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ORIGINAL ARTICLE

Optimization of enzymatic hydrolysis conditions for the production of antioxidant peptides from muscles of *Nemipterus japonicus* and *Exocoetus volitans* using response surface methodology

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Abstract In the present study, protein of muscles of commercially important marine fishes *Nemipterus japonicus* and *Exocoetus volitans* were extracted by trypsin and their hydrolysis conditions viz., temperature, time, and enzyme to substrate concentration on degree of hydrolysis were studied by response surface methodology. The optimum values for *N. japonicus* was found as temperature, 30°C, hydrolysis time of 100 min an enzyme/substrate concentration of 1.59% whereas, for *E. volitans* muscle protein, optimum hydrolysis conditions were temperature, 30°C, hydrolysis time of 115 min and enzyme/substrate concentration of 1.67%. Furthermore, amino acid sequence of antioxidant peptides derived after chromatographic purification was identified by ESI–MS/MS. The analysis of peptides showed sequences as Glu-Ser-Asp-Arg-Pro (620.3 Da) and Gly-Trp-Met-Gly-Cys-Trp (747.3) for *N. japonicus* and *E. volitans* muscle, respectively. The peptides contained important antioxidant amino acids and acted as good antioxidant peptides to scavenge free radicals.

Keywords *Nemipterus japonicus* · *Exocoetus volitans* · Antioxidant peptides · Amino acids

Introduction

Oxygen is an essential element for life to perform biological functions such as catabolism of fats, proteins and carbohydrates during metabolism and during respiration in aerobic organisms in order to generate energy for growth and other activities. Oxygen, though not dangerous by itself is involved in the generation of various kinds of "reactive oxygen species" (ROS) such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH·), and non-free radical species such as hydrogen peroxide (H^2O_2) and singlet oxygen ($^1O_2$) are formed (Gulçin 2009). When free radicals are produced in excess or are produced and not eradicated from the body, they can attack the closest molecules subtracting electrons starting in a chain reaction in which the new molecule with a missing electron attacks other molecules (Kaur and Kapoor 2001). Free radicals are believed to be involved in the oxidation of lipids and oxidative decomposition of unsaturated fatty acids. Lipid peroxidation is of great concern to the food industry and consumers because it leads to the development of undesirable off-flavors, odors, and potentially toxic reaction products (Lin and Liang 2002). Free radicals modify DNA, proteins, and smaller cellular molecules and are implicated in the aetiology of various age-related chronicle diseases like cardiovascular diseases, neurodegenerative disorders, diabetes and certain types of cancer (Ames 1983). Bioactive peptides are short polymers with 3–20 amino acid units that are inactive in parent protein (Pihlanto-Leppala 2000). These bioactive peptides can be released from different protein by digestive enzymes during gastrointestinal digestion or by in vitro proteolytic hydrolysis with exogenous proteases. Several bioactive peptides can exert more than one physiological effect in the human body (Erdmann et al. 2008), and some of these include : antihypertensive, immunomodulating, anti-thrombotic, antioxidative, opioid, cholesterol lowering, anticancer, and antimicrobial activities (Vercruysse et al. 2005). The peptides exhibited several bioactivities like inhibition of lipid peroxidation and elimination of free radicals produced in vivo and the

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activity depends on their amino acid composition and sequences (Hook et al. 2001).

Enzymatic hydrolysis is influenced by several factors, namely, temperature, pH, enzyme/substrate concentration and time that cooperatively influence the enzyme activity thereby making the process more controllable and hence offer possibilities to control the process (Viera et al. 1995; Liaset et al. 2000). It has been reported that the choice of substrate, protease used and degree of hydrolysis generally affects the physicochemical properties of the resulting hydrolysates. Response surface methodology is a frequently used statistical method that can use all the variables simultaneously and determine the optimum conditions required. RSM uses quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate problems (Madamba 2002; Giovanni 1983).

The objectives of this study were (a) to optimize the hydrolysis conditions like temperature, enzyme/substrate ratio and hydrolysis time with the purpose of obtaining degree of hydrolysis of *Nemipterus japonicus* and *Exocoetus volitans* muscle protein by trypsin and to evaluate antioxidant activities of peptides derived after purification; (b) sequencing of antioxidant peptides derived from these two commercially important fishes.

Materials and methods

Trypsin, pepsin, and papain for enzymatic hydrolysis were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade commercially available.

Sample collection

Marine fishes *N. japonicus* and *E. volitans* were collected from the Royapuram sea coast (13°6′26″N 80°17′43″E), Chennai, Tamil Nadu, India, and muscles were separated from the fishes, minced and stored in plastic bags at −20°C until used.

Preparation of protein hydrolysates

To produce antioxidant peptides from fish backbone, enzymatic hydrolysis was performed using enzyme trypsin (buffer, 0.1 M Na_2HPO_4–NaH_2PO_4; pH 8.8; temperature, 37°C) at enzyme/substrate ratio (1/100 w/w). Substrate and enzyme were mixed thoroughly, and the mixture was incubated for 4 h with continuous stirring and heated in a boiling water bath at 100°C for 10 min to inactivate enzyme activity. The samples were then centrifuged at 10,000*g* for 15 min at 4°C, and the supernatant was lyophilised.

Determination of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the percentage ratio of the number of peptide bonds broken (h) to the total number of peptide bonds per unit weight (h_{tot}), in each case, was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis (Alder-Nissen 1986) as given below:

$$\mathrm{DH}(\%) = \frac{BN_b}{M_p \alpha h_{tot}} \times 100$$

where B is the amount of NaOH consumed (ml) to keep the pH constant during the reaction, N_b is the normality of the base, MP is the mass of protein (in grams, % $N \times 6.25$), $1/\alpha$ is the calibration factor for pH–stat, and h_{tot} is the content of peptide bonds (Alder-Nissen 1986).

Optimization of hydrolysis conditions by response surface methodology

Response surface methodology was used to predict the optimal hydrolysis conditions of fish protein hydrolysis by using trypsin. Optimization of three variables temperature (X_1), time (X_2) and *E*/*S* ratio (X_3) for the degree of hydrolysis of *N. japonicus* muscle (Y_1) and *E. volitans* muscle (Y_2) was performed using Design-expert 7.0 software package (Stat-Ease Inc., USA). Each variable had five levels and totally 20 runs were employed. The response functions (Y_1, Y_2) were related to the coded variables (X_1, X_2, X_3) by a second order polynomial (Eq. 1) using the method of least squares.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{23} X_1 X_2 + \beta_{31} X_3 X_1. \quad (1)$$

Purification of protein hydrolysates

Ion exchange chromatography

The lyophilized protein hydrolysate (80 mg/ml) was dissolved in 20 mM sodium acetate buffer (pH 4.0), and loaded onto fast protein liquid chromatography (FPLC) on DEAE XK 26/20 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 62 ml/h. Each fraction collected at a volume of 4 ml was monitored at 280 nm; pooled fractions were then concentrated using a rotary evaporator, and antioxidant activities were investigated. Further, the fraction having strong antioxidant properties was lyophilized and subjected to next separation.

Gel filtration chromatography

The lyophilized fraction was further purified on Sephadex G-25 gel filtration column equilibrated with distilled water. The column was eluted with distilled water, and 4 ml of fractions was collected at a flow rate of 0.8 ml/min. The fractions were detected at 280 nm and subjected to antioxidant activity assays. A strong antioxidant fraction was lyophilized for further investigation.

Assays of electron spin resonance (ESR) spectrometer

DPPH radical scavenging assay

DPPH radical scavenging activity was measured using the methodology by Nanjo et al. (1996). A 60 μl peptide solution (or ethanol itself as control) was added to 60 μl of DPPH (60 μM) in ethanol solution. After mixing vigorously for 10 s, the solution was then transferred into a 100 μl quartz capillary tube, and the scavenging activity of peptide on DPPH radical was measured using ESR spectrometer after 2 min. The experimental conditions were as follows: magnetic field, 3,475 G; power, 5 mW; modulation frequency, 100 kHz; amplitude, 2 × 1,000; sweep time, 30 s. Radical scavenging ability (RSA) was calculated based on the following equation, in which H and H_0 were the height of the third resonance peak for samples with and without protein, respectively.

$$\mathrm{RSA} = (H_0 - H)/H_0 \times 100.$$

Hydroxyl radicals scavenging activity

Hydroxyl radicals were generated by iron-catalyzed Fenton Haber–Weiss reaction and they reacted rapidly with nitrone spin trap DMPO (Rosen and Rauckman 1984). The resultant DMPO-OH adducts were detectable with an ESR spectrometer. The peptide solution (20 μl) was mixed with DMPO (0.3 M, 20 μl), $FeSO_4$ (10 mM, 20 μl), and H_2O_2 (10 mM, 20 μl) in a phosphate buffer solution (pH 7.4), and then transferred into a 100 μl quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. The experimental conditions were as follows: magnetic field, 3,475 G; power, 1 mW; modulation frequency, 100 kHz; amplitude, 2 × 1,000; sweep time, 4 min. The RSA was calculated as above with H and H_0 representing the height of the second resonance peak for samples with and without protein, respectively.

Superoxide anion radical scavenging activity

Superoxide anion radicals were generated by UV irradiated riboflavin/EDTA system (Guo et al. 1999). The reaction mixture containing 0.3 mM riboflavin, 1.6 mM EDTA, 800 mM DMPO and indicated concentration of peptide fraction was irradiated for 1 min under a UV lamp at 365 nm. The reaction mixture was transferred to a 100 μl quartz capillary tube of the ESR spectrometer for measurement. The experimental conditions were as follows: magnetic field, 336.5 ± 5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 1,000; sweep time, 1 min. The RSA was calculated as above with H and H_0 representing the height of the first resonance peak for samples with and without protein, respectively.

Identification of peptide by ESI–MS/MS

The highest antioxidative activity fraction after ion exchange gel filtration chromatography purification was dissolved in a buffer of 75% acetonitrile/25% water of HPLC grade and then loaded into a FIA type 3200 QTRAP mass spectrometer (Applied Biosystem). The sample was passed at a flow rate of 20 μl/min which was operated in the positive electrospray ionization (ESI +ve) mode via the electro spray interface. The Drying (35 psi) and ESI nebulizing gas (45 psi) used was high purity nitrogen. Spectra were recorded over the mass/charge (m/z) range 200–1,000. About three spectra were averaged in the MS and multiple MS (MS/MS) analyses. The peptide sequencing was performed by manual calculation.

Statistical analysis

All the data analysis was performed using SPSSS 10.0 statistical software. A one-way analysis of variance (ANOVA) was conducted for the analysis of response values obtained by the RSM model.

Results and discussion

Determination of degree of hydrolysis

The hydrolysis of muscles of *N. japonicus* and *E. volitans* was carried out with trypsin by pH–stat method for 4 h. The hydrolysis proceeded at a high rate during the initial 15 min and slowed down thereafter and then reached a steady state when no apparent hydrolysis took place. The degree of hydrolysis reflects the percentage of peptide bonds cleaved by protease and peptides with greater degree of hydrolysis exerting important effects on their antioxidant properties (Li et al. 2007).

Optimization of hydrolysis conditions by response surface methodology

The optimal hydrolysis conditions of *N. japonicus* and *E. volitans* were studied by RSM. Observed values for

degree of hydrolysis (DH) at different combinations of independent variables are listed in Table 1. The range of the degree of hydrolysis for *N. japonicus* and *E. volitans* muscle protein was from 29.12 to 42.15% and 21.21 to 43.21%, respectively. In this study the value of degree of (DH) for both fish muscle protein was higher than DH of Grass carp skin, 1.1–15.2% (Wasswa et al. 2008) and silver catfish frame, 6.25 to 21.38% (Amiza et al. 2011) but lower than degree of hydrolysis of Catla viscera, 34.23–49.65% (Bhaskar et al. 2007). The difference in degree of hydrolysis maybe because of the difference in the part of fish used in hydrolysis, difference in fish species and difference in enzyme used (Bhaskar et al. 2007).

The results of regression analysis for both fish muscle protein showed that temperature, time and enzyme concentration had a linear effect on DH values while as temperature and enzyme concentration had quadratic effect. Interaction for the DH was observed between temperature ($p < 0.01$) and enzyme concentration ($p < 0.01$) for both fishes. In case, the highest positive value of esteemed regression coefficient was observed for the enzyme concentration ($X_3 = 4.6155$) for *N. japonicus* muscle protein whereas, in the case of *E. volitans* muscle protein the highest value for esteemed regression coefficient was found for temperature ($X_1 = 7.6753$) as shown in Table 2.

Table 1 Enzymatic hydrolysis experimental design of independent variables along with the observed values for the response variable, degree of hydrolysis for *N. japonicus* (Y_1) and *E. volitans* (Y_2)

Run no.	Temperature (X_1)	Time (X_2)	*E/S* (X_3)	Y_1	Y_2
1	25.00	50.00	0.500	31.00	29.78
2	35.00	50.00	0.500	30.41	30.12
3	25.00	150.00	0.500	31.48	31.21
4	35.00	150.00	0.500	32.27	31.88
5	25.00	50.00	1.500	34.51	33.67
6	35.00	50.00	1.500	37.11	34.21
7	25.00	150.00	1.500	35.00	33.71
8	35.00	150.00	1.500	38.12	38.18
9	21.59	100.00	1.00	29.12	34.22
10	38.40	100.00	1.00	30.51	33.86
11	30.00	15.91	1.00	33.10	21.21
12	30.00	184.09	1.00	35.03	35.33
13	30.00	100.00	0.15	31.50	30.21
14	30.00	100.00	1.84	42.15	43.35
15	30.00	100.00	1.00	36.11	36.42
16	30.00	100.00	1.00	36.11	36.42
17	30.00	100.00	1.00	36.11	36.42
18	30.00	100.00	1.00	36.11	36.42
19	30.00	100.00	1.00	36.11	36.42
20	30.00	100.00	1.00	36.11	36.42

The regression equation for the degree of hydrolysis for *N. japonicus* (Y_1) and *E. volitans* (Y_2) muscle proteins as function of three independent variables temperature, time and enzyme concentration (X_1, X_2 and X_3) and their interactions was derived as follows

$$\begin{aligned} Y_1 = {} & -36.1.11 + (1.017 \times X_1) + (0.8736 \times X_2) \\ & + (4.6155 \times X_3) + (-6.1008 \times X_1^2) \\ & + (-1.8501 \times X_2^2) + (0.9121 \times X_3^2) \\ & + (0.0671 \times X_1X_2) + (0.0955 \times X_1X_3) \\ & + (-0.02999 \times X_2X_3) \end{aligned}$$

$$\begin{aligned} Y_2 = {} & -26.0061 + (7.67534 \times X_1) \\ & + (0.2074 \times X_2) + (-1.7694 \times X_3) \\ & + (-0.0334 \times X_1^2) + (-0.0011 \times X_2^2) \\ & + (0.5265 \times X_3^2) + (0.0213 \times X_1X_2) \\ & + (-0.2000 \times X_1X_3) + (0.0041 \times X_2X_3) \end{aligned}$$

The results for analysis of variance (ANOVA) for degree of hydrolysis of muscle proteins of *N. japonicus* and *E. volitans* by trypsin demonstrated that the models for both fishes are highly significant at 99% confidence level ($p < 0.0001$) showed in Tables 3 and 4. The desirability value was close to 1 and the model fitted the experimental data with an acceptable determination coefficient (*N. japonicus*, $r^2 = 0.9910$ and *E. volitans*, $r^2 = 0.9963$) and therefore can be suggested that the conditions were most suitable to obtain the optimum DH. The suggested optimum hydrolysis conditions for *N. japonicus* muscle protein were temperature, 30°C, hydrolysis time of 100 min an enzyme/substrate concentration of 1.59% whereas, for *E. volitans* muscle protein, optimum hydrolysis conditions

Table 2 Model coefficients for independent variables and their interactions by linear regression for the DH values for *N. japonicus* and *E. volitans*

Factor	Coefficients	
	N. japonicus	*E. volitans*
Constant	−36.1011***	−26.0061***
Linear		
Temperature (X_1)	1.0172**	7.6753***
Time (X_2)	0.8736*	0.2074**
E/S concentration (X_3)	4.6165*	−1.7694**
Quadratic		
Temperature (X_1^2)	−6.1008**	−0.0334**
Time (X_2^2)	−1.850	−0.0011
E/S concentration (X_3^2)	0.9121*	0.5265*
Interactions		
X_1X_2	0.06711**	0.00213**
X_1X_3	0.09510	−0.2000
X_2X_3	−0.0299	0.00410

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Table 3 Results of ANOVA for degree of hydrolysis (DH) for *N. japonicus*

Factors	SS	*df*	MS	*F*	*p*
Model	398.61	9	34.9	146.01	0.0001
Linear					
Temperature (X_1)	140.02	1	140.02	101.32	0.0003
Time (X_2)	15.03	1	15.03	32.27	0.008
E/*S* concentration (X_3)	132.01	1	132.01	68.64	0.000
Quadratic					
Temperature (X_1^2)	45.22	1	45.22	29.97	0.0021
Time (X_2^2)	11.31	1	11.31	10.21	0.032
E/*S* concentration (X_3^2)	0.91	1	0.91	12.63	0.068
Interactions					
X_1X_2	1.83	1	1.83	10.21	0.027
X_1X_3	0.97	1	0.97	1.46	0.261
X_2X_3	0.82	1	0.82	0.74	0.3162
Error	9.51				
Total	408.12				

p level of significance

Table 4 Results of ANOVA for degree of hydrolysis (DH) for *E. volitans*

Factors	SS	*df*	MS	*F*	*p*
Model	421.61	9	38.20	141.27	0.0001
Linear					
Temperature (X_1)	153.72	1	153.72	111.53	0.0007
Time (X_2)	139.26	1	139.26	74.74	0.0027
E/*S* concentration (X_3)	34.63	1	34.63	98.76	0.0015
Quadratic					
Temperature (X_1^2)	14.27	1	14.27	4.36	0.0081
Time (X_2^2)	0.98	1	0.98	2.52	0.0051
E/*S* concentration (X_3^2)	2.11	1	2.11	5.74	0.0414
Interactions					
X_1X_2	0.87	1	0.87	1.44	0.0064
X_1X_3	0.76	1	0.76	0.86	0.3251
X_2X_3	0.17	1	0.17	0.43	0.3612
Error	4.41				
Total	417.28				

p level of significance

were temperature, 30°C, hydrolysis time of 115 min and enzyme/substrate concentration of 1.67%.

Figure 1a shows response surface plot as function of temperature and enzyme/substrate concentration and it was concluded that increase in concentration of enzyme increased degree of hydrolysis. This may be due to increase in enzyme molecules increase more chances for hydrolysis to occur. Response surface plot for *N. japonicus* as a function of temperature and hydrolysis time is depicted in Fig. 1b. It was observed that DH increased with increase in temperature and hydrolysis time and above optimal temperature 30–35°C, DH decreased that may be due denaturation and inactivation of enzymes at higher temperature. It was found that hydrolysis time and enzyme/substrate concentration had linear effect on degree of hydrolysis (Fig. 1c).

Response surface plot for *E. volitans* muscle protein is shown in Fig. 2. It was observed that temperature and enzyme/substrate concentration had linear effect on DH and both factors increased DH (Fig. 2a). Further, it was found that increase in temperature with respect to hydrolysis time increased degree of hydrolysis to 35% and showed gradual decrease to 33% with increase in hydrolysis time (Fig. 2b). Moreover, it was observed that DH increased up to 42% as a function of hydrolysis time and enzyme/substrate concentration and finally showed sudden decrease of DH to 24% at 170 min. Therefore, the findings has proved that the RSM approach is appropriate for optimizing the hydrolysis of fish proteins conditions by proteases for production of interested biological peptides.

In this study, we found that DH exerts important effect on the antioxidant property of hydrolysates, as low molecular peptides contribute more to the inhibitory activity than polypeptides (Ren et al. 2008). Trypsin hydrolysate of *N. japonicus* muscle protein sat highest DH (42.15%) exerted scavenging effect on DPPH radicals, hydroxyl radicals and superoxide radicals with values 45.3, 48.4 and 42.1%, respectively. Whereas, in case of *E. volitans* muscle hydrolysate, highest DH (43.35%) showed inhibitory effect on DPPH radical, hydroxyl radical and superoxide radical with values of 46.2, 50.1 and 44.1%, respectively. The results were supported by the reports of Ren et al. (2008) who also reported that antioxidant activity increased considerably with increase in DH for grass carp sarcoplasmic protein hydrolysate. Furthermore, Li et al. (2007) also found that radical scavenging capacity of the porcine collagen hydrolysate improved with increase in DH as long as DH does not exceed more than 85%.

Purification and antioxidant activity of peptides

Trypsin protein hydrolysates of *N. japonicus* and *E. volitans* with maximum degree of hydrolysis obtained by optimum hydrolysis conditions were purified by ion exchange chromatography followed by gel chromatography. Both hydrolysates were dissolved in sodium acetate buffer (20 mM, pH 4.0) at 80 mg/ml and loaded on DEAE XK 26/20 XK 26/20 anion exchange column. Trypsin hydrolysates of both fish muscle protein were fractionated into three fractions and all the fractions were tested for their radical scavenging activities. Fraction II of *N. japonicus* (DPPH radical, 46.8%; hydroxyl radical, 49.2% and

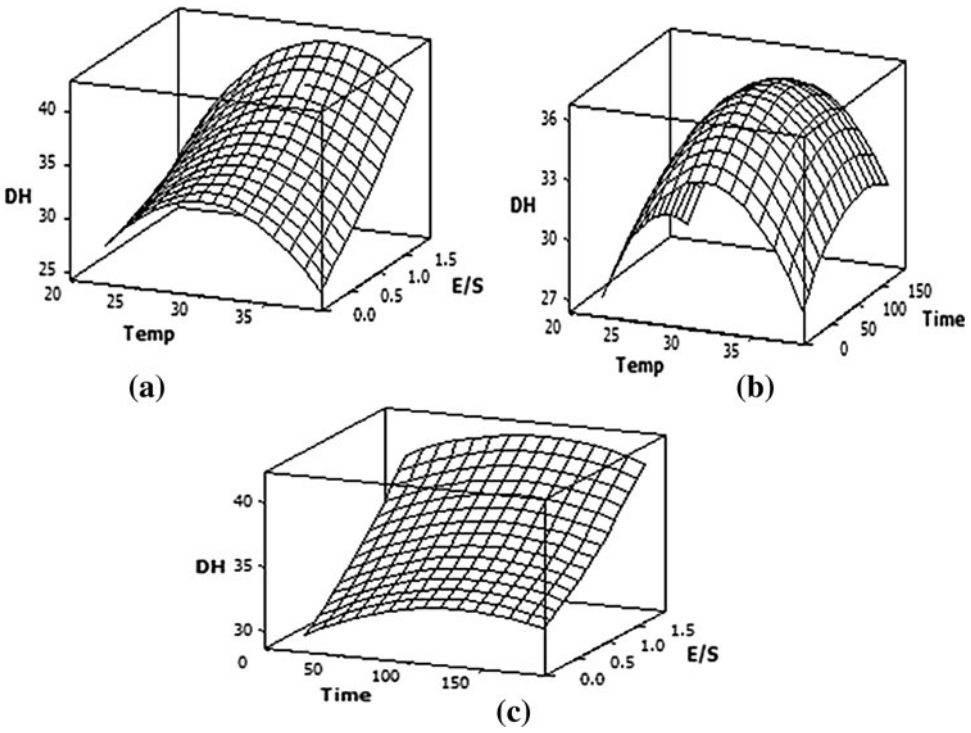

Fig. 1 The three-dimensional plot of effect of (**a**) temperature and *E*/*S* (**b**) temperature and time (**c**) time and *E*/*S* on DH of *N. japonicus* muscle protein

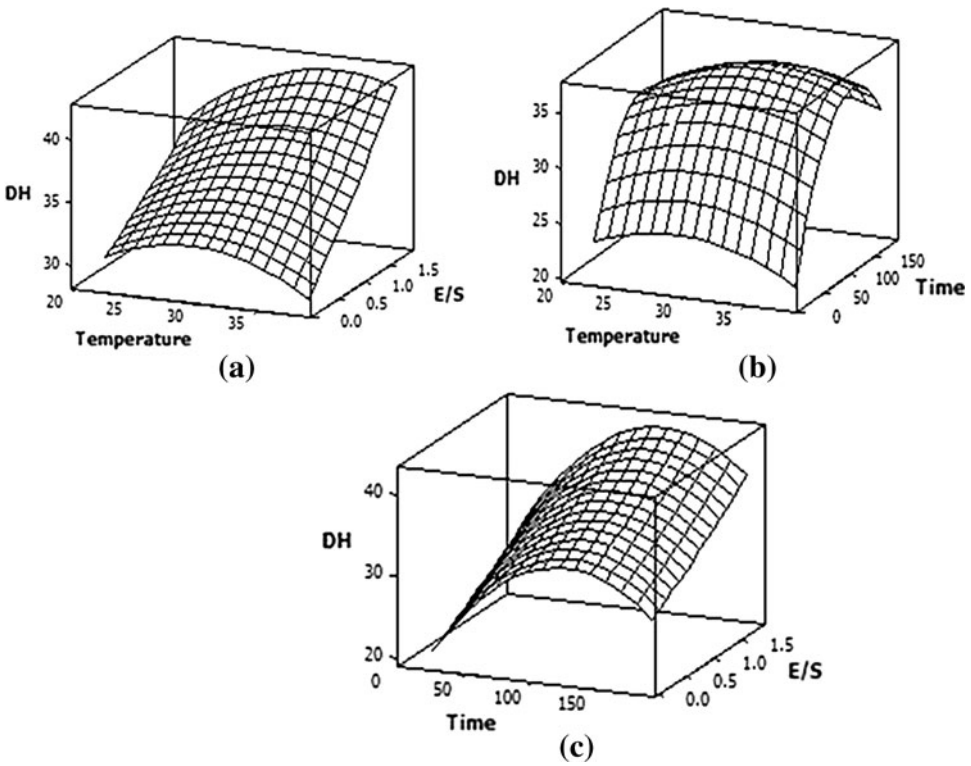

Fig. 2 The three-dimensional plot of effect of (**a**) temperature and *E*/*S* (**b**) temperature and time (**c**) time and *E*/*S* on DH of *E. volitans* muscle protein

superoxide radical, 43.1%) and fraction III of *E. volitans* (DPPH radical, 48.1%; hydroxyl radical, 52.8% and superoxide radical, 47.1%) showed maximum radical scavenging activity than other fractions (Shabeena and Nazeer 2010). These potent fractions were further purified by gel filtration chromatography and were fractionated into two and three peaks for *N. japonicus* and *E. volitans*, respectively. Fraction IIa (DPPH radical, 48.7%; hydroxyl radical, 51.2% and superoxide radical, 44.2%) for *N. japonicus* muscle and fraction IIIb (DPPH radical, 51.1%;

hydroxyl radical, 56.6% and superoxide radical, 50.3%) for *E. volitans* were more potent to suppress free radicals than other fractions (Shabeena and Nazeer 2010).

Identification of peptide by ESI–MS/MS

The active fractions of muscles of *N. japonicus* and *E. volitans* obtained after chromatography were subjected ESI MS/MS for sequencing peptides. The MS spectrum of purified peptides of *N. japonicus* muscle and *E. volitans* muscle showed molecular mass of 620.3 and 747.3 Da, respectively (Fig. 3a, b). The analysis of each mass gave following sequences, *viz.* Glu-Ser-Asp-Arg-Pro and Gly-Trp-Met-Gly-Cys-Trp for *N. japonicus* and *E. volitans* muscle, respectively (Fig. 4a, b). The sequences of peptides have hydrophobic amino acid proline which is expected to favor oxidation inhibition. Hydrophobic amino acid residues such as, Leu, Gly and Val are presumed to inhibit lipid peroxidation by increasing solubility of peptides in lipid and thereby facilitating better interaction with radical species (Rajapakse et al. 2005). It is commonly believed that His, Met, and Cys are very important to the radical scavenging

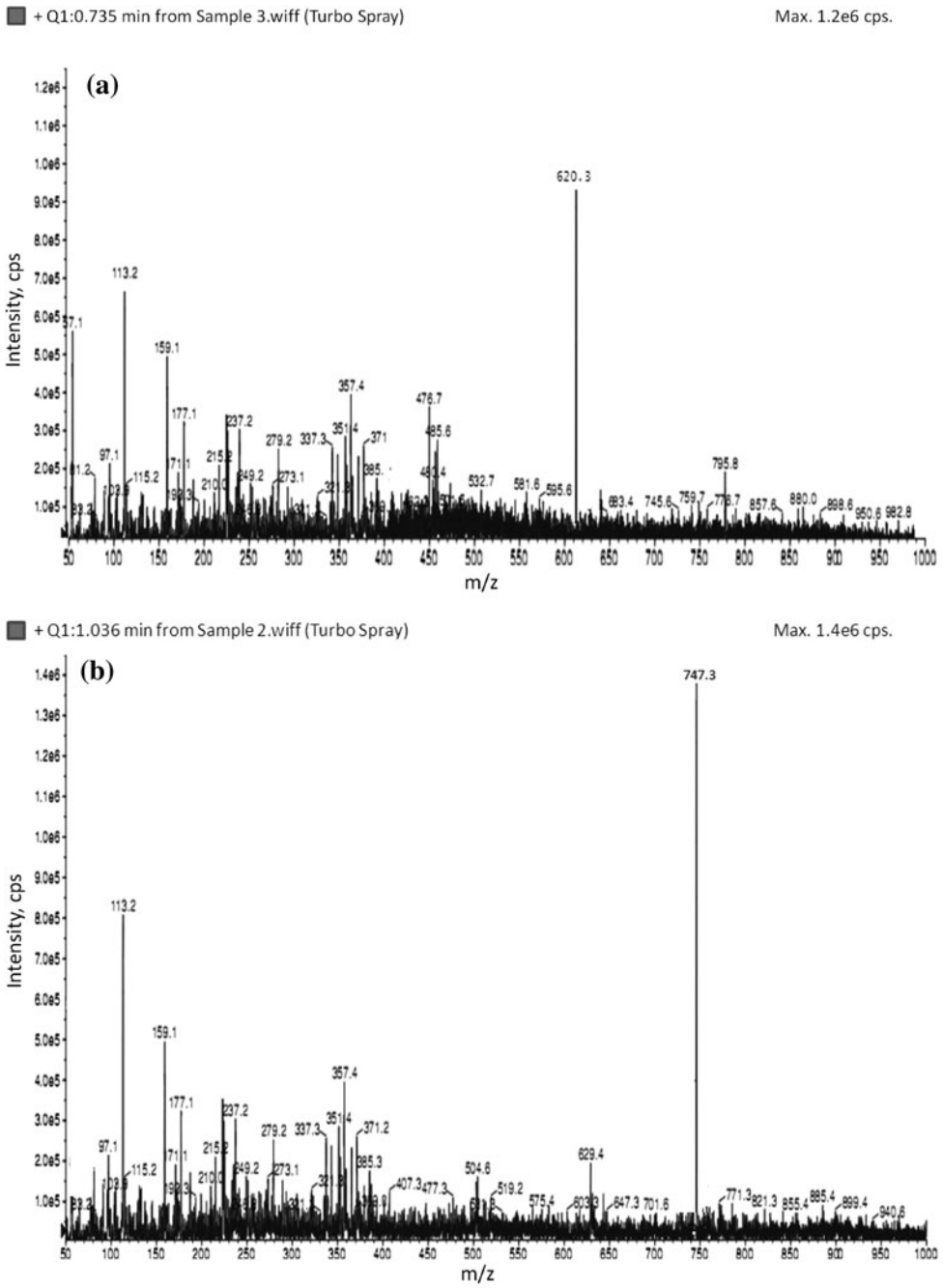

Fig. 3 **a** MS spectrum of the chromatographic fraction of *N. japonicus* muscle protein. **b** MS spectrum of the chromatographic fraction of *E. volitans* muscle protein

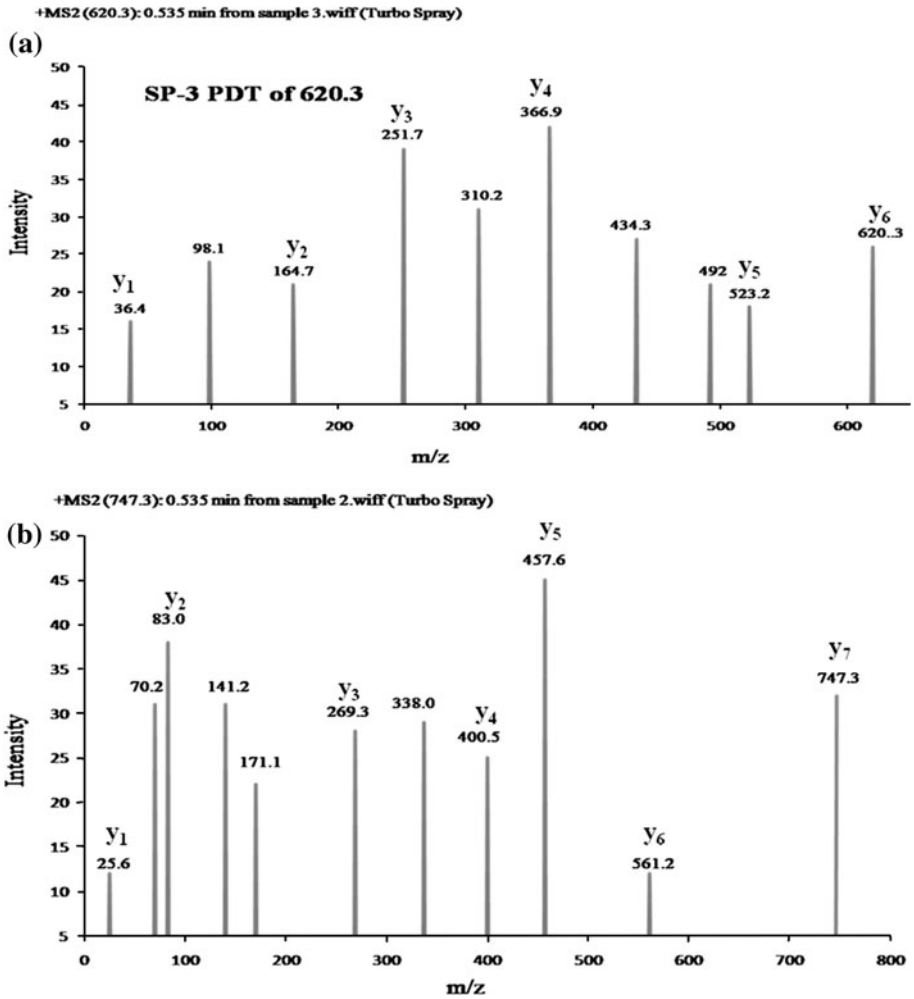

Fig. 4 **a** MS/MS spectrum of the ion *m/z* 620.3. By manual calculation the sequence of this peptide was Glu-Ser-Asp-Arg-Pro. **b** MS/MS spectrum of the ion *m/z* 747.3. By manual calculation the sequence of this peptide was Gly-Trp-Met-Gly-Cys-Trp

activity of peptides due to their special structure of characteristics: the imidazole group in His has the proton-donation ability (Tsuge et al. 1991); Met is prone to oxidation of the Met sulfoxide (Hernandez-Ledesma et al. 2005); Cys donates the sulfur hydrogen (Hernandez-Ledesma et al. 2005). The results were supported by Suetsuna et al. (2000), reported antioxidant peptide from a peptic hydrolysate of casein possessed a primary structure of Tyr-Phe-Tyr-Pro-Glu-Leu and acted as potential antioxidant. Moreover, Li et al. (2007) identified the peptide Gln-Gly-Ala-Arg, which exhibited the highest antioxidant activity from porcine skin collagen hydrolysates.

Conclusion

Based on the current results it can be concluded that degree of hydrolysis of *N. japonicus* and *E. volitans* muscle protein hydrolysed by trypsin was significantly influenced by temperature, enzyme/substrate concentration and time. The suggested hydrolysis conditions for obtaining the optimum DH using trypsin was found as temperature, 30°C; hydrolysis time, 100 min; enzyme/substrate concentration, 1.59% and temperature, 30°C; hydrolysis time; 115 min; enzyme/substrate concentration, 1.67%; for *N. japonicus* and *E. volitans* muscle proteins, respectively. Moreover, it was found peptides purified from *N. japonicus* and *E. volitans* muscle has important amino acids with potency to scavenge free radical and could help to protect against oxidative damage in living systems. However, further detailed studies on these purified peptides are needed in regard of in vivo antioxidant activities.

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Conflict of interest No competing financial interests exist.

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