight distribution of abalone viscera subcritical water (AVS)-assisted enzymatic hydrolysis with papain (AVSE-P), bromelain (AVSE-B), neutral protease (AVSE-N), and flavourzyme (AVSE-F)

Table 3 Relative proportion (%) of molecular weight distribution of abalone viscera subcritical water (AVS)-assisted enzymatic hydrolysis with papain (AVSE-P), bromelain (AVSE-B), neutral protease (AVSE-N), and flavourzyme (AVSE-F)

	> 1000 Da	500–1000 Da	180–500 Da
AVS	13.23 ± 1.27^{a}	48.53 ± 3.45^{b}	38.24 ± 2.92^{c}
AVSE-P	27.01 ± 2.32^{b}	49.30 ± 3.57^{b}	23.69 ± 2.28^{b}
AVSE-B	44.89 ± 5.12^{c}	40.76 ± 2.77^{a}	14.35 ± 1.32^{a}
AVSE-N	12.87 ± 1.29^{a}	48.51 ± 3.41^{b}	38.62 ± 2.47^{c}
AVSE-F	31.47 ± 2.87^{b}	43.69 ± 2.88^{ab}	24.84 ± 1.88^{b}

Any two means in the same column followed by the same letter are not significantly different ($P > 0.05$)

observed in the relative proportions of molecular weight distribution between AVS and AVSE-N (Table 3). However, the peak shapes noted in the HPLC differed (Fig. 1). When hydrolyzed by neutral protease, the peptide fractions above 1000 Da decreased, and peptide fractions of 500–1000 Da and 180–500 Da became dominant. These results indicate that AVSE obtained by subcritical water-assisted neutral protease hydrolysis features a lower molecular weight than other enzymes.

Antioxidant Activities

The antioxidant activities of AVS and AVSEs were evaluated by FRAP assay and according to the scavenging abilities of DPPH$^{\bullet}$, ABTS$^{\bullet+}$, DMPD$^{\bullet+}$, and $^{\bullet}$OH. Table 4 lists the obtained results.

The IC_{50} value in the FRAP of AVS totaled 5.51 mg/mL, which was higher ($P < 0.05$) than that of AVSE hydrolyzed by any of the enzymes, thereby suggesting that enzymatic hydrolysis can enhance the reducing power of AVS. Such tendency was observed in the antioxidant activities of DPPH$^{\bullet}$, ABTS$^{\bullet+}$, DMPD$^{\bullet+}$, and $^{\bullet}$OH during scavenging. However, the FRAP values of AVSEs were lower ($P < 0.05$) than those of abalone viscera hydrolysates derived by the corresponding proteases (Zhou et al. 2012). The IC_{50} values for DPPH$^{\bullet}$ scavenging abilities of AVSEs varied from 0.98 to 0.45 mg/mL, which were lower than those of abalone viscera hydrolysates (Zhou et al. 2012) and blue mussel protein hydrolysates (Wang et al. 2013). AVSE-N and AVSE-F showed similar IC_{50} values for DPPH$^{\bullet}$ scavenging ability; the values were lower ($P < 0.05$) than those of the others. A similar trend was observed in ABTS assay. These suggested that AVSE-N and AVSE-F exhibited good antioxidant activities in terms of DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging abilities. In the case of DMPD$^{\bullet+}$ scavenging ability, IC_{50} values ranged from 1.61 to 0.72 mg/mL, with AVSE-B yielding the lowest value ($P < 0.05$). Furthermore, AVSE-B and ASVE-P presented the same IC_{50} values for $^{\bullet}$OH scavenging ability, and the obtained value was lower ($P < 0.05$) than that of the others.

On one hand, FRAP reflects the capability to donate an electron (Chandrasekara and Shahidi 2010), whereas DPPH and ABTS$^{\cdot+}$ reflect the capability to donate electrons or hydrogen atoms (Soares et al. 1997; Liu et al. 2014). On the other hand, DMPD$^{\bullet+}$ and $^{\bullet}$OH scavenging abilities are used to determine the antioxidant activities of hydrophilic samples (Asghar et al. 2007). Therefore, FRAP, and DPPH$^{\bullet}$, and ABTS$^{\bullet+}$ scavenging abilities showed similar trends, whereas the highest DMPD$^{\bullet+}$ and $^{\bullet}$OH scavenging abilities were observed in AVSE-B (Table 4).

Interestingly, similar distributions and patterns of molecular weight were observed between AVSE-N and AVSE-F and between AVSE-P and AVSE-B (Fig. 1). These tendencies were also found in the results on antioxidant activities (Table 4). These findings suggest that the antioxidant activities of AVSE might be related to molecular weight distribution. Several studies also indicated that free radical scavenging abilities of protein hydrolysates are closely related to molecular weight (He et al. 2013; Bamdad et al. 2011). However, no similar phenomena were observed in this study based on the correlation analysis between molecular weight and antioxidant activities of AVSE (data not shown).

Single free radical assays cannot reflect multiple mechanisms by which antioxidants retard or inhibit lipid oxidation in food systems (Pan et al. 2016). Therefore, lipid peroxidation

Table 4 IC_{50} values (mg/ml) in the antioxidant activities of abalone viscera subcritical water (AVS)-assisted enzymatic hydrolysis with papain (AVSE-P), bromelain (AVSE-B), neutral protease (AVSE-N), and flavourzyme (AVSE-F)

	FRAP assay	DPPH·scavenging	ABTS$^{\cdot+}$ scavenging	DMPD$^{\cdot+}$ scavenging	·OH scavenging
AVS	5.51 ± 0.20^{d}	2.93 ± 0.18^{d}	1.48 ± 0.08^{d}	1.61 ± 0.08^{d}	3.72 ± 0.24^{d}
AVSE-P	1.23 ± 0.04^{b}	0.56 ± 0.03^{b}	0.56 ± 0.02^{b}	0.84 ± 0.05^{a}	0.92 ± 0.03^{a}
AVSE-B	4.21 ± 0.24^{c}	0.98 ± 0.09^{c}	0.70 ± 0.03^{c}	0.74 ± 0.05^{a}	0.98 ± 0.05^{a}
AVSE-N	1.03 ± 0.03^{a}	0.46 ± 0.01^{a}	0.41 ± 0.01^{a}	0.95 ± 0.06^{b}	1.20 ± 0.05^{b}
AVSE-F	1.13 ± 0.03^{a}	0.45 ± 0.01^{a}	0.40 ± 0.01^{a}	1.26 ± 0.08^{c}	1.71 ± 0.04^{c}

IC_{50} value is the effective concentration at which DPPH·, ABTS$^{\cdot+}$, DMPD$^{\cdot+}$, and OH radicals were scavenged by 50%, or the absorbance was 0.5 at 700 nm for reducing power

Any two means in the same column followed by the same letter are not significantly different ($P > 0.05$)

Table 5 Inhibition of linoleic acid peroxidation by abalone viscera subcritical water extract (AVS)-assisted enzymatic hydrolysis with papain (AVSE-P), bromelain (AVSE-B), neutral protease (AVSE-N), and flavourzyme (AVSE-F), at a concentration of 1 mg/mL

	6 h	12 h	24 h	36 h	48 h	60 h
Control	0.39 ± 0.01^{dA}	0.60 ± 0.02^{dB}	1.01 ± 0.03^{fC}	1.67 ± 0.04^{gD}	1.92 ± 0.09^{gE}	2.01 ± 0.08^{dE}
AVS	0.35 ± 0.01^{cA}	0.42 ± 0.02^{cB}	0.53 ± 0.02^{eC}	0.65 ± 0.03^{fD}	0.77 ± 0.03^{fE}	0.85 ± 0.05^{cF}
AVSE-P	0.22 ± 0.01^{bA}	0.42 ± 0.02^{cB}	0.39 ± 0.03^{dB}	0.50 ± 0.02^{dC}	0.54 ± 0.02^{dC}	0.61 ± 0.03^{bD}
AVSE-B	0.22 ± 0.01^{bA}	0.25 ± 0.01^{cB}	0.41 ± 0.02^{dC}	0.59 ± 0.02^{eD}	0.68 ± 0.02^{eE}	0.65 ± 0.03^{bE}
AVSE-N	0.22 ± 0.01^{bA}	0.23 ± 0.01^{bA}	0.27 ± 0.01^{cB}	0.29 ± 0.02^{cB}	0.29 ± 0.01^{cB}	0.30 ± 0.02^{aB}
AVSE-F	0.22 ± 0.01^{bA}	0.21 ± 0.01^{bA}	0.23 ± 0.01^{bA}	0.23 ± 0.01^{bA}	0.22 ± 0.01^{bA}	0.26 ± 0.02^{aB}
Ascorbic acid	0.02 ± 0.01^{aA}	0.04 ± 0.01^{aB}	0.05 ± 0.01^{aB}	0.13 ± 0.01^{aC}	0.18 ± 0.01^{aD}	0.65 ± 0.03^{bE}

Any two means in the same column followed by the same small letter are not significantly different ($P > 0.05$)

Any two means in the same row followed by the same capital letter are not significantly different ($P > 0.05$)

inhibition was determined to further characterize the antioxidant activities of AVS and AVSEs (Table 5). The absorbance value of linoleic acid without antioxidants (control) was the highest ($P < 0.05$) and consistently increased ($P < 0.05$) within 60 h of storage at 37 °C in the dark. When linoleic acid was added with AVS, the increased absorbance was retarded obviously after 6 h, thus suggesting that AVS can inhibit lipid oxidation. The effect of AVSEs, particularly AVSE-N and AVSE-F, on linoleic acid peroxidation inhibition was significantly ($P < 0.05$) better than that of AVS. The inhibition efficiency of AVSE-N and AVSE-F was close to that of ascorbic acid at the same concentration after 60 h of storage, thereby indicating that AVSE-N and AVSE-F exhibit excellent antioxidant activities in the linoleic acid system and feature development potential. Furthermore, the tendency of inhibition effect on linoleic acid peroxidation of AVS and AVSEs (Table 5) was similar to that of FRAP, and DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging abilities but different from that of DMPD$^{\bullet+}$ and $^{\bullet}$OH scavenging abilities (Table 4). These results suggest that lipid oxidation could be mainly inhibited by donating electrons or hydrogen atoms. Studies also reported that the hydrophobicity of peptides could enhance their solubility in lipids and promote interactions between peptides and radicals from lipid oxidation (Guo et al. 2015). Therefore, the hydrophobicity of AVSE-N and AVSE-F might be larger than that of AVSE-P and AVSE-B (Tables 4 and 5).

The antioxidant activities of hydrolysates are usually related to amino acid composition and sequence (Chi et al. 2015b). Therefore, the relationship between antioxidant activities and amino acid contents was also investigated in this study (Table 6). High correlation coefficients were observed between lipid peroxidation inhibition and contents of hydrophobic amino acids including glycine, tyrosine, valine, methionine, phenylalanine, leucine, and isoleucine. Similar tendencies were observed between hydrophobic amino acid contents and FRAP, and DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging abilities, and the correlation coefficients ranged from 0.72 to 0.99, except between isoleucine and FRAP assay. By contrast, the relationship of most hydrophobic amino acids with DMPD$^{\bullet+}$ and $^{\bullet}$OH scavenging abilities was lower mainly because DMPD$^{\bullet+}$ and $^{\bullet}$OH scavenging abilities were used as determinants for hydrophilic samples. This tendency was similar to that in reports on oysters protein hydrolysates (Wang et al. 2014), squid muscle protein hydrolysates (Rajapakse et al. 2005), and zein hydrolysates (Zhu et al. 2008).

Table 6 Correlation coefficients between antioxidant activities (IC_{50} values) and amino acid contents

Amino acids	Lipid peroxidation inhibition	FRAP assay	DPPH scavenging	ABTS$^{\cdot+}$ scavenging	DMPD$^{\cdot+}$ scavenging	$^{\cdot}$OH scavenging
Glycine	0.79	0.86	0.98	0.98	0.62	0.41
Tyrosine	0.73	0.99	0.98	0.79	0.43	0.19
Valine	0.92	0.80	0.91	0.98	0.80	0.59
Methionine	0.83	0.72	0.85	0.99	0.77	0.61
Phenylalanine	0.82	0.92	0.98	0.96	0.61	0.38
Leucine	0.86	0.82	0.75	0.94	0.88	0.76
Isoleucine	0.81	0.60	0.92	0.99	0.67	0.48

Conclusions

The AVS mainly consisted of proteins and carbohydrates. When AVS was digested by a protease, the obtained AVSEs mainly comprised of proteins irrespective of the protease used. AVSE-N and AVSE-F showed higher lipid peroxidation inhibition efficiency, FRAP, and DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging abilities, whereas AVSE-P and AVSE-B showed better DMPD$^{\bullet+}$ and $^{\bullet}$OH scavenging abilities. The present results demonstrated that the antioxidant activities of hydrolysates obtained from AVS could be improved by digestion with proteases and are related to amino acid composition. As the results of this study have been obtained by small batch production, the present approach is applicable to large-scale preparation. Furthermore, the obtained antioxidant hydrolysates from abalone viscera by subcritical water-assisted enzymatic hydrolysis could be applied in nutraceutical and pharmaceutical industries.

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