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RESEARCH ARTICLE

Purification and Identification of Antioxidant Peptide from Black Pomfret, *Parastromateus niger* (Bloch, 1975) Viscera Protein Hydrolysate

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Abstract To utilize fish waste, black pomfret, *Parastromateus niger* viscera was analysed for its proximate and amino acid composition followed by hydrolysis using various proteases to extract antioxidant peptide. Antioxidant activities of the crude hydrolysate was evaluated using DPPH (54%), metal chelating (78.6%) at a concentration of 1 mg/mL, whereas the reducing power assay was done with different concentration (0.5-2.5 mg/mL) and the activity also increased with increasing concentration (0.021-0.068). Furthermore, the hydrolysate was purified by diethylaminoethyl (DEAE) ion-exchange and Sephadex G-25 gel filtration chromatography. Finally, the purified peptide had a mass of 701.9 Da, and the amino acid sequence was identified as Ala-Met-Thr-Gly-Leu-Glu-Ala using electrospray ionization-tandem mass spectrometry (ESI-MS/MS). Moreover, the protection ability of the peptide toward hydroxyl radical-induced oxidative DNA damage and inhibiting lipid peroxidation was evaluated and compared with natural antioxidant α-tocopherol.

Keywords: antioxidant peptide, pomfret, electrospray ionization-tandem mass spectrometry (ESI-MS/MS), lipid peroxidation, DNA damage

Introduction

Free radical-mediated lipid peroxidation, oxidative stress, and antioxidants are widely discussed in many current research areas. Reactive oxygen species (ROS) and free radicals play an important role in many diseases (1). Under normal conditions, ROS and free radicals are effectively eliminated by the antioxidant defence systems such as antioxidant enzymes and non-enzymatic factors. However, under pathological conditions, the balance between the generation and elimination of ROS is broken, as a result of these events, biomacromolecules including DNA, membrane lipids, and proteins are damaged and this damage causes aging, cancer, and several diseases (2).

In order to reduce damage of ROS, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ) are used (3). However, BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis (4). So, in recent years, there has been increasing interesting in finding natural antioxidants, which can protect the human body from free radicals and retard the progress of many chronic diseases (5).

Recently several studies have described the antioxidative activity of hydrolysates such as, round scad protein (6) and flying fish protein (7). However, little information about antioxidative peptide from fish byproduct was available until now.

Therefore, the aim of this study was to isolate antioxidant peptide and to evaluate antioxidant properties of protein hydrolysate of black pomfret *Parastromateus niger* (Bloch, 1975) viscera, which is normally discarded as a waste in the process of fish industry and also human consumption.

Materials and Methods

Proteases for enzymatic hydrolysis (pepsin, trypsin, and α-chymotrpsin), α-linoleic acid, ammoniam thiocyanate, α-tocopherol, DPPH, $FeSO_4$, and H_2O_2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals

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and reagents were of analytical grade commercially available.

Sample preparation Twelve (2 kg) adult black pomfrets (*Parastromateus niger*) were purchased from the Royapuram (13°6'26"N 80°17'43"E) coast, Tamilnadu, India. Body length was 16±2.12 cm, and wet body weight was 215± 31.7 g. Sample fish were divided into 3 groups (4 fishes/ group). Visceral mass dissected separately and stored in freezer at –20°C until further use.

Proximate composition analyses Proximate (moisture, ash, protein, and lipid) composition was determined on wet weight basis (w.w.b.). Moisture content was determined by placing approximately 2 g of sample into a pre weighted aluminum dish (8). Samples were then dried in an oven at 105°C until a constant mass was obtained. Ash content was estimated by charring the pre-dried sample in a crucible at 600°C until a white ash was formed (8). The total crude protein (N×6.25) in raw material was determined using the Kjeldahl method (8) and lipid content was determined gravimetrically using Bligh and Dyer method (9).

Amino acid analyses The amino acid composition was identified by using the methods of Yanhong *et al.* (10) with small modifications. The sample viscera was digested separately with HCl (6 N) at 110°C for 24 h and neutralized with NaOH and loaded onto HPLC. HPLC analysis was carried out in an Agilent 1100 assembly system after precolumn derivatization with *O*-phthaldialdehyde (OPA). Each sample (1 μL) was injected on a Zorbax 80 A C_{18} 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 % of protein.

Preparation of protein hydrolysate by gastrointestinal digestion The digestion process was carried out using the method described by Kapsokefalou and Miller (11) with necessary modification. A 100 mL of 4%(w/v) protein isolating solution (0.1 M phosphate buffer) was brought to the desired pH to represent the stomach digestion using 1 and 10 M HCL and NaOH under thorough mixing. Pepsin was added at the enzyme to substrate ratio of 1/100 (w/w), at pH 2.5 then incubated at 37°C on a shaker for 2 h. After 2 h the pH was set to 8 to obtain the conditions of small intestine digestion. Similarly trypsin and α-chymotrypsin were supplemented both at the enzyme to substrate ratio of 1/100 (w/w). Then the solution was further incubated at 37°C for 2.5 h. When samples were taken at the end of digestion, the pH was adjusted to 6.5. Samples were centrifuged at 10,000×g for 15 min at 4°C and the supernatant was stored at –80°C. The frozen samples were lyophilized to obtain the dry powder.

Antioxidant assay

***Scavenging effect on DPPH free radical*:** The scavenging effect of the hydrolysate on DPPH free radical was measured by Burits and Bucar (12) with some modifications. Aliquots of sample at concentrations 0.5-2.5 mg were taken in different test tubes and were dissolved in 1 mL of ethanol followed by 4 mL of 0.004% of DPPH in methanol solution in each test tube. The test tubes were incubated at room temperature for 30 min and absorbance was read at 517 nm. A lower absorbance represented a higher DPPH scavenging activity.

$$\text{Radical scavenging activity (\%)}=[(A_{control}-A_{sample})/A_{control}]\times 100$$

where, A_{sample} is the absorbance of sample and $A_{control}$ is the absorbance of the control at 517 nm.

***Reducing power*:** The reducing power of hydrolysate was measured according to the method of Yildirim *et al.* (13). Different concentrations of the hydrolysate ranging 0.5-2.5 mg/mL were taken in different test tubes and were dissolved in 1 mL of methanol. A 2.5 mL of phosphate buffer and 2.5 mL of 1% potassium ferricyanide were added to each test tube and incubated for 20 min at 50°C. After incubation 2.5 mL of 10% trichloroacetic acid was added to each test tube and centrifuged at 900×g for 10 min. A 2.5 mL of the upper layer was mixed with equal volume of distilled water and 0.5 mL of 0.1% ferric chloride and the absorbance was read at 700 nm. The reducing power tests were run in triplicate. Increased absorbance of the reaction mixture indicated increased reducing power.

***Determination of chelating activity on Fe^{2+}*:** The chelating activity of Fe^{2+} was measured using the method of Dinis *et al.* (14). Samples were added to a solution of 2 mM $FeCl_2$ (0.05 mL), the reaction was initiated by addition of 5 mM ferrozine (0.2 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm. All analyses were done in triplicate. The % inhibition of ferrozine-Fe^{2+} complex formation is given by the formula:

$$\text{Chelating (\%)}=[(A_0-A_1)/A_0]\times 100$$

where, A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the samples and standard. The control contained $FeCl_2$ and ferrozine, complex formation molecules.

Lipid peroxidation inhibition assay The lipid

peroxidation inhibition activity of the hydrolysate was measured in α-linoleic acid emulsion system according to the methods of Osawa and Namiki (15). Briefly, a sample (1 mg) of the hydrolysate was dissolved in 10 mL of 50 mM phosphate buffer (pH 7.0), and added to a solution of 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 flask with a screw cap at 40±2°C in a dark room, and the degree of oxidation was evaluated by measuring the ferric thiocyanate (FTC) method according to Mitsuda *et al.* (16). The reaction solution (100 μL) incubated in the α-linoleic acid emulsion 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 color development with $FeCl_2$ and thiocyanate at different intervals during the incubation period at 40±2°C.

Purification of antioxidant peptide

***Ion exchange chromatography* (*IEC*):** The lyophilized black pomfret viscera protein hydrolysate (20 mg/mL) was dissolved in 20 mM sodium acetate buffer (pH 4.0), and loaded onto a fast protein liquid chromatography (FPLC) on a XK 26 DEAE anion exchange column (16×100 mm) 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 was monitored at 280 nm, collected at a volume of 4 mL and concentrated using a rotary evaporator; antioxidant activity was also investigated. A strong antioxidant fraction was lyophilized, and size exclusion chromatography was used as the next step for further purification.

***Size exclusion chromatograph*:** The lyophilized fraction was further purified on Sephadex G-25 size exclusion column (2.5×75 cm) equilibrated with distilled water. The column was eluted with distilled water, and 3 mL of fractions was collected at a flow rate of 1 mL/min. Each fraction was detected at 280 nm and antioxidant activity was also investigated. A strong antioxidant fraction was lyophilized, and subjected to electrospray ionization-tandem mass spectrometry (ESI-MS/MS) to determine the amino acid sequence and molecular mass determination.

Determination of the amino acid sequence of the purified peptide Accurate molecular mass and peptide sequencing of the purified peptide was determined by positive ion mode using ESI-MS and MS/MS. The purified peptide was dissolved in 75% acetonitrile/25% water of HPLC grade, then loaded into FIA type 3200 QTRAP mass spectrometer (Applied Biosystem Inc., Foster City, CA, USA). The sample was passed at a flow rate of 20 μL/min. The drying (35 psi) and ESI nebulising gas (45 psi) used was high purity nitrogen. Spectra were recorded over the mass/charge (*m/z*) range 200-1,000. About 3 spectrum were averaged in the MS and multiple MS (MS/MS) analyses. The peptide sequencing was performed by manual calculation.

Protective effect of the purified peptide against hydroxyl radical induced DNA damage To study the protection effects of the purified peptide on DNA damage by hydroxyl radical, the reaction was conducted in an Eppendorf tube at a total volume of 12-μL containing 0.5 μg of pBR 322 plasmid DNA in 3 μL of 50 mM phosphate buffer (pH 7.4), 3 μL of 2 mM $FeSO_4$, and 2 μL of the purified peptide at various concentrations. Then, 4 μL of 30% H_2O_2 was added, and the mixture was incubated at 37°C for 30 min. The mixture was subjected to 0.8% agarose gel electrophoresis. DNA bands (supercoiled, linear, and open circular) were stained with ethidium bromide.

Statistical analysis All experiments were performed in triplicate. Data were presented as mean±standard deviation (SD). The statistical analysis was performed using SPSS 10.0 software (SPSS Inc. Chicago, IL, USA). The significant difference was determined with 95% confidence interval ($p<0.05$).

Results and Discussion

Proximate composition The proximate composition *viz*., moisture, ash, crude protein, and lipid of the black pomfret viscera is shown are tabulated as per wet weight basis (Table 1). The viscera showed a 74% of moisture, 3.4% ash, 14.4% of protein, and 3.9% of lipid. These results are similar to those reported by Kyrana and Lougovois (17) for sea bass (76.72% moisture, 1.23% ash, 19.43% protein, and 4.81% fat) and values for sea bream (74.4% water, 1.30% ash, 20.03% protein, and 3.90% fat) have been reported by Grigorakis *et al.* (18). Generally fish is known for its high protein content and ranges from 8 to 21% in marine fishes and 13 to 17% in fresh water fishes (19). The

Table 1. Proximate composition (%,w/w) of viscera of black pomfret

Black pomfret	Proximate composition (%)
Moisture	74±0.5[1)]
Ash	3.4±0.4
Protein	14.4±0.5
Lipid	3.9±0.3

[1)]Values are mean±SD, n=3.

Table 2. Amino acid composition (%,w/w) of black pomfret viscera

Amino acid	Viscera
Aspartic acid	8.9
Threonine[1)]	2.6
Serine	5.8
Glutamic acid	9.9
Proline	8.7
Glycine	15.5
Alanine	10.3
Valine[1)]	4.7
Methionine[1)]	5.4
Isoleucine[1)]	4.2
Leucine[1)]	2.3
Tyrosine[1)]	3.4
Phenylalanine	1.9
Lysine[1)]	2.9
Histidine[1)]	2.8
Arginine[1)]	5.4
Cysteine	2.7
Tryptophan[1)]	2.4

[1)]Essential amino acids

current observations also showed protein content in the same direction and black pomfret muscle (16.5%) showed high protein content followed by skin (16.1%), viscera (15.7%), and bone (14.8%) respectively, these results from total proximate composition of black pomfret are proving it as a proteinacious fish.

Amino acid composition The amino acid composition of viscera of black pomfret is shown in Table 2. Black pomfret protein contained a high amount of histidine, valine, alanine, and glycine in visceral mass and importance of these amino acids are known from decades. Histidine, being an aromatic compound and is capable of making free radicals stable by transferring of 1 electron. Whereas, valine contains a non-polar aliphatic group has the nature of ready reactivity to hydrophobic polyunsaturated fatty acid (PUFA) (20). Presence of glutamine (9.9%) increases the quality of black pomfret viscera because; it is the most abundant free amino acid in the body and acts as a donor of nitrogen in the synthesis of purines and pyrimidines, it is essential for the proliferation of cells (21). Black pomfret viscera protein is also rich in lucine, isolucine, proline, and well-balanced in both essential and non essential amino acid composition.

DPPH radical scavenging activity DPPH radical scavenging activity of black pomfret viscera hydrolysate at a concentration of 1 mg/mL showed good scavenging ability as depicted (Fig. 1) and compared with the standard α-tocopherol. Peptides have been reported to have

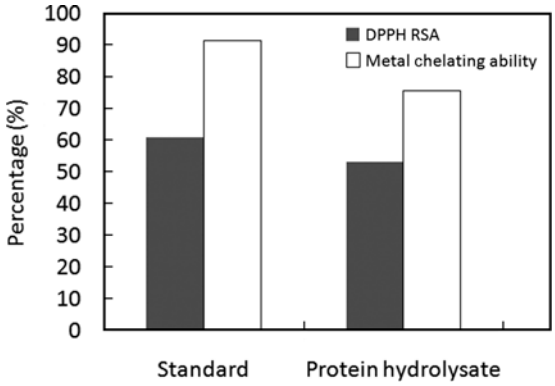

Fig. 1. DPPH radical scavenging activity (RSA) and metal chelating ability of protein hydrolysate obtained from black pomfret viscera. α-Tocopheral and EDTA are used as standard for DPPH RSA and metal chelating, respectively.

Table 3. Reducing power ability of black pomfret crude protein hydrolysate

Concentration (mg/mL)	α-Tocopherol	Protein hydrolysate
0.5	0.028±0.009[1)]	0.021±0.001*
1.0	0.046±0.005	0.034±0.002
1.5	0.062±0.005	0.045±0.004
2.0	0.087±0.006	0.057±0.004
2.5	0.112±0.008	0.068±0.003

[1)]Values are mean±SD, n=3; *Crude activity was significant with the standard at p<0.05.

antioxidative activity (1,7) and the results indicated that the peptides in the black pomfret viscera hydrolysate acted as a good electron donor and could react with free radicals to terminate the radical chain reaction.

Reducing power The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron or hydrogen (13). The reducing activity of viscera protein hydrolysate and the reference compounds increases steadily with an increase in concentration (Table 3). Bougatef *et al.* (22) demonstrated that peptide size and concentration clearly influenced on reducing power activity. However, viscera protein hydrolysate showed lower reducing power than α-tocopherol.

Chelating activity on Fe^{2+} Metal chelating activity of black pomfret viscera protein hydrolysate were determined at a sample concentration of 1.0 mg/mL. EDTA was used as a positive control to compare the activity of viscera protein hydrolysate. Ferrozine produces a violet complex with Fe^{2+}, this complex formation is interrupted in the presence of a chelating agent, resulting in decreased violet color. Therefore, measurement of the rate of color reduction estimates the chelating activity of the coexisting chelator (7). The chelating ability of viscera protein hydrolysate

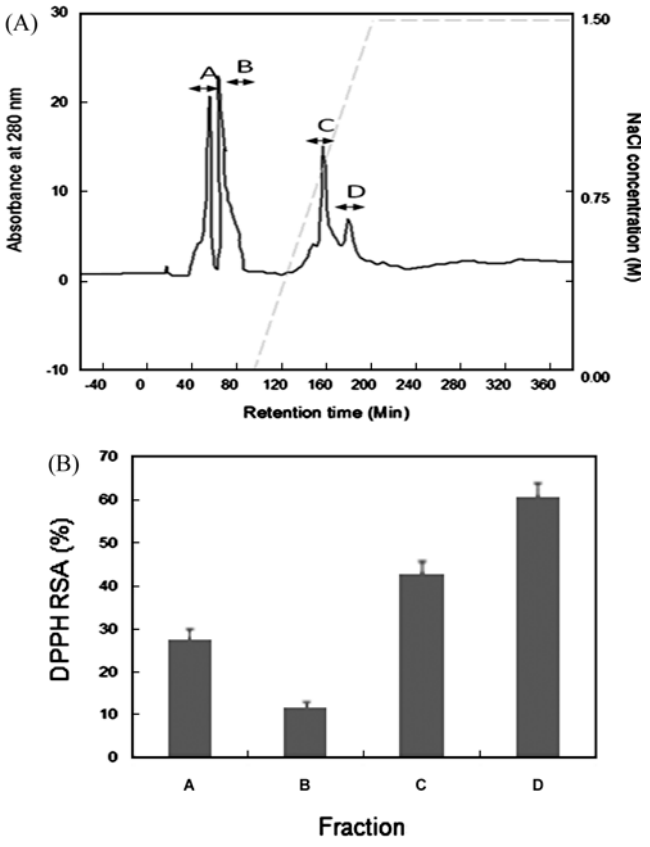

Fig. 2. Purification scheme of antioxidant peptides obtained from hydrolysates of black pomfret viscera by ion-exchange chromatography on a Hiprep 16/10 DEAE cellulose anion exchange column (A) and DPPH assay (indicated by DPPH RSA %) for obtained fraction A, B, C, and D (B).

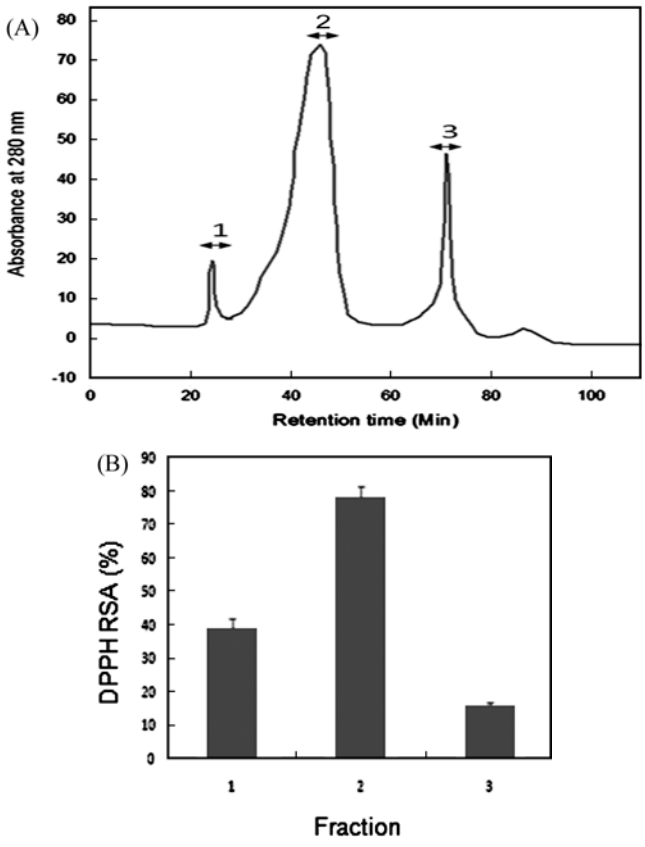

Fig. 3. Purification scheme of fraction D peptides obtained from IEC by gel filtration chromatography on Sephadex G-25 column (A) and DPPH assay (indicated by DPPH RSA %) for obtained fraction 1, 2, and 3 (B).

was strongest but far lower than that of EDTA (Fig. 1). Our results were thus similar to those reported for hemp and alfalfa leaf protein hydrolysates (23). Moreover, the lower molecular weight distribution of hydrolysates played an important role in the metal chelating activity.

Purification of antioxidant peptide The lyophilized visceral protein hydrolyste (20 mg/mL) was dissolved in 20 mM sodium acetate buffer (pH 4.0), and loaded onto a Hiprep 16/10 DEAE cellulose anion exchange column equilibrated with 20 mM sodium acetate buffer (pH 4.0), and eluted with a linear gradient of NaCl (0-2.0 M) in the same buffer at a flow rate of 1 mL/min. Each fraction was monitored at 280 nm (Fig. 2A), collected at a volume of 4 mL/tube. The collected fractions were lyophilized separately and measured for DPPH radical savanging activity (Fig. 2B). Fraction D showed strong antioxidant activity and further purified on Sephadex G-25 gel filtration column. Purification of peptides by Sephadex G-25 is always promising and helps to desalt the fractions (20,24). The Sephadex G-25 column was eluted with distilled water, and 3 mL of fractions was collected at a flow rate of 1 mL/min.

Table 4. DPPH radical scavenging activity (RSA) for fractions collected from ion exchange and size exclusion chromatography

S. No.	Fraction	DPPH RSA (%)
i	A	28.7±1.2
ii	B	19.2±0.9
iii	C	43.4±2.3
iv	D	61.7±2.6
v	1	39.6±1.9
vi	2	78.6±2.1
vii	3	14.3±0.7

The fractions were detected at 280 nm (Fig. 3A) and each fraction was examined against DPPH radical scavenging activity (Fig. 3B), and the fraction 2 found with strong antioxidant activity as tabulated (Table 4).

Lipid peroxidation inhibition assay To evaluate lipid peroxidation inhibition activity, the purified sample was employed in the linoleic acid emulsion system. In this model system, peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals, derived from the pre-existing lipid peroxide, were engaged to initiate lipid peroxidation in the controlled conditions (25). As shown in Fig. 5, peptide of black pomfret viscera

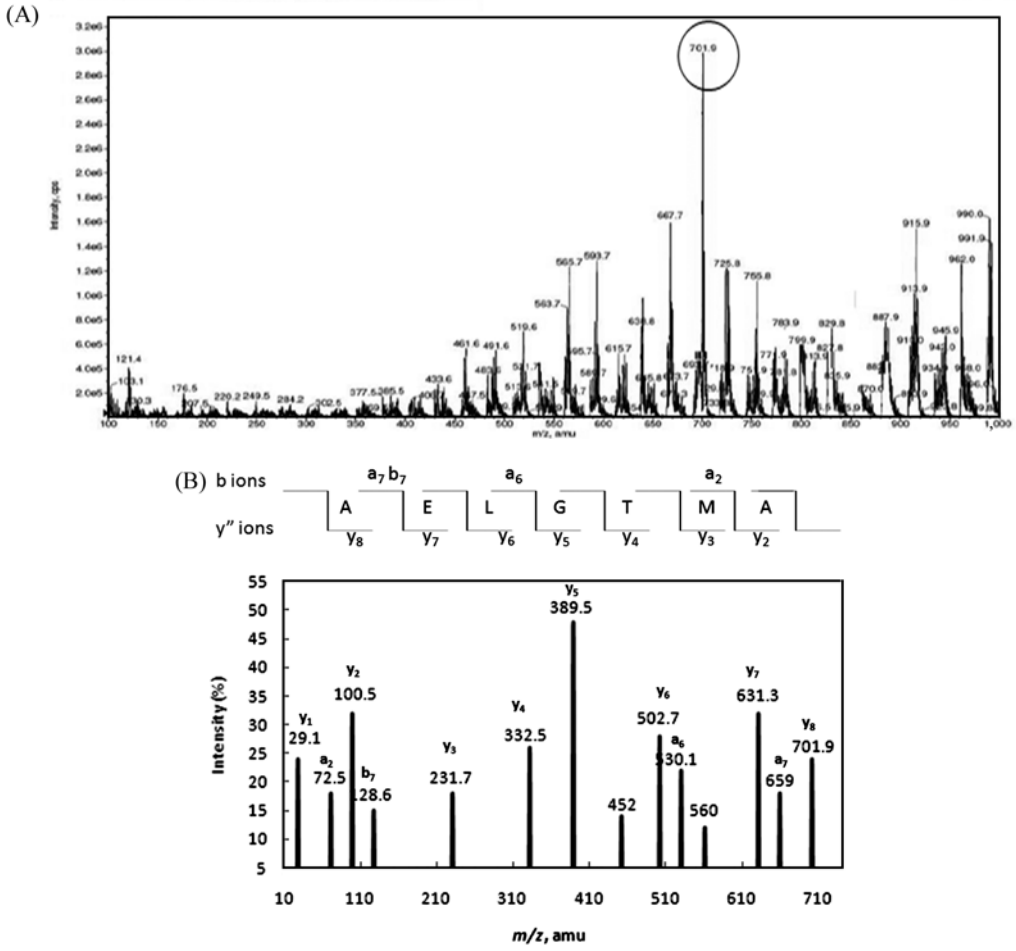

Fig. 4. Mass spectrum of chromatography fraction 2 (A) and MS/MS spectrum of ion *m/z* 701.9 (B).

significantly inhibited lipid peroxidation and the activity was higher than that of α-tocopherol for a period of 7 days.

Cheng *et al.* (26) reported that phenolic compounds afforded their protective actions in lipid peroxidation by scavenging the lipid-derived radicals (R•, RO•, or ROO•) to stop the chain reactions in a heterogeneous lipid phase. To inhibit lipid peroxidation in this model, it may be an important hydrophobic property of the purified peptide sequence to scavenge on lipid derived radicals. Due to this property, black pomfret viscera peptide could interact with lipid molecules and could scavenge by donating protons to lipid derived radicals.

Protective effect of the purified peptide against hydroxyl-radical-induced DNA damage DNA damage by ROS can initiate carcinogenesis or affect the pathogenesis of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease; among ROS, the hydroxyl radical is recognized as a DNA-damaging agent of physiological significance (27). In this study, the active peptide was subjected to investigate the protective effect of the purified peptide on hydroxyl radical induced plasmid pBR 322 DNA damage *in vitro*. The mixture was subjected to 0.8% agarose gel electrophoresis, DNA bands (supercoiled, linear, and open circular) were stained with ethidium bromide. The effect of black pomfret viscera peptide on free-radical induced DNA damage was investigated as shown in Fig. 6. DNA was completely damaged form under the treatment of hydroxyl radical generated from a Fenton reaction (lane 2), and it was compared with plasmid DNA (lane 1). DNA single-strand cleavage has also been reported in other free radical-involved circumstances, such as c-radiation and peroxyl radical (28). Several studies have already observed that some peptides derived from different protein sources possess protective ability of hydroxyl radical induced DNA damage (29). According to the result, DNA treated with peptide of black pomfret viscera, protected hydroxyl radical induced DNA damage (lane 3), indicating an antioxidant effect.

Identification of peptide by ESI-MS/MS The lyophilized active fraction 2 of gel filtration chromatography is considered for further examination of peptide sequence using ESI-MS/MS. The sequence of the purified peptide

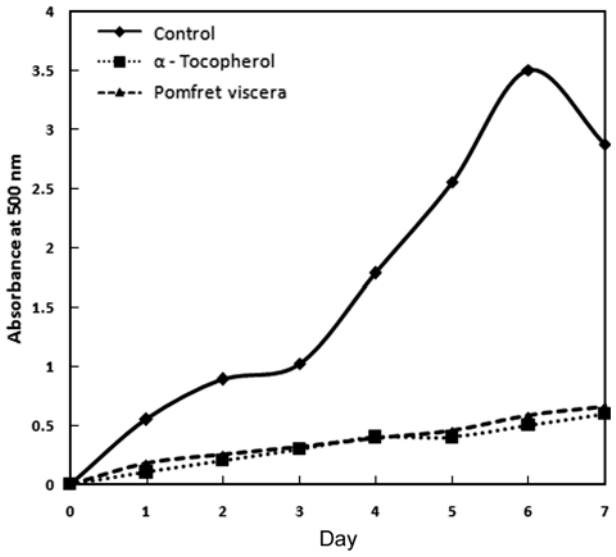

Fig. 5. Lipid peroxidation inhibition activity of black pomfret viscera protein hydrolysate peptide. Activity was measured in the linoleic acid emulsion system using α-tocopherol as positive control.

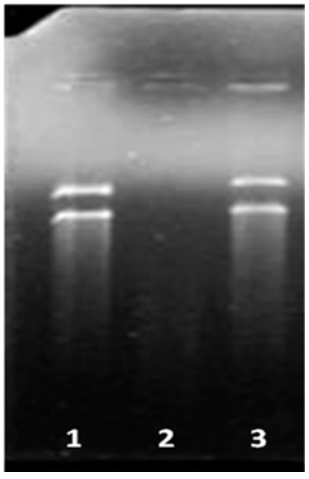

Fig. 6. Agarose gel electrophoretic patterns of plasmid DNA breaks by hydroxyl radical in the presence of purified peptide. Lane 1, plasmid DNA; lane 2, hydroxyl radical damage (DNA damage control); lane 3, pomfret viscera peptide

identified with a molecular mass of 701.9 Da was determined by MS (Fig. 4A). Further the mass focuses the sequence of Ala-Met-Thr-Gly-Leu-Glu-Ala (Fig. 4B). It is commonly believed that His, Met, and Cys are very important to the radical scavenging activity of peptides due to their special structural characteristics, Met is prone to oxidation of the Met sulfoxide. In addition this peptide contains glycine, which seems to play a vital role as observed in several antioxidative peptides (25). Alanine and leucine are also important to the radical scavenging activity reported by Bougatef *et al.* (22). Moreover, the mass of antioxidant peptides generally vary from 400 to 1,500 kDa (20,22) and the activity of these peptides are influenced by the presence and sequence of potential amino acids (7). Therefore the sequence of this purified peptide makes it a potential antioxidant and was able to quench the free radicals.

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