Antioxidant and anti-hypertension activities of protein hydrolysate from sea cucumber, Holothuria parva using enzymatic hydrolysis

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Abstract

Bioactive peptides of marine organisms have recently attracted great attention from scientists and industries. In this study, tissue protein of sea cucumber *Holothuria parva* muscle were hydrolyzed using bacterial alcalase enzyme and then sea cucumber protein hydrolysis (SPH) was separated by ultrafiltration membranes (30, 10 and 3 kDa) and four fractions with different molecular weight; FI (> 30 kDa), FII (10 < MW < 30 kDa), FIII (3 < MW < 10 kDa) and FIV (< 3 kDa) were obtained. The antioxidant and ACE-inhibitory of SPH and four fractions products FI, FII, FIII, and FIV were evaluated. The results show that the protein content of four fractions FI, FII, FIII, and FIV were 47.33, 57.08, 47.66, and 15.5 mg/ml, respectively. The results of biological analyzes showed that SPH exhibited the highest hydroxyl radical scavenger (48% at 10 µg/ml) and ferric ions (229.03% µg/mg) followed by FIV by (35% at 10 µg/ml). The highest ferric reducing was seen at SPH with (229.03 % at 10 µg/ml) followed by FIV (146/94% at 10 µg/ml). The DPPH scavenging activity of SPH, FI, and FII were 7.32%, 27.4%, and 6.77% at 10 µg/ml, respectively. The Angiotensin-I-converting enzyme (ACE) inhibitory activity of 1mg FI, FII, FIII, and FIV were 7.60, 3.73, 7.86, and 13.60 mg/ml, respectively. The fraction with molecular weight below 3 kDa exhibited higher ACE inhibitory activity. The results of the current study showed that the *H. parva* muscle protein and their hydrolysis products has a suitable capacity for use in medical purposes.

Introduction

There is a growing interest worldwide to find new bioactive peptides from natural resources. Marine proteins are excellent sources of bioactive peptides, which have attracted the attention of the pharmaceutical, nutraceutical and medicinal industries because of their various biological activity, including antioxidant, antimicrobial, antidiabetic, antihypertensive and immunomodulatory properties (Chai et al. 2017; Xu et al. 2018; Mada et al. 2020). Research on sea cucumbers were provided evidences of the health benefits of their bioactive peptides compounds (Anderson et al. 2011). Antioxidant and Angiotensin-converting enzyme inhibitory peptides were obtained from the protein hydrolysate of sea cucumber (Zhao et al. 2007; Forghani et al. 2012; Sadegh Vishkaei et al. 2016; Chim-Chi et al. 2017; Li et al. 2018; Auwal et al. 2019; Dewi et al. 2020). Due to the negative effects of using synthetic ACE inhibitors such as captopril, enalapril, and lisinopril which causes several side effects, including dry cough, allergic reactions, hyperkalemia, hypotension, renal failure, decrease in white blood cells, and angioedema (Wood 1995), the use of efficient natural substitutes is demanded by consumers (Balti et al. 2015; Ngo et al. 2015; Sadegh Vishkaei et al. 2016; Li et al. 2018; Aluko 2019).

The bioactive peptides from different marine source can be extracted with several different techniques including non-enzymatic (ultrasound, high hydrostatic pressure, and supercritical fluids), enzymatic method, fermentation methods, and organic solvents. Among these techniques, hydrolysis using digestive or commercial enzymes is the preferred method in food industry. Several antioxidant peptides were obtained from sea cucumber by the hydrolysis process (Wang et al. 2010; Zhou et al. 2012; Zheng et al. 2012; Ghanbari et al. 2015; Guo et al. 2020; Safari and Yaghoubzadeh 2020). Food-derived bioactive peptides which dad 2–50 amino acid residues exert more beneficial effects on the body due to their low

toxicity and antioxidant activity (Vijaykrishnaraj and Prabhasankar 2015; Daliri et al. 2017; Zhou et al. 2017; Mada et al. 2020).

One of the most common and available sea cucumbers species in Persian Gulf and Oman sea in Iranian coastal waters is *Holothuria parva* (Fatemi et al. 2011). So far, several studies have been conducted on the properties of bioactive compounds of this species (Ehsanpour et al. 2015; Seydi et al. 2015; Ebrahimi et al. 2018; Yousefzadi and Mashjoor 2019; Shadi and Oujifard 2019). *H. parva* is an edible sea cucumber that belongs to the phylum Echinoderms, which is commonly on sale in Asian markets, but it is not usually consumed as food in Iran. Therefore, its use in the medical sector may be considered more in Iran. Therefore, the objective of this study was to evaluate in vitro antioxidant and ACE-inhibitory activities of hydrolysate peptide from *H.parva* muscle tissue.

Material and methods

Samples

Sea cucumber, *H. parva* were collected from Hormuz Island coast, Persian Gulf. After removing the internal organs, the muscles were cut on site. The samples were kept in an ice box at 4°C, and transferred to Tarbiat Modares University laboratory.

Preparation of sea cucumber protein hydrolysates

Enzymatic hydrolysis was performed according to the method of Vasudevan Ramakrishnan et al (2013) with slight modification. Prior to extraction, sea cucumber muscle was cut into 1–2 cm pieces and ground well using a grinder. The amount of 50 g sample was first placed in a 500 ml glass bottle and put in a water bath (Memmer, Germany) at 85 C for 10 min to deactivate the endogenous enzymes. Then, 50 ml of distilled water was added to the mixture and mixed using a magnetic stirrer (RHD, IKA, Germany). The pH of the mixture was adjusted to the desired level (7.5) with 1N NaOH. The enzymatic hydrolysis was started by adding 1.5% (by weight of raw material) alcalase and placed in a shaker incubator (Comecta, Spain) operating at 200 rpm and 55°C for 4 h. At the end of hydrolysis, the reaction was stopped by heating the mixture to 85 C for 10 min to inactivate the enzymes. Afterward, the mixture was cooled at room temperature and centrifuged at 6000 rpm for 30 min. Paper filters and vacuum pumps were used to completely separate the solid particles from the liquid phase.

Determination of the degree of hydrolysis

The degree of hydrolysis (DH) was determined according to the method described by Hoyle and Merritt (1994) Merritt with some modifications. An aliquot of 500 mL hydrolysate was thoroughly mixed with 500 mL of 20% trichloroacetic acid (TCA), and the mixture was subjected to centrifugation at 8000 g for 10 min. The DH was calculated according Eq. (1):

$$\mathrm{DH} = \left(\frac{\mathrm{N}_2}{\mathrm{N}_1}\right) \times 100\%.$$

1

Where N2 is 10% TCA soluble nitrogen, N1 is sample soluble nitrogen.

Determination of soluble protein content

The amount of 5.5 g of sample was mixed with 11 ml of distilled water and then homogenized with a homogenizer. The sample was centrifuged and the supernatant was separated as total protein. Protein content of samples was determined by bicinchoninic acid (BCA) protein assay according to the manufacturer's protocol using bovine serum albumin as the standard. The absorbance of the samples was recorded by ELISA microplate reader (Epoch, Biotech, USA) at 562 nm.

Ultrafiltration

Protein hydrolysate was fractionated using UF membranes (Amicon, Millipore) with a range of molecular weight cutoffs of 3, 10, and 30 kDa, resulting in four fractions; FI (MW > 30 kDa), FII (10 < MW < 30 kDa), FIII (3 < MW < 10 kDa) and FIV (MW < 3 kDa). Afterward, the amount of protein in each fraction was determined by BCA. The fractions were collected to determine their antioxidant and ACE-inhibitory activities.

Antioxidant assay

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of hydrolysates was determined according to the method of Je et al (2009). Hydroxyl radicals were generated by a Fenton reaction in the presence of FeSO4. A volume of 200 µl diluted sample was mixed with a mixture containing 200 µl of 10 mM FeSO4, 200 µl of 100 mM EDTA, 200 µl of 10 mM 2-deoxyribose, and 1 ml of 0.2 M phosphate buffer (pH 7.4). Then, 200 µl of 10 mM H2O2 was added and the mixture was incubated at 37 C for 4 h. After incubation, 1 ml of 2.8% TCA and 1.0% TBA were added to the mixture, which was then placed in a boiling water bath for 15 min. The absorbance was measured at 532 nm. The hydroxyl radical scavenging activity was calculated as follows Eq. (2):

$$\mathrm{hydroxylradicalscavrngingactivity}\,(\%) = 1 - \left(\frac{\mathrm{A}_{\mathrm{sample}}}{\mathrm{A}_{\mathrm{blank}}}\right) \times 100.$$

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Ferric reducing power

The ferrous reducing power was determined by the method described by Chalamaiah et al (2015). A volume of 1 ml diluted sample was mixed with 2.5 ml potassium ferrate (1%). Then the mixture was incubated at 50 C in dark for 30 min. Then, 2.5 ml of 10% TCA was added to the mixture and incubated in room temperature for 10 min. A volume of 2.5 ml of incubated mixture was gently mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. After 10 min reaction, the absorbance of the final solution was measured at 700 nm. Ferrous ion chelating activity was calculated using the Eq. (3):

$$\mathrm{ferrouschelatingactivity}\,(\%) = 1 - \left(\frac{\mathrm{A_{sample}}}{\mathrm{A_{blank}}}\right) \times 100$$

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DPPH radical-scavenging assay

The DPPH radical scavenging activity of hydrolysates was determined accordingly to previously reported method Yang et al (2019). A volume of 1.5 ml of sample solution with different protein concentrations (0.1, 0.2, 0.5, 1, and 1.7 mg.ml) was added to 1.5 ml of 0.1 mM DPPH in 95% ethanol. The mixture was shaken and kept at room temperature in dark for 30 min, and the absorbance of the sample was recorded at 517 nm by ELISA microplate reader (Epoch, Biotech, USA). Then, DPPH radical scavenging activity was calculated according to Eq. (4):

$$\mathrm{DPPHinhibition}\,(\%) = 1 - \left(\frac{\mathrm{A_{sample}}}{\mathrm{A_{blank}}}\right) \times 100. \,(4)$$

Angiotensin-converting enzyme-inhibitory (ACE-I) activity

The ACE inhibitory activity was determined according to literature (Lee et al. 2009). A mixture of 50 µL hydrolysate solution and 50 µl of ACE solution (25 munits/ml) pre-incubated at 37 C for 5 min. Then, the mixture was incubated with 150 µl of substrate (8.3 mM Hip-His-Leu in 50 mM sodium borate buffer) at 37 C for 60 min. The reaction was terminated by adding 250 µL HCl 1 M. The resulting hippuric acid was extracted with 0.5 ml of ethyl acetate. After centrifugation (3000 rpm, 15 min), 0.2 mL of the upper layer was transferred into a test tube, and dried at 80 C for 1 h. The hippuric acid was dissolved in 0.5 ml of distilled water and absorbance was measured at 228 nm using ELISA microplate reader (Epoch, Biotech, USA).

Statistical analysis

Statistical analyses were performed with SPSS statistical software, version 17 in triplicate. One-way analysis of variance (ANOVA) test was applied to analyze differences between the means of each group. The data are reported as the mean ± standard deviation (SD).

Result and discussion

There are more than 1100 varieties of sea cucumbers, of which about 40 are available in commercial markets. Sea cucumbers usually have high protein and low lipid content. In recent years, marine protein-derived hydrolysates have received too much attention because of their health benefits. In the current study, the protein content of *H. parva* fresh tissue and enzymatic hydrolysis product was analyzed (Table 1). The result showed that the protein content of SPH was 63.75 mg/g which was close to other sea cucumber species, such as *Holothuria polii* (86.6 mg/g), *Holothuria tubulosa* (88.2 mg/g) and *Holothuria mammata* (78.8 mg/g), and it was higher than the protein content of *Stichopus horrens* (28.3 mg/g) (Chang-Lee et al. 1989; Forghani et al. 2012; Ghanbari et al. 2012). Thus, *H. parva* protein could be expected to be a suitable source of protein. The protein contents of four faction peptides after ultrafiltration are summarized in Table1. The enzymatic hydrolysis is one of the best methods for preparing marine protein that can facilitate the production of short chain peptides (Vijaykrishnaraj and Prabhasankar 2015). In this study, hydrolysis of sea cucumber *H. parva* muscle by alcalase enzyme was performed at 55°C for 3 hours, which resulted in about 45.9 degree of hydrolysis. Similarly, degree of hydrolysis for sea cucumber by alkaline enzyme in various studies has been reported as 42.2% at 120 min (Chim-Chi et al. 2017), 39.8 (Forghani et al. 2012), 48.37 (Safari and Yaghoubzadeh 2020), 83.35 (Ghanbari et al. 2012). Therefore, hydrolysis of *H. parva* muscle by alcalase enzyme has the potential to produce peptide fractions.

Table.1. Protein content of *H. parva*fractions after ultrafiltration (mg/ml).

Fractions	FI	FII	FIII	FIV
Protein content (mg/ml)	47.33	57.08	47.66	15.5

Anti-oxidative activities

Many biological activities in sea cucumbers have been reported. Their activities are often species-specific and closely are related with the bioactive compounds contained in sea cucumbers.Sea cucumbers exert strong capability of scavenging ROS. Among reactive oxygen species (ROS), is an important reactive oxygen species with the strongest chemical reaction. It reacts easily with amino acids, DNA, and membrane components, resulting in extensive biological damage. Hydroxyl radical scavenging activity of sea cucumber hydrolysates were reported by researches (Wang et al. 2010; Zhou et al. 2012; Zhang et al. 2017; Safari and Yaghoubzadeh 2020). Fig.1 shows hydroxyl radical scavenging activity of SPH and its fractions. The results indicated that SPH has the strongest hydroxyl radical scavenger (48% at 10 µg/ml)

followed by FIV (35% at 10 µg/ml), FIII (22% at 10 µg/ml), FII (15% at 10 µg/ml), and then FI (13% at 10 µg/ml). FIV exhibited the strongest scavenging activity on hydroxyl radicals among fractions. According to previous reports protein hydrolysates from sea cucumbers exhibit anti-oxidant activities depending on molecular weight, amino acids composition and peptide hydrophobicity. The results of this study showed that the peptide fractions which had the low molecular weight showed the highest hydroxyl radical scavenging activity (You et al. 2010; Chi et al. 2014). Similarly, UF fractions with the lowest molecular mass range < 3-kDa, which were obtained from the sea cucumber exhibited high hydroxyl radical scavenging activity (Zhou et al. 2012; Safari and Yaghoubzadeh 2020). The results indicated that FIV fraction possibly contained more substrates, which were electron donors and could react more easily with free radicals to make them more stable products and terminate radical chain reactions. With enzymatic extraction followed by UF method can target specific sites of proteins to produce desirable bioactive peptides

Ferric (Fe^{3+}) reducing antioxidant power (FRAP)

The FRAP is often used to evaluate the ability of antioxidant compounds to donate an electron or hydrogen, and some research have indicated that there is a direct correlation between antioxidant activities and reducing power of peptide (Wiriyaphan et al. 2012; Chalamaiah et al. 2015). As shown in Fig.2 SPH and its fractions exhibited strong ferric reducing power. Among the samples SPH exhibited the highest ferric reducing power (229.03 % at 10 µg/ml) followed by FIV (146/94% at 10 µg/ml) and FIII (72/64% at 10 µg/ml), while the lowest value were obtained from FI (54.5% at 10 µg/ml) and FII (48% at 10 µg/ml). It is suggesting that the higher ferric reducing power detected in SPH may have resulted from additive or synergistic actions of the four peptide fractions in total hydrolysate. Similar results have revealed that the low molecular weight fraction have higher reducing power than high molecular weight fraction (Zheng et al. 2012; Chai et al. 2015; Taheri and Bakhshizadeh G 2020). Compounds with higher reducing power have better abilities to donate electron or hydrogen and use as an antioxidant (Je et al. 2009). Therefore, strong reducing power of sea cucumber hydrolysate may be attributed to the increased availability of hydrogen ions due to release of the peptide fractions by enzymatic hydrolysis

DPPH radical-scavenging assay

DPPH has been widely used as substrate to evaluate antioxidant properties of compounds as free radical scavengers or hydrogen donors. When DPPH radical encounter a proton-donating substance such as an antioxidant, the radical is scavenged and the absorbance is reduced by changing color from purple to yellow (Liu et al. 2009). The DPPH scavenging activity of SPH, FI, and FII were 7.32%, 27.4%, and 6.77% at 10 µg/ml, respectively (Fig.3). The results are in line with previous researches reported by Zhang et al (2017) and Guo et al (2020) who reported that the DPPH scavenging activity of sea cucumber hydrolysate. Moreover, the results clearly indicated that FI fraction with high molecular weight exhibited the highest DPPH radical scavenging activity, which is contrary to some previous studies (Zheng et al. 2012; Safari and Yaghoubzadeh 2020).

Angiotensin-converting enzyme-inhibitory activity

Angiotensin-I converting enzyme (ACE) plays a crucial role in the regulation of blood pressure and widely utilized in clinical applications to prevent angiotensin II generation from angiotensin I in cardiovascular diseases. The result of present study indicated that ACE-inhibitory activity protein hydrolysates SPH, FI, FII, FIII, and FIV of *H .parva* were about 8.80, 7.60, 3.73, 7.86, and 13.60% at 1 mg/ml respectively (Fig.4). these result suggested that alcalase enzyme have good ability to release ACE-inhibitory peptides from *H. parva* muscle. The review of Lee and Hur (2017) focusing on animal-, marine-, and plant-origin ACE inhibitors, have concluded that optimal hydrolysis treatment for marine organisms is microbe-extracted enzymes (e.g., alcalase, neutrase, thermolysin). Furthermore, various studies have reported that alcalase is one of the most effective enzymes to isolate ACE inhibitory peptides from sea cucumber species (Zhao et al. 2007; Forghani et al. 2012; Ghanbari et al. 2015; Dewi et al. 2020). FIV fraction with molecular weight below 3 kDa exhibited the most potent activity compared to SPH and other fractions, probably because the lower molecular weight peptides were thought to be much easier to reach the active site of ACE to realize the inhibitory activity (Alemán et al. 2011). These results are consistent with previous studies reporting greater ACE-inhibitory activity of low molecular weight marine peptides rather than those with high molecular weight (Jung et al. 2006; Wu et al. 2008; Lee et al. 2010; Dewi et al. 2020). Similarly, Zhao et al (2007) have reported that the ACE inhibitory activities of sea cucumber hydrolysates varied with the molecular weight, and the fraction with molecular weight below 1 kDa exhibited higher ACE inhibitory activity. On the other hand, Silvestre et al (2012) have reported that the ultrafiltration may result in retention of peptides with structures (aromatic amino acids or proline at the C-terminal position) that enhance ACE-inhibitory activity.

Conclusion

Alcalase-hydrolysed protein from *H. parva* (45.9% DH) contained bioactive peptide fractions possessing antioxidant and ACE inhibitory properties. SPH exhibited the highest hydroxyl radical and ferric ion scavenging activities, whilst FI fraction showed the highest DPPH radical scavenging activity. Furthermore, FIV exhibited the highest ACE inhibitory activity. However, further research will be needed to identify and purify bioactive peptide fractions in *H. prva* hydrolysate.

Declarations

Funding

This study had the support of Faculty of Marine Science, Tarbiat Modares University, Iran.

Conflict of Interests

The authors declare no competing interests.

Contributions

Asghar Mohamadzadeasl was Masters student and involved in thesis project conceptualization, conducted the bioactivity assays, performed the extractions and wrote the manuscript **and** Saber Khodabandeh was Supervisor, provided scientific supervision during all stages of this project.

Data Availability

The datasets of the current study are available from the corresponding author on reasonable request.

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Figures

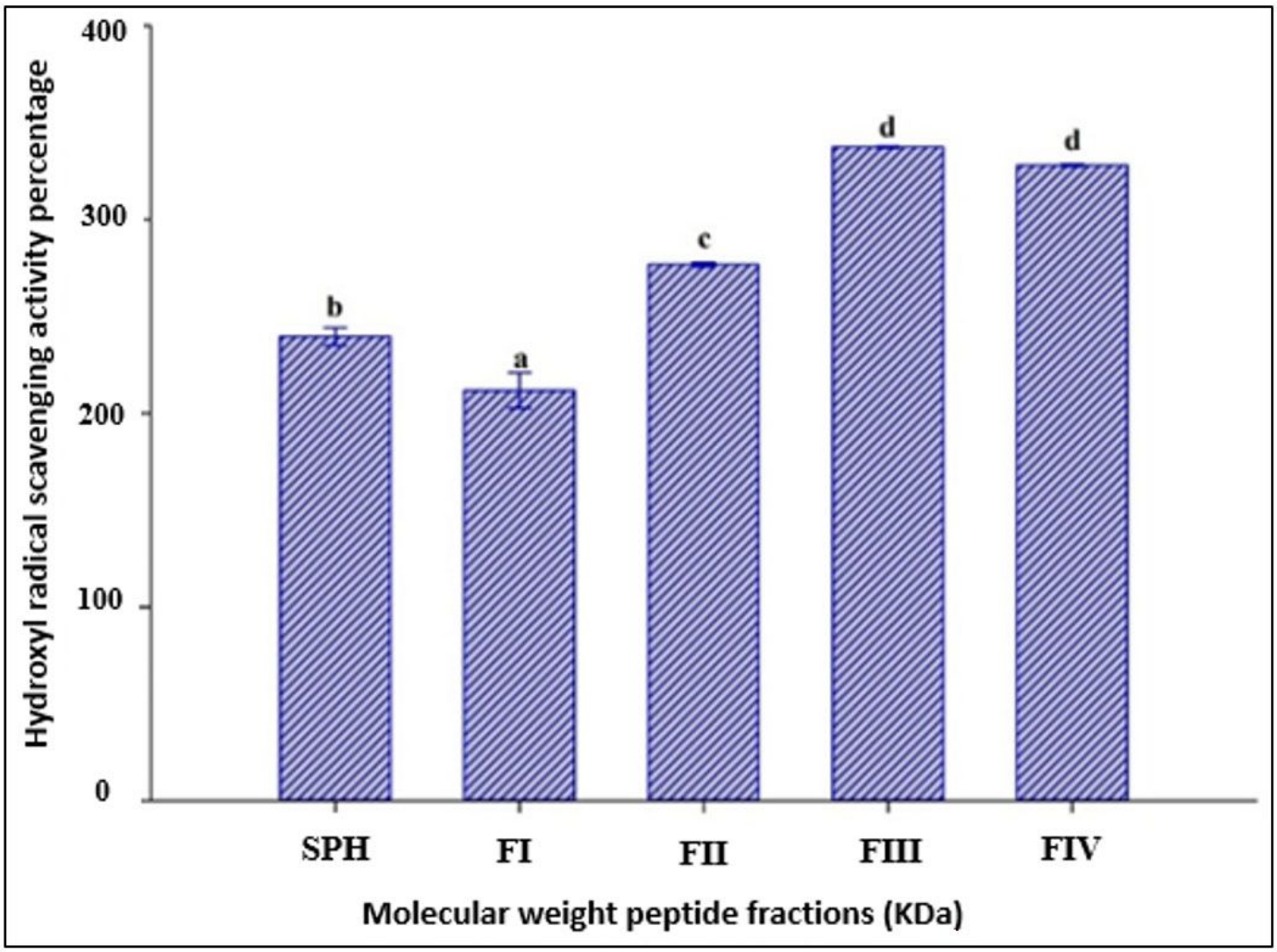

Figure 1

Hydroxyl radical scavenging activity of *H.parva*hydrolysate and its fractions. Different letters (a, b, c, …) indicate significant differences ($p<0.05$).

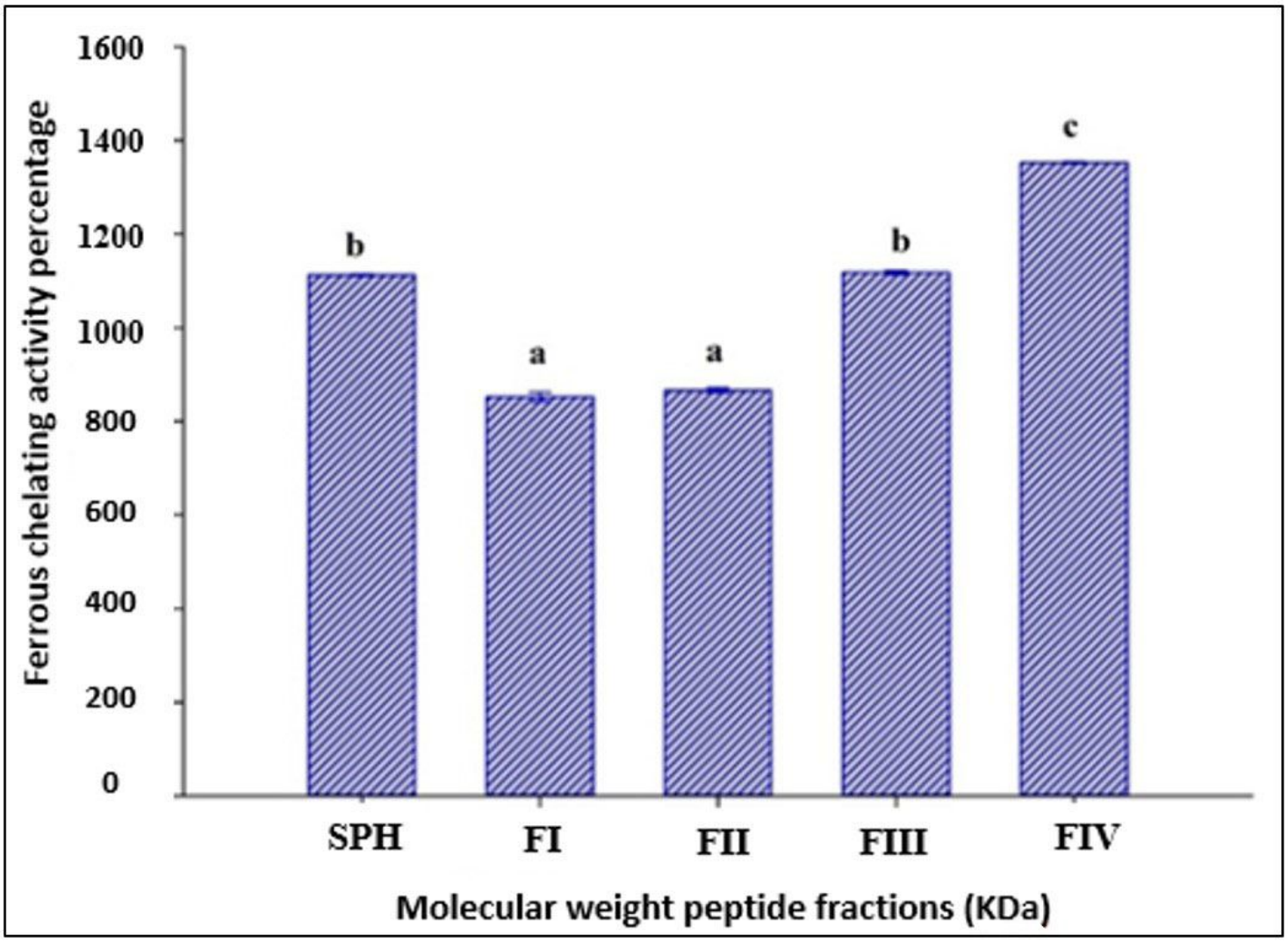

Figure 2

Ferrous reducing antioxidant power of *H.parva* hydrolysate and its fractions. Different letters (a, b, c, …) indicate significant differences ($p<0.05$).

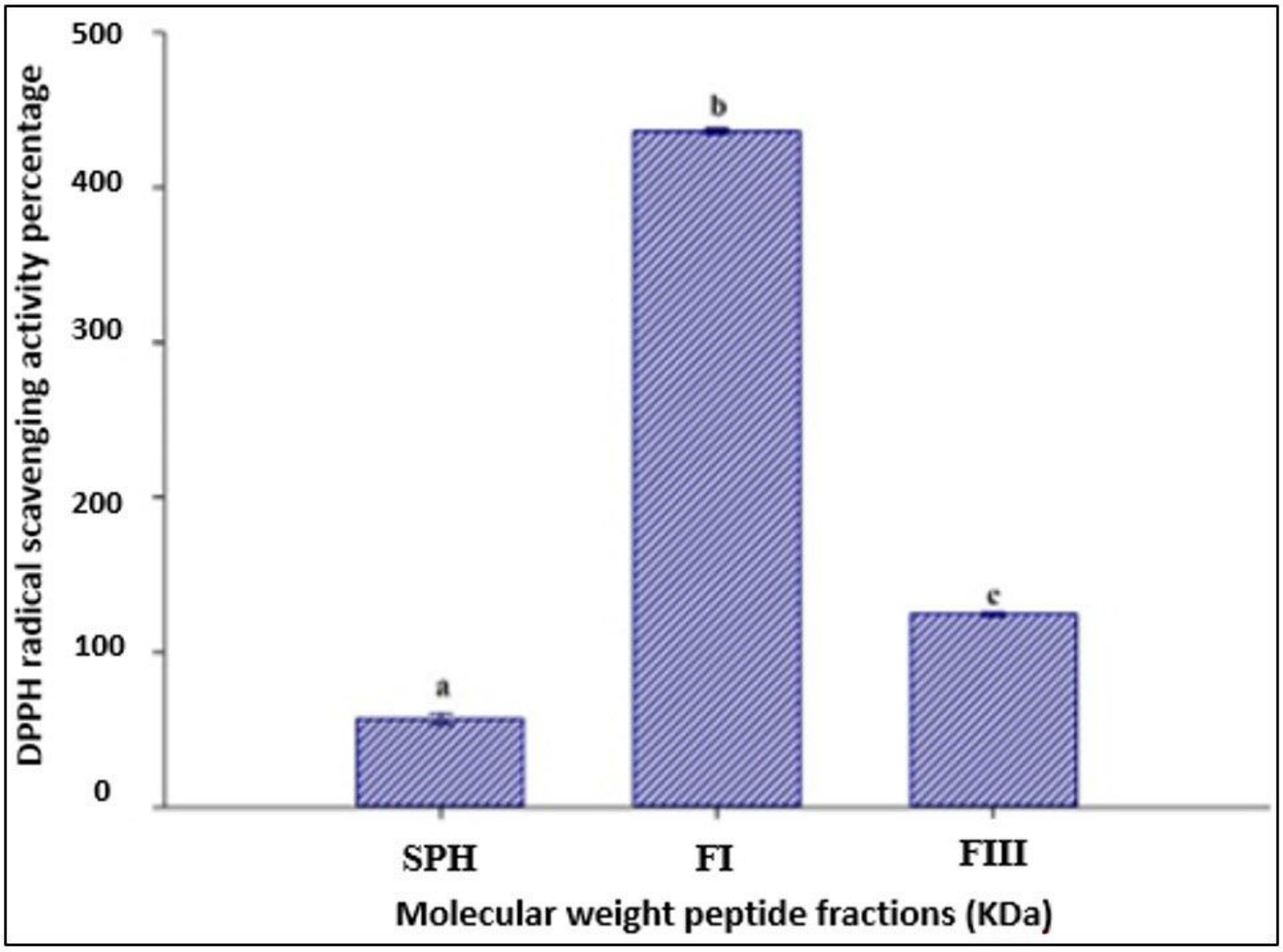

Figure 3

DPPH radical scavenging activity of *H.parva* hydrolysate and its fractions. Different letters (a, b, c, …) indicate significant differences ($p<0.05$).

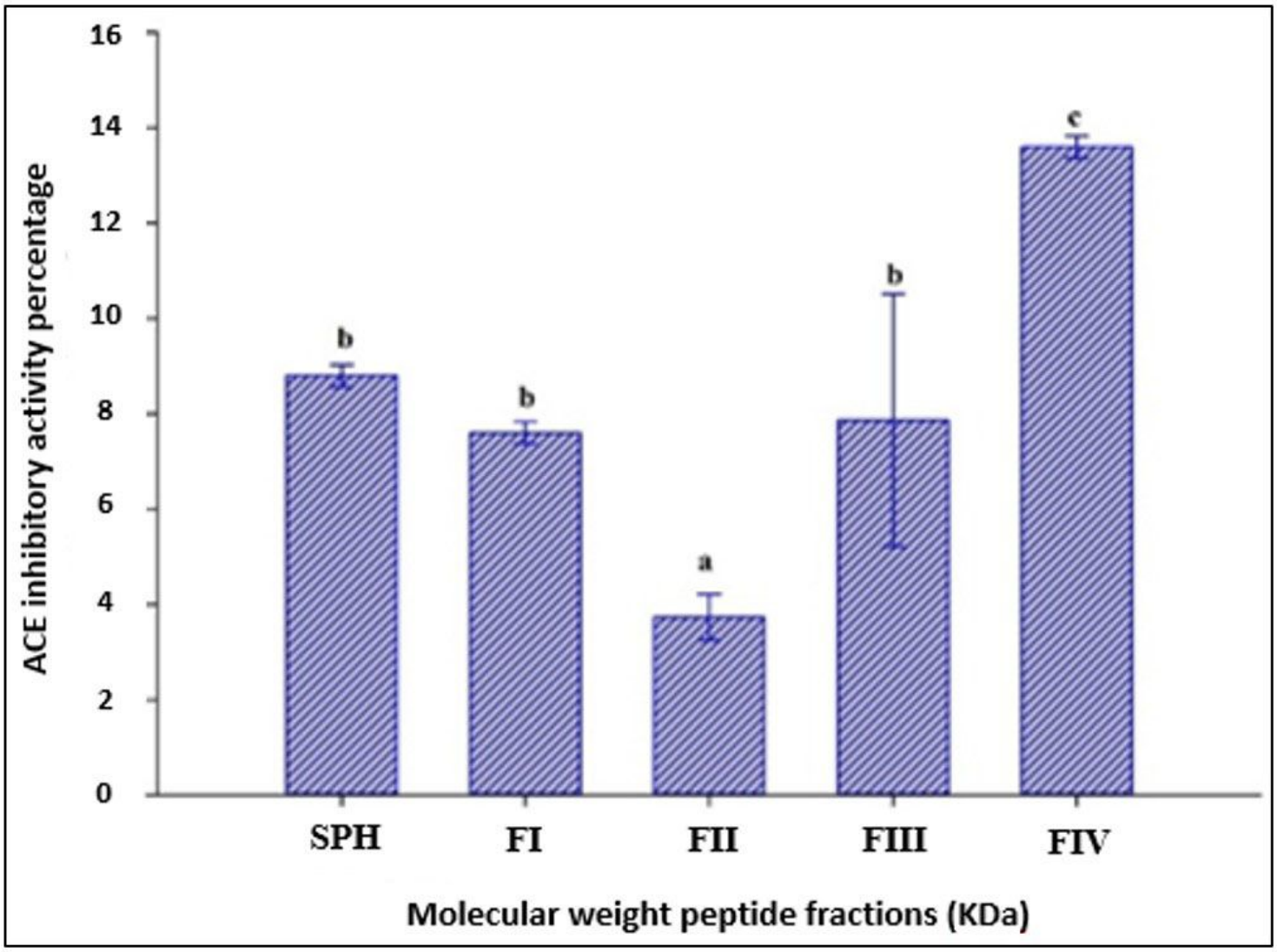

Figure 4

ACE-inhibitory activity of *H.parva* hydrolysate and its fractions. Different letters (a, b, c, …) indicate significant differences ($p<0.05$).