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Relationship between antioxidative potential and amino acids composition of the bioactive peptides prepared from Indian squid *Uroteuthis* (*Photololigo*) *duvaucelii* (d'Orbigny, 1835) using alcalase

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ABSTRACT

Biopeptides derived from fish protein hydrolysates are gaining popularity as active ingredients in functional foods. In this study, squid protein hydrolysates (SPH) prepared from *Uroteuthis* (*Photololigo*) *duvaucelii,* using alcalase and their biopeptides fractionated using ultrafiltration were investigated for their antioxidant properties in relation to the changes in their amino acid profiles. Squid mantle hydrolysed within 30 min with a degree of hydrolysis (DH) of 13.7%. SPH had 89% 2, 2-diphenyl-1- picrylhydrazyl (DPPH) inhibition, 94% 2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulphonicacid) (ABTS) inhibition and 96% hydroxyl inhibition at 10 mg ml^{-1} concentration. In the squid peptides, DPPH inhibition increased with the decrease in the molecular size of peptides from 51% to 95% (3 Ka), ABTS radical inhibition was <42% for peptides with >5KDa size and >95% for peptides with <5KDa. Hydroxyl radical inhibition increased from 75-98% with decrease in molecular size. Increase in glycine, alanine, leucine and taurine was noticed in peptides with MW <5KDa along with a decrease in methionine and arginine. Histidine, asparagine and tyrosine had also contributed to the activities of peptides with MW 3-5 KDa sizes. This study indicated that peptides with MW <5KDa had more free radicals scavenging activities with more hydrophobic amino acids, tyrosine and taurine which are mainly responsible for antioxidative properties. Therefore, squid peptides with MW <5KDa prepared using alcalase have potential to serve as nutraceuticals.

Keywords: Alcalase, Amino acid, Antioxidative, Biopeptides, Hydrolysate, Indian squid

Introduction

Fish are rich source of structurally diverse bioactive compounds used to promote consumer health as well as to improve the shelf life of food products. Fish protein hydrolysates (FPH) and their peptides exert various biological activities. Antioxidative properties of several fish protein hydrolysates derived from muscle, skin and bone have been reported by several authors (Byun and Kim, 2001; Wu *et al.*, 2003; Mendis *et al.*, 2005; Je *et al.*, 2007; Thiansilakul *et al.*, 2007; Yang *et al.*, 2008 a,b; Gimenez *et al.*, 2009; Hsu *et al.,* 2009). Studies on the antioxidative properties of squid protein hydrolysates (SPH) and their bioactive peptides derived from Indian squid in relation to their amino acid composition are quite limited. Studies in this line have been carried out earlier on the giant squid skin (Mendis *et al.*, 2005, Gimenez *et al.*, 2009) as well as muscle (Rajapakse *et al.*, 2005).

Peptides, that are usually inactive within the parent protein, can be released as an active compound by the action of plant, animal and microbial enzymes (Hoyle *et al.*, 1994; Simpson *et al.*, 1998; Aspmo *et al.*, 2005; Viera *et al.*, 1995). Alcalase is a microbial alkaline enzyme produced by *Bacillus licheniformis* that is highly efficient in the production of bioactive compounds (Kristinsson and Rasco, 2000). It hydrolyses the peptide bonds of aliphatic or aromatic amino acids such as leucine, phenylalanine, tyrosine and tryptophan (Rao *et al.*, 1998). Studies carried out with different enzymes have proven that FPH prepared using alcalase exhibited high antioxidative properties (Thiansilakul *et al*., 2007). Peptides isolated from FPH usually consist of 3-20 amino acid residues (Pihlanto-Leppala, 2001) and their activities are based on their structural properties, molecular size, amino acid composition and their sequence (Arihara, 2004; Kim and Wijesekara, 2010).

Amino acid residues such as histidine, leucine, tyrosine, methionine and cysteine are associated with radical scavenging activity while hydrophobic amino acids such as proline and hydroxyproline appear to play an important role in the inhibition of lipid peroxidation (Mendis *et al.*, 2005; Sarmadi and Ismail, 2010). Free radical quenching has been reported to be the main antioxidative mechanism of the peptides due to the amino acids, especially proline, alanine, valine and leucine (Mendis *et al.*, 2005). The antioxidant

properties of fish peptides are not attributable to a single antioxidant mechanism as the properties derived by the peptides were mainly due to the presence of different amino acids favouring one mechanism over others. Taking into consideration all the above aspects, the present study was undertaken with the aim to utilise the Indian squid *Uroteuthis* (*Photololigo*) *duvaucelii* to prepare squid protein hydrolysate (SPH) and their biopeptides using alcalase, as a maiden study. The antioxidative properties of the squid peptides in relation to the changes in the amino acid composition were also examined.

Materials and methods

The Indian squid *U.* (*P.*) *duvaucelii* caught by trawl net were obtained from Thoothukudi Fishing Harbour, Tamil Nadu, India. Alcalase enzyme was obtained from Novozyme, Bagsvaerd, Denmark. Chemicals such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH); 2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulphonicacid) (ABTS); 2,4,6-tripyridyl-s-triazine (TPTZ) and 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma-Aldrich, St. Louis, Missouri, USA. Nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide sodium salt monohydrate (NADH), phenazine metho-sulphate (PMS), potassium persulphate, sodium salicylate, L-leucine and potassium ferricyanide were procured from Hi-Media Laboratories India Pvt. Ltd., Mumbai. Ferrous chloride ($FeCl_2$) was purchased from Alfa-acer Heysham, England. Amino acid standards H were procured from Thermoscientific Pvt. Ltd., Meridian Rd., Rockford, U.S.A. Nanosep® Centrifugal tubes with molecular weight cutoffs (MWCO) 30 KDa and 10 KDa were purchased from Pall Life Sciences Ann Arbor Michigan, Mexico and Vivaspin molecular weight cutoffs (MWCO) 5 KDa and 3 KDa were purchased from Sartorius Stedim Biotech, Gottingen, Germany. All other chemicals like ferric chloride ($FeCl_3$), trichloroacetic acid (TCA), ferrous sulphate, hydrogen peroxide, potassium ferric cyanide, sodium acetate, sodium hydroxide, sodium dihydrogen phosphate, disodium hydrogen phosphate, butylated hydroxyl anisole (BHA) and Vitamin C were commercially available and were of analytical grade.

Optimisation of enzyme hydrolysis

Squids were gutted and deskinned manually to separate the mantle tissues and skin for the enzyme hydrolysis. Raw muscle tissue was hydrolysed using alcalase at a ratio of 1:100:200 (enzyme:sample : 50 mM sodium phosphate buffer), under optimum pH of 8.0 and 50°C (Klompong *et al.*, 2007). Hydrolysis was initially carried out in a serological water bath for a total duration of 180 min. Samples were drawn after every 5 min to test the degree of hydrolysis. The reaction was finally terminated by increasing the temperature to 70°C for 15 min. The hydrolysate was filtered through Whatman No.1 filter paper and centrifuged at 10,000 rpm for 15 min at 4°C in a refrigerated centrifuge (Hettich, Germany).

Degree of hydrolysis

Degree of hydrolysis (DH) was calculated by determination of free amino group after reaction with TNBS following the method described by Adler-Nissen (1986) and Crowell *et al.* (1985) with slight modification. About 0.25 ml of SPH was pipetted out into 2.0 ml of aqueous 1% SDS and incubated at 70°C for 15 min. From this, 0.25 ml was transferred into test tube containing 2.0 ml of 0.2 m sodium phosphate buffer (pH 8.2). A blank was set using 0.25 ml of 1% SDS. Then, 2 ml of TNBS reagent (0.1% w/v) was added, mixed well, covered with aluminum foil and incubated in dark at 50°C for 50 min. The reaction was then terminated by the addition of 4.0 ml of 0.1N HCl and cooled at room temperature for 30 min. The absorbance was read at 420 nm in a UV-Vis. spectrophotometer (Model V-531, JASCO, Japan). The amount of free amino group liberated was expressed as L-leucine equivalent. The DH was determined following the equation given by Beak and Cadwallader (1995).

$$DH = [(L_t - L_0)/(L_{max}-L_0)] \times 100$$

where L_t corresponds to the amount of α-amino acid released at time t; L_0 is the amount of α-amino acid in original muscle; L_{max} is the maximum amount of α-amino acid in muscle.

To determine the total α-amino acids, 0.5 g of mantle tissue taken in a test tube was mixed with 4.5 ml of 6N HCl, flushed with nitrogen gas, sealed tightly and hydrolysis was carried at 100°C for 24 h in a hot air oven (Dalal, New Delhi). After the hydrolysis, it was cooled, filtered and then neutralised with 6N NaOH prior to the determination by TNBS reagent.

Biopeptides fractionation

Biopeptides were separated from the SPH based on their molecular weight cutoffs (MWCOs), using ultrafiltration centrifugal tubes. SPH (0.5 ml) was taken in the centrifugal tube having MWCO <30 KDa filter and centrifuged at 10,000 rpm for 10 min, to obtain >30 KDa and <30 KDa peptide fractions. The <30 KDa fraction was again transferred into the centrifugal tube having MWCO 10 KDa filter and centrifuged at 10,000 rpm for 10 min to get 30-10 KDa and <10 KDa biopeptides fractions. The <10KDa fraction was further separated in similar manner to obtain 10-5 KDa, <5KDa, 5-3KDa and <3 KDa peptide fractions using the respective MWCO filters. The concentration of biopeptides in each fraction was estimated following Lowry *et al.* (1969).

Total amino acid profile

Amino acid composition was determined by first hydrolysing the samples according to the method of Ishida *et al.* (1981). Peptide samples were taken in tubes,

sealed in a stream of nitrogen gas and hydrolysed using 6N HCl by placing the tubes in a hot air oven (Technico, India) set at 110ºC for 24 h. After hydrolysis, the samples were filtered through Whatman No.1 filter paper to remove the unhydrolysed debris and neutralised with 6N NaOH. Neutralised samples were filtered again through a PTFE syringe filter of 0.2 µm diameter (Cole Parmer, East Bunker Court Vernon Hills, USA). Filtered samples were derivatised using the AccQ. Tag Ultra precolumn derivatisation kit. For derivatisation, 10 µl of filtrate was mixed with 70 µl of AccQ. Tag Ultra Borate buffer and 20 µl of reconstituted Acc Q Tag Ultra derivatisation reagent. Derivatised samples were transferred into auto sampler maximum recovery vials of capacity 2 µl.

Amino acid derivatives were then separated in the Waters ACQUITY-UPLC (Waters Corporation, Milford, USA) fitted with ACCQ-TAG ULTRA C18, 1.7 µm, 2.1x100 mm column by gradient elution. Mobile phase consisted of two buffers, ACCQ-TAG ULTRA Eluent A containing acetonitrile and formalin and Eluent B containing acetonitrile. Flow rate was set at 0.5 ml min^{-1}. Column temperature was maintained at 55ºC. Gradient elution programme was followed setting a flow of 99.9% Eluent A initially and then progressing to 78.8% at 10.34 min; 40.4% at 11.26 min and 10% at 11.27 min. The total run time was 13.30 min. Amino acids were quantified based on their absorbance at 260 nm with the help of tunable UV detector and analysed using Empower 2 Software. Amino acid standards (Thermo Scientific, Rockford, USA) were also run simultaneously for calibration. Amino acids were identified with the help of authentic standards and represented in g 100 g $protein^{-1}$.

Antioxidant properties

To determine the antioxidant properties of the squid muscle hydrolysates during the hydrolysis reaction, 10-15 ml of hydrolysate was withdrawn after every 5 min interval in the test tube and the reaction was terminated by incubation to 70°C for 15 min. The reaction mixture was then centrifuged at 8000 rpm for 10 min at 4°C, neutralised to pH 7.0 using either HCl or NaOH and filtered through syringe filter having 0.2 µm dia (Polysulfone, Whatman, Florham Park, USA) to obtain a clear supernatant.

DPPH radical scavenging activity

DPPH radical scavenging activity was determined as per Wu *et al*. (2003) with slight modifications. SPH and their peptides (1.5 ml) mixed with 1.5 ml of 0.1 mm DPPH in 95% methanol were allowed to stand in dark for 30 min; and the absorbance was measured at 517 nm, 50 mM sodium phosphate buffer (pH 7.2) was used as the control. Standards were prepared using BHA and vitamin C at different concentration for comparison. All the activities were determined in triplicate.

$$\text{DPPH radical scavenging activity (\%)} = [(A0 - A1)/A0] \times 100\%$$

where, A0 is the absorbance of control and A1 is the absorbance of sample or standard.

ABTS radical scavenging activity

ABTS radical scavenging activity was determined as per the method of Shalaby (2013) with slight modification. Stock ABTS solution was prepared by incubating 7.0 mM of ABTS with 2.45 mM potassium persulfate for 12 h in dark, which was further diluted at the ratio of 1:30 using methanol to obtain an absorbance of 0.700 = 0.02 at 734 nm. ABTS solution was freshly prepared before use. For the assay, SPH and their peptides (150 µl) mixed with 2.85 ml of diluted ABTS solution were left for 2 h in dark and the absorbance was measured at 734 nm. 50 mM sodium phosphate buffer (pH. 7.2) served as the control. Standards were prepared using BHA and vitamin C at different concentrations for comparison. All the activities were determined in triplicate.

$$\text{ABTS radical scavenging activity (\%)} = [(A0 - A1)/A0] \times 100\%,$$

where, A0 is the absorbance of control and A1 is the absorbance of sample or standard.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured according to the method developed by Smirnoff and Cumbes (1989). SPH and their peptides (0.5 ml) were mixed with 1.0 ml of 1.5 mM $FeSO_4 \cdot 7H_2O$, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and 1.0 ml of distilled water and incubated at 37°C for 1 h prior to measurement of the absorbance at 562 nm. A control was prepared with 50 mM sodium phosphate buffer (pH 7.2). A salicylate blank was set similar to control but without sodium salicylate. Standards were prepared using BHA and vitamin C at different concentrations for comparison. All the activities were determined in triplicate.

$$\text{Hydroxyl radical scavenging activity} = [1-(A1-A2)/A0] \times 100$$

where, A0 is the absorbance of the control (without sample); A1 is the absorbance of the sample or standard; A2 is the absorbance of the control without sodium salicylate.

Statistical analysis

Results are presented as mean±standard error and significance of the difference between mean values was determined by one-way analysis of variance (ANOVA) coupled with Duncan's multiple range test using windows based statistical software SPSS 10. p-value of less than 0.05 was considered to be significant (Christensen, 1996).

Results and discussion

Degree of hydrolysis

Biological activities of the SPH and biopeptides depend on the degree of hydrolysis (DH). DH curve of SPH prepared using alcalase at 50°C and pH 8.0 is given in Fig. 1. The DH increased significantly to 13.7% within 30 min ($p<0.05$); and thereafter, no further increase in DH was observed. In tuna skin and squid tunic gelatin hydrolysates produced using alcalase, the maximum DH achieved was 47.52% recorded after 150 min of hydrolysis, of which, 25-30% occurred within the first 5 min (Gomez Guillen *et al.*, 2010). The round scad muscle hydrolysates prepared using alcalase had much higher DH of 60% (Thiansilakul *et al.*, 2007), but in the muscle hydrolysate prepared from yellow stripe trevally, using alcalase, the maximum DH was only 20% after 30 min of hydrolysis (Klompong *et al.*, 2007). In squid, tuna and halibut gelatin hydrolysates prepared using alcalase, the DH recorded were 31, 26 and 19%, respectively after 180 min of hydrolysis (Aleman *et al.*, 2011).

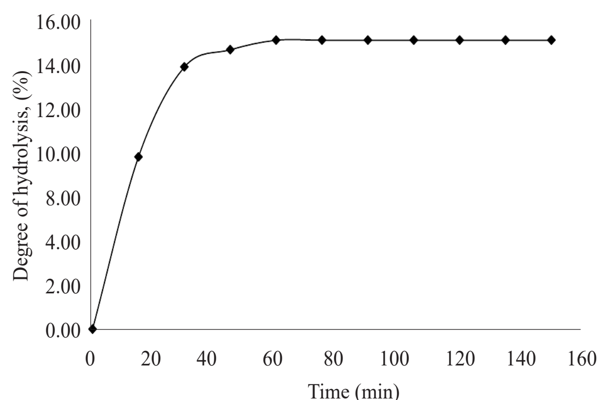

Fig.1. Degree of hydrolysis of SPH prepared using alcalase

The rate of hydrolysis by the alcalase on any substrate was quite fast in the initial stages and later reached a plateau, where no apparent change took place. We report DH either in terms of total α-free amino acids or total nitrogen. The DH represented in terms of total nitrogen usually gave much higher values than those represented as α-free amino acids. As recently, it has been indicated that DH has to be represented in terms of α-free amino acids (Adler-Nissen,1986; AOAC, 1995) the same has been followed in this study.

Amino acid profile

The standard UPLC method could separate all the seventeen amino acids within the stipulated runtime with the retention times of histidine at 3.275 min, serine 4.477 min, arginine 4.717 min, glycine 4.911 min, aspartate 5.388 min,. glutamate 6.067 min, threonine 6.613 min, alanine 7.143 min, proline 7.93 min, cystine 9.102 min, lysine 9.178 min, tyrosine 9.356 min, methionine 9.560 min, valine 9.717 min, isoleucine 10.582 min, leucine 10.684 min and phenylalanine at 10.808 min. The linearity of the method is 0.25 to 2.5 µmol of each amino acid. The LOD (Level of Detection) and LOQ (Level of Quantitation) of ACQUITY-UPLC are 0.1 µmol and 2.5 µmol respectively.

Amino acid profile of SPH and their peptide fractions were examined to understand their role in relation to the antioxidative potential. Squid mantle consisted predominantly of glycine (15 mg 100 mg^{-1}), glutamic acid (12 mg 100 mg^{-1}), lysine (9 mg 100 mg^{-1}), alanine (8.5 mg 100 mg^{-1}), leucine (7.8 mg 100 mg^{-1}) and aspartic acid (7.5 mg 100 mg^{-1}) (Table.1). After hydrolysis, there were changes in the proportions of few amino acids. The amino acids lost upon hydrolysis included histidine, arginine, aspartic acid, glutamic acid and methionine. Some of the amino acids also increased upon hydrolysis *viz*., glycine, alanine, tyrosine, serine and taurine. There was no significant variation in the proportions of the remaining amino acids ($p>0.05$).

In the purified peptide fractions, loss of histidine was predominant except in the peptide fraction with MW 3-5 KDa. Hydroxy-lysine 1, increased from 0.91 mg 100 mg^{-1} to 0.49 mg 100 mg^{-1}, but later reduced in peptide fraction with MW <3KDa. Lysine remained almost constant. Arginine reduced in the peptides with lower MW, aspartic acid reduced in the peptides with MW 5-10 KDa, while glutamic acid reduced by 40-50% in the peptide fractions. Asparagine showed slight variation in the peptides.

Tyrosine increased from 1.2 mg 100 mg^{-1} to a maximum of 8.3 mg 100 mg^{-1} in the peptides with MW <5 KDa and further decreased to 2.63 mg 100 mg^{-1} in <3KDa MW peptides. Phenylalanine and proline remained more or less similar in the peptides. Serine, on the other hand, increased from 0.92 mg 100 mg^{-1} to maximum of 1.62 mg 100 mg^{-1} in peptide fractions with MW <3 KDa. Cysteine did not show any change except in <3KDa fraction with 0.04 mg 100 mg^{-1}. Methionine reduced significantly with the reduction in the molecular size of peptides, particularly from 0.9 mg 100 mg^{-1} to 0.07 mg 100 mg^{-1} in <3KDa peptide fraction. Threonine did not increase or decrease significantly in the peptides. Proline is the major cyclic acid present in appreciably higher concentration (6.5 mg 100 mg^{-1}) in squid, SPH and in their peptide fractions.

Glycine increased from 14.85 mg 100 mg^{-1} to a maximum of 20.6 mg 100 mg^{-1} in peptides. Peptides below MW <5 KDa contained more glycine. Alanine also increased in a similar way from 8.5 mg 100 mg^{-1} to 12.6 mg 100 mg^{-1}. Valine and leucine showed slight increase in proportion, while isoleucine remained more or less constant in the peptides with the decrease in molecular size. Proportion of

Table 1. Changes in the amino acid profile (g 100 g^{-1}) of squid muscle, SPH and their peptide fractions

Amino acid	Muscle	SPH	<30Kda	<10Kda	<5KDa	<3KDa
His	0.70±0.02	0.13±0.01	0.77±0.02	0.06±0.01	0.63±0.01	0.00±0.01
Lys	8.91±0.11	8.55±0.12	8.24±0.11	8.10±0.13	8.16±0.12	9.70±0.14
HyLys1	0.19±0.01	0.49±0.04	0.42±0.07	0.39±0.05	0.39±0.04	0.23±0.02
Arg	4.81±0.05	3.31±0.04	2.79±0.6	2.72±0.02	2.21±0.01	1.37±0.02
Basic	14.62	12.49	12.22	11.27	11.39	11.31
Asp	7.51±0.12	3.63±0.11	7.31±0.15	5.56±0.13	6.64±0.12	7.05 ±0.16
Glu	11.97±0.13	5.86±0.14	5.92±0.17	7.32±0.12	6.32±0.16	5.78±0.13
Acidic	19.48	9.49	13.23	12.88	12.96	12.83
Gln	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0
Asn	1.17±0.01	0.81±0.02	0.89±0.01	0.56±0.01	1.07±0.02	0.43±0.02
Neutral	1.17	0.81	0.89	0.56	1.07	0.43
Phe	2.92 ±0.07	2.01±0.01	1.96±0.08	2.05±0.07	2.31±0.10	2.59 ±0.12
Tyr	1.26±0.04	3.81±0.06	4.29±0.09	3.07±0.10	6.52±0.08	2.63 ±0.12
Aromatic	4.17	5.82	6.25	5.12	8.83	5.22
Pro	6.39±0.12	6.51±0.11	6.30±0.11	6.13±0.12	6.48 ±0.13	6.50±0.12
Ser	0.92±0.03	1.24 ±0.01	1.08±0.01	1.35±0.04	1.11±0.02	1.60±0.08
Cys	0.11±0.01	0.00±0.0	0.11±0.01	0.17±0.01	0.15±0.02	0.04±0.01
Thr	2.13±0.09	1.95±0.10	1.79±0.12	2.42±0.11	2.53±0.14	2.60±0.11
Met	0.90±0.06	0.38±0.02	0.33±0.03	0.39±0.01	0.08±0.01	0.07±0.01
Cyclic	10.45	10.08	9.61	10.46	10.36	10.80
Gly	14.85±0.16	14.26±0.12	14.77±0.12	15.28±0.11	17.98±0.09	20.65±0.01
Ala	8.55±0.12	8.73±0.11	8.66±0.11	9.96±0.15	10.60±0.12	12.60±0.02
Val	4.71±0.11	4.70±0.13	4.58±0.11	5.19±0.12	5.18±0.11	5.51±0.11
Ile	4.61±0.12	4.59±0.11	4.49±0.13	4.92±0.11	4.68±0.11	4.75±0.13
Leu	7.77±0.14	7.83±0.12	7.65±0.12	8.40±0.16	8.77±0.19	9.58±0.12
Aliphatic	40.50	40.11	40.16	43.75	40.88	53.09
Tau	1.97±0.09	1.66±0.10	1.57±0.11	1.97±0.12	2.31±0.09	3.51±0.08
Sulphonic	1.97	1.66	1.57	1.97	2.31	3.51
Total	92.36	80.46	83.93	86.01	87.80	97.19

taurine in squid was quite high (1.9 mg 100 mg^{-1}) and later it increased to 3.5 mg 100 mg^{-1} in peptides with MW <3KDa.

DPPH radical scavenging activity

DPPH radical scavenging activities of the SPH and their peptide fractions are given in Fig. 2. SPH exhibited 89% DPPH inhibition after 30 min of hydrolysis. Peptide fractions with MW >30 KDa had 51% DPPH inhibition, while those with MW <30KDa had 63% DPPH inhibition. DPPH inhibition further increased with the decrease in molecular size of the peptides and those fractions with MW <3 KDa showed the maximum DPPH inhibition of 95% at 10 mg ml^{-1} of protein concentration. Results showed that peptide fraction with MW <3 KDa had better DPPH inhibition than SPH, proving that purification to lower molecular size provide peptides with more antioxidative activities. DPPH inhibition expressed by squid peptides were much higher than 100 ppm of BHA (70%). Similar to our findings, mackerel muscle hydrolysates with MW of 1.4 KDa also possessed a stronger antioxidant activity (Wu *et al.*, 2003).

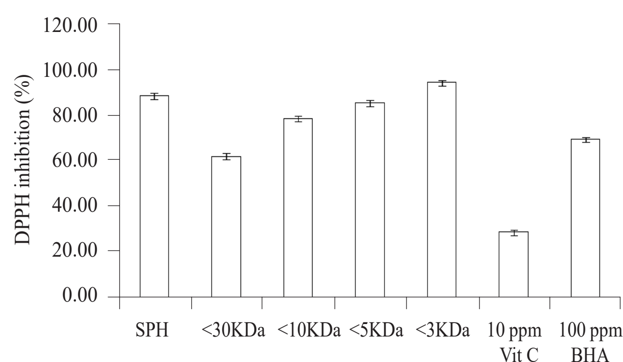

Fig. 2. DPPH radical scavenging activities of TPH and their peptide fractions prepared using alcalase in comparison with commercial antioxidants

Antioxidant activity of FPH has been reported to be mainly dependent on the enzymes, hydrolysis conditions, size of the peptides and their sequences (Clare, 2000; Arihara, 2004). Initially, alkaline (basic) amino acids were reported to provide strong DPPH antioxidant activity in royal jelly and grass carp muscle hydrolysates (Ren *et al.*, 2008;

Guo *et al.*, 2009). But, in SPH and their peptides, alkaline (basic) amino acids decreased with decrease in the molecular size of the peptides and are therefore not responsible for this activity. Later, studies on the peptide profile of hemp protein hydrolysates indicated that hydrophobic amino acids exhibited DPPH inhibition (Xiansheng *et al.*, 2009). In support to this, the proportion of these amino acids in SPH increased from 40 to 53% in the squid peptides and more specifically glycine, alanine, leucine and valine. Xiaoqing *et al.* (2013) identified that among the hydrophobic amino acids, leucine, phenylalanine and valine had contributed towards high DPPH inhibition in clam (*Paphia undulata*) hydrolysates. In this study, phenylalanine did not increase, to contribute for the high DPPH inhibition. But an increase in tyrosine, serine and taurine was observed with increase in molecular size of squid peptides, which could also contribute towards the antioxidant properties in addition to the hydrophobic amino acids.

ABTS radical scavenging activity

ABTS radical scavenging activities of SPH and their peptide fractions are given in Fig. 3. Unlike DPPH inhibition, significant differences in the ABTS radical scavenging activities were observed between SPH and their peptide fractions with MW <5KDa and those with MW between 10-30 KDa (p>0.05). ABTS inhibition was generally higher in SPH (94%) and it got reduced in peptide fractions with higher MW 10-30 KDa to 42%. However, the activity further increased in peptides with MW <5KDa and was found to be maximum in 3KDa peptides with 97%. The ABTS inhibition expressed by squid peptides with <3KDa was much higher than the activity of 10 ppm BHA (70%) and almost equal to 100 ppm vitamin C (100%).

The presence of hydrophilic amino acids has been reported to be responsible for higher ABTS activity in the ornate threadfin bream muscle hydrolysates prepared with pepsin extracted from skipjack tuna (Nalinanona

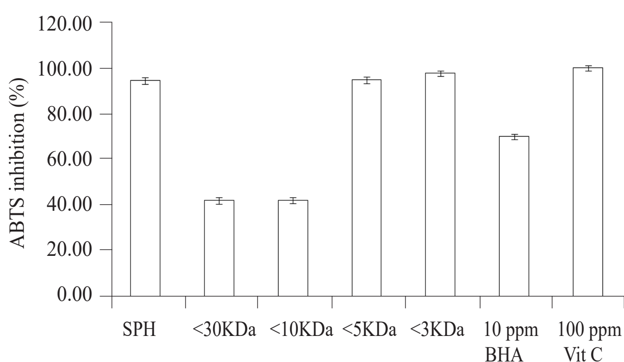

Fig. 3. ABTS radical scavenging activities of TPH and their peptide fractions prepared using alcalase in comparison with commercial antioxidants

et al., 2011). In this study, the hydrophilic amino acids *viz*., aspartic acid, tyrosine and asparagine have been identified as the major amino acids responsible for ABTS inhibition, as the proportion of these amino acids was similar in SPH and the peptide fractions with MW <5 KDa. Hydrophobic amino acids and taurine could also contributed to ABTS radical inhibition.

Hydroxyl radical scavenging activity

Hydroxyl radicals are generally formed from superoxide anion and hydrogen peroxide in the presence of transition metal ions such as Fe^{2+} and Cu^{2+}. SPH exhibited 75% hydroxyl radical inhibition with 96% at 10 mg ml^{-1} concentration (Fig. 4). Hydroxyl radical scavenging activities of shrimp muscle hydrolysates at 5 mg ml^{-1} concentration have been reported (Cao *et al.*, 2009). Peptide fractions with higher MW generally have lower hydroxyl radical inhibition than those with lower MW. Hydroxyl radical inhibition increased from 74% to a maximum of 97% with the decrease in MW from >30 KDa to <3KDa. In an earlier report, purified peptides derived from Alaska pollack (*Theragra chalcogramma*) exhibited only 35% inhibition (Je *et al.*, 2005), but the

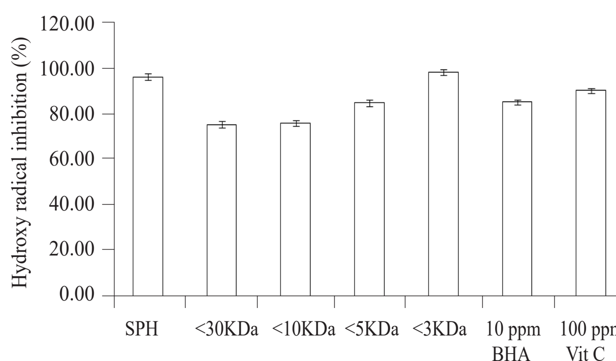

Fig. 4. Hydroxyl radical scavenging activities of TPH and their peptide fractions prepared using alcalase in comparison with commercial antioxidants

purified squid peptides showed more inhibition than the SPH itself. Similar to our findings, peptide fraction with 3KDa of grass carp muscle hydrolysate prepared using alcalase exhibited higher hydroxyl radical scavenging activity (Ren *et al.*, 2008). Hydroxyl radical scavenging activities were also reported to be much higher in puffer fish skin gelatin hydrolysates and low MW peptides with <5KDa than vitamin C and E (Zhu *et al.*, 2008). In this study, the hydroxyl activity expressed by squid peptides was higher than 10 ppm of BHA (85%) and 10 ppm of vitamin (90%).

Suetsuna *et al.* (2000) suggested that the phenolic groups in phenylalanine and tyrosine contribute more for the hydroxyl radical scavenging activity through electron donation. According to Li *et al.* (2003), the presence of

high phenylalanine in the peptides showed high hydroxyl radical scavenging activity due to the reaction of aromatic ring with hydroxyl radical to form a stable compound. More specifically, tyrosine at C-terminal was also found to be responsible for hydroxyl radical inhibition based on the three peptides sequenced from royal jelly protein hydrolysates (Guo, 2009). In this study, phenylalanine did not show any increase in the peptides of smaller MW, instead tyrosine increased in the squid peptides with the decrease in MW (except <3KDa) and hence, the findings partially support the earlier report. In addition, the hydrophobic amino acids, serine and taurine that had increased in peptides contribute for the hydroxyl inhibition by the squid peptide fractions.

Very high DPPH (95%), ABTS (98%) and hydroxyl radicals (98%) scavenging activities of squid peptides with MW <3KDa could be related to some unusual changes in the amino acid profile. For instance, the proportion of hydrophobic amino acid was 53% in the squid peptides as against 40% in SPH. Increase in hydrophobic amino acids is mainly because of the catalytic sites of alcalase, which are peptide bonds of aliphatic or aromatic amino acids *viz*., leucine, phenyl alanine, tyrosine and tryptophan (Rao *et al*., 1998). Loss of histidine as well as asparagine was very prominent. Gain of serine, glycine, alanine and taurine was found quite unique. Taurine has been identified to be involved in radical scavenging activities, membrane regulation, osmoregulation and regulation of calcium homeostasis (Larsen *et al.*, 2007). Also, taurine has been reported to lower cholesterol absorption due to enhancement of cholesterol degradation and excretion of the bile acid (Yokogoshi *et al.*, 1999). Increase in taurine in squid peptides thus provides additional benefits apart from antioxidative properties. In squid peptides of lower molecular size, histidine did not contribute to any antioxidative activity, however, increase in the proportions of hydrophobic amino acids in addition to serine and taurine was found responsible for the radical scavenging activities in squid peptides.

Purified peptides with MW <5 KDa possessed more DPPH, ABTS and hydroxyl radical scavenging activities than SPH and their peptides with MW >5KDa. Amino acid profile showed differences among raw squid, SPH and their peptides fractions. Predominance of lysine, aspartate, glutamate, proline, glycine, alanine, leucine, valine, isoleucine and taurine was noticed in the squid peptides of lower molecular weights. Hydrophobic amino acids taurine and serine were responsible mainly for the antioxidative properties. Cyclic amino acid, proline is a unique one in squid contributing for special antioxidative property unlike fish peptides. This study thus helped to understand the changes in the different amino acid profiles of the different molecular size peptides of squid and their resultant antioxidant activities. In addition, it also indicated that the increase in taurine, provides cholesterol lowering effect in addition to antioxidative properties in squid peptides. These low molecular weight squid peptides can be prepared commercially using tangential flow filter (TFF) systems, cost effectively for incorporation in functional food products.

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