Recovery of functional materials with thermally stable antioxidative properties in squid muscle hydrolyzates by subcritical water

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Abstract Subcritical water hydrolysis was carried out to produce functional materials from squid muscle using a batch reactor. The reaction temperatures and pressures for hydrolysis of thermal dried squid muscle were maintained from 160 to 280 °C and 6 to 66 bar for 3 min. The ratio of material to water for hydrolysis was 1:25 (w/v) and it was stirred at 140 rpm. Hydrolysis yield was increased after increasing the temperature and pressure while the protein in hydrolyzate decreased with the rise of temperature. The reducing sugar yield was high at temperature 220 °C in subcritical water hydrolysis of squid muscle. Low molecular weight peptides were found in all hydrolyzates by SDS-PAGE. The highest yield of free and structural amino acid in hydrolyzate was 421.53 ± 1.24 and 380.58 ± 2.25 mg/100 g, respectively at 250 °C. All essential amino acids were identified in muscle hydrolyzates and it was high at 220 °C. Among the essential amino acids, leucine was the most abundant. Antioxidative properties were found in all hydrolyzates and it was high at 220 °C. More than 98 ± 0.26 % ABTS antioxidant activity was retained in hydrolyzates after long time heat treatment.

Keywords Subcritical water · Hydrolyzate · Squid muscle · Amino acid · Reducing sugar · Antioxidative properties

Introduction

Hydrolysis is needed to obtain functional materials. Current industrial hydrolysis methods include chemical (acid, alkali or catalytic) hydrolysis and enzymatic hydrolysis. But the

chemical hydrolysis needs violent reaction conditions and often brings serious pollution of the environment. Enzymatic hydrolysis takes long time for completing production cycle and is also expensive. Sub-critical water can provide a new reaction medium in many chemical reactions. Changes in physical and chemical properties of water under sub-critical conditions, especially in hydrogen bond, ion product and dielectric constant, could facilitate reactions with a wide range of organic compounds resulting in many valuable materials (Yoshii et al. 2001; Laria et al. 2004; Tomita and Oshima 2004; Yagasaki et al. 2005). Most of biomass is easily hydrolyzed in super or subcritical water which is structurally different from normal water. Without any pollution, hydrolysis in subcritical water is environment friendly technology (Cheng et al. 2008). Recently growing attention has led to extensive research activities using subcritical water for hydrolysis and conversion of biomass to useful compounds (Yoshida et al. 1999; Kruse and Gawlik 2003; Bicker et al. 2005; Tavakoli and Yoshida 2006; Salak Asghari and Yoshida 2007; Uddin et al. 2010). The thermal protein hydrolysis is gaining in importance in economical as well as ecological aspects.

Squid (*Todarodes pacificus*) belongs to the family of ommastrephidae is a commercially important species for Japan, Korea and China (Kang et al. 2005). It is one of the most heavily exploited squid species in the world (FAO 2003). This fish contains a lot of protein, carbohydrates, fats and other bioactive compounds.

Food proteins have long been recognized for their nutritional and functional properties. The nutritional properties of proteins are associated with their amino acid content in conjunction with the physiological utilization of specific amino acids upon digestion and absorption (Friedman 1996; Korhonen and Pihlanto 2006). Amino acids are used for a variety of applications in industry, but their main use is as additives to animal feed. This is necessary, since many of

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the bulk components of these feeds, such as soybeans, either have low levels or lack some of the essential amino acids: lysine, methionine, threonine, and tryptophan are most important in the production of these feeds. Hydrolyzate of fish muscle contains free amino acids (FAAs), structural amino acids (SAAs) and peptides, which are not incorporated in proteins. The FAAs and SAAs have been implicated to be responsible for the characteristic taste of seafood (Fuke 1994). In addition, FAAs play important roles in physiological functions such as osmoregulation and buffer capacity in the tissues of aquatic animals (Van Waarde 1988). The biological activity of a peptide is widely recognized to be based on amino acid composition (Korhonen and Pihlanto 2003). Peptides could be used in the formulation of functional foods and nutraceuticals to prevent damage related to oxidative stress in human disease conditions. Moreover, natural antioxidants are desirable because they can be used at higher concentrations without the toxic side effects associated with the use of synthetic equivalents (Aluko 2008; Li et al. 2008).

Many carbohydrates are excellent scavengers for metal ions. Reducing sugars (Glucose, fructose) have the ability to block the reactive sites of ions such as copper, iron and, to a lesser extent, cobalt. This characteristic of monosaccharides aids in food preservation by retarding catalytic oxidation reactions. Additionally, reducing sugars are known to have antioxidant properties in food systems. For this reason, it has been employed in the food industry as food additives for biscuits, cookies and sausages (Clarke 1997).

Therefore, the aim of this study was to produce functional materials (protein, peptides, amino acids and reducing sugar) in the hydrolyzates from thermal dried squid muscle using subcritical water. Additionally, the antioxidative properties in the hydrolyzates were evaluated for quality functional food product.

Material and methods

Materials The washed squid muscle samples were collected from F & F Co., Busan, Korea and brought to the laboratory in iced condition. All reagents used in this study were of analytical or high performance liquid chromatography (HPLC) grade.

Sample preparation The squid muscle samples were dried in a thermal dryer at 80 °C for 24 h. The complete dried sample was crushed by mechanical blender and then stored at −20 °C until further use and analysis. For economical benefit squid muscle was dried by thermal dryer instead of freeze dryer.

Proximate composition analysis The moisture, ash and crude protein content were determined according to AOAC

(1990) and lipid content was measured by conventional soxhlet extraction using hexane as solvent for 24 h. Non protein content was estimated by subtracting the sum of weight of moisture, ash, protein and lipid from total weight.

Subcritical water hydrolysis (SWH) The SWH was carried out in 200 mL of a batch reactor made of 276 Hastelloy with temperature control. Figure 1 shows a schematic diagram of the SWH apparatus. Exactly, 6 g of thermal dried samples were suspended separately in 150 ml of distilled water (material to water ratio of 1:25 w/v) and charged into the reactor. The reactor was then closed and heated by an electric heater to the desired temperature (160–280 °C). The pressures were estimated based on saturated steam table to be between 6 and 66 bar for the temperature range studied (Haywood 1968). The temperature and pressure in reactor of each experiment was measured by temperature controller and pressure gauge, respectively. The sample was stirred by stirrer at 140 rpm. The reaction time for each sample was 3 min. The heat-up time for reaching the desired temperature took from 32 to 64 min (heat-up time is the amount of time that a device or system require to go from a cold start to operating temperature). After rapid cooling to room temperature, the hydrolyzed samples from the reactor were collected and filtered using a filter paper (Advantec No. 5A). The liquid portion called hydrolyzate was analyzed for protein, amino acids, reducing sugar and antioxidant activity.

Protein measurement The protein content of the hydrolyzate was analysed according to Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) SDS-PAGE of different hydrolyzates were

Fig. 1 Flow diagram of subcritical water hydrolysis experimental apparatus

carried out by the method of Laemmli (1970) using 4.4 % (w/v) stacking gel and 10 % (w/v) separating gel. Hydrolyzates were mixed with sample buffer at the ratio of 1:1.5 (v/v). Then 20 μ L of the sample was loaded in each well. Electrophoresis was performed using a Mini-Protein III cell module (Bio-Rad Laboratories, CA, USA) at a constant voltage (30 mA for 1.5 h). Gels were fixed, stained with 0.1 % Coomassie Brilliant Blue R-250 (dissolved in 45 % methanol and 10 % glacial acetic acid) for 40 min and destained with 30 % methanol and 40 % acetic acid. Molecular weights of the polypeptides were estimated by using molecular weight calibration markers (Sigma Chemical Co., St. Louis, Mo., USA).

Reducing sugar measurement Reducing sugars content was analysed by 3,5-dinitrosalicylic (DNS) acid method (Miller 1959) with slight modification. D-glucose was used as a standard. 0.1 mL of hydrolyzate was diluted up to 3 mL by HPLC grade water and then mixed 3 mL of DNS reagent for each sample. The mixture was heated in boiling water for 5 min. After cooling the room temperature, 1 mL of 40 % potassium sodium tartarate (Rochelle salt) solution was added to stabilize the colour. After 10 min, the spectrophotometric reading was taken at 510 nm.

Amino acid analysis The protein concentration of the hydrolyzates of squid muscle obtained by subcritical water hydrolysis were filtered and loaded onto a S430 (SYKAM) and S433-H (SYKAM) amino acid auto analyzer for free and structural amino acid analysis. Cation separation column LCA K07/Li (4.6 $\times$ 150 mm) and LCA K06/Na (4.6 $\times$ 150 mm), column temperature (37–74 °C) and (57–74 °C), buffer pH range (2.90–7.95) and (3.45–10.85) were used for free and structural amino acid analysis. The mobile phase was 5 mM of *p*-toluenesulfonic acid solution at a flow rate of 0.45 mL/min. A mixture of 5 mM *p*-toluenesulfonic acid, 20 mM of bis-tris and 100 mM of EDTA was used as post column reagent at a flow rate of 0.25 mL/min. Excitation and emission wavelength was kept at 440 and 570 nm for both operational conditions.

Antioxidant activity measurement

DPPH free radical scavenging assay The scavenging effects of crude methanolic extracts were determined by the method of Yen and Chen (1995) with slight modification. Briefly, 3.9 mL of 0.1 mM DPPH solution (in methanol) was added to the test tube containing 0.1 mL of hydrolyzate sample. The mixture was vortexed for 10 s and kept at room temperature for 30 min in the dark. The absorbance of all the sample solutions was measured at 517 nm. The percentage of DPPH free radical scavenging capacity was calculated using this formula:

Table 1 Proximate compositions of thermal dried squid muscle

^a Composition (%)	Thermal dried squid muscle
Moisture	7.35 $\pm$ 0.06
Ash	6.18 $\pm$ 0.05
Protein	73.26 $\pm$ 0.25
Lipid	6.90 $\pm$ 0.09
Non protein	6.31 $\pm$ 0.07

^a Data are the mean value of three replicates $\pm$ SD

$[1 - (As/Ac)] \times 100\%$ (As=absorbance of crude extract at 517 nm; Ac=absorbance of blank at 517 nm). Sample blank was performed according to the method.

ABTS free radical scavenging assay ABTS assay was performed by the method of Zheleva-Dimitrova et al. (2010) with modification. ABTS was dissolved in water to make a concentration of 7 mM/L. ABTS⁺ was produced by reacting the equal volume of ABTS stock solution with 2.45 mM/L potassium persulfate and allowing the mixture to stand in the dark at room temperature for 16 h before use. For the test of samples, the ABTS⁺ stock solution was diluted with 80 % methanol to an absorbance of 0.70 $\pm$ 0.02 at 734 nm. After the addition of 3.9 mL of diluted ABTS⁺ to 0.1 mL of hydrolyzate samples, the mixture was kept in dark environment at room temperature for 6 min. The absorbance of all the sample solutions was measured at 734 nm. The percentage of ABTS⁺ free radical scavenging activity was calculated using this formula: $[1 - (As/Ac)] \times 100\%$ (As=absorbance of crude extract at 734 nm; Ac=absorbance of blank at 734 nm). Sample blank was performed according to the method.

Reducing power assay Reducing power was determined by the method of Oyaiza (1986). The sample solution (1 mL)

Fig. 2 Comparison of hydrolysis yield obtained by subcritical water at different temperatures

Table 2 Protein yield from thermal dried squid muscle hydrolyzates at different temperatures

Temperature (°C)	^a Thermal dried squid muscle hydrolyzate (mg/mL)
160	12.66±0.34
190	12.59±0.29
220	10.39±0.27
250	10.21±0.24
280	10.12±0.28

^a Data are the mean value of three replicates ± SD

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 20 min. An aliquot (2.5 mL) of 10 % trichloroacetic acid was added to the mixture, followed by centrifugation at 3,000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with 2.5 mL of distilled water and 2.5 mL of 0.1 % ferric chloride and the absorbance was read at 700 nm. Increased absorbance of the reaction mixture indicates increasing reducing power.

Thermal stability of hydrolyzates To determine thermal stability, 2 mL of hydrolyzates were transferred into a screw-capped test tube. The tube was capped tightly and placed in a boiling water bath (100 °C) for 0, 25, 50, 75, 100, 125 and 150 min. The sample without incubation (25 °C) was used as the control. The residual antioxidative activities were determined by ABTS assay.

Statistical analysis All experiments were carried out in triplicate. The data are expressed as the mean ± standard

Fig. 3 SDS-PAGE from thermal dried squid muscle hydrolyzates at different temperatures **Fig. 4** Reducing sugar yields from thermal dried squid muscle hydrolyzates at different temperatures. Data are the mean value of three replicates ± SD **Fig. 5** Total and essential amino acids yield from thermal dried squid muscle hydrolyzates at different temperatures. Data are the mean value of three replicates ± SD

deviation. Differences ($P<0.05$) between means were identified by descriptive statistics using SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA).

Results and discussion

Proximate analysis of thermal dried squid muscle The proximate compositions of thermal dried squid muscle are shown in Table 1. The protein was found 73.26 ± 0.25 % in thermal dried squid muscle. Uddin et al. (2010) reported that 45.76 % protein was present in freeze dried squid viscera which are much lower than thermal dried squid muscle. The moisture, ash, lipid and non protein were present 7.35 ± 0.06 , 6.18 ± 0.05 ,

6.90 ± 0.09 and 6.31 ± 0.07 %, respectively in thermal dried squid muscle.

SWH conversion yield Superheated water is liquid water under pressure at temperatures between the usual boiling point, 100 °C (212 °F) and the critical temperature, 374 °C (705 °F). It is also known as “subcritical water”. The hydrolysis yield at different temperatures after SWH from thermal dried squid muscle is shown in Fig. 2. The residual muscle powder recovered after SWH was dried and weighed [W (g)], Conversion yield of squid muscle, X, was evaluated from the weight change of squid muscle, as: $X = \frac{W_0 - W}{W_0} \times 100$.

Where, W_0 is the total amount of squid muscle introduced in reactor, which is nearly 6 g. It was found that the hydrolysis

Table 3 Individual free amino acids yield from thermal dried squid muscle hydrolyzates at different temperatures

^a Amino acid compositions	Squid muscle hydrolyzate (mg/100 g)				
	160 °C	190 °C	220 °C	250 °C	280 °C
Phosphoserine	4.30±0.04	3.37±0.03	2.00±0.04	1.41±0.02	0.92±0.02
Taurine	30.25±0.35	39.74±0.39	60.16±0.48	27.63±0.18	16.57±0.13
Aspartic acid	2.67±0.03	7.15±0.08	4.60±0.06	6.36±0.07	0.51±0.03
Threonine	0.68±0.01	0.86±0.02	2.07±0.03	1.12±0.02	0.80±0.04
Serine	ND	ND	ND	ND	0.23±0.01
Glutamic acid	6.08±0.06	1.61±0.03	0.83±0.02	1.14±0.03	2.62±0.05
Proline	18.33±0.14	11.64±0.13	10.16±0.12	34.41±0.32	16.33±0.14
Glycine	13.77±0.12	19.83±0.15	40.54±0.38	68.49±0.52	34.09±0.28
Alanine	14.93±0.11	32.05±0.32	53.55±0.46	77.95±0.58	43.82±0.32
α-aminobutyric acid	0.82±0.01	0.35±0.01	1.27±0.04	4.69±0.06	2.40±0.05
Valine	11.12±0.12	24.52±0.17	43.45±0.28	25.95±0.16	14.78±0.14
Cystine	3.58±0.03	3.06±0.03	0.40±0.02	2.56±0.05	1.80±0.04
Methionine	1.59±0.02	1.31±0.02	8.71±0.06	13.15±0.12	6.74±0.08
Cystathionine	0.26±0.01	10.18±0.10	20.02±0.15	17.81±0.15	10.70±0.12
Isoleucine	12.96±0.10	3.55±0.05	27.38±0.19	10.80±0.10	6.54±0.06
Leucine	25.62±0.18	39.13±0.34	54.23±0.42	42.89±0.33	21.27±0.18
Tyrosine	1.28±0.02	0.93±0.02	23.50±0.17	12.32±0.11	6.74±0.07
Phenylalanine	14.02±0.15	19.75±0.14	27.33±0.20	17.63±0.14	9.79±0.10
β-alanine	0.03±0.01	0.04±0.01	0.06±0.01	0.14±0.02	0.16±0.01
β-aminoisobutyric acid	0.44±0.01	0.64±0.02	0.34±0.01	4.71±0.05	2.19±0.03
γ-amino-n-butyric acid	0.16±0.02	0.13±0.01	0.37±0.02	0.05±0.01	0.06±0.01
Histidine	0.90±0.03	0.82±0.02	8.14±0.08	6.76±0.06	2.37±0.02
Tryptophan	2.05±0.05	4.62±0.06	6.70±0.07	5.15±0.06	2.76±0.04
Hydroxylysine	N.D	N.D	0.19±0.01	1.13±0.03	0.25±0.01
Ornithine	0.53±0.02	0.08±0.01	4.33±0.05	13.36±0.12	9.74±0.09
Lysine	0.23±0.01	5.34±0.07	10.84±0.11	21.26±0.20	14.17±0.12
Ethanolamine	ND	ND	ND	2.70±0.04	4.81±0.06
Arginine	2.05±0.07	0.69±0.03	2.25±0.04	ND	0.73±0.02

ND not detected

^a Data are the mean value of three replicates ± SD

yield increased with the increase in temperature in the vessel (high pressure reactor). The highest hydrolysis yield in squid muscle was 99.18 ± 0.60 % at 280 °C. Similar results were reported by Watchararujij et al. 2008; Park et al. 2012 for subcritical water hydrolysis of rice bran and *Laminaria japonica*. Generally, protein normally has low solubility in water at ambient temperature due to strong aggregation through hydrophobic interactions. However, the solubility of new materials such as protein in water increases at higher temperature. In addition, at high temperature the hydrolysis yield increased due to the increase rate of hydrolysis caused by the raise in water ionization constant.

Protein yield in hydrolyzate Hydrolyzed squid muscle protein may be used in the food industry for protein enrichment and modification of functional properties. The amount of protein content in squid muscle hydrolyzates at different temperatures and pressure are shown in Table 2. The highest protein yield in thermal dried squid muscle hydrolyzate was 12.66 ± 0.34 mg/mL at 160 °C. After increasing the temperature, the protein yield was decreased. Similar pattern was observed in scallop waste, soybean and freeze dried squid viscera (Tavakoli and Yoshida 2006; Watchararujij et al. 2008; Uddin et al. 2010). This may suggest that protein molecules were decomposed to water soluble low molecular weight organic compound at elevated temperature.

SDS-PAGE Results of SDS-PAGE for different hydrolyzates are shown in Fig. 3. A large number of different subunit and polypeptides with masses in range from 6.25 to 97.4 KDa

were showed by SDS-PAGE in different hydrolyzates but hydrolyzates at 280 °C was not shown any peptide band. It can be due to high temperature may produce very little peptide (below 3.5 KDa) or decomposes peptide to other organic compounds. Among the all hydrolyzate samples, hydrolyzates at 220 °C was found more low MW peptide band and after increasing the temperature peptide band was decreased. Similar observations have been reported in scallop viscera waste (Tavakoli and Yoshida 2006).

Reducing sugar yield Carbohydrates are the most abundant class of organic compounds found in living organisms when it reacts with hydronium and hydroxide ions in aqueous solution produces reducing sugars. This sugar can be used in the food industries as food additives. The reducing sugar content in thermal dried squid muscle hydrolyzates are shown in Fig. 4. The highest amount of reducing sugar was found 2.30 ± 0.05 mg/mL at 220 °C. The amount of reducing sugar in thermal dried squid muscle was decreased after increasing the temperature at 220 °C. Ali Nehari et al. (2011) reported that reducing sugar was increased up to the temperature at 280 °C which is higher than squid muscle. This means that reducing sugar from squid muscle is not stable at high temperature.

Total and essential amino acid yield Amino acids and proteins are the building blocks of life. When proteins are digested or broken down, amino acids are left (Trumbo et al. 2002). The tastes of amino acids are sweet and bitter. Nevertheless, they contribute to the flavor of foods. Amino

Table 4 Individual structural amino acids yield from thermal dried squid muscle hydrolyzates at different temperatures

^a Amino acid compositions	Squid muscle hydrolyzate (mg/100 g)				
	160 °C	190 °C	220 °C	250 °C	280 °C
Aspartic acid	3.83 ± 0.06	9.34 ± 0.10	6.12 ± 0.08	8.08 ± 0.09	0.58 ± 0.03
Threonine	0.50 ± 0.02	1.11 ± 0.03	2.40 ± 0.04	0.17 ± 0.02	0.28 ± 0.02
Glutamic acid	4.17 ± 0.07	1.09 ± 0.02	0.40 ± 0.02	2.95 ± 0.05	2.70 ± 0.06
Proline	22.79 ± 0.21	15.84 ± 0.14	10.26 ± 0.12	37.81 ± 0.32	17.38 ± 0.15
Glycine	15.09 ± 0.16	10.39 ± 0.10	43.22 ± 0.32	73.69 ± 0.52	35.65 ± 0.28
Alanine	15.13 ± 0.14	28.26 ± 0.22	54.02 ± 0.44	78.25 ± 0.54	45.24 ± 0.38
Cystine	2.18 ± 0.04	2.52 ± 0.05	4.70 ± 0.06	13.76 ± 0.12	6.27 ± 0.07
Valine	11.46 ± 0.12	4.05 ± 0.06	45.42 ± 0.38	31.54 ± 0.28	15.37 ± 0.14
Methionine	2.28 ± 0.05	1.07 ± 0.03	7.81 ± 0.08	5.06 ± 0.07	5.94 ± 0.09
Isoleucine	12.37 ± 0.11	3.48 ± 0.04	28.01 ± 0.22	7.65 ± 0.9	6.55 ± 0.07
Leucine	24.42 ± 0.21	47.88 ± 0.42	56.65 ± 0.42	42.64 ± 0.33	21.79 ± 0.17
Tyrosine	3.52 ± 0.07	2.15 ± 0.06	25.71 ± 0.14	12.89 ± 0.12	6.81 ± 0.08
Phenylalanine	16.72 ± 0.14	26.21 ± 0.20	29.74 ± 0.18	25.23 ± 0.21	13.49 ± 0.11
Histidine	8.40 ± 0.09	11.95 ± 0.12	14.10 ± 0.11	18.29 ± 0.17	9.89 ± 0.10
Lysine	0.72 ± 0.02	0.29 ± 0.02	11.75 ± 0.10	22.59 ± 0.20	17.20 ± 0.15

^a Data are the mean value of three replicates $\pm$ SD

acids and protein hydrolyzates are therefore useful additives in food industries (Rogalinski et al. 2005). The total and essential (free and structural) amino acid yields in thermal dried squid muscle hydrolyzate are shown in Fig. 5. It was found that within total amino acids, free amino acid yield was higher than structural amino acids in hydrolyzates. However, within essential amino acids it was shown opposite direction. The highest yields of free and structural amino acids in the hydrolyzates were 421.53 ± 1.24 and 380.58 ± 2.25 mg/100 g at 250 °C, respectively for total amino acids and 188.83 ± 0.84 and 195.86 ± 0.80 mg/100 g at 220 °C, respectively for essential amino acids. Amino acid yield was decreased after increasing the temperature at 250 °C. In addition, high temperature causes the decomposition of amino acids into organic acids or other products (Sato et al. 2004). Kang and Chun

(2004) reported that the significant decrease in the amino acid production from a hydrothermal process of fish wastes was due to the decomposition of amino acids into organic acids or volatile materials.

The yields of individual free and structural amino acids in hydrolyzates at different temperatures are shown in Tables 3 and 4. Among the recovered amino acids glycine was the most abundant and it was 68.49 ± 0.52 and 73.69 ± 0.52 mg/100 g of free and structural amino acids at 250 °C. All essential amino acids were found in thermal dried squid muscle hydrolyzates. Within the free and structural amino acids, the highest individual essential amino acids were leucine 54.23 ± 0.42 & 56.65 ± 0.42 mg/100 g, isoleucine 27.38 ± 0.19 & 28.01 ± 0.22 mg/100 g, valine 43.45 ± 0.28 & 45.42 ± 0.38 mg/100 g and phenylalanine

Fig. 6 Antioxidant activities measured from thermal dried squid muscle hydrolyzates at different temperatures by (a) DPPH radical scavenging assay, (b) ABTS radical scavenging assay and (c) Reducing power assay. Data are the mean value of three replicates $\pm$ SD

27.33±0.20 & 29.74±0.18 mg/100 g at 220 °C, respectively. One of the best known essential amino acid is tryptophan, which performs several critical functions for people. It helps induce normal sleep, reduce anxiety, depression, and artery spasm risk; and helps produce a stronger immune system. Tryptophan in the thermal dried squid muscle hydrolyzates was found and it was 6.70±0.07 mg/100 g of free amino acids. After increasing the temperature at 250 °C, the individual amino acids were decreased. Cheng et al. (2008); Uddin et al. (2010) reported that most of amino acids gave maximum yield at the reaction temperature range of 180 to 220 °C and 200 to 290 °C. It may be differ due to the different sample sources, processing and experimental operational conditions. Some other works have been carried out in which the thermal degradation of amino acids occur at temperature above 250 to 300 °C, depending on the raw protein and corresponding contact time (Daimon et al. 2001; Quitain et al. 2001; Yoshida et al. 2003).

Antioxidant activity

DPPH free radical scavenging effect DPPH has been widely used for free radical-scavenging assessments due to its ease and convenience. DPPH is a stable free radical with an absorbance maximum at 517 nm in methanol. In the present study, all hydrolyzates were found to be effective scavengers against DPPH radical (Fig. 6a). The maximum scavenging effect was found 50.96±2.10 % at 220 °C. After increasing this temperature scavenging effect was decreased. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged and the absorbance is reduced (Shimada et al. 1992). The result reveals that the thermal dried squid muscle hydrolysates potentially contained substances which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

ABTS free radical scavenging effect ABTS is a good method to evaluate liophilic as well as hydrophilic antioxidants, it seems logical that this method gave higher values, registering the activity of both types of antioxidant present in the hydrolyzates. The scavenging capacities of the different extracted hydrolyzates for the ABTS radical were measured and compared (Fig. 6b). As can be seen, the scavenging effect of all hydrolyzate was almost similar. Among them the hydrolyzate at 250 °C is little higher and the scavenging effect was up to 99.42±1.20 %. During hydrolysis at high temperature and pressure, a wide variety of smaller peptides and free amino acids is generated. Changes in size, level and composition of free amino acids and small peptides affect the antioxidative activity (Wu et al. 2003).

Reducing power Reducing power is one symbol of antioxidant ability and may serve as a significant indicator of potential antioxidant activity. Several studies have indicated that the antioxidant effect is related to the development of reductones (Yen and Duh 1993). In the present study, all hydrolyzate samples showed higher reducing power (Fig. 6c). Hydrolyzate at 220 °C, reducing power was very high than other hydrolyzates. After increasing the temperature at 220 °C, reducing power was decreased. From this result, it appears that hydrolyzate from thermal dried squid muscle could function by donating electrons to the free radicals and high temperature may cause decomposes peptide to other organic compounds.

Thermal stability of hydrolyzates For hydrolyzate production used high temperature in short reaction time (3 min) and then showed high antioxidant activities in all hydrolyzates. Therefore, thermal stability of hydrolyzates was monitored by ABTS radical scavenging activities is shown in Fig. 7. ABTS radical scavenging activities of hydrolyzates were quite stable when heated at 100 °C up to 150 min, where activities of more than 98±0.26 % were retained. A small loss in ABTS radical scavenging activity might be due to aggregation of some antioxidant peptide, caused by long time heat treatment. In general, proteins were vulnerable to heat treatment, leading to the aggregation of protein and the exposure of hydrophobic domain (Sikorski and Naczki, 1981). Peptides derived from many protein sources with increased hydrophobicity have been reported to correlate with antioxidant properties (Faithong et al. 2010). The result indicated that peptides with low MW were most likely stable following heat treatment. Therefore, the antioxidant peptides from hydrolyzates can be incorporated in cooked food systems without significant loss of their antioxidant activities.

Fig. 7 Thermal stabilities of thermal dried squid muscle hydrolyzates at different temperatures measured using different time. Data are the mean value of three replicates ± SD

Conclusion

The result presented in this study demonstrated that subcritical water hydrolysis technique has great potential for practical application because it was not only energy saving, environmentally friendly and cost effective but also produced many functional materials, especially amino acids and reducing sugar with zero emission from thermal dried squid muscle. At 250 °C, the maximum yield of free and structural amino acid (421.53 ± 1.24 and 380.58 ± 2.25 mg/100 g) was obtained, while maximum essential amino acids were found at the temperature of 220 °C. Among the essential amino acids leucine was abundant (54.23 ± 0.42 and 56.65 ± 0.42 mg/100 g of free and structural amino acid). Reducing sugar produced by this method could be considered as valuable materials because it may be used as an energy source and food additives. The material balance based on the analytical results was estimated to be at least 99.18 ± 0.60 % of conversion from thermal dried squid muscle at 280 °C. Antioxidant activity was found in all hydrolyzates and it was varied with different temperatures. Therefore, thermal dried squid muscle hydrolysate can be used in food systems as a natural additive possessing antioxidative properties.

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