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Assessment of antioxidant properties of membrane ultrafiltration peptides from mungbean meal protein hydrolysates

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Background Bioactive peptides can prevent damage associated with oxidative stress in the human body when consumed regularly. Recently year, peptides have attracted immense interest because of their beneficial functional properties, safety and little or no side effects when used at high concentration. Most antioxidant peptide has small size less than 1 kDa and contains high proportion of hydrophobic amino acid. Particularly Tyr, Leu, Ala, Ile, Val, Lys, Phe, Cys, Met and His exhibited high antioxidant activity. Mungbean protein contain high abundance of protein and hydrophobic amino acid contents, investigating its bioactivity is an important aspect of adding value to this by-product obtained from a growing industry. Therefore, the objectives of this study were to optimize the conditions used to generate MMPH with antioxidant activity form bromelain and to investigate the antioxidant activities of each molecular weight peptide fraction.

Methods Response Surface Methodology (RSM) was used for screening the optimal conditions to produce Mungbean meal protein hydrolysate (MMPH). After that optimal MMPH was fractionated using ultrafiltration membranes with different molecular weight (MW) distribution. Crude-MMPH and four peptide fractions were investigated for five antioxidant activities: DPPH scavenging activity, Hydroxyl scavenging activity, Superoxide scavenging activity, Ferric reducing antioxidant power and metal ion chelation activity.

Results The optimal condition of crude-MMPH production was 12 % (w/w) of bromelain and hydrolysis time for 12 h. The EC_{50} of DPPH was the highest for the F4 peptide fraction (MW<1 kDa) at 0.5320 mg/mL. Metal ion chelating activity was generally weak, except for the F4 that had a value of 43.94% at a protein concentration of 5 mg/mL. The F4 also exhibited high hydroxyl and superoxide radical scavenging activities (54 and 65.1%), but poor activity for ferric reducing antioxidant power (0.102 mM Fe^{2+}/mg protein) compared to other peptide fractions and crude-MMPH. Molecular weight and amino acid were the main factors that determined the antioxidant activities of these peptide fractions. Results show that F4 have high antioxidant potentials.

Discussion The lowest MW Fraction (less than 1 kDa) showed the highest DPPH activity, superoxide-, hydroxyl-scavenging activity and metal chelation activity. On the other hand, this fraction had poor ferric reducing power. This showed that low molecular weight has an important effect on antioxidant activities. According to the mechanism of the reaction, the potential of antioxidant activity was divided into two main groups: hydrogen atom transfer (HAT) and single electron transfer (SET). Therefore, this finding suggests that the antioxidant mechanism of peptides obtained mungbean could react with many species of free radicals by multiple mechanisms. Mungbean meal peptide can be developed into multiple functional foods which possess both antioxidant properties and aroma/taste.

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ABSTRACT

Background

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Introduction

Bioactive peptides are usually obtained by enzymatic hydrolysis of food proteins. Normally, peptides can not exhibit bioactive function within their parent protein chain but these functions can be activated after cleavage by endo/exo – proteases (Chi et al., 2014). Apart from enzymatic hydrolysis, these peptides can also be cleaved from parent proteins by microbial enzymes and during food processing (Pownall, Udenigwe & Aluko, 2010). Enzymatic hydrolysis is a suitable way to produce bioactive peptides without losing nutritional value (He et al., 2013). Additionally, the use of enzymatic hydrolysis of protein can help to control the end products, thereby producing desirable target peptides (Adler-Nissen, 1986). It is an inexpensive method for cleaving proteins to peptides and free amino acids and, the solubility of the peptide products is usually higher than that of the parent protein with their amino acid profile can remain essentially unchanged or probably enhanced in some fractions (Cumby et al., 2008).

An antioxidant is a substance that counteracts the oxidation reaction caused by free radicals (Lobo et al., 2010). The most widely used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) but the use of synthetic antioxidants is under strict regulation because they are toxic to human health (Ng, Tan & Khor, 2017). In recent years, natural antioxidants have attracted immense interest because of their beneficial functional properties and safety. Natural antioxidants have little or no side effects when used at high concentration (Pownall, Udenigwe & Aluko, 2010; Li et al., 2008). Bioactive peptides are used in the formulation of functional food that can prevent damage associated with oxidative stress in the human body when consumed regularly.

Recently, many studies are interested in generating bioactive peptides from plant protein sources. Some factors that influence the bioactivity of peptide are size, amino acid composition and sequence (Arise et al., 2016). Generally, bioactive peptides have two to twenty amino acid residues per chain and often contain high proportion of hydrophobic amino acid. Almost all bioactive peptide that contain Tyr, Leu, Ala, Ile, Val, Lys, Phe, Cys, Met and His exhibited high antioxidant activity (Wattanasiritham et al., 2016). However, the antioxidant properties of hydrolysates depend on the protease specificity, the degree of hydrolysis (DH), and the nature of the released peptides (molecular weight (MW) and amino acid composition) (Arise et al. 2016).

Mungbean meal is a by-product of vermicelli or green bean noodle industry and has a protein content of 70% (w/w). Owing to its high protein and hydrophobic amino acid contents and abundance, investigating its bioactivity is an important aspect of adding value to this by-product obtained from a growing industry. Bioactive peptides from mungbean protein were hydrolysed using Alcalase, Neutrase and Virgibacillus sp. The peptides derived from these enzymes, possessed antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities (Lapsongphon & Yongsawatdigul, 2013; Li et al., 2006). Previous studies have shown that mungbean hydrolysed using bromelain could generate flavour peptides that have umami flavour characteristics and also antioxidant properties (Sonklin, Laohakunjit & Kerdchoechuen, 2011; Sonklin et al., 2018). While the antioxidant properties from mungbean protein hydrolysate have been reported, the capability of the peptide fractions to retain these activities lacks of information. Therefore, the objectives of this study were to optimize the conditions

used to generate MMPH with antioxidant activity form bromelain and to investigate the antioxidant activities of each molecular weight peptide fraction.

Materials and Methods

Materials

Mungbean meal and bromelain was provided from Sittinun Co., Ltd., Thailand and K-Much-Industry Co., Ltd. (Bangkok, Thailand). Mungbean meal was defatted using hexane based on a previously described method (Sonklin, Laohakunjit & Kerdchoechuen, 2011). All chemical reagents were of analytical grade. 2,2-diphenyl-1-picrylhydrazyl (DPPH), glutathione reduced (GSH), 1-10-Phenanthroline, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate (ferrozine) and pyrogallol were purchased from Sigma Aldrich Missouri, USA. All other reagents were purchased from Fisher Scientific except stated otherwise.

Screening conditions to produce enzymatic MMPH using Response Surface Methodology (RSM)

Response Surface Methodology (RSM) was used for screening the optimal conditions used to produce mungbean protein hydrolysate according to the method described by Sonklin et al. (2018). The optimal condition was selected using enzyme content and hydrolysis time which contributed to the highest degree of hydrolysis, DPPH and ABTS scavenging activities. Defatted mungbean meal 10 g was mixed in 100 mL of distilled water, and acidified to pH 6.0 using 2 M HCl. Bromelain was added to samples at 5, 10, 15 and 20% (w/w). All samples were hydrolysed

for 6, 12, 18 and 24 h respectively. After complete reaction at each hydrolysis time, enzyme reaction was terminated by heating at 95 °C for 15 min, cooled in a water bath, filtered, freeze dried and analysed for DH (Flavia & Maria, 1998), DPPH and ABTS (Sonklin et al., 2018). For the RSM step, enzyme concentration (x_1) and hydrolysis time (x_2) were independent variables. Three responses (y) were analysed DH, DPPH and ABTS. Each reponse can be described by the equation below (Khunri & Cornell, 1987).

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2$$

Where: b_0 was an offset term.

b_1 and b_2 linear effects and

b_{12} interaction effects.

The experiment plan was designed and statistically analysed for the effects of the independent variables using the SPSS 22.0 program. Statistica program 5.0 (StatSoft Inc., 1995, USA) was used to build the surface plots of each variables.

Preparation of peptide fractions with different molecular weight distributions

MMPH (crude-MMPH) from the optimized hydrolysis condition was selected for the ultrafiltration step. The crude-MMPH was fractionated through a series of ultrafiltration membranes with molecular weight cut-off (MWCO) of 10, 5 and 1 kDa (Millipore, Germany). This process yielded four fractions: F1 (MW>10 kDa), F2 (MW 5-10 kDa), F3 (MW 1-5 kDa) and F4 (MW <1 kDa). The fractions were freeze-dried and stored at -20 °C until used.

Determination of the amino acid compositions

Total and free amino acid of crude-MMPH and free amino acid of peptide fractions (F1, F2, F3, and F4) were analysed according to the method described by Li et al. (2008). Pre-treatment of total amino acid analysis, sample was carried out by hydrolysing samples using 6 M HCl at 110 °C for 22 h, samples were vigorously shaken and filtered. The acid in the permeate dried using a desiccator and re-dissolved using 1 mL of 0.02 HCl. The dried samples were kept at 4 °C until it was injected into the HPLC. For the pre-treatment of free amino acid analysis, trichloroacetic acid (TCA) was added to samples at ratio 1:1 and incubated for 2h. After incubation, samples were filtered and centrifuged at 6000 g for 10 min. Supernatant was collected and stored at 4 °C before injection. After pre-treatment step, each samples were injected in to reversed-phase, high performance liquid chromatography (RP-HPLC) (Agilent Technologies, Agilent 1100, USA). The following conditions were used for analysis. O-phthaldialdehyde (OPA) was added into column (Zorbax 80 A C18 column (4.6 i.d. × 180 mm, Agilent Technologies, Palo Alto, CA, USA)) for pre-column derivatization. Samples (1 µL) were injected into the column using two mobile phases. Mobile phase A was 7.35 mM of 7.2. sodium acetate : triethylamine : tetrahydrofuran at ratio 500 : 0.12 : 2.5 (v/v/v) and acidified to pH 7.2 with acetic acid. Mobile phase B was 7.35 mM sodium acetate : methanol : acetonitrile with ration 1:2:2 (v/v/v) and adjusted to pH 7.2. The system was operated at 40 °C and peaks detected at two wave-lengths 338 and 262 nm. Eighteen amino acids (Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val and Trp) were used as external standards.

Antioxidant properties of peptide fractions

DPPH scavenging activity

166 The scavenging activity of peptide samples against DPPH radical was determined using the
167 described method of Girgih et al. (2011). The EC_{50} (peptide fractions that inhibited 50 % of
168 DPPH activity) of the samples were determined. The EC_{50} was calculated using a non-linear
169 regression from a plot of percentage DPPH activity versus peptide concentrations (0.2, 0.4, 0.6,
170 0.8 and 1.0 mg/mL).
171
172 **Hydroxyl radical scavenging activity**
173 The hydroxyl radical scavenging assay was modified using the method described by Ajibola et
174 al. (2011). Samples and standard (GSH) were dissolved in 0.1 M sodium phosphate buffer (pH
175 7.4) to a final concentration of 2 mg/mL. The reactions were carried out in a microplate well
176 plate. Fifty microliters of samples or buffer (Blank) was mixed with 50 µL of 3 mM 1, 10-
177 phenanthroline in 0.1 M sodium phosphate buffer (pH 7.4) and 50 µL of 3 mM $FeSO_4$. To initiate
178 the Fenton reaction, 50 µL of 0.01% hydrogen peroxide (H_2O_2) was added and absorbance read
179 at 536 nm every 10 min for 1 h while incubating at 37 °C with continuous shaking. The
180 percentage hydroxyl radical scavenging activity was calculated using the following equation:

181 $$\text{Hydroxyl radical scavenging activity } (\%) = \frac{(\Delta\text{Amin}^{-1}(\text{blank}) - \Delta\text{Amin}^{-1}(\text{sample})}{\Delta\text{Amin}^{-1}(\text{blank})} \times 100$$

182 Where ΔA is the change in absorbance.
183
184 **Superoxide radical scavenging activity**
185 A range of sample concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL final) were prepared by
186 dissolving them in 50 mM Tris–HCl buffer, pH 8.3 containing 1 mM EDTA. Samples (80 µL) were

pipetted into clear microplate well, buffer was used for the blank well. The reaction was measured immediately using a spectrophotometer at 420 nm after adding 40 µL of 1.5 mM pyrogallol in 10 mM HCL for 4 min at intervals of 1 min (Xie et al., 2008). The superoxide scavenging activity was calculated using the following equation:

$$\text{superoxide scavenging activity } (\%) = \frac{(\Delta\text{Amin}^{-1}(\text{blank}) - \Delta\text{Amin}^{-1}(\text{sample})}{\Delta\text{Amin}^{-1}(\text{blank})} \times 100$$

Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed according to the method of Benzie and Strain (1996) as described by Karamać and co-worker (2016). The FRAP reagent was prepared by mixing 0.3 M acetate buffer, 10 mM TPTZ at pH 3.6 in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ pH 3.6 at ratio of 5:1:1 (v/v/v). Deionized water was used to hydrate samples (2mg/mL). The reaction was carried out in a 96-well microplate. Sample and FRAP reagent were incubated at 37 °C before adding 40 µL of samples to 200 µL of the FRAP reagent and absorbance was measured at 593 nm. A standard curve was generated using $FeSO_4.7H_2O$ (0.03 – 0.9 µmol/mL) and the absorbance's of the samples reported as mmol of Fe^{2+} reduced per g of peptide fractions from the regression slope of the standard curve.

Metal ion chelation activity

The metal ion chelating activity of peptide fractions was measured using the method described by Xie et al. (2008). Samples were prepared to final concentrations of 0.5, 1.0, 3.0 and 5.0 mg/mL using DDQ, which served as the blank. Samples were mixed with 0.05 mL of 2 mM

$FeCl_2$, 1.85 mL of Milli-Q water, 0.1 mL of 5 mM of ferrozine solution in a reaction tube for 10 min at room temperature. Subsequently, 20 µL of mixed solution were pipetted into 96 well microplates and absorbance measured at 562 nm. The percentage chelating activity was calculated using the following equation:

$$\text{Metal chelating activity } (\%) = \frac{(A_b - A_s)}{A_b} \times 100$$

Where A_b is the absorbance values of blank

A_s is the absorbance values of sample

Statistical analysis

All assays were determined in triplicate and means (n-3) of data analysed using the analysis of variance (ANOVA), Duncan's multiple range test (DMRT) of variables was used to separate the means and significant differences was accepted at ($p \leq 0.05$) using the SAS program Version 6.0 (SAS Institute, 1997, USA).

Results

Screening of optimum conditions to produce MMPH by bromelain using RSM

The optimum bromelain treatment conditions used to produce MMPH was determined using the RSM. The effect of different enzyme treatment conditions on DH, DPPH and ABTS are reported using a quadratic equation. Enzyme concentration (x_1) and hydrolysis time (x_2) a statistically significant interaction ($p \leq 0.05$) on the DH, DPPH and ABTS value are explained in the equations for each variable are shown below:

229

230 $$DH: y = 8.401 + 3.305X_1 + 2.318X_2 - 0.0986X_1^2 - 0.0654X_2^2 - 0.0256X_1X_2 \text{ ------ eq. 1}$$

231 $$DPPH: y = -2.967 + 8.468X_1 + 3.429X_2 - 0.254X_1^2 - 0.0951X_2^2 - 3.070\times10^2X_1X_2 \text{ ----- eq. 2}$$

232 $$ABTS: y = 0.238 + 9.255X_1 + 1.418X_2 - 0.310X_1^2 - 0.00296X_2^2 - 0.0007961X_1X_2 \text{ ---- eq. 3}$$

233

234 The equations showed that enzyme concentration (X_1) was the most important factors affecting
235 DH, DPPH and ABTS values than hydrolysis time (X_2) because an estimated regression
236 coefficient of X_1 presented the higher value. The determination coefficients (R^2) of the DH,
237 DPPH and ABTS were 0.818, 0.925 and 0.969, respectively. The equations were used to build
238 response surface graphs to predict the critical points and effectiveness of DH (Figure 1A), DPPH
239 (Figure 1B) and ABTS (Figure 1C). The DH, DPPH and ABTS increased by combined effects of
240 increasing enzyme concentration and hydrolysis time until the critical point, all of three
241 variables were constant and slightly decreased.
242 An optimum condition for the production of MMPH was selected from an overlay of Figure 1, 2
243 and 3 and choosing the range of enzyme concentration and hydrolysis time which gave the
244 maximum DH, DPPH and ABTS activity. Results showed that the optimal enzyme concentration
245 was 10-18 % and hydrolysis time 8-24 h. Therefore, 15% of bromelain and 12 h hydrolysis time
246 were chosen to produce MMPH. The models used were confirmed by observed and predicted
247 values as showed in Table 1. Results showed that the predicted values which were derived the
248 models were close to the observed value.

249

250 **Amino acid composition of peptide fractions**

The crude-MMPH was analysed for both total amino acids and free amino acids, but F1, F2, F3 and F4 were used to analyse only total amino acids. High content of Glu, Asp, Lys, Arg, Leu, Ser, Pro and Phe were found in the crude-MMPH (162.66, 104.39, 72.11, 67.00, 62.93, 54.27, 48.01 and 45.61 mg/g protein, respectively) (Table 2). The predominant amino acids were hydrophobic amino acids (HHA) group. Amino acids of crude-MMPH were in peptide forms. Consequently, F1-F4 fractions of crude-MMPH were analysed only for total amino acids because they are already in peptide form. Asp and Glu were the highest in all peptide fractions (Table 2). In particular, F4 had the highest content of Glu, Arg, Gly, Leu, Met, Tyr, Phe, Trp and Ser. All peptide fractions had high amounts of total HAA, and the contents were quite similar in each fraction. The proportion of aromatic amino acid contents (Tyr, Phe, Trp) in fraction F4 was also found to be the highest compared to the other peptide fractions.

DPPH radical scavenging activity

The activity of crude-MMPH and its membrane fractions to scavenge the DPPH radical is presented as the EC_{50} value (Figure 2). Crude-MMPH and its peptide fractions scavenged the radical to a 50% inhibition at a range of 0.4 – 0.9 mg/mL. All peptide fractions had low EC_{50} values of DPPH than the hydrolysate. EC_{50} value of fraction 4 showed the greatest EC_{50} as 0.53 mg/mL, followed by F1, F3 and F2 with EC_{50} as 0.63, 0.68 and 0.69, respectively. EC_{50} value of fraction 4 (0.53 mg/mL) was quite close to the EC_{50} value of standard which was glutathione (0.41 mg/mL).

Hydroxyl radical scavenging activity

Hydroxyl radicals scavenging activity of crude-MMPH and peptide fractions were analysed for triplicate in each samples. Crude-MMPH and its peptide fractions exhibited different hydroxyl scavenging activities, as shown in Figure 2. The <1 kDa (F4) fraction had superior ability when compared to crude-MMPH and all other fractions. F4 showed 54.50 % of hydroxyl scavenging activity, followed by F1, crude-MMPH, F2 and F3 which were 49.64, 39.91, 35.18 and 26.61, respectively. Glutathione which was a standard in this study showed the clearly high as 98.68 %.

Superoxide radical scavenging activity

Superoxide radicals scavenging activity assay was used to determine the antioxidant activity of crude-MMPH and peptide fractions. Figure 3A shows that the superoxide radical scavenging activity of crude-MMPH and its fractions increased distinctly with increasing concentration from 1.2 to 2.0 mg/mL. However, the activity was below 50% even at 2 mg/mL for all samples, except for F4 (<1 kDa). The superoxide radical scavenging activity at 2 mg/mL for F4 was 65.10% which is close to the activity observed for the standard (GSH = 63.06%) at 1.8 mg/mL.

Ferric reducing activity power (FRAP)

FRAP is related to the generated Fe^{2+} as shown in mM Fe^{2+} /mg protein. As depicted in Figure. 3B, FRAP of crude-MPH and its fraction increased significantly with increasing protein concentration from 0.2 - 1 mg/mL. Moreover, FRAP of peptide fractions in this study increased with increases in the molecular weight of peptides. F1 (>10 kDa), which had a HMW, had the greatest reducing power at every concentrations. F1 generated Fe^{2+} as 0.061, 0.107, 0.118,

294 0.231 and 0.242 mM Fe^{2+} /mg protein when used protein concentration at 0.2, 0.4, 0.6, 0.8 and
295 1.0 mg/mL protein, respectively.
296
297 **Metal chelation activity**
298 Metal chelation activity of crude-MMPH and it fractions had poor activity, even as at high
299 concentrations (5 mg/mL), their metal chelation activity value was less than 50% (Figure 3C).
300 Glutathione showed a low metal chelation activity (53.25 %) as well. The highest metal
301 chelation activity was observed for only F4, and the greatest activity was at 5 mg/mL at 43.94%.
302 The metal chelation activity of F3 and F4 at 5 mg/mL did not different from 4 mg/mL. Results
303 can identify that 5 mg/mL was the limited concentration of MMPH peptides for metal chelation
304 activity. The metal chelation activity of other peptide fractions was inconsistent, but the trend
305 was slightly increased with decreased molecular weights.
306
307 **Discussion**
308 The optimum condition for production of MMPH was investigated using response surface
309 graphs of DH, DPPH and ABTS which were built by statistically accurate equations. Because the
310 determination coefficients (R^2) of the DH, DPPH and ABTS equations were closed to 1.000 which
311 indicated that the regression models were appropriate and accurate. In addition, the predicted
312 values were close to the observed value (Table 1). At the end of DH, DPPH and ABTS response
313 surface graphs (Figure 1A-C), they showed constant and slightly decreased might be cause of an
314 over saturation of the protease enzyme. The over saturation of the protease enzyme occurred
315 when using higher enzyme concentration and hydrolysis time brought about decreasing in

316 hydrolysis which caused a decrease in its biological activity. The DPPH and ABTS value were also
317 decreased (Ovissipour et al., 2009).
318
319 The crude-MMPH which derived from the optimal condition from RSM was analysed amino acid
320 composition. Crude-MMPH contained highly hydrophobic amino acids (HAA) due to the
321 hydrolysis using bromelain which is an endoprotease which cleaves at hydrophobic position
322 inside the protein chain. Bromelain has specific cleavage preference at Arg, Lys and Phe sites,
323 resulting in the rich HAA observed in this study (Adler-Nissen, 1986; Sonklin, Laohakunjit &
324 Kerdchoechuen, 2011). F4 had the highest proportion of aromatic and hydrophobic amino acids
325 when compared to other peptide fractions and combined with F4 contained low molecular
326 weight peptide less than 1 kDa. Therefore, it can be assumed that F4 will exhibit the highest
327 antioxidant activity. Previous studies have shown that LMW peptides rich in aromatic and
328 hydrophobic amino acids have high antioxidant potentials (Ajibola et al., 2011).
329
330 In order to confirm the antioxidant abilities and to understand type of antioxidant mechanism
331 of peptides from mungbean meal protein hydrolysate, this study performed to analyse five
332 antioxidant activities. All peptide fractions had low EC_{50} values of DPPH than that of the
333 hydrolysate. This indicates that purified peptides exhibited stronger antioxidant activity based
334 on their molecular weight as the F4 (≤1 kDa) had a significantly lower EC_{50} value than other
335 peptide fractions and crude-MMPH. The low molecular weight (LMW) peptides scavenged
336 DPPH activity better than the high molecular weight (HMW) peptides. Peptide fractions from
337 canola meal protein, barley hordein and hemp protein hydrolysate have shown similar activities

and trends to those observed in this study (Alashi et al., 2014; Bamdad & Chen, 2013; Girgih et al., 2011). EC_{50} value of fraction 4 was quite close to the glutathione. It implied that the peptide fraction of MMPH that have molecular weight less than 1 kDa was an effective antioxidant peptides which could scavenge DPPH radical and prevent the free radical associated with oxidative stress. The reaction is based on a colour change from purple (DPPH) to clear yellow colour due to DPPH gaining the electron and becoming a stable radical molecule (Liu et al., 2010). Besides MW, the amino acid composition is an important factor that affects antioxidant properties. Peptide fraction F4, which exhibited the highest DPPH scavenging activity, had high contents of total HAA and AAA. Peptides that are composed of the amino acids in their chain can easily scavenge the DPPH radical. Moreover, the HAA and AAA group improved the solubility of peptide in non-polar environment (Kim et al., 2007).

Hydroxyl radicals are highly active when compared to other reactive oxygen radicals. The hydroxyl radical can react with almost all biomolecules in cells, affecting ageing and chronic diseases. Consequently, the defence of the hydroxyl radical is necessary for the protection of many metabolic disorders associated with the hydroxyl radical (Ajibola et al., 2011). The high activity observed for F4 may also be an influence of the LMW peptides, as reported in various studies (Cheung et al., 2012; Ajibola et al., 2011). If the hydroxyl radical scavenging activity was strictly based on molecular weight, F4 should show the highest activity followed by F3, F2 and F1, respectively. However, in this study, F1 had higher activity than F2 and F3. The reaction of hydroxyl radicals and MMPH peptides could be predicted based on information from Lee, Koo and Min (2004). The hydrophobic and aromatic amino acid in MMPH peptides may act on the

double bond in the hydroxyl radical molecule to become hydroxycyclohexadienyl radicals which are stable molecules, resulting in the termination of the reaction. Additionally, the results showed that hydroxyl radical scavenging activity of peptide fraction F2 and F3 were less than that of the crude-MMPH. This might be due to the potential synergistic effect of the crude-MMPH.

A lot of biological reactions can produce the superoxide radicals ($O_2^{\cdot-}$), which it is a harmful species. Superoxide radicals are very important precursor to generate various highly reactive radicals such as hydroxyl radical and can be the necessary radical for the initiation of lipid oxidation. Therefore, superoxide radicals are used to determine the antioxidant activity of hydrophilic and hydrophobic compounds (Kanatt , Chander & Sharma, 2007). Although superoxide is a similar radical oxygen species as hydroxyl radicals which can be generated by biological reactions, hydroxyl radicals are more potent reactive species than the superoxide radicals. This high tendency to react with biomolecules can lead to a situation where the ions are not readily available to accept protons from the peptides (Cacciuttolo et al., 1993). This is evident in the observed activity as superoxide radical scavenging activity slightly higher than those of the hydroxyl radical scavenging activity at 2 mg/mL (Cacciuttolo et al., 1993). However, results obviously indicated that MMPH is antioxidant peptide which good ability to prevent oxygen specie radical for cell damage protection.

FRAP assay is used to detect the ability of antioxidant substances to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The results are presented as Fe^{2+} reducing power which is the ability of the

peptides to be electron donors and to stop the chain reaction that leads to the continuous production of free radicals (Carrasco-Castilla et al., 2012). The FRAP of peptide fractions in this study increased with increases in the molecular weight of peptides, in contrast to results obtained for DPPH, hydroxyl and superoxide radical scavenging activity. This implies that the molecular weight might not be the most important contributing factor to the ferric ion reducing power. This might be due to the variation in the mechanism of reaction. FRAP reactions could indicate the ability of antioxidants to donate electrons, but DPPH, hydroxyl and superoxide radical scavenging activity could indicate proton donations. This finding suggested that MMPH and its fractions were antioxidant that high ability for proton donation than electron donation. This correlates with the amino acid composition. Asp (acidic), Lys (basic), Ile (hydrophobic), Pro (hydrophobic) and Cys (aromatic) were found to be less in the F4 fraction comparison to other peptide fractions. These amino acids can react with free radical by proton donation, to bring about a decrease in the FRAP for the F4 (<1 kDa) fraction, these results are consistent with previous work from Udenigwe and Aluko (2011). They reported that sulfur and hydrophobic amino acid can improve ferric reducing potential. In addition, Cys was lower in F4 had in comparison to the other peptide fractions. This might be one of the contributing factors affecting the reduction in the ferric reducing activity power observed for F4 as sulfur in the Cys structure has the ability to donate its sulfur hydrogen (Rajapakse et al., 2005). In addition, reports have also supported the influence of Met, Cys, Tyr, Met, Trp, His and Lys on FRAP (Carrasco-Castilla et al., 2012).

Chelation of metal ions is an assay used to measure antioxidant ability, which the reactive oxygen species (ROS) are catalysed by transition metal iron and copper derived from chelation of metal ions. ROS stimulate the oxidation reaction of unsaturated lipid and promotion of oxidative damage at different levels (Saiga, Tanabe & Nishimura, 2003). The result from this study and some previous studies suggested that metal ion chelation activity may not depend on molecular weight, but might be associated with small molecule and the coordination site which can act as an iron chelator (Kong & Xiong 2006). F4, which had the highest metal ion chelation activity, had a high proportion of Asp and Glu, and these amino acids were also high in the crude-MMPH and its fractions as well. However, Asp and Glu were both high so they probably were able to contribute extra electrons to improve electrostatic and ionic interaction between themselves and metal ion (Zhu et al., 2008). F4 showed the highest His which has an imidazole group known for its good chelating ability. Gly, Ala, Leu, Met, Try, Phe and Ser were also found to be higher in F4 compared to other fractions. These might have also contributed to its metal chelating activity.

Conclusion

MMPH and its ultrafiltration fractions had antioxidant activities with the potential to inhibit oxidation reactions. The LMW peptide fractions had antioxidant activities that were higher than the crude-MMPH, except for ferric reducing activity. Fraction 4, which has a molecular weight less than 1 kDa, showed the highest DPPH radical scavenging activity, superoxide scavenging activity, hydroxyl scavenging activity and metal chelation activity, on the other hand, this fraction had poor ferric reducing power. This showed that LMW has an important effect on

antioxidant activities. According to the mechanism of the reaction, this work determined the potential of antioxidant activity was divided into two main groups: hydrogen atom transfer (HAT) and single electron transfer (SET). Therefore, our findings suggested that the antioxidant mechanism of peptides obtained mungbean could react with many species of free radicals by multiple mechanisms. In addition, peptides from MMPH exhibited the chelation of transition metal activity as well. Amino acid compositions of these peptides were related to results from many assays of antioxidant activities which showed high amounts of hydrophobic amino acids and aromatic amino acids in all peptide fractions and their original whole hydrolysate (crude-MMPH). However, to determine the specific mechanism of antioxidant activity, the sequences will be further studied to understand the correlation between the peptide structure and specific antioxidant mechanisms. This study shows that mungbean protein can be developed as a value added commercial functional food ingredient, which it will increase its economic value

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559 Table lists

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564 Figure lists

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566 Figure 1: Response surfaces for the effects of enzyme concentration and hydrolysis time on (a)
567 the degree of hydrolysis (DH), (b) DPPH radical-scavenging activity (DPPH) and (c) ABTS radical-
568 scavenging activity (ABTS).

569

570 Figure 2: The effective concentration that scavenged 50% (EC_{50}) values for DPPH scavenging
571 activity values and the percentage of hydroxyl scavenging activity (2 mg/mL protein) of crude-
572 MMPH and its ultrafiltration peptide fraction.

573

574 Figure 3: Antioxidant activities of crude-MMPH and its ultrafiltration peptide fraction; (a)
575 superoxide scavenging activity, (b) ferric reducing antioxidant power (FRAP), and (c) metal
576 chelation activity.

Table 1(on next page)

Observed and Predicted Values

Observed and Predicted Values for optimizing the hydrolysis condition.

Table 1:

Trial	Conditions[a]	DH[b]		DPPH[b]		ABTS[b]	
		obs	pre	obs	pre	obs	pre
1	x_1 = 10%, x_2 = 18 h	48.9	47.5	79.1	81.6	81.2	77.8
2	x_1 = 15%, x_2 = 24 h	40.1	44.3	78.4	83.3	82.2	86.5
3	x_1 = 20%, x_2 = 24 h	43.1	40.8	79.0	77.6	78.0	78.7

obs = observed, pre = predicted, [a] x_1 represents enzyme concentration. x_2 represents hydrolysis time., [b] Mean ± SD (n = 3)

Table 2(on next page)

Amino acid composition

Amino acid composition of crude-MMPH and its ultrafiltration peptide fractions.

1 Table 2:

Group	Content (mg / g Protein)				Total amino acid (% w/w Protein)			
	Amino acid	Total amino acids	Free amino acids	amino acid in peptide form	F1	F2	F3	F4
Acidic	Asp	104.39±3.93	1.06±0.04	103.33±3.89	14.23	13.8	13.7	11.77
	Glu	162.66±2.04	16.92±0.51	145.70±2.55	18.30	17.9	18.2	18.72
Basic (+ charge)	Lys	72.11±6.12	28.94±0.20	43.17±5.93	8.15	8.32	8.00	6.89
	Arg	67.00±1.39	22.10±0.27	44.91±1.12	6.63	6.71	6.72	6.85
	His	20.01±0.76	11.58±0.59	8.81±0.16	3.55	3.07	2.75	3.02
Hydrophobic	Gly	36.78±2.52	5.49±0.86	31.29±3.38	3.19	3.78	3.73	3.98
	Ala	40.02±1.26	10.36±0.23	29.66±1.03	4.12	3.97	3.91	4.88
	Val	36.48±1.50	6.68±0.25	29.80±1.75	4.48	5.18	5.30	5.02
	ILe	36.86±1.84	3.63±0.17	33.23±2.01	4.72	4.64	4.67	3.56
	Leu	62.93±0.15	22.29±0.14	40.64±0.11	6.48	6.63	6.70	8.60
	Pro	48.01±1.09	3.57±0.10	44.44±0.99	4.97	5.59	6.17	3.62
	Met	8.90±1.39	4.60±0.11	4.30±1.50	1.00	1.13	1.09	1.60
	Cys	3.97±0.34	0.19±0.23	3.78±0.57	2.27	0.83	0.60	0.30
Aromatic	Tyr	29.03±2.72	5.24±1.15	23.79±1.58	3.73	3.37	3.44	3.81
	Phe	45.61±1.39	7.06±0.16	38.55±1.54	4.73	5.08	5.17	6.58
	Trp	6.05±0.31	1.18±0.17	4.87±0.48	0.70	0.74	0.78	0.83
Hydrophilic	Ser	54.27±2.38	13.70±0.88	40.57±3.25	5.48	5.76	5.67	6.50
	Thr	33.41±1.71	5.93±0.27	27.48±1.44	3.34	3.46	3.37	3.52
Total*		868.51	170.54	697.98	100	100	100	100

2

Figure 1(on next page)

Response surfaces for the effects of enzyme concentration and hydrolysis time

Response surfaces for the effects of enzyme concentration and hydrolysis time on (a) the degree of hydrolysis (DH), (b) DPPH radical-scavenging activity (DPPH) and (c) ABTS radical-scavenging activity (ABTS).

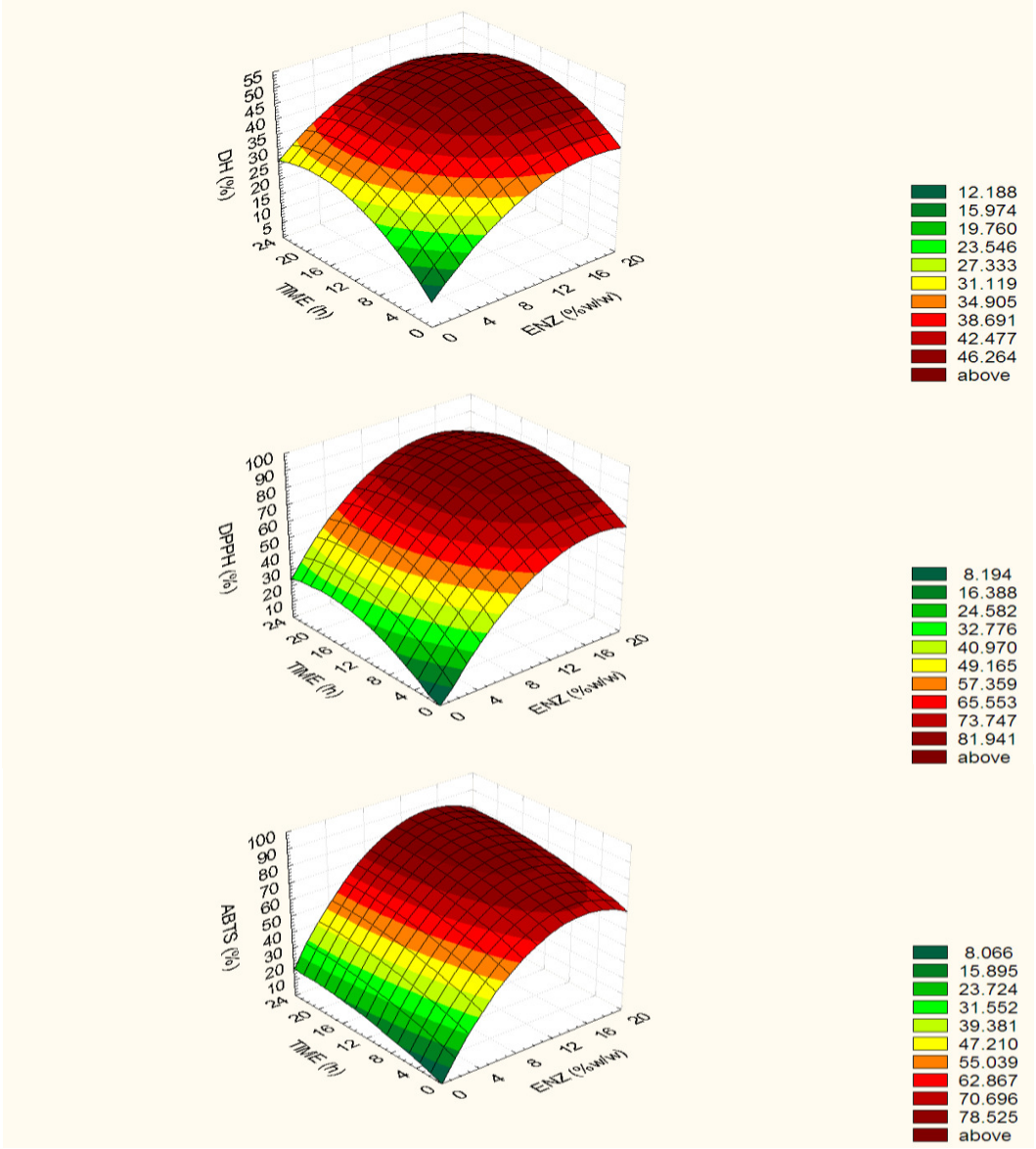

Figure 1:

Figure 2(on next page)

The effective concentration that scavenged 50% (EC_{50}) values

The effective concentration that scavenged 50% (EC_{50}) values for DPPH scavenging activity values and the percentage of hydroxyl scavenging activity (2 mg/mL protein) of crude-MMPH and its ultrafiltration peptide fraction.

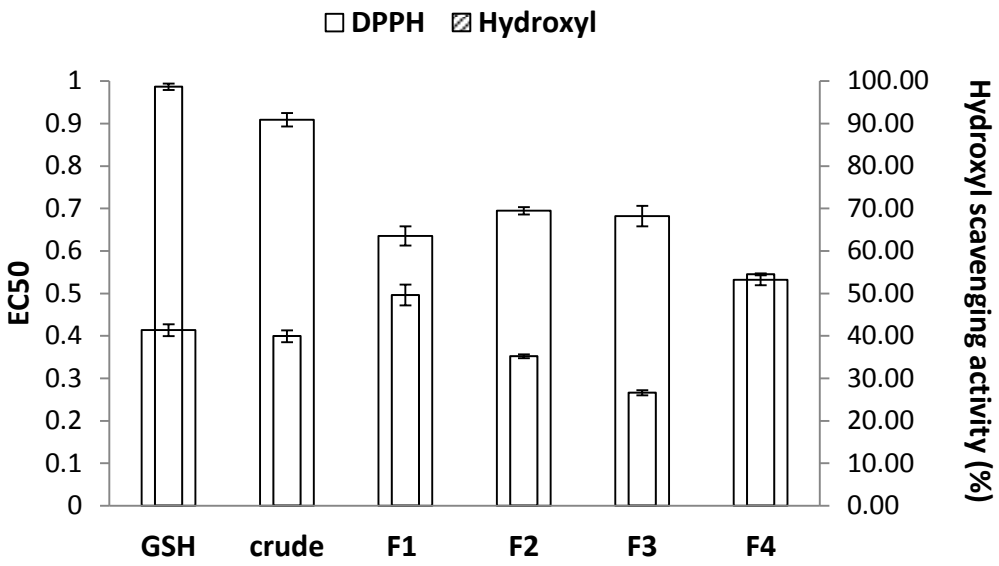

Figure 2:

Figure 3(on next page)

Antioxidant activities of crude-MMPH and its ultrafiltration peptide fraction

Antioxidant activities of crude-MMPH and its ultrafiltration peptide fraction; (a) superoxide scavenging activity, (b) ferric reducing antioxidant power (FRAP), and (c) metal chelation activity.

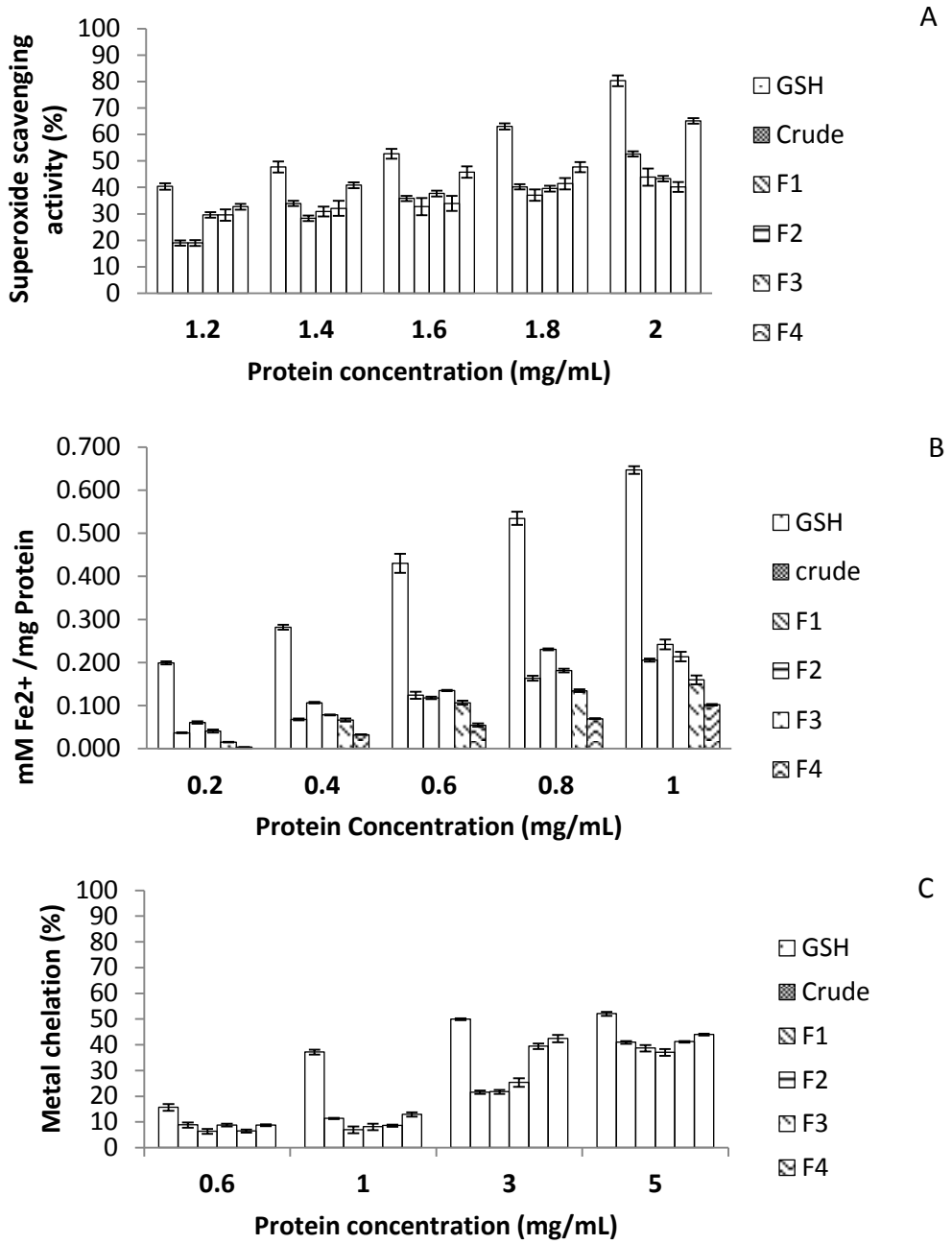

Figure 3: