Radical scavenging and amino acid profiling of wedge clam, *Donax cuneatus* (Linnaeus) protein hydrolysates

R. A. Nazeer · M. A. V. Saranya · Shabeena Yousuf Naqash

Revised: 8 September 2012 / Accepted: 7 November 2012 / Published online: 16 November 2012
© Association of Food Scientists & Technologists (India) 2012

Abstract Body, foot and viscera of *Donax cuneatus* (Linnaeus) were hydrolyzed using commercial proteases (pepsin, trypsin and papain) and tested for their antioxidant activity by DPPH scavenging ability and reducing power assays. In comparison between all the hydrolysates, papain viscera (28.513 ± 0.165 & 0.186 ± 0.008) and foot (33.567 ± 0.132 & 0.166 ± 0.013) hydrolysates showed highest **DPPH and reducing power ability respectively**. The active hydrolysates were purified with DEAE- cellulose followed by Sephadex G-25 columns connected to FPLC. Further, the isolated active fractions were loaded onto HPLC for their amino acid profiling and found with the presence of potential amino acids viz., histidine, cysteine, alanine etc. These results suggest that the isolated antioxidant peptide from viscera and foot hydrolysate of *D. cuneatus* can be used in treating human diseases where free radicals and oxidative damage are involved.

Keywords Antioxidant · *Donax cuneatus* · ROS · ESR Spectrometer · Protein hydrolysate

Introduction

Reactive oxygen species (ROS) such as singlet oxygen ($^1\text{O}_2$), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical ($^*\text{OH}$) are often generated as **byproducts** of biological reactions or from exogenous factors (Curti 1991). The involvement of these species in the pathogenesis of a large number of diseases including rheumatoid arthritis, atherosclerosis, skin-aging, nephritis,

reperfusion injury, asthma, diabetes mellitus and carcinogenesis are well documented (Harman 1998). On the other hand oxidative reactions in foods lead to the deterioration of quality attributes such as flavor, aroma, texture, and color (Park et al. 2001). In lipid peroxidation, these reactions typically involve free radical pathways that result in the formation of lipid hydroperoxides, which are eventually decomposed to low molecular weight carbonyls in the presence of prooxidants (Elias et al. 2008). A potent scavenger of these species may serve as a possible preventive intervention for free radical mediated diseases (Gutteridge and Halliwell 2010). But, synthetic antioxidant such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are restricted to use in food products because of their induction of DNA damage and toxicity (Ito et al. 1986). So, natural antioxidants have been the focus of considerable interest in recent times and in our previous work we examined the antioxidant activity of the peptides isolated from *Nemipterus japonicas* and *Exocoetus volitans* (Shabeena and Nazeer 2010, 2011).

In India, bivalve mollusc comprises major marine fishery resources and is traditionally fished for food and shell. The annual production of gastropods and bivalves is estimated at about 1, 00,000 tones. Fishing for molluscs provides employment to thousands of people, particularly to those living in coastal rural areas (Shanmugam et al. 2007). The farming of bivalves is fast picking up, and during 2002, the production of oysters was estimated at 350 tones and mussels 1,250 tones (Narasimham 2005). Of the several species of wedge clams of the family Donacidae that occur on the east and west coasts of India in the surf-beaten sands, *Donax cuneatus*, is considered as food source by many people (Nayar 1952). It forms the food of a large number of fishermen along the coast when the sea is rough and the general fishery conditions are poor. However, at present there is no regular fishery of these clams for food or any

R. A. Nazeer (✉) · M. A. V. Saranya · S. Y. Naqash
Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur,
603 203, Chennai, Tamilnadu, India
e-mail: nazeer.ra@ktr.srmuniv.ac.in

organized lime burning industry, with the result that this valuable resource is wasted without being much utilized. Therefore, the aim of this study was to identify the antioxidative potentials and amino acid composition of peptides derived from the wedge clam *Donax cuneatus* protein hydrolysate. For this purpose, the wedge clam protein was enzymatically hydrolyzed and the resulting peptides were assessed for their free radical scavenging ability.

Materials and methods

Materials

1,1-diphenyl-2-picrylhydrazyl (DPPH), methanol, ethanol, linoleic acid, ammonium thiocyanate, ferric chloride, α -tocopherol, potassium ferricyanide, trichloroacetic acid, trypsin, papain, pepsin, sodium carbonate were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Sample collection

Wedge clam, *Donax cuneatus* (L.) was collected from Kovalam Beach (13° 6' 15" N and 30° 17' 30" E), Chennai, Tamilnadu. The shells were removed and the sample was dissected into foot and viscera and stored at −20 °C before further assays.

Enzymatic hydrolysis

To extract antioxidant peptide from different parts of clams, enzymatic hydrolysis was performed using various enzymes [pepsin (0.1 M Glycine buffer, pH 2.0 at 37 °C), trypsin (0.1 M Sodium phosphate buffer, pH 8.0 at 37 °C) and papain (0.1 M Sodium phosphate buffer, pH 6.0 at 37 °C)] at their optimal conditions. At enzyme/substrate ratio of 1/100 (w/w), 1 % substrate and enzyme were mixed. The mixture was incubated for 6 h at each optimal temperature with stirring and then heated in a boiling water bath for 10 min to inactivate the enzyme. Aliquot from each hydrolysate was centrifuged at 12000 rpm for 10 min and supernatant was lyophilized as discussed by and stored under −20 °C until use (Je et al. 2007).

Scavenging ability on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals

DPPH radical scavenging activity was determined according to the method of Yen and Hsieh (1995). An aliquot of sample (1 mg/ml) was mixed with 3 ml of methanol and then added to 1 ml of 0.15 mM DPPH in methanol. The mixture was then mixed vigorously and

allowed to stand at room temperature in the dark for 30 min. The absorbance of the mixture was measured at 517 nm using a UV-Vis spectrophotometer. The control was conducted in the same manner but methanol was used instead of sample. DPPH radical scavenging activity was calculated as follows:

Radical scavenging activity (%)

$$= (\text{control} - \text{sample}) / \text{control} \times 100,$$

Where, sample is the absorbance of sample and control is the absorbance of the control at 517 nm.

Reducing power assay

The ability of the hydrolysate to reduce iron (III) was determined according to the method of Yen and Chen (1995). An aliquot of 1 mg/ml sample of each hydrolysate was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1 % potassium ferricyanide. The mixture was incubated at 50 °C for 30 min, followed by addition of 2.5 ml of 10 % (w/v) trichloro acetic acid. The mixture was then centrifuged at $1,650 \times g$ for 10 min. Finally, 2.5 ml of the supernatant solution were mixed with 2.5 ml of distilled water and 0.5 ml of 0.1 % (w/v) ferric chloride. After 10 min reaction the absorbance of the resulting solution was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Measurement of the antioxidative activity in linoleic acid model system

The antioxidative activity was measured in α -linoleic acid model system according to the methods of Osawa and Namiki (1985). Briefly, a sample (1.3 mg) was dissolved in 10 ml of 50 mM phosphate buffer (pH 7.0), and added to a solution containing 0.13 ml of linoleic acid and 10 ml of 99.5 % ethanol. Then the total volume was adjusted to 25 ml with distilled water. The mixture was incubated in a storage bottle with a screw cap at 40 ± 2 °C in a dark room and the degree of oxidation was evaluated by measuring the ferric thiocyanate values. The ferric thiocyanate value was measured according to the method of Mitsuta et al. (1996). The reaction solution (100 μ l) incubated in the α -linoleic acid model system was mixed with 4.7 ml of 75 % ethanol, 0.1 ml of 30 % ammonium thiocyanate, and 0.1 ml of 2×10^{-2} M ferrous chloride solution in 3.5 % HCl. After 3 min, the thiocyanate value was measured by reading the absorbance at 500 nm following colour development with FeCl_2 and thiocyanate at different intervals during the incubation period.

Table 1 DPPH radical scavenging activity and reducing power ability of wedge clam protein hydrolysates

Body parts	Type of enzyme	DPPH	Reducing power assay
Foot	Trypsin	25.213±0.114 ^c	0.156±0.013 ^c
	Pepsin	20.827±0.126	0.124±0.012
	Papain	33.567±0.132 ^b	0.166±0.013 ^b
Viscera	Trypsin	27.783±0.105 ^b	0.155±0.008 ^b
	Pepsin	19.463±0.150 ^c	0.132±0.009 ^c
	Papain	28.513±0.165 ^a	0.186±0.008 ^a

Mean±Standard Deviation, $n=3$;
P values; ^a 0.00, ^b 0.01, ^c 0.05

Purification of the active fractions

The lyophilized clam viscera and foot protein hydrolysate (20 mg/ml) was on a XK 26 DEAE anion exchange column equilibrated with 20 mM sodium acetate buffer (pH 4.0), and eluted with a linear gradient of NaCl (0–1.5 M) in the same buffer at a flow rate of 1 ml/min. Each fraction collected at a

volume of 6 ml was monitored at 280 nm, pooled fractions were then concentrated using a rotary evaporator; and antioxidant activities were investigated. The fraction having strong antioxidant properties were lyophilized, and loaded onto a Sephadex G-25 gel filtration (GE Healthcare, Sweden) column (2.5×75 cm) which was previously equilibrated with distilled water and set into at a flow rate of 1 ml/min. Each

Fig. 1 Purification of the antioxidant peptide from viscera protein hydrolysate of wedge clam (a) By XK26 DEAE anion exchange chromatography (b) By Sephadex G-25 gel filtration chromatography

fraction collected at a volume of 3 ml was monitored at 280 nm, pooled fractions were then concentrated using a rotary evaporator; and antioxidant activities were investigated.

Amino acid composition

The amino acid composition was identified by using the methods of Huesgen (1999). The lyophilized active fractions from clam viscera and foot were digested with HCl (6 N) at 110 °C for 24 h and neutralized with NaOH and loaded onto High performance liquid chromatography (HPLC). The analysis was carried out in an Agilent 1100 assembly system after precolumn derivatization with o-phthalaldehyde (OPA). Each sample (1 µl) was injected on a Zorbax 80 A C₁₈ column at 40 °C with detection at 338 and 262 nm. Mobile phase A was 7.35 mmol/l sodium acetate/triethylamine (500:0.12, v/v), adjusted to pH 7.2 with acetic acid, while mobile phase B (pH 7.2) was 7.35 mmol/l sodium acetate/ methanol/ acetonitrile (1:2:2, v/v/v). The amino acid composition was expressed as percentage of protein.

Fig. 2 Purification of the antioxidant peptide from foot protein hydrolysate of wedge clam (a) By XK26 DEAE anion exchange chromatography (b) By Sephadex G-25 gel filtration chromatography

Statistical analysis

Experimental results were expressed as mean of triplicate $\pm$ standard deviation. Statistical analysis of the result was performed using SPSS 10.1 for Windows (SPSS, Chicago, IL, USA).

Results and discussion

Preparation of clam protein hydrolysates and their antioxidant properties

Recently, protein hydrolysates obtained by enzymatic digestion of various marine resources tuna liver (Je et al. 2009), pink perch (Shabeena and Nazeer 2010), Nile tilapia (Ngo et al. 2010), gastropod (Nazeer and Srividhya 2011) and sea urchin (Qin et al. 2011) were reported to have antioxidant activity. In the present study viscera and foot **were chosen and processed further for hydrolysis** using commercial

enzymes (pepsin, trypsin and papain) and the prepared hydrolysates were evaluated for their antioxidant activity using DPPH radical scavenging activity and reducing power ability assay. As shown in Table 1 papain viscera (28.513 ± 0.165) and papain foot (33.567 ± 0.132) effectively quenched DPPH radicals and basing on reducing power assay papain viscera (0.186 ± 0.008) and foot (0.166 ± 0.013) showed highest activity than remaining hydrolysates. Papain is a cysteine protease of peptidase family, which can cleave the protein and produce peptides and the peptides prepared using this enzyme on porcine myofibril (Saiga et al. 2003), black-bone silky fowl (Liu et al. 2011) and loach (You et al. 2011) has shown similar strong antioxidant activity. Based on DPPH radical scavenging and reducing power ability tests the two active protein hydrolysates were chosen and preceded further for purification.

Purification of active fraction

The two active papain viscera and foot protein hydrolysates were employed for the purification and identification of antioxidant peptide. Hydrolysates were loaded onto DEAE anion exchange column and three fractions (I, II and III) of each were collected as shown in chromatogram (Figs. 1a and 2a). Generally, purification of antioxidant active peptide by ion exchange is common fashion (Qian et al. 2008; Shabeena and Nazeer 2010, 2011) and the collected fractions were concentrated and tested against DPPH radical scavenging activity (Table 2). As shown in figure, the active fraction of viscera (II – 45.32 ± 0.144) and foot (III – 43.43 ± 0.066) was further purified using the Sephadex G-25, open column connected to FPLC (Figs. 1b and 2b). Since, *D. cuneatus* being a marine animal and moreover after ion exchange chromatography it is preferred to opt for a gel filtration column, which facilitates the samples to purify and also desalt (Byun et al. 2009; Shabeena and Nazeer 2010; Nazeer and Srividhya 2011). As observed in Figs. 1 b and 2 b, clear peaks labeled as A–D were identified and compared for their antioxidant activity similar as after ion exchange.

Table 2 DPPH radical scavenging activity of Fast protein liquid chromatography fractions collected from wedge clam protein hydrolysates

Fractions	Viscera	Foot
I	12.08 ± 0.055	19.090 ± 0.017^b
II	45.32 ± 0.144	36.183 ± 0.014^a
III	22.25 ± 0.079^b	43.43 ± 0.066^b
A	73.62 ± 0.226^b	53.474 ± 0.015^a
B	36.553 ± 0.096^b	79.491 ± 0.237
C	22.700 ± 0.131	31.135 ± 0.014^a
D	—	12.168 ± 0.026^b

Mean $\pm$ Standard Deviation, $n=3$; P values; ^a 0.00, ^b 0.01

Table 3 Amino acid composition (% w/w) of the active fractions of purified protein hydrolysates

Amino acid	Viscera	Foot
Glycine	1.16	7.81
Histidine	9.41	1.21
Glutamic acid	6.79	7.79
Threonine ^a	5.92	11.24
Arginine	12.76	2.83
Aspartic acid	3.46	2.25
Valine ^a	3.32	3.23
Leucine ^a	2.16	3.21
Alanine	3.74	5.68
Lysine ^a	13.89	13.49
Serine	1.31	2.87
Tyrosine	9.72	8.62
Phenylalanine ^a	10.92	7.79
Proline	4.65	6.37
Isoleucine ^a	2.75	2.13
Methionine ^a	3.78	4.48
Cysteine	2.32	5.56
Tryptophan ^a	1.70	2.86

^a Essential amino acids

Active fractions of viscera (A – 73.62 ± 0.226) and foot (B – 79.491 ± 0.237) showing high antioxidant activity (Table 2) were finally loaded on an analytical HPLC and their amino acid sequences were determined.

Amino acid Composition

After recording the activity of isolated antioxidant peptides from wedge clam viscera and foot protein hydrolysates, it is possibly due to the presence of amino acids, which functioned as electron donors and could react with free radicals to form more stable products. To conform the quality and

Fig. 3 Antioxidant activity of purified peptide in a linoleic acid emulsion system measured by the ferric thiocyanate method. Mean $\pm$ Standard Deviation, $n=3$

quantity of amino acids available in isolated peptides from viscera and foot papain hydrolysates were subjected to HPLC for amino acid composition, as tabulated (Table 3). Bioactive peptides contain 2–20 amino acid residues per molecule (Pihlanto-Leppala 2000) and wedge clam peptides also showed the presence in same. Lysine (13.89 & 13.49 %), threonine (5.92 & 11.24 %), tyrosine (9.72 & 8.62 %) and phenylalanine (10.92 & 7.79 %) are the major amino acids and serine (1.31 & 2.87 %), aspartic acid (3.46 & 2.25 %) and valine (3.32 & 3.23 %) showed low percentage in the amino acid profiling of viscera and foot respectively. These compositions of amino acids were also observed in active antioxidant fractions of fishes and gastropod (Nazeer et al. 2011; Nazeer and Srividhya 2011). The above results showed the presence of essential amino acid contents of both the samples, which was higher than the recommended values for human adults (FAO/WHO 1990). And presence of amino acids viz., glutamic acid, histidine, threonine, arginine in good percentage explains the reason behind the high antioxidant activity (Shabeena and Nazeer 2011). Moreover, amino acids such as, tyrosine, methionine, histidine, lysine and tryptophan, were generally accepted as antioxidants (Chen et al. 1996) and presence of all these amino acids makes the wedge clam peptide a potent antioxidant.

Measurement of the antioxidative activity in α -linoleic acid model system

As shown in Fig. 3, both viscera and foot peptides were considerable inhibitors of lipid peroxidation and exhibited a nonlinear pattern. These peptides, has slow downed the lipid oxidation successfully for a period of six days and the results were compared with natural antioxidant α -tocopherol. Even though the exact mechanism of peptides to act as antioxidant is not clearly known but, the aromatic amino acids and histidine are reported to play a vital role for the activity (Chen et al. 1996). And more over presence of hydrophobic amino acids also help the peptides to interact and stop the lipid peroxidation. Since, lipid oxidation is of a great concern of the food industry and among consumers because it leads to the development of undesirable off-flavors and potentially toxic reaction products (Park et al. 2001). Therefore, the peptides isolated from wedge clam may have potential applications in the food industries as well as in pharmaceuticals.

Conclusion

Based on these results, the protein hydrolysates prepared from the foot and viscera of *Donax cuneatus* by enzymatic hydrolysis has potent antioxidant properties. And the purified fractions from viscera and foot papain protein

hydrolysates possess high antioxidant activity along with lipid peroxidation inhibition ability, which would be a great concern for nutraceutical industries. However, extensive studies like peptide sequencing and *in vivo* level evaluation are required to understand the complete nature of these peptides.

Acknowledgment We gratefully acknowledge Dr. K. Ramasamy, Dean, School of Bioengineering, SRM University, for his support throughout the work. We extend our gratitude to the management, SRM University for providing the facilities.

References

- Byun HG, Lee JK, Park HG, Jeon JK, Kim SK (2009) Antioxidant peptides isolated from the marine rotifer, *Brachionus rotundiformis*. *Process Biochem* 44:842–846
- Cerutti PA (1991) Oxidative stress and carcinogenesis. *Eur J Clin Invest* 21:1–11
- Chen HM, Muramoto K, Yamauchi F, Nokihara K (1996) Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soyabean protein. *J Agri Food Chem* 44:2619–2623
- Elias RJ, Kellerby SS, Decker EA (2008) Antioxidant activity of proteins and peptides. *Crit Rev Food Sci Nutr* 48:430–441
- FAO/WHO (1990) Protein quality evaluation. Report of a joint FAO/WHO expert consultation held in Bethesda, Md. 1989 Dec 4–8. FAO/WHO, Rome
- Gutteridge JMC, Halliwell B (2010) Antioxidants: molecules, medicines, and myths. *Biochem Biophys Res Comm* 393:561–564
- Harman D (1988) Free radical theory of ageing: current status. In: Nagy IZ (ed) *Akademiai Kiado. Budapest and Elsevier Science Publishers, Amsterdam*, pp 3–21.
- Huesgen AG (1999) Sensitive and reliable amino acid analysis in protein hydrolysate using Hp1100 Series HPLC. *Hewlett Packard, Palo Alto, CA*, pp 1–12
- Ito N, Hirose M, Fukushima S, Tsuda H, Shirai T, Tatematsu M (1986) Studies on antioxidants: their carcinogenic and modifying effects on chemical carcinogenic (carcinogenesis). *Food Chem Toxicol* 24:1099–1102
- Je JY, Park PJ, Kim SK (2007) Antioxidant activity of peptide isolated from Alaska Pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Food Res Intern* 38:45–50
- Je JY, Lee KH, Lee MH, Ahn CB (2009) Antioxidant and antihypertensive protein hydrolysates produced from tuna liver by enzymatic hydrolysis. *Food Res Intern* 42:1266–1272
- Liu JH, Tian YG, Wang Y, Nie SP, Xie MY, Zhu S, Wang CY, Pan Z (2011) Characterization and *in vitro* antioxidation of papain hydrolysate from black-bone silky fowl (*Gallus gallus domesticus* Brisson) muscle and its fractions. *Food Res Intern* 44:133–138
- Mitsuta HK, Yasumoto K, Iwami K (1996) Antioxidative action of indole compounds during the autoxidation of linoleic acid. *Eiyoto Shokuryo* 19:210–214
- Narasimham KA (2005) Molluscan fisheries of India. *Vedamsbooks. Delhi, B.R. Pub.* 14, 362
- Nayar NK (1952) Studies on the growth of the wedge clam, *Donax (Latona) cuneatus* Linnaeus. *Ind J Fish* 2:325–349
- Nazeer RA, Srividhya TS (2011) Antioxidant peptides from the protein hydrolysates of *Conus betulinus*. *Intern J Pep Res Therap* 17:231–237

- Nazeer RA, Deeptha R, Jaiganesh R, Sampathkumar NS, Shabeena YN (2011) Radical scavenging activity of seela (*Sphyræna barracuda*) and ribbon fish (*Lepturacanthus savala*) backbone protein hydrolysates. *Intern J Pep Res Therap* 17:209–216
- Ngo DH, Qian ZJ, Ryu BM, Park JW (2010) *In vitro* antioxidant activity of a peptide isolated from Nile tilapia (*Oreochromis niloticus*) scale gelatin in free radical-mediated oxidative systems. *J Funct Foods* 2:107–117
- Osawa T, Namiki M (1985) Natural antioxidant isolated from eucalyptus leaf waxes. *J Agri Food Chem* 33:777–780
- Park PJ, Jung WK, Nam KS, Shahidi F, Kim SK (2001) Purification and characterization of antioxidative peptides from protein hydrolyzate of lecithin-free egg yolk. *J American Oil Chem Soc* 78:651–656
- Pihlanto-Leppala A (2000) Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory. *Trends Food Sci Tech* 11:347–356
- Qian ZJ, Jung WK, Kim SK (2008) Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin *Rana catesbeiana* Shaw. *Bioresource Tech* 99:1690–1698
- Qin L, Zhu BW, Zhou DY, Wu HT, Tan H, Yang JF, Li DM, Dong XP, Murata Y (2011) Preparation and antioxidant activity of enzymatic hydrolysates from purple sea urchin (*Strongylocentrotus nudus*) gonad. *LWT - Food Sci Tech* 44:1113–1118
- Saiga A, Tanabe S, Nishimura T (2003) Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. *J Agri Food Chem* 51:3661–3667
- Shabeena YN, Nazeer RA (2010) Antioxidant activity of hydrolysates and peptide fractions of *Nemipterus japonicas* and *Exocoetus volitans* muscle. *J Aqua Food Product Tech* 19:180–192
- Shabeena YN, Nazeer RA (2011) Evaluation of bioactive properties of peptide isolated from *Exocoetus volitans* backbone. *Intern J Food Sci Tech* 46:37–43
- Shanmugam A, Palpandi C, Sambasivam S (2007) Some valuable fatty acids exposed from wedge clam *Donax cuneatus* (Linnaeus). *African J Biochem Res* 1:14–18
- Yen GC, Chen HY (1995) Antioxidant activity of various tea extracts in relation to their antimutagenicity. *J Agri Food Chem* 43:27–32
- Yen G, Hsieh P (1995) Antioxidant activity and scavenging effects on active oxygen of xylose-lysine Maillard reaction products. *J Sci Food Agri* 67:415–420
- You L, Zhao M, Liu RH, Regenstein JM (2011) Antioxidant and antiproliferative activities of loach (*Misgurnus anguillicaudatus*) peptides prepared by papain digestion. *J Agri Food Chem* 59:7948–7953