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# Physicochemical properties of hydrolysates from enzymatic hydrolysis of pumpkin (*Cucurbita moschata*) protein meal

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**Abstract**

Pumpkin protein meal was hydrolysed by alcalase, flavourzyme, protamex and neutrase. Physicochemical characteristics were evaluated by previously published methods using pumpkin protein hydrolysates (PPHs) and pumpkin protein meal (PPM) as a control. Hydrolysis had significantly changed the physicochemical characteristics of protein meal and its functionality as well. Different enzymes had different specificity towards proteins which resulted into peptides of different molecular weight, size and sequence of amino acids that determined surface properties. Hydrolysates prepared by alcalase (HA) showed highest protein content (92.22%), best molecular weight distribution of peptides (98%) over the range of 180-5000Da, highest degree of hydrolysis (14.20%), yield (53.29%), solubility (93.40%) and emulsification capacity (61.1 $m^2/g$) at pH11, foaming capacity (64.39%) , fat absorption capacity (3.28mg/ml) and best gelation properties (2.00%). However, Hydrolysates prepared by flavourzyme (HF) showed highest emulsification (73.20%) and foaming stability (47.94%) after 120 minutes. Hydrolysates prepared by protamex (HP) showed the best water holding capacity (2.24 mg/mL), while hydrolysates prepared by neutrase (HN) had highest content of total amino acid (76.01%). The results suggest that pumpkin oil processing by-product can be converted into hydrolysates which are a good source of protein fortification for a variety of food products as well as a potential food ingredient.

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## Introduction

China is the world largest producing country of pumpkins (*Cucurbita moschata*). It produces over 6 millions of tonnes per annum, accounting for 27.44% of world production (*http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor*). Large quantity of pumpkin seeds is used for extraction of oil, leaving a large amount of residue as pumpkin seed meal, which contains 60-70% of high value protein (Vaštag *et al*., 2011). Unfortunately, this by-product remains largely underutilized as animal feed and fertilizers. However, the usage of plant proteins, especially from cereals and oilseeds has been growing intensively over the last decades. They have been used as the alternative for animal proteins in human nutrition, functional agents and bioactive components in food as well as in pharmaceutical products (Dagorn-Scaviner *et al*., 1987; Gujska and Khan, 1991; Ogunwolu *et al*., 2009; Boye *et al.*, 2010).

Pumpkin seed flours were used as protein supplements in a variety of local foods (Bekebain, 1992). Besides their wide use as food ingredients, they have pharmacological activities too, such as antidiabetic (Quanhong, 2003), antifungal (Wang, 2003), antibacterial and anti-inflammation activities (Caili, 2006). Moreover, research carried out in last few decades showed that they have antioxidant effects (Nkosi, 2006). Since most native proteins do not show functional properties desirable for food industries, their modification for improvement of these properties, especially solubility, need to be addressed (Moure, 2006).

The most important feature affecting the functional and organoleptic properties of a protein is its surface structure. Surface structure affects the interaction of a protein with water or other proteins. Thus, by modifying the structure of the protein, particular functional and organoleptic properties are obtained. Enzymatic hydrolysis is the most important protein structure modification process in the food industry, known to improve functional properties of dietary protein without affecting its nutritive value by converting it into peptides with desired size, charge and surface properties (Rafik Balti, 2010). Moreover, enzymatic hydrolysis is also reported to be helpful in removing antinutritional factors (Moure, 2006).

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The peptides produced by hydrolysis have smaller molecular masses and less secondary structure than intact proteins. This can lead to improved functionality such as increased solubility near the isoelectric point (Kong *et al*., 2007) increased heat stability (Molina Ortiz and Wagner, 2002), emulsification (Xiong *et al*., 2008) and increased whipping (Molina Ortiz and Wagner, 2002) ability which makes hydrolysates advantageous for use in many food products. Choosing the right proteolytic enzyme (Hammershøj *et al*., 2008; Zhao *et al.*, 2012), environmental conditions (Ovissipour *et al*., 2009) for hydrolysis and degree of hydrolysis (Klompong *et al.*, 2007; Wasswa *et al.*, 2007; Jamdar *et al*., 2010) is crucial for enhancing the physicochemical and functional properties of proteins as well as reducing bitterness of resulted peptides. Various enzymes such as alcalase, flavourzyme, protamex and neutrase have been used to produce hydrolysates of improved different characteristics (Damrongsakkul *et al*., 2008; You *et al*., 2009; Yust *et al*., 2010; Tsou *et al.*, 2010).

Past studies on pumpkin seeds hydrolysis have focused on hydrolysis process optimization using response surface methodology (Peričin *et al*., 2009; Vaštag *et al*., 2010), and investigation of bioactivity of hydrolysates (Vaštag *et al*., 2011). To the best of our knowledge, the pumpkin protein hydrolysates have not been the subject of study for surface properties elsewhere. In this work, the effect of enzymatic hydrolysis and enzyme type on the physicochemical characteristics of pumpkin protein hydrolysates were evaluated comparing to pumpkin protein meal as control, by considering the proximate composition, hydrolysis degree, surface hydrophobicity, thermal property, molecular weight distribution, and amino acid composition. For practical purposes, four commercially available and low cost proteases were selected to ensure the suitability of the outcomes for industrial food manufacture in order to maximize their utilization and to avoid waste disposal problems.

## Material and Methods

*Material, enzymes and reagents*

Pumpkin (*Cucurbita moschata*) protein meal (PPM) was kindly provided by Qinghai General Health Bio-Science Co., LLC. This meal had been obtained on a large scale from pumpkin seeds, through de-hulling, disintegrating and de-fatting. It was stored at 4°C, ground and passed through 60 mesh sieve. Food grade enzymes used were purchased in Novo Nordisk's Enzyme Business in Wuxi, China. These are alcalase endonuclease from *Bacillus subtilis* with specific activity of 2.4 AU/g, flavourzyme from *Aspergillus oryzae* with activity of 500 LAPU/g, neutrase from *Bacillus subtilis* strain with activity of 1.5 AU/g, protamex, a *Bacillus protease* complex with activity of 1.5 AU/g and they were stored at 4°C. 1-anilino-8-naphthalene sulphonate (ANS) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other solvents/chemicals used were of analytical grade and obtained from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China).

*Enzymatic hydrolysis*

Pumpkin protein meals were hydrolysed for five hours by commercially available enzymes: alcalase 2.4 L, flavourzyme 500 LAPU/g, protamex 1.5 AU/g and neutrase 0.8 L. Hydrolytic reaction was carried out in a 400 mL jacketed reactor at optimum hydrolysis conditions with magnetic stirring throughout the reaction. Substrate concentration was 4%, enzyme/substrate ratio (w/w) of 1%. The reaction temperature was set and kept at 58°C for alcalase, 50°C for flavourzyme, protamex and neutrase. The substrate dispersion was preheated at optimal temperature for 30 min before enzyme addition. The pH was adjusted by 1N NaOH or 1N HCl to 8.0 for alcalase, 7.0 for flavourzyme and neutrase and 6.5 for protamex. The enzymatic hydrolysis was stopped at selected periods of incubation time by heating the dispersion at 95°C in water bath for 10 min, cooled and centrifuged at 10,000 x g for 30 minutes at 4°C. The supernatant was used to measure the degree of hydrolysis (DH). The 5-hour hydrolysates supernatant obtained were freeze-dried and then preserved at -18°C for subsequent analysis.

*Degree of Hydrolysis measurement*

The DH was determined by formaldehyde titration method according to Song *et al*. (2013), as follows: Five milliliters of hydrolysates supernatant were diluted with 60 ml distilled water, while magnetically stirring, and titrated by 0.05M NaOH (standard titration solution) to pH 8.2 and volume recorded. Then, 10 ml formaldehyde (8-14%) were added into the beaker and continued for titration with 0.05 M NaOH to reach pH 9.2. And, the consumed volume of NaOH was also recorded. The value of DH was calculated according to the following equation:

$$DH = \frac{C \times (V1 - V2) \times V/5}{m \times \text{percentage of protein in raw material} \times 8} \times 100 \quad (1)$$

C—the concentration of standard titration solution of NaOH(0.05M)

V1—the consumed volume of 0.05 M NaOH titrating up to pH 9.2

V2—the consumed volume of 0.05 M NaOH titrating up to pH 8.2
V—the total volume of pumpkin protein hydrolysate
m—the mass of the raw material

*Proximate analysis and yield*

Determination of crude protein (using nitrogen conversion factor of 6.25), fat, ash, moisture content and total carbohydrates (by difference) of pumpkin protein meal and hydrolysates was estimated as per AOAC (1995). The protein and fat contents were expressed on a dry weight basis. The protein recovery (yield) was calculated as percentage of the ratio of weight of protein dispersed during hydrolysis to the weight of protein in the hydrolysates recovered after freeze drying.

*Estimation of molecular weight distribution*

Molecular weight (MW) distribution of hydrolysates was analysed by gel filtration chromatography under isocratic conditions using a Shodex Protein KW-802.5 column (8.0 mm × 300 mm) fitted with a Protein-Pak 125 Sentry Guard Column (Waters Pty, Sydney, Australia) on an HPLC system. Hydrolysates were dissolved in phosphate buffer saline (50 mM $Na_2HPO_4/NaH_2PO_4$ and 150 mM $Na_2SO_4$, pH 7.0) to prepare a 1 mg/mL solution and filtered through 0.22 μm syringe filter with 100 μL being injected into the column. Elution was at room temperature, 0.8 mL/min flow rate and peak absorbance was monitored at 214 nm. Each sample was run in triplicate. Bovine serum albumin (66.0 kDa), ovalbumin (44.3 kDa), β-lactoglobulin A (18.3 kDa), cytochrome C (12.4 kDa) and cytidine (0.243 kDa) were run as standards.

The percentage abundance (area under the peak) of the determined molecular weight was obtained from the HPLC software (Varian STAR chromatography workstation version 6.41, Varian Inc., Victoria, Australia).

$$\text{LogMW} = -0.2087T + 6.6753 \qquad R^2 = 0.9964 \qquad (2)$$

MW means molecular weight and T means elution time.

*Amino acid composition*

The amino acid composition of PPM and PPHs was determined according to the method of Yang *et al.* (2011) with a slight modification. Amino acid composition was determined by high performance liquid chromatography (Waters, Milford, MA) equipped with a PICO.TAG column. The total amino acid composition was determined after hydrolysis at 110°C for 24 h with 6 M HCl prior to the derivatization with phenyl isothiocyanate. Alkaline hydrolysis at 105°C for 24 h with 4M NaOH was also done for determination of tryptophan (Trp) level. External standards were used for quantification. The amino acid standards included l-alanine (Ala), l-arginine (Arg), l-aspartic acid (Asp), l-cystine (Cys), l-glutamic acid (Glu), l-glycine (Gly), l-histidine (His), l-isoleucine (Ile), l-leucine (Leu), l-lysine (Lys), l-methionine (Met), l-phenylalanine (Phe), l-proline (Pro), l-serine (Ser), l-threonine (Thr), l-tyrosine (Tyr), l-valine (Val), l-tryptophan (Try) and ammonium chloride.

*Water absorption capacity*

The water absorption capacity (WAC) of PPM and PPHs was measured following the method of Cumby *et al.* (2008) with a minor modification. One milligram PPM and PPHs was taken into a 10 mL centri-fuge tube and 1 ml water was added. The mixture was thoroughly vortexed for 10 min at 26°C and centrifuged at 1431 g for 20 min at room temperature. The water absorbed by the sample was determined from the difference in weights and expressed as grams of water absorbed per g of PPM and PPHs.

*Fat absorption capacity*

For the determination of fat absorption the method described by Tang (2007) was used. Samples (0.1 g) were mixed with 1mL of sunflower oil. After 10 minutes mixing, the mixture was centrifuged at 1600 ×g for 25 min and the weight of absorbed oil was recorded by difference in weights. The oil absorption capacity was expressed as the number of grams of oil retained by g of material at pH 7.

*Emulsifying properties*

Emulsifying properties were determined according to the method of Jamdar *et al.* (2010) with some minor modifications. Vegetable (sunflower) oil (2 mL) and 6 mL of 0.1% protein solution were mixed and the pH was adjusted to 2.5, 5.0, 7.5 and 10.0. The mixture was homogenized using a Fluko FA25 homogenizer (Shanghai, China) at a speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50 mL) was pipetted from the bottom of the container at 0 and 10 min after homogenization and mixed with 5 mL of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured at 500 nm using a spectrophotometer. The absorbance measured immediately ($A_0$) and 10 min ($A_{10}$) after emulsion formation were used to calculate the emulsifying activity index (EAI) and emulsion stability (ES) as follows:

$$\text{EAI}(m^2/g) = (2 \times 2.303 \times A_0 \times \text{Dilution})/C \times (1-\phi) \times 10^4 \qquad (3)$$

$$ES\ (min) = (A_o \times 10)/A_0\text{-}A_{10} \quad (4)$$

where $A_{10}$ and $A_0$ represent the absorbances at 500 nm after 10 min and time zero, respectively, at 100 times dilution, C represents the sample concentration (g/mL) before emulsification, and ϕ is the oil volume fraction (v/v) of the emulsion (ϕ = 0.25).

*Foaming properties*

Foaming capacity (FC) and foaming stability (FS) of PPM and PPHs were determined according to the method of Arogundade (2006). An aliquot (25 mL) of 0.3% sample solution (in 10 Mmol/L PBS pH 7) was homogenized at a speed of 16,000 rpm (Philip-HR1731 homogenizer), to incorporate air for 2 min at ambient temperature. The whipped sample was immediately transferred into a 25-mL cylinder and the total volume was read after 30 seconds. The FC was calculated according to the following equation:

$$FC(\%)=(A_0\text{-}B) \times 100/B \text{ by Naczk } \textit{et al}.,(1985). \quad (5)$$

Where $A_0$ is the volume after whipping (ml), B is the volume before whipping (mL).

The whipped sample was allowed to stand at 25°C for 10, 30, 60, 90 and 120 min and the volume of whipped sample was then recorded. Foam stability was calculated as follows:

$$FS\ (\%) = (A_t\text{-}B) \times 100/B \quad (6)$$

Where $A_t$ is the volume after standing (mL), and B is the volume before whipping (mL).

*Gelation properties*

LGC of PPM and PPHs was determined using the method described by Adebowale and Lawal (2003) with little modification. Samples were mixed with 5 mL of distilled water in a centrifuge tube to obtain 2-20% (w/v) concentrations. The centrifuge tube was heated for 1 h in a boiling water bath, cooled rapidly under running tap water and further cooled for 2 h in a refrigerator at 4°C. The least gelation concentration was regarded as the concentration at which the sample from the inverted tube did not fall or slip.

*Protein solubility*

Protein solubility was determined according to the method of Kim *et al.* (2004). PPM and PPH dispersions (1%, w/v) in deionised water were prepared and adjusted to pH 3, 5, 7, 9, and 11 with either 1M HCl or 1M NaOH. They were magnetically stirred for 30 min at room temperature (approximately 25°C) and centrifuged at 5000 rpm for 15 min. PPM and PPH dispersions (1%, w/v) in 0.2M NaOH were also prepared. After appropriate dilution, the protein contents in the supernatants and in the overall suspensions were then measured by Lowry's method Lowry (1951) using bovine serum albumin as the standard. Protein solubility was calculated as the percent distribution of protein in the supernatant over the total protein content in the dispersion.

*Differential scanning calorimetry (DSC)*

DSC of PPM and PPH were performed according to Molina Ortiz and Wagner (2002) with slight modification. Twenty percent dispersions of lyophilized samples in 0.01 M phosphate buffer pH 7.0 were hermetically sealed in aluminum pans. Samples were analysed at 5°C $min^{-1}$ in a range of 20–125°C using DSC Polymer Laboratories equipment (Rheometric Scientific, Weston Road), using an empty double pan as a reference. Transition temperatures and areas below the endothermic curves were measured to calculate the corresponding thermal denaturation enthalpies (ΔH in Joules per gram of dry weight) from the thermogram by the TA Instrument Universal Analysis 2000 data processing software (Arogundade *et al*., 2009). Rescan experiments were conducted to assess the extent of denaturation after the first heating cycle.

*Surface hydrophobicity ($H_o$)*

Surface hydrophobicity ($H_0$) of the protein hydrolysates was measured as described by Zhao *et al.* (2012) using 1-anilino-8-naphthalene sulphonate (ANS) as the hydrophobic fluorescence probes. The 8 mM solution of ANS in 10 mM phosphate buffer at pH 7.0 was prepared before measurements. Protein solutions (4 mL) with various concentrations from 0.005% to 0.025% (w/v) in 10 mM phosphate buffer at pH 7.0 were thoroughly mixed with 20 µl of freshly prepared ANS. The mixtures were shaken vigorously and stored for 10 min while stored in the dark. The fluorescence intensity (FI) of each sample was measured at 390 nm (excitation) and the emission within the range of 300–800 nm using an F-4500 model fluorometer (Hitachi Co., Tokyo, Japan).

The final FI value at each protein concentration was obtained by subtracting the FI of the blank from the FI of each sample in buffer. The initial slope of FI versus protein concentration (%, w/v) was calculated by a linear regression analysis and used as an index of $H_0$.

*Statistical analysis*

All the tests were conducted in triplicate. The results obtained were subjected to one-way analysis of variance. Duncan's new multiple range test was

performed to determine the significant difference between samples within the 95% confidence interval using SPSS 11.5 software (SPSS Inc., Chicago, Illinois, USA).

## Results and Discussion

*Enzymatic hydrolysis of pumpkin protein meal*

The hydrolysis of pumpkin protein meal was carried out by different commercial proteases; alcalase, flavourzyme, protamex and neutrase. Rapid hydrolysis was observed within the first 30 min. Thereafter, a slower hydrolysis rate was found up to 300 min (Figure 1(A)). Indeed for the first stage where the reaction rate is high, corresponds to an easy breakdown of peptide bonds. In the second stage the reaction rate is reduced due to hydrolysis of more compact peptide bonds. In addition to that, inhibition can occur due to lipid oxidation or presence of impurities other than protein, or increase in peptides which in turn act as effective substrate competitors due to undigested or partially digested proteins. During first stage alcalase and protamex showed a higher DH than flavourzyme and neutrase treated hydrolysates did.

However after 5-hour hydrolysis, alcalase and flavourzyme were most efficient with 13.9 and 11.5 respectively. This is due to the fact that alcalase is an endoprotease characterized by a very broad specificity in peptide cleavage. Flavourzyme is a fungus-origin enzyme containing a mixture of exopeptidases and endoproteases and has been used to prepare short chain peptides through extensive hydrolysis of food proteins (Pedroche *et al.*, 2002). The efficient hydrolysis observed for flavourzyme and alcalase demonstrates their high proteolytic activity towards pumpkin proteins as it was also found by Bamdad *et al.* (2011). The low degree of hydrolysis of protamex and neutrase hydrolysates can be attributed to their inability to hydrolyse the pumpkin protein peptide bond efficiently. Moreover, some partial enzyme inactivation may also have occurred. When comparing DH of all hydrolysates produced from pumpkin protein meal and full fat pumpkin flour, the hydrolysates from the latter had a higher DH (data not shown). The protein in defatted flour were most likely denatured. As a consequences, the protein substrates were less susceptible to hydrolysis by the added enzymes due to poor wettability, thereby reducing the dispersibility and hence accessibility of enzymes to the substrate (Hoyle, 1994; Balamurugan and Ignacimuthu, 2011). During the defatting process, endogenous proteinases in pumpkin seeds might undergo the denaturation.

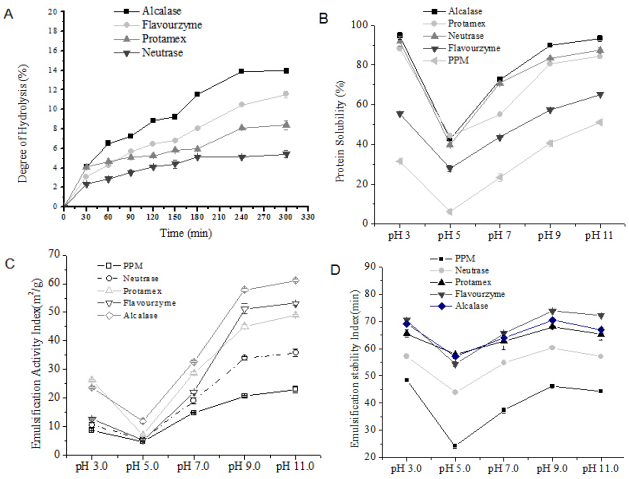


Figure 1. (A)Hydrolysis Degree (DH) at different time, (B) Protein solubility(PS),(C)Emulsification Activity Index (EAI) and (D) Emulsification Stability Index (ESI) of Pumpkin Protein Meal (PPM) and Pumpkin Protein Hydrolysates (PPHs) prepared by Alcalase, Flavourzyme, Protamex and Neutrase. PS, EAI, and ESI were measured at different pH (3, 5, 7, 9 and 11). Values are given as mean from triplicate determinations.

The high temperatures used in the defatting process might inactivate endogenous enzymes and hence reduce the rate of hydrolysis, thus leading to a lower DH in hydrolysate from defatted meal. Endogenous enzymes provide additional proteolytic effect to added enzymes during hydrolysis (Mackie, 1982). Low hydrolysis degree was also found during hydrolysis of cuttle fish (*sepia oficinalis*) by-products (Rafik Balti AB, 2010).

*Proximate composition and protein recovery*

Table 1 shows the proximate composition and protein yield of PPM and PPHs. Hydrolysates had higher protein but lower fat, ash and carbohydrates content. This is due to dissolution of protein during hydrolysis followed by centrifugation to separate insoluble and undigested matter. However, the hydrolysis had not efficiently reduced the fat content. The emulsion formed in conjunction with protein and peptides during the hydrolysis may account for the retention of fat in hydrolysates (Jamdar *et al.*, 2010). However, to avoid rancidity during long term storage of hydrolysates, complete removal of fat either before or after hydrolysis is needed. The yield is also an important issue as maximum protein hydrolysates recovery is desired. Alcalase treated hydrolysates have significantly shown higher protein recovery than others ($p < 0.05$), probably because of high solubility. Zhao Qiang *et al.* (2012) have found that alkaline proteases had greater capability to dissolve protein compared to neutral and acidic proteases.

Table1. PPM and PPHs proximate composition (g/100 g) and yield

| | Protein | Fat | Moisture | Ash | Carbohydrate | Yield |
|---|---|---|---|---|---|---|
| PPM | 67.87±0.44 | 4.33±0.16 | 3.48±0.09 | 9.87±0.29 | 14.98±0.97 | - |
| Alcalase | 92.22±0.62 | 2.43±0.00 | 1.77±0.34 | 2.20±0.15 | 2.20±0.33 | 53.29±0.69 |
| Flavourzyme | 83.53±0.49 | 3.33±0.18 | 2.69±0.30 | 3.26±0.20 | 7.53±0.30 | 38.52±0.73 |
| Protamex | 81.07±0.38 | 3.60±0.06 | 3.19±0.11 | 2.18±0.21 | 10.89±0.77 | 41.64±1.76 |
| Neutrase | 84.63±0.81 | 2.60±0.08 | 1.76±0.01 | 3.55±0.33 | 9.27±0.08 | 35.72±1.58 |

Note: Values are given as mean ± SD from triplicate determinations (n = 3), PPM : Pumpkin Protein Meal, PPHs: Pumpkin Protein Hydrolysates

Table 2 . Molecular weight distribution (%) of PPHs with different fractions

| | >10,000Da | 10000-5000Da | 5000-1000Da | 1000-180Da | <180Da |
|---|---|---|---|---|---|
| Alcalase | 0.91±0.03$^{a}$ | 1.48±0.03$^{a}$ | 23.21±0.27$^{a}$ | 57.20±0.99$^{d}$ | 17.76±0.79$^{c}$ |
| Neutrase | 0.86±0.03$^{a}$ | 7.04±0.00$^{c}$ | 38.04±0.13$^{c}$ | 34.43±0.31$^{b}$ | 19.84±0.30$^{d}$ |
| Flavourzyme | 28.94±0.88$^{c}$ | 11.59±0.13$^{d}$ | 21.72±0.48$^{a}$ | 25.83±1.07$^{a}$ | 12.32±0.56$^{a}$ |
| Protamex | 2.24±0.30$^{b}$ | 2.64±0.27$^{b}$ | 28.41±0.06$^{b}$ | 50.90±0.51$^{c}$ | 15.69±0.18$^{b}$ |

Note: Values are given as mean ±SD from triplicate determinations (n = 3), Different letter in the same column means significantly differences between samples ($P < 0.05$). PPHs: Pumpkin Protein Hydrolysates and Da: Dalton.

Table 3.Total amino acid composition (g/100 g) of PPM and PPHs

| | PPM | Alcalase | Flavourzyme | Protamex | Neutrase | FA0 |
|---|---|---|---|---|---|---|
| **Essential amino acid** | | | | | | |
| Isoleucine | 2.125±0.18$^{a}$ | 2.716±0.12$^{a}$ | 3.000±0.08$^{a}$ | 2.570±0.53$^{a}$ | 2.734±0.15$^{a}$ | 2.8 |
| Leucine | 4.412±0.25$^{a,b}$ | 4.699±0.21$^{b}$ | 3.702±0.06$^{a}$ | 4.432±0.70$^{a,b}$ | 4.874±0.10$^{b}$ | 6.6 |
| Lysine | 2.362±0.32$^{a}$ | 2.436±0.03$^{a}$ | 2.195±0.07$^{a}$ | 2.580±0.38$^{a}$ | 2.492±0.10$^{a}$ | 5.8 |
| Tryptophan | 0.257±0.06$^{a}$ | 0.518±0.10$^{b}$ | 0.773±0.10$^{c}$ | 0.152±0.04$^{a}$ | 0.163±0.04$^{a}$ | 1.1 |
| Histidine | 1.484±0.00$^{ab}$ | 1.369±0.04$^{ab}$ | 1.427±0.31$^{ab}$ | 1.172±0.03$^{a}$ | 1.596±0.12$^{b}$ | 1.9 |
| Threonine | 1.800±0.06$^{ab}$ | 1.878±0.09$^{ab}$ | 1.591±0.14$^{a}$ | 1.742±0.15$^{a}$ | 1.768±0.11$^{a}$ | 1.4 |
| Valine | 2.877±0.10$^{ab}$ | 3.087±0.01$^{b}$ | 2.554±0.02$^{a}$ | 2.553±0.01$^{a}$ | 3.566±0.39$^{c}$ | 3.5 |
| Methionine | 1.472±0.30$^{a}$ | 2.053±0.76$^{a}$ | 1.526±0.24$^{a}$ | 1.376±0.08$^{a}$ | 1.826±0.10$^{a}$ | 2.5 |
| Tyrosine | 1.923±0.05$^{abc}$ | 2.332±0.31$^{bc}$ | 1.754±0.11$^{a}$ | 1.836±0.08$^{ab}$ | 2.428±0.31$^{c}$ | 6.3 |
| **Non-Essential Amino Acids** | | | | | | |
| Phenylalanine | 3.557±0.43$^{b}$ | 3.683±0.23$^{b}$ | 2.856±0.05$^{a}$ | 2.982±0.02$^{a}$ | 3.771±0.12$^{b}$ | |
| Aspartic Acid | 5.504±0.62$^{a}$ | 6.474±0.65$^{a}$ | 5.472±0.53$^{a}$ | 5.717±0.33$^{a}$ | 6.436±0.29$^{a}$ | |
| Glutamic Acid | 12.667±0.40$^{a}$ | 15.808±0.32$^{c}$ | 15.088±0.01$^{b}$ | 16.181±0.00$^{c}$ | 16.865±0.02$^{d}$ | |
| Serine | 3.550±0.62$^{a}$ | 3.736±0.31$^{a}$ | 3.387±0.23$^{a}$ | 3.720±0.25$^{a}$ | 3.788±0.28$^{a}$ | |
| Glycine | 3.601±0.36$^{a}$ | 3.855±0.14$^{ab}$ | 4.283±0.09$^{b}$ | 3.923±0.03$^{ab}$ | 4.063±0.02$^{ab}$ | |
| Arginine | 9.619±0.05$^{a}$ | 11.602±0.34$^{b}$ | 11.824±0.10$^{b}$ | 11.777±0.17$^{b}$ | 13.652±0.30$^{c}$ | |
| Alanine | 2.745±0.16$^{a}$ | 3.410±0.55$^{a}$ | 2.702±0.21$^{a}$ | 2.814±0.13$^{a}$ | 3.105±0.25$^{a}$ | |
| Cysteine-S | 0.577±0.43$^{a}$ | 0.287±0.01$^{a}$ | 0.463±0.17$^{a}$ | 0.380±0.10$^{a}$ | 0.551±0.19$^{a}$ | |
| Prolamine | 2.910±0.07$^{b}$ | 2.043±0.02$^{a}$ | 1.841±0.15$^{a}$ | 1.825±0.10$^{a}$ | 2.331±0.43$^{a}$ | |
| Total | 63.44 | 71.99 | 66.44 | 67.73 | 76.01 | |
| %EAA/TAA | 36.01 | 34.81 | 32.88 | 32.14 | 33.90 | |

Note: Mean ± S.D. in the same column with different letters were significantly different by Duncan's multiple range test ($p < 0.05$), PPM: Pumpkin Protein Meal, PPHs: Pumpkin Protein Hydrolysates, FAO: Food and Agriculture Organization, EAA: Essential Amino Aci , TAA: Total amino acid.

*Distribution of molecular weight*

Chain length of peptides is of special interest because a number of functional properties depend in part on their molecular size (Wasswa *et al*., 2007). Hydrolysing protein into shorter peptides changes the molecular weight distribution, and usually exposes some of the hydrophobic groups folded inside the intact native protein molecule to the aqueous phase. This is accompanied by the structural re - arrangement, thus improving functional properties. The molecular weight distribution of PPHs of 5-hour hydrolysis were analysed by high performance liquid chromatography and results are presented in the Table 2.

In general, the hydrolysates were mainly composed of lower molecular weight (<1000 Da) while PPM had 12,000 – 36,000 Da protein(EC, 1979b). This indicates that all hydrolysates had substantially degraded the pumpkin proteins.

However the molecular weight distributions of protein hydrolysates obtained by individual proteases were noticeably distinct, reflecting the differences in peptide chain lengths and exposure of the terminal amino groups, which could greatly influence the functional properties of the hydrolysates. Flavourzyme treated hydrolysate had the highest percentage (40.93%) of >5,000 Da peptide fraction than other hydrolysates. However, the percentages of its <5000 Da fractions were lower. On the other hand neutrase treated hydrolysates showed highest percentage (38%) of 5000-1000 Da protein fraction followed by protamex (28%) and alcalase (23%). The range of 180–5000 Da was the main molecular weight fraction for all the hydrolysates, which accounted for approximately 98%, 95%, 92% and 60% respectively for alcalase, protamex, neutrase and flavourzyme. The high efficiency of molecular distribution of alcalase and protamex might be due to their broad specificity and their alkaline hydrolysis. Zhao qiang *et al.* (2012) has suggested that alkaline proteases were much more effective in producing smaller peptides of rice dreg protein than other proteases which might be the same case for pumpkin proteins. Poor molecular distribution of flavourzyme treated hydrolysates were also observed from pumpkin hydrolysates (Vaštag *et al*., 2011) and *Phaseolus vulgaris* hydrolysates (Torruco-Uco, 2009) .

*Surface hydrophobicity*

Surface hydrophobicity ($H_0$) reflects the number of hydrophobic groups on the surface in contact with the polar aqueous environment. Changes in $H_0$ as a result of enzymatic hydrolysis influence the interfacial properties of hydrolysates. Hydrolysis of protein into shorter peptides may result in an increase or decrease of hydrophobicity, due to the exposure of hydrophobic groups that are folded inside the intact native protein molecule (Liu, 2010). The $H_o$ value of PPM and PPHs prepared with four different proteases for 5 hours are shown in Table 4. The PPHs exhibit significantly lower $H_o$ than PPM ($P < 0.05$). The high surface hydrophobicity of PPM could be explained by the detection of peptides/proteins at higher molecular weight. Similar results have been reported for protein hydrolysates hybrid catfish frame (Kwanruedee Wachirattanapongmetee, 2009) and sardine protein hydrolysate (Quaglia, 1990). Low surface hydrophobicity suggests that the small peptides in the hydrolysates had fewer hydrophobic binding sites for the ANS or that the binding sites for the probe in the peptides were modified during hydrolysis. Additionally, the peptides released from the native structure of protein may adopt a conformation with hydrophilic groups more exposed outwards. Hydrolysates have also exhibited different Ho according to the type of production enzyme in the decreasing order of alcalase, protamex, flavourzyme and neutrase. This phenomenon is probably due to the fact that enzymes break the protein in different ways and at different sites, resulting in different numbers of hydrophobic groups. However the high $H_0$ of alcalase treated hydrolysates might be attributed to its molecular distribution while for flavourzyme and protamex, the hydrolysis occurred from the exterior to the interior of the protein molecules, and as a consequence, more hydrophobic clusters were gradually exposed (Tang *et al.*, 2009).

*Amino acid composition of PPM and PPHs*

The amino acid composition (g/ 100 g of sample) of PPM and PPHs at 5 h of hydrolysis time are shown in Table 3. It was found that all samples contained essential amino acids present at high levels compared with the FAO Pattern (Tidjani *et al*., 2011) for adults except for histidine and with comparable level for children. Therefore, the obtained protein hydrolysates could possibly be a dietary protein supplement to poorly balanced dietary proteins. Hydrolysis has changed slightly the amino acid composition except for arginine, glutamic acid and asparitic acid, which probably reflects the higher solubility of these amino acids and this can impart the acidic behavior of protein or peptides. It is due to breakdown of the enzyme causing slight addition of non-soy protein amino acids. Alternatively, the clarification step after hydrolysis removes aggregates that can account for the differences in amino acid concentrations. Otherwise, if a large number of insoluble peptides had been generated as product, and lost during the clarification step, the amino acid composition of the hydrolysates would not have coincided with that of starting material. However there were some differences in amino acid composition between PPHs, mainly due to the difference in specificity of the enzymes used and in protein solubility from different hydrolysis environment. Considering the content of amino acids with some characteristics, neutrase treated hydrolysates had highest percentage in hydrophobic, hydrophilic, charged and uncharged polar amino acid (22.98%, 41%, 39%, and 12.59% respectively), while flavourzyme had lowest percentage in hydrophilic (36.4%), charged (34.56%) and uncharged polar (11.46%) amino acids. Protamex was poorest in hydrophobic amino acids (19.06%). Bitterness of protein hydrolysates is associated with the release of peptides containing hydrophobic amino acid residues (FitzGerald, 2006).

However the smaller peptides are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water and increase solubility (G. A. Gbogouri and 2004). The differences in the amino acids may thus result in differences in some physiochemical for themselves and for peptides or proteins that they form (Tidjani Amza *et al*., 2011). The nutritional qualities of PPM and PPHs were evaluated by EAA/TAA (Table 3). Both PPM and PPHs were higher than the ideal protein pattern criterion (as recommended by FAO/WHO), with EAA/TAA values being well above 32% (Zhao Qiang *et al.*, 2012).

*Differential scanning calorimetry*

The thermal transition of pumpkin protein meal and its four enzymes treated hydrolysates was investigated by DSC, and the related DSC characteristics are listed in Table 5. Protein meal and its hydrolysates exhibited different DSC patterns. PPM presented a typical endothermic peak with thermal denaturation temperature (Td) of about 88 $^{o}$C. In the hydrolysates, endothermic peaks with higher Tp than that of PPM were observed. Similar results have been obtained for hydrolysates of native and modified soy protein isolates (Molina Ortiz and Wagner, 2002).

The onset denaturation temperature of endothermic peak significantly ($p < 0.05$) increased from 74.5 for PPM to about 80, 84, 89, 90$^{o}$C respectively for HP, HN, HF AND HA when DH increased from 5.06% to 13.9%, thus the denaturation temperature was related with the DH change. Moreover, lack of conspicuous minor endothermic peak as DH increases, compared to the endothermic peak suggested that there was an extensive unfolding of PPM proteins during hydrolysis process and that only a little trace of native structure persisted in the proteins (Arogundade *et al*., 2008). Luckily, the signal of the minor endotherm gradually increased with increasing DH showing that the phenomena were also DH dependent. The presence of soluble and insoluble aggregates in hydrolysates, may account for these two endothermic events. The ΔH represents the extent of ordered structure of a protein (Molina Ortiz and Wagner, 2002). Thus, the data suggest that the extent of the ordered structure of hydrolysates was unrelated to the extent of hydrolysis but nearly correlated to surface hydrophobicity (Grothe and Park, 2000) of the samples. Furthermore, the width at half peak height of endothermic peak ($T_{1/2}$), indicative of the cooperativity of the thermal transition was nearly unaffected by the enzymatic hydrolysis (data not shown).Therefore the hydrolysates were more heat stable than PPM even if they had gone through enzymatic and thermal treatment.

*Protein solubility*

Solubility is one of the most important physicochemical property of proteins and protein hydrolysates, surface-active properties and rheological or hydrodynamic properties. In many protein based formulations, for instance, emulsions, foams and gels, good solubility for the protein is usually required (Nice, 1979). High solubility of a product is necessary for its use in many manufactured foods to improve other functional properties such as emulsification and foaming (Yust MdM, 2010). The solubility profiles of PPM and PPHs as a function of pH are presented in Figure 1 (B). Both PPM and PPH were practically pH-dependent, and they both had a minimum solubility at around pH 5, which is the isoelectric point of pumpkin protein. However PPM was less soluble than the hydrolysates, indicating that enzymatic hydrolysis considerably improved ($P < 0.05$) the solubility of pumpkin protein meal at all pH values tested. After hydrolysis, protein solubility at pH 7.0 increased from 23 for control PPM to 43, 54, 70 and 72% respectively for flavourzyme, protamex, neutrase and alcalase. Improvement in protein solubility due to enzyme hydrolysis is well documented (Sorgentini and Wagner, 2002; Tsumura *et al.*, 2005).

Generally protein solubility depends on several factors such as pH, polarity, molecular size, hydrophilic sites (WHC). The increased protein solubility could be due to smaller molecular peptides being produced by hydrolysis. In addition, enzymatic hydrolysis could lead to unfolding of protein molecules, both polar and non-polar amino acid groups buried inside protein molecules could be exposed on the surface of protein molecules after unfolding. These exposed polar amino acids may interact with water molecules through hydrogen bonds and electrostatic interactions, resulting in increased protein solubility. However, the lowest solubility of PPHs observed at pH 5.0 could be attributed to both net charge of peptides, which increases as pH moves away from pI, and surface hydrophobicity, that promotes the aggregation via hydrophobic interaction. The pH affects the charge on the weakly acidic and basic side chain groups, and hydrolysates generally showing low solubility at their isoelectric points (Chobert, 1988). HA, HP, and HN exhibited higher solubility than HF, and a significant difference was observed for all tested pH values ($P < 0.05$). This may be due to difference in molecular weight distribution where HA, HP and HN contained

smaller peptides than HF (Table 2). Additionally, Tsumura *et al.* (2005) have found that hydrolysis of proteins increased the solubility, but balance between hydrophilic and hydrophobic forces scores over DH. Slight increase in protein solubility at pH 3, 9, and 11 were observed with PPM and PPHs. This shows that at these pH values, both unmodified PPM and PPHs carried negative (pH 9 or 11) and positive (pH 3) electric charges, thus contributing to solubility. The effects of smaller peptides and of exposure of hydrophilic groups on protein solubility were minor compared to those of electric charges at pH 3, 9, and 11.

*Emulsification properties*

The emulsifying activity (EAI) and stability (ESI) of PPM and PPHs with different commercial enzymes at various pH (3–11) was determined (Figure1(C) and (D)). On whole, PPHs had higher EAI and ESI than PPM reflecting that the hydrolysis process has significantly ($p < 0.05$) increased the emulsifying properties. All the samples showed minimal EAI and ESI values at pH 5.0. The influence of type of enzyme, extent of hydrolysis and molecular weight distribution on the EAI and ESI were dependent upon the pH at which the EAI was measured; at pH 3, HP had best EAI while at pH 5, 7, 9 and 11, AH showed highest EAI. NH has shown poor EAI for all tested pH values but FH had the best ESI. The poor emulsification activity of NH might be explained by its low DH (Figure 2 (A)). These results were in agreement with those reported by Balti et al. (2010) where he found that the EAI of protein hydrolysates from cuttlefish (*Sepia officinalis*) by-products increased as the degree of Hydrolysis increases.

The mechanism to generate the emulsion system is attributed to the adsorption of peptides on the surface of freshly formed oil droplets during homogenization and the formation of a protective membrane that inhibits coalescence of the oil droplet. Hydrolysates are surface-active materials and promote oil-in-water emulsion because of their hydrophilic and hydrophobic groups with their associated charges (Dagorn-Scaviner *et al.*, 1987). In order to exhibit good emulsifying activity, the protein and/or peptides must be able to migrate rapidly to the water/oil interface and then unfold and rearrange rapidly at the interface (Kotlar *et al*., 2013). Although the peptides with low molecular weight can migrate rapidly to the interface, the hydrophobic/hydrophilic balance of these small peptides is not good enough to stabilize emulsions (Deng *et al*., 2011). This justifies the highest ESI of flavourzyme treated hydrolysates as it had higher molecular weight (Table 3). Dagorn-Scaviner *et al.* (1987) analyzed amino acid sequence at an oil/water interface and concluded that amphiphilic character was more important than was peptide length for emulsion properties. The flexibility of protein or peptide structure may also be a vital factor governing the emulsifying properties.

Table 4. Water Holding Capacity (mg/ml), Oil Holding Capacity (mg/ml), Least Gelation Concentration (%), Bulk Density (g/ml), Surface Hydrophobicity , Foam Capacity (%) and Foam Stability (%) of PPM and PPHs

| | Alcalase | Flavourzyme | Protamex | Neutrase | PPM |
|---|---|---|---|---|---|
| WHC | $1.68\pm0.03^{a}$ | $1.98\pm0.03^{a}$ | $2.24\pm0.04^{b}$ | $1.94\pm0.1^{a}$ | $1.72\pm0.03^{a}$ |
| OHC | $3.28\pm0.26^{d}$ | $1.72\pm0.12^{b}$ | $2.8\pm0.02^{c}$ | $2.76\pm0.11^{c}$ | $1.08\pm0.04^{a}$ |
| LGC | $2\pm0.1^{a}$ | $6.5\pm0.85^{c,d}$ | $4\pm0.23^{b}$ | $5\pm0.9^{b,c}$ | $8\pm0.12^{d}$ |
| BD | $0.66\pm0.009^{d}$ | $0.39\pm0.001^{a}$ | $0.57\pm0.002^{c}$ | $0.51\pm0.003^{b}$ | $0.34\pm0.005^{a}$ |
| $H_o$ | $2703\pm21^{d}$ | $1751\pm24^{b}$ | $1908\pm32^{c}$ | $1304\pm41^{a}$ | $3714\pm33^{e}$ |
| F.C | $64.39\pm1.3^{c}$ | $60.57\pm2^{c,d}$ | $61.84\pm0.92^{a}$ | $53.17\pm1.32^{d}$ | $52.57\pm21.73^{b}$ |
| F.S | | | | | |
| 10 min | $60.72\pm2.8^{d}$ | $58.23\pm2.4^{c}$ | $53.98\pm2.8^{b}$ | $44.92\pm2.9^{a}$ | $44.03\pm2.4^{a}$ |
| 30 min | $50.93\pm1.4^{c}$ | $55.43\pm1.9^{b}$ | $50.47\pm2.3^{c}$ | $41.87\pm1.9^{d}$ | $37.46\pm2.1^{a}$ |
| 60 min | $43.4\pm2.1^{c}$ | $51.48\pm1.3^{b}$ | $43.88\pm2.1^{c}$ | $35.44\pm2.2^{d}$ | $31.38\pm1.8^{a}$ |
| 90 min | $40.33\pm1.3^{c}$ | $48.43\pm1.7^{b}$ | $40.37\pm1.8^{c}$ | $32.29\pm2.32^{d}$ | $27.49\pm1.3^{a}$ |
| 120min | $37.48\pm2.1^{c}$ | $47.94\pm1.5^{b}$ | $39.04\pm1.4^{d}$ | $31.18\pm1.23^{e}$ | $23.44\pm0.7^{a}$ |

Note: Mean ± S.D. in the same row with different letters were significantly different by Duncan's multiple range test ($p < 0.05$), PPM: Pumpkin Protein Meal, PPHs: Pumpkin Protein Hydrolysates, min: minutes, WHC: Holding Capacity, OHC: Oil Holding Capacity,
LGC: Least Gelation Concentration, BD: Bulk Density, $H_o$: Surface Hydrophobicity, FC: Foam Capacity and FS: Foam Stability

Table 5. Thermal properties of PPM and PPHs

| | $T_o$(°C) | $T_p$(°C) | ΔH($Jg^{-1}$) |
|---|---|---|---|
| PPM | $74.50\pm0.02^{a}$ | $88.92\pm0.64^{a}$ | $4.10\pm0.03^{d}$ |
| Alcalse | $90.03\pm1.43^{d}$ | $93.00\pm0.04^{c}$ | $1.61\pm0.08^{c}$ |
| Flavourzyme | $89.25\pm1.10^{d}$ | $91.73\pm0.44^{b}$ | $0.93\pm0.07^{b}$ |
| Neutrase | $84.36\pm0.60^{c}$ | 89.63±0.57a | $0.50\pm0.01^{a}$ |
| Protamex | $80.73\pm0.88^{b}$ | $91.13\pm0.42^{b}$ | $4.33\pm0.00^{d}$ |

Note: Mean ± S.D. in the same column with different letters were significantly different by Duncan's multiple range test ($p < 0.05$),PPM :Pumpkin Protein Meal, PPHs: Pumpkin Protein Hydrolysates, To: Onset denaturation temperature, Tp: Peak denaturation Temperature, ΔH: Enthalpy, °C: Degree Centigrade

The low EAI and ESI found at pH 5, correlates with solubility decrease (Figure (1B)). Since the lowest solubility occurred at pH 5, peptides could not move rapidly to the interface. Additionally, the net charge of peptide could be minimized at pH 5. The higher EAI of hydrolysates accompanied their higher solubility. Hydrolysates with high solubility can rapidly diffuse and adsorb at the interface (Deng *et al*., 2011). Hydrolysates that had better EAI were not necessarily having better ESI for all pH values tested suggesting that the sequence and composition of amino acids in peptide between hydrolysates might be different, leading to varying charge of the resulting peptides at a particular pH. Emulsifying properties were influenced by specificity of enzyme as also demonstrated by Wasswa *et al.* (2007).

*Foaming properties*

The results of foaming capacity (FC) and Foam stability (FS) of pumpkin protein meal and

hydrolysates measured at pH 7 are given in Table 4. There was a significant ($p < 0.05$) increase in the foaming capacity and stability of the four hydrolysates compared with PPM. FC were increased from 47.24% for PPM to 53.1%, 60.3%, 61.65% and 63.9% respectively for NH, FH, PH and AH. Foam expansion after whipping was monitored for 120 min to indicate the foam stability of protein hydrolysates. Within the first 10 min, alcalase prepared hydrolysate showed the highest foam stability at the beginning but the trend was not consistent as flavouzyme treated hydrolysates showed good FS after 120 min. Foam formation requires the ability of a protein to quickly adsorb at the water/air interface, thereby lowering the surface tension. Hence, the adsorption rate, together with the ability to unfold and re-arranging at the interface have been reported as one of the most important factors for foam formation (EC, 1979a).

Thus the small size of PPHs peptides allowed them to adsorb quickly to the air–water interface, lowering the surface tension and giving rise to a foam expansion. However, the foam expansions obtained in this study were lower than those reported for hydrolysates from cucurbitin (EC, 1979b) and protein hydrolysate from grass carp (Ctenopharyngodon idella) skin (Kotlar *et al*., 2013), but higher than those reported for gelatin hydrolysates obtained from skin of sole and squid (Nice, 1979).

The adsorption rate to the air–water interface may be influenced by the molecular size, protein structure and hydrophobicity of the hydrolysates (Yust MdM, 2010), which are highly dependent on the parent protein from which they are obtained and the hydrolysis procedure. There was a full positive correlation for FC and $H_o$ among all the hydrolysates and partial correlation with WHC. Foam stability mainly depends on the extent of protein–protein interactions within the matrix of the films surrounding the air bubbles and the flexibility of protein or peptide structure (Tsumura *et al*., 2005). Therefore, the size and charge of peptides may be different for hydrolysates produced by different enzymes. HF exhibited superior foam stability most because it contained larger size of peptides (table 2) which could form flexible films around the air bubbles, as evidenced by higher foam stability.

*Oil holding capacity*

Oil holding capacity expresses the quantity of fat/oil directly bound by the proteins. The capacity of hydrolysates to absorb fat/oil is an important functional characteristic of ingredients used in the meat and confectionery industries. Oil holding capacity of PPHs was determined and compared with that of PPM. All PPHs exhibited fat absorption capacity greater than that of PPM (Table 4). This may be attributed to the unfolding of protein structure, as well as exposure of more hydrophobic groups allowing the physical entrapment of oil. AH had the highest fat absorption (3.28 g/mL) followed by PH (2.8 g/mL) then NH (2.76 g/mL) while FH had lowest value (1.72 g/mL) among hydrolysates but higher than that of casein (1.3 g/ml) (Rafik Balti and Nasri, 2010). Several factors may affect the ability of hydrolysates to bind fat, such as bulk density of the protein (JE, 1976), degree of hydrolysis (Sorgentini and Wagner, 2002) and enzyme–substrate specificity (Chobert, 1988). However, the present results show that there was no correlation between OHC and DH, or between OHC and $H_o$ the same trend was observed by protein hydrolysates of catfish frame (Kwanruedee Wachirattanapongmetee, 2009). Some reports demonstrated that the different enzymes used for hydrolysis of salmon muscle protein resulted in different fat absorption ability (Chobert, 1988). The results showed that PPHs exhibited good fat absorption thus could be very useful in the meat and confectionary industries.

*Water holding capacity*

The functional properties of proteins in food systems depend in part on water–protein interaction. WHC refers to the ability of the protein to imbibe and retain the water against gravitational forces within a protein matrix. It is therefore positively correlated with water binding capacity. As shown in Table 4, hydrolysis has slightly but significantly increased the WHC of PPM, this may be attributed to the increased presence of polar groups such as –COOH and $–NH_2$ during enzymatic hydrolysis.

Among the four hydrolysates, protamex hydrolysate has highest water holding capacity and hydrolysates prepared with alcalase had lowest but higher than that of casein (Rafik Balti and Nasri, 2010). The WHC of the hydrolysates was independent of DH (Figure (1A)). In this case, the improvement of WHC was attributed to the content of non-protein components in the samples (Table 1) (Guan *et al*., 2007). A contrary result on whey protein hydrolysates was observed (Sinha, 2007). The type of enzyme used to produce hydrolysate has also been reported to affect the WHC (Cumby *et al.*, 2008).

*Gelation capacity*

Effects of enzymatic hydrolysis and enzyme type on gelation capacity are presented in Table 4. Taking the least gelation concentration (LGC) as the index of gelation capacity, lower LGC means better gelation

capacity. Gel formation of protein is the result of a two-step process involving the partial denaturation of individual proteins to allow more access to reactive side groups within the protein molecules, and the aggregation of these proteins by means of reactive side groups into a three-dimensional network structure capable of retaining significant amounts of water (Lawal, 2004). The hydrolysate significantly improved the gelation capacity of unhydrolysed PPM from 8.0 to 6.5, 5.0, 4.0 and 2.0 respectively for HF, HN, HP and HA. Differences in gelation capacity are due to the difference in prevalent surface charge of the proteins/peptides. At pH 7 where LGC were measured, the surface charge is large and significant repulsive forces prevent aggregation of protein molecules and formation of an ordered network structure. The result indicates improvement in gelation capacity as the ionic strength of the protein solution increases among hydrolysates and they were in agreement with those found by other researchers (Otte, 1999; Martinahrčková, 2002; Ljiljana, 2011) .

*Bulk density*

Bulk density is the measure of heaviness of the powder. Moreover BD is an important parameter that determines the packaging requirement of a product. Furthermore bulk density signifies the behavior of a product in dry mixes. It also varies with the fitness of the particles. PPM and PPHs had varying bulk densities of 0.33 and 0.37, 0.46, 0.55, 0.66 for PPM and HF, HP, HN, HA respectively (Table 4). Present results were similar to those of hydrolysates of grass carp skin (Wasswa *et al*., 2007). The low bulk density of PPM was due to its low particle density and large particle size. It is worthy to note that BD was positively correlated to OHC, however the reason behind was not yet known.

## Conclusion

This research has shown that pumpkin protein meal could potentially serve as a good source of desirable quality peptides and amino acids at low salt content, to be used as natural additives, such as emulsifiers. Enzymatic hydrolysis of PPM has been a suitable route to improve its physicochemical properties although they were determined by the type of enzyme used for hydrolysis. Here, we observed the use of four commercial proteases for the enzymatic hydrolysis of PPM. The EAA/TAA values for the obtained PPHs were > 34%, with protein recovery ranging from 35.72% to 53.29%.

Alcalase was most effective in producing smaller peptides and in recovering the proteins, which enabled its hydrolysates to exhibit best solubility, emulsification activity, foaming activity, oil holding capacity and bulk density. On the other hand the hydrolysates by flavourzyme showed the best emulsification and foaming stability. Protamex treated hydrolysates were best in water holding capacity with low ash content of 2.18%. Further studies should investigate the optimum combination of alcalase or protamex in sequence with flavourzyme to improve the hydrolysis efficiency of PPM thereby maximizing its utilization and minimizing waste disposal problems.

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