MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society For Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

EFFECT OF DIFFERENT PRE-SOAKING TECHNIQUE ON PHYSICOCHEMICAL PROPERTIES OF *Canavalia ensiformis* PROTEIN HYDROLYSATE

Nur Aina Shazwani Sukri[1], Zamzahaila Mohd Zin[1], Mohd Aidil Adhha Abdullah[2], Nor Dini Rusli[3,4], Katherine Louise Smedley[5] and Mohamad.Khairi Zainol[1]*

[1]*Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030 Mengabang Telipot, Kuala Nerus, Terengganu*
[2]*Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu*
[3]*Faculty of Agro-Based Industry, Universiti Malaysia Kelantan, Jeli Campus, 17600 Jeli, Kelantan, Malaysia*
[4]*Institute of Food Security and Sustainable Agriculture, Universiti Malaysia Kelantan, Jeli Campus, 17600 Jeli, Kelantan, Malaysia*
[5]*Strafford School, Stratford-upon-Avon, Warwickshire, CV37 9DH, United Kingdom*

*Corresponding author: mkhairi@umt.edu.my

History
Received: 4th December 2020
Accepted: 2nd February 2021

Keywords:

Antioxidant activities, Canavalia ensiformis, protein hydrolysate, soaking treatments

Abstract

Canavalia ensiformis or Kacang Koro (Jack Beans) is one of the under-exploited tropical beans that contains between 25-35% crude protein content. Pre-treatments such as soaking helps to maintain the desired properties of protein hydrolysate, such as antioxidants, structure, high water capacity, oil-water capacity, and removing antinutrients. However, there is little information on the effect of soaking using different solvents on physicochemical properties of *C. ensiformis*. The main objectives of this study are to determine the effect of pre-treatments (A= lime juice, B=in 50% w/v tamarind solution (1:2) for 15 min and rinsed in distilled water, C=3% salt solution (1:3) and 50% (w/v) activated carbon solution (1:2), D= in distilled water (1:2) as control.) on physicochemical properties of *C. ensiformis* protein hydrolysate. The hydrolysates were evaluated based on moisture content, protein content, water holding capacity (WHC), oil holding capacity (OHC), emulsifying properties, solubility, degree of hydrolysis (DH), foam stability, foaming capacity and antioxidant properties using 2,2-diphenyl-2-picrylhydrazyl (DPPH) assay for antioxidant activities. The highest protein content (6.89±0.71%) was observed in treatment D. Treatment C exhibited the highest degree of hydrolysis water, oil holding capacity, emulsifying stability index (ESI), solubility and DPPH. The size of *C. ensiformis* protein hydrolysate analysed using scanning electron microscope (SEM) varies due to different pH soaking treatments between pH 5 to pH 10. *C. ensiformis* protein hydrolysate treated in treatment B showed the highest inhibition towards DPPH (27.23±4.65%). Overall, this study showed that the best technique for extracting a high quality *C. ensiformis* protein hydrolysate that can be used in food or pharmaceutical products was pre-soaking in salt and activated carbon, thus promoting the use of this under-utilized legume.

INTRODUCTION

The quest for new protein-rich foods due to the growing human population, particularly based on plant origin, is now necessary because of the increased population and high price of animal-based protein [1]. Legume offers one of the

cheapest protein products with excellent qualities [2]. Legumes are also rich in carbohydrates, low in serum cholesterol, high in fibre, low fat, high concentration of polyunsaturated fatty acids and long shelf-life [3].

Similar to soybeans, *Canavalia ensiformis* (Kacang Koro) has the potential to become a major source of protein with a protein content of 20-30% [4]. This legume also has a higher protein content than maize, parboiled rice, and egg [3]. Protein hydrolysate is a mixture of polypeptides, oligopeptides, and amino acids produced by hydrolysis of physical (heat or shear) or chemical (acid, alkali, or enzyme) from different sources of animal and plant protein. Since many years ago, this protein hydrolysate has been commonly used in processed food for human consumption like the soy sauce of today [5].

Pre-treatment processes involve primarily the efficient separation of complexly interlinked fractions and the accessibility of each part, thus constituting an important step in a wide range of applications, particularly for the valorisation of biomass [6]. Several conditions can be adopted as a pre-treatment approach and the selected process must avoid reducing the size of biomass particles, retaining the hemi-cellulose fraction, minimising the production of degradation products, minimising energy demands and needing a low-cost pre-treatment catalyst and/or low-cost recycling catalyst and high-value regeneration [7]. Pre-treatment of seeds from dry-food legumes (pulses) prior to cooking includes mainly extracting foreign material, washing, decorticating and soaking. Different processing techniques are required to inactivate or eliminate antinutritional factors, thereby improving legumes' nutritional quality [8].

A successful pre-treatment method can maintain desirable hydrolysate properties, such as antioxidants, structure, high water capacity, oil-water capacity. Legume soaking before cooking is a common practice to soften and tenderise the texture of beans. As the beans contain certain complex sugars that are indigestible by enzymes, soaking the legume seeds before cooking can solve the problem [9].

Soaking allows for distribution of water within the legumes among starch and protein fractions [10]. This provides nutritional and health benefits and enables health professionals to consume more beans [11]. Since it is well known that different pre-treatment will have different impacts on protein structure and functionality, it is anticipated that pre-treatment may also have an impact on *C. ensiformis* protein physicochemical properties. Therefore, this study are aimed to determine the effect of pre-treatment (soaking in different mediums) on physicochemical properties of *C. ensiformis*'s protein hydrolysate. This study will provide a new way to preserve *C. ensiformis* protein's beneficial physicochemical properties, which will enable its potential as a new, cheap source of protein.

MATERIALS AND METHODS

Raw materials

Four months old *C. ensiformis* fruit pods were collected from a farm in Kuala Berang, Terengganu. The seeds were removed from their pods, air-oven dried at 60 °C for 26 h and ground into flour before enzymatic hydrolysis. Alcalase enzyme (Merck, USA) was used to extract the protein hydrolysate.

Sample preparation

Pre-treatment

The dried *C. ensiformis* seeds were pre-soaked in four different methods using four different types of solutions which include Treatment A: *C. ensiformis* seeds was initially soaked in lime juice (1:2 ratio of seeds to solution); Treatment B: *C. ensiformis* seeds soaked in 50% w/v tamarind solution (1:2) for 15 min and rinsed in distilled water; Treatment C: *C. ensiformis* seeds were soaked in 3% salt solution (1:3) and 50% (w/v) activated carbon solution (1:2) and Treatment D: *C. ensiformis* seeds were soaked in distilled water (1:2) as control. All soaked samples were continuously stirred for 6 h. The designated pre-treatment solutions were changed every 2 h until the 6th hour.

Protein extraction and hydrolysis

The pH of the solubilized proteins were adjusted with 1 N HCl to their isoelectric points, which is 4.9 for *C. ensiformis* seeds [12]. The suspension was then centrifuged at 1317*g* for 12 min. The sample was mixed with a 100 ml sodium phosphate buffer (1:2 w/v) for 5 min at 25 °C. The solution was adjusted to pH 8 (optimal pH for Alcalase enzyme) using 1 N NaOH. Alcalase was added at concentration 2.0% (w/w) of raw material. Heat treatment was applied to denature enzymes using a water bath at 90°C for 10 min [13]. The resulting solution was then centrifuged at 9000 rpm for 25 min. The sample was filtered and collected, rapidly pre-frozen at -80°C and freeze dried using a freeze dryer at -40ºC.

Physical properties of dried protein hydrolysate Yield

The centrifuged solution was filtered using filter paper and the amount was recorded as (a). The yield of powdered protein hydrolysate was obtained by weighing the powdered collected after freeze drying was recorded as (b). The percentage of yield after drying was calculated as shown in [Equation 1].

$$\text{Yield} = \frac{powder\ collected\ after\ drying(b)}{liquid\ protein\ hydrolysate\ (a)} \text{ x } 100\% \text{ [Equation 1]}$$

Water holding capacity (WHC) and oil holding capacity (OHC)

A suspension of 0.5 g protein hydrolysate (dry weight) was added to 5 ml of distilled water. The mixture was left to stand at 30 min. After that, it was centrifuged (Hettich Universal 32, Germany) at 5000g for 30 min. The free water was discarded and the tube was drained for 10 min. The pallet left in the centrifuge tube was weighed and calculated using the equation [Equation 2] [15].

Water holding capacity (WHC) of *C. ensiformis* protein hydrolysate (%) = $\frac{Weight\ of\ pallet}{Weight\ of\ powder}$ x 100 [Equation 2]

Oil holding capacity (OHC) was conducted according to a method modified from [14]. Palm oil of 10 mL was added to 0.5 g *C. ensiformis* protein hydrolysate and centrifuged in 5000g for 30 mins at 25°C. The unbound oil was decanted off, and oil absorption capacity was determined from the difference in weight of the hydrolysate samples and expressed as gram of oil bound per gram sample [Equation 3].

OHC of *C. ensiformis* protein hydrolysate (%) = $\frac{Weight\ of\ oil\ held}{Weight\ of\ powder}$ x 100 [Equation 3]

Morphological properties-SEM

Microstructure determination using a Scanning Electron Microscope(Jeol-6360, USA). The sample was applied on the surface of the sticker on a specimen holder. The sample was coated with 99% pure gold using Auto Fine Coater prior to analysis of the SEM. The specimen was viewed using 5000X magnification [15].

Foaming capacity and foam stability

A 20 ml sample of 5% (w/v) suspension was prepared with an added to sodium phosphate buffer. The suspension was placed in 100 ml beaker and was homogenized at 17 500 rpm for 2 min (IKA, Germany), and the foam volume was recorded after 30 s. Foaming capacity was expressed as the percentage increase in foam volume measured at 30 s [Equation 4]. Foam stability was determined according to residual foam volume at 3 min after homogenized. Both properties were determined as a function of pH [12]. Foam stability (FS) was determined as the volume of foam that remained after 3 min expressed as a percentage of the initial foam volume [Equation 5].

Foaming capacity = $\frac{volume\ of\ foam\ after\ 30s}{original\ volume\ of\ protein\ solution}$ x 100 [Equation 4]

Foam stability = $\frac{volume\ of\ foam\ after\ 3\ min}{volume\ of\ foam\ after\ 30\ s}$ x 100 [Equation 5]

Solubility

Protein hydrolysates solubility were determined using the established Biuret method [16]. Two hundred milligrams of protein hydrolysate were dispersed in 20 ml of deionized water and pH of the mixture was adjusted to 2, 3, 4, 5, 6 7, 8, 9, 10, 11, 12 with 1 or 6 N HCl and 1 or 6 N NaOH. The mixture was then stirred and agitated at 25°C for 30 min and centrifuged at 7500g for 15 min. Total protein content in the sample was determined after solubilization of the sample in 0.5 N NaOH. Protein solubility was calculated based on equation 6:

$$Solubility = \frac{protein\ content\ in\ supernatant\ total}{protein\ content\ in\ sample}\ x\ 100$$

[Equation 6]

pH value

pH value was determined at room temperature (25°C). The pH meter used was calibrated at pH 4 and pH 7. Then the pH meter was rinsed with distilled water and wiped before inserted into the sample.

Colour profile

Colour (L*, a*, and b* values) of the samples were determined using Colourimeter (Minolta Chroma CR 300, Japan). Colour was measured by spectrophotometer (trismulus colour machine with CIE lab colour scale) (Hunter, Lab Scan X E, Reston VA). The sample was crushed coarsely and flattened prior to colour determination.

Structural properties of protein hydrolysate

Fourier Transform Infrared Spectroscopy (FTIR) (Nicolet, Thermo Electron, USA) was used to study the structural properties of *C. ensiformis* protein hydrolysate. The lyophilized protein hydrolysate was measured for its infrared spectra in the absorbance of 4000-500 cm^{-1} by FTIR spectrophotometer. The scanning was performed with a resolution of 4 cm^{-1} and the FTIR spectra were an average of 32 scans [17]. The functional group and mode of vibrations were identified based on the peak of interest at specific wavelength and absorbance [15].

Chemical properties of dried hydrolysate

Determination of moisture and crude protein content

The moisture and crude protein content were determined according to the Association of Official Analytical Chemists' procedure (AOAC) [18].

Degree of hydrolysis

The degree of hydrolysis of *C. ensiformis* protein hydrolysate was determined using trichloroacetic acid (TCA) method [14]. Two grams of lyophilized hydrolysate was used in this analysis. One gram of *C. ensiformis* protein hydrolysate was determined through the Kjeldahl method [18]. As for the 10% TCA soluble nitrogen determination, 1 g of *C. ensiformis* protein hydrolysate was added into 10 mL distilled water, respectively. Then, 10 mL of 20% TCA was added into the sample and mixed by vortex. The samples were left to stand for 30 mins, in order to allow the protein to precipitate. The samples were then centrifuged at 8000 rpm for 5 mins (High-Speed centrifuges 1580R, Cryozen Co, Ltd, Korea). Protein content in the collected supernatant byproduct using Kjeldahl method [18]. The protein content was calculated using equation and:

Percentage of nitrogen (%) = $\frac{(T-B)X\ N\ X\ 14.007X100}{weight\ of\ the\ sample\ (mg)}$ [equation 7]

Percentage of DH (%) = $\frac{Percentange\ of\ nitrogen(A)}{Total\ percentage\ of\ nitrogen}$ x 100 [equation 8]

Where,
T = Titration volume for the sample (ml)
B = Titration for the control (ml)
N = Concentration of hydrochloric acid (HCL)
F = Protein factor (6.25)

Emulsifying properties

The emulsifying activity index (EAI) and emulsion stability index (ESI) of the samples were determined using the method described by Klompong et al. [19] without the pH adjustment. Approximately 10 ml of corn oil was mixed with 30 ml of 1% protein sample. The mixture was then homogenized at 20,000 rpm for 1 min. An aliquot of the emulsion (50 µl) was pipetted from the bottom of the container at 0 and 10 min after homogenization and diluted in 5 ml of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured immediately at 0min (A_0) and 10min (A_{10}) at 500nm using a spectrophotometer.

Emulsifying stability index (ESI) (min) = $\frac{A0\ X\ \Delta t}{\Delta A}$

Where,
A_0 is the absorbance at 0 min after homogenization;
A_{10} is the absorbance at 10 min after homogenization;
Δt is 10 min and ΔA is the difference of absorbance at 0 min and 10 min (A0 – A10).

Antioxidant activity based on 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was evaluated according to the method described by [20] with slight modification. 6.1×10^{-5} M solution of DPPH was prepared in ethanol. A 75 µL of the diluted extract was added with 3 mL of the DPPH solution. The absorbance was taken at 515 nm using methanol with DPPH as the negative control, while quercetin as a positive control after letting it stable for 1 h. All analyses were conducted in dark or dim light. The inhibition percentage (IP) of the DPPH by the extract was calculated according to this formula:

IP = [(A0 min - A60 min) / A0 min] x 100

A0 min is the absorbance of the blank at t = 0 min, and 60 min is the absorbance of samples at 60 min. The result expressed as µmol Trolox equivalent (TE) per gram of sample on a dry basis, through a dose-response curve for Trolox (0-350 µM).

Statistical analysis

All the data were obtained from triplicates analysis and analysed using one-way ANOVA. The statistical analysis for protein hydrolysate was completed using MINITAB 19 software at the confidence level at $\rho \leq 0.05$. The statistical comparison was performed with one-way analysis (ANOVA) using Fisher's Least Significant Difference (LSD) test and values of $\rho < 0.05$ were considered significant [21].

RESULTS AND DISCUSSION

Yield of powder hydrolysate

The determination of yield demonstrates the efficiency and efficacy of the extraction process. Table 1 shows that treatment D (soaked in distilled water) was found to have a higher yield (4.13%) than other samples. This result showed a contrast data to a previous study [22] who mentioned that acid treatment removed certain acid-soluble proteins, lipids, and other unwanted components, disrupted some cross-links of collagen molecules, and caused skin swelling gelatine extraction more efficient. The use of excess acid over hydrolyzed proteins such as collagen molecules and the loss of recoverable protein during later washing processes could be due to the high excessive acid concentration which maximise protein extraction [23]. An increase in the yield value in treatment D also resembles protein polymer arrangement, as they have different physical and chemical properties, which reacted differently towards acidic treatments.

Table 1: Chemical composition of powdered protein hydrolysate *C. ensiformis*.

Sample	Yield (%)	Moisture (%)	Protein (%)	Emulsifying activity (m^2/g)	Emulsifying stability (min)	Degree of hydrolysis (%)	Dpph (%)
A	1.73±0.53[a]	0.48±0.00[a]	6.89±0.71[a]	0.76±0.03[b]	3.31±0.41[b]	6.12±0.43[b]	21.68±4.33[a]
B	2.52±0.66[a]	0.44±0.01[b]	2.59±1.22[b]	2.11±0.01[a]	2.53±0.05[b]	7.27±1.11[b]	27.23±5.31[a]
C	3.81±0.02[a]	0.48±0.01[a]	0.34±0.01[c]	0.44±0.02[b]	6.07±0.07[a]	21.38±3.01[a]	25.55±2.07[a]
D	4.13±0.21[a]	0.47±0.01[a]	4.08±0.49[b]	1.51±0.27[ab]	3.62±0.11[ab]	9.04±0.84[b]	13.11±4.44[b]

Note: A= lime juice, B=in 50% w/v tamarind solution (1:2) for 15 min and rinsed in distilled water, C=3% salt solution (1:3) and 50% (w/v) activated carbon solution (1:2), D= in distilled water (1:2) as control. [different pre-treatment solution]. All values given are means of triplicate results. Standard deviation (mean ± SD) is included for each average. Means with different letter are significantly different ($p < 0.05$) within the same column.

Chemical properties of *C. ensiformis* hydrolysate

Table 1 shows the moisture content of the powdered hydrolysate extracted from *C. ensiformis* following soaking using different soaking solutions. There were no significant differences between solutions except treatment B.

The moisture content of pre-soaked *C. ensiformis* in distilled water (0.48±0.00%) was higher than Treatment B ($p>0.05$) but slightly different in pre-treatment with salt and activated carbon, where it contains 0.48±0.01% and a nearly similar moisture content of *C. ensiformis* impregnated with any solution.

The protein content of treatment A (6.89±0.71%) was found to be the highest protein among the others. The protein content in pre-treated *C. ensiformis* obtained from the current study was lower compared to previous findings [24]. This might be due to treatments of the solution that affect the protein in the *C. ensiformis*. Moreover, the protein in pre-treatment salts and activated carbon showed the lowest protein content where 0.34±0.0%, because activated carbon was used to extract small molecules from protein solutions and often a dextran coating was used to avoid interaction between the proteins and the surface of the activated carbon [25].

Emulsifying properties of powdered *C. ensiformis* protein hydrolysate

Table 1 also represents the emulsifying activity index (EAI) and emulsifying stability index (ESI) of powdered protein hydrolysate of *C. ensiformis* for different treatments. The EAI of treatments A, B, C and D were 0.76, 2.1, 0.4 and 1.5 m^2/g, respectively. The ESI of treatments A, B, C and D were 3.32, 2.46, 5.96 and 3.65 min, respectively. Treatments C and D significantly high compared to treatments A and B. There was a significant difference ($p<0.05$) between the treatments of different solutions. A protein with ideal qualities as an emulsifier for an oil-in-water emulsion would have relatively low molecular weight, a balanced amino acid composition with charged, polar and non-polar residues, good water solubility, well-developed surface hydrophobicity and a relatively stable conformation [5].

The results also showed that ESI values were proportional to the pH of treatment used. Similar results as reported by Naqash and Nazeer [26] that an increase in the pH will increase the ESI which was accompanied by their higher solubility but in contrast for EAI. High EAI does not generally mean high ESI depending on the hydrolysis condition (pH, temperature, and enzyme), other processing steps and emulsifying test conditions (pH, concentration, temperature, and others) [27].

Degree of hydrolysis (DH) of powdered C. ensiformis protein hydrolysate

The degree of hydrolysis plays an important role in deciding the functional properties of protein hydrolysate since protein structure hydrolysis induces a decrease in protein molecular mass (MM) and an increase in both the amount of ionizable groups and the accessibility of hydrophobic regions in the protein structure [27].

The hydrolysis of the *C. ensiformis* protein hydrolysate resulted in a degree of hydrolysis of 6%, 7.3%, 20.6% and 9.4 % for treatment A, B, C and D of different solutions, respectively. Treatment C was significantly higher than other treatments which showed that the degree of hydrolysis protein hydrolysate of all different treatments made a significant difference ($p<0.05$).

The muscle cell membranes tend to round up and form insoluble vesicles during hydrolysis, leading to the removal of membrane-structured lipids [28]. The inconsistent degree of hydrolysis along the hydrolysis may be due to reduced enzyme activity due to the exhaustion of the enzyme as a substrate over time. In addition, prolonged hydrolysis may denature molecules of protein. The decrease in DH could be

due to the denaturation of protein molecules, subsequently reducing its biological activities [29].

Antioxidant activity (AOA)-Scavenging effect on DPPH free radical

The results showed that DPPH scavenging activity in treatment B (27.2%) followed by treatment C (22.5%), treatment A (21.6%) and treatment D (13.2%), respectively. Yet no significant was recorded in treatment A, B and C, furthermore, treatment D significant the lowest. This might be because of the denaturation of amino acids that can inhibit DPPH or cleaving of peptides into amino acids [12]. A study by [30] reported a higher DPPH value (56.78%) were observed in methanolic extract *C. ensiformis* seeds. Antioxidant production is affected by changes in length, rate and composition of free amino acids and small peptides [14]. Peptides composed of hydrophobic and aromatic amino acids can effectively scavenge the radical DPPH [15]. They also reported that *Canavalia gladiata* (59.23 µg/ml), exhibited higher DPPH value that of *C. ensiformis* (34.35 µg/ml), suggesting higher concentration of flavonoids and phenolic compounds, will exhibit stronger antioxidative activity. The different results compared to *C. ensiformis* protein hydrolysate because of the different presence of amino acid and peptides in different beans [12].

Physical properties of powder hydrolysate

Microstructure of powdered *C. ensiformis* protein hydrolysate

Figure 1 presents the microstructure of powder protein hydrolysate extracted from *C. ensiformis* in different pre-treatments solutions. Figure 1 illustrates the image of *C. ensiformis* protein hydrolysate with 5000X magnification. All of the *C. ensiformis* protein hydrolysate at different hydrolysis times exhibited irregular shape. This might be due to the effect of the freeze drying process that causes the change of samples into powder form. The freeze dried protein hydrolysate can be referred to as "collapse-building" shape [31]. The irregular shape might influence the functional properties of the sample like absorbing oil and water in its molecule where it also might help in exposing more amino acids in outwards to entrap water and oil molecules. The changes in morphological characters may affect the functional properties of protein hydrolysates [15].

The protein hydrolysate D pre-treatment microstructure is larger in size than other pre-treatments. The size and structure had a major impact on the hydrolysate protein's functional properties. In pre-treatment D, the protein hydrolysate microstructure is greater in size while in pre-treatment B, protein hydrolysate is smaller in size. Drying protein does, however, cause few stresses that can denature protein by modifying protein structures [32]. Pre-treatment A was more compact and clumped together where it may also suggest that other bonds, such as covalent, hydrogen and disulfide, still bind the protein molecules together even after the soaking process. These characteristic compacts and clumps together play an important role in the formation of gel [33]. Furthermore, the smooth ellipsoids surface of pre-treatment B of *C. ensiformis* protein hydrolysate can cause rupture because the structure of starch granules with amorphous and crystalline areas was described as semi-crystalline, causing oxidation and acetylation to strike the amorphous area, separating the region from the starch layer, resulting in the breakup of the granules [34]. Overall, the morphological structure such as the size and shape of *C. ensiformis* protein hydrolysate might have a great influence on functional properties.

Functional groups of *C. ensiformis* protein hydrolysate

Fourier Transform Infra-Red (FTIR) was used to test changes in the frequency of vibration in the functional groups of the *C. ensiformis* protein hydrolysate. The spectra of *C. ensiformis* powder protein hydrolysate were measured within the 500-4000 cm^{-1} wavenumber range by the FTIR spectrometer. The FTIR spectrum reveals the dynamic structure of the adsorbent due to the presence of a greater number of peaks (Figure 2). Table 2 shows a peak at 3500-3200 cm-1 (Region 1) for the presence of hydroxyl in flavonoids, 3200-2800 cm^{-1} (Region 2) for the presence of C-H groups, 1800-1600 cm-1 (Region 3) for the C = O group, 1900-1500 cm^{-1} (Region 4) for the C = C groups, 1300-800 cm^{-1} (Region 5) for the C-O group and 1300-900 cm^{-1} (Region 6) for the fingerprint area. Protein hydrolysate presents three area wavenumbers where Amide I (1700–1600 cm^{-1}) induced by C = O stretching vibration of the protein component, Amide II (1560–1520 cm^{-1}) occurs mainly from C-N stretching with N-H bending vibrations of the protein backbone, and Amide III (1240–1430 cm−1) bands are formed by C-N stretching [35]. The Amid I band is the most sensitive IR spectral region to predict protein secondary structural components and is largely attributed to C = O stretching vibrations (about 80 percent) with certain N-H bending and C-H stretching modes in the plane. In particular, C = O stretching protein vibrations depend primarily on different secondary structures and intra-or inter-molecular effects, including hydrogen bonding patterns and molecular geometry [36].

Water and oil holding capacity of powdered protein hydrolysate

Water holding capacity (WHC) and oil holding capacity (OHC) are important features to evaluate the texture properties of food products such as juiciness, mouthfeel and tenderness. Water holding capacity is the absorption capacity possessed in protein compounds [37]. Data in Table 3 showed WHC there was a significant difference ($p<0.05$)

between *C. ensiformis* proteins that soaked in different solutions.

Protein hydrolysate in treatment C showed the highest water holding capacity (71.2%) as compared to other samples. However, the water holding capacity was lower in treatment A (39.7%). This could be due to the high concentration of polar groups present in the sample. The interaction between protein and water also depends on the sample's polar groups and hydrophilicity and several studies have shown that protein hydrolysis with the increased availability of polar groups can have a good impact on WHC [27]. The sudden increase in WHC can occur as hydrophilic amino acid balances with hydrophobic amino acids. The WHC ultimately depends on the relationship between protein and water, the size of the protein molecule and the equilibrium between hydrophilic and hydrophobic groups [12].

Oil holding capacity (OHC) is known as the amount of oil that protein retains where the oil linked to the hydrophobic interaction of oil and protein [38]. Table 3 data showed that there was a significant difference between *C. ensiformis* proteins in different pre-treatments. *C. ensiformis* protein hydrolysate in treatment B shows the highest OHC (1.5%) compared to other treatments because more hydrophobic classes allow to the physical imprisonment of oil [39]. Protein hydrolysate in treatment A shows the lowest oil holding capacity (0.5%) might happen because of the releases of hydrophobic groups from the hydrolysis process. This illustrates that OHC is heavily dependent on the release from the hydrolysis phase of hydrophobic groups. He et al. [40] reported that OHC origin was due to the combination of oil physical entanglement and substance hydrophobicity.

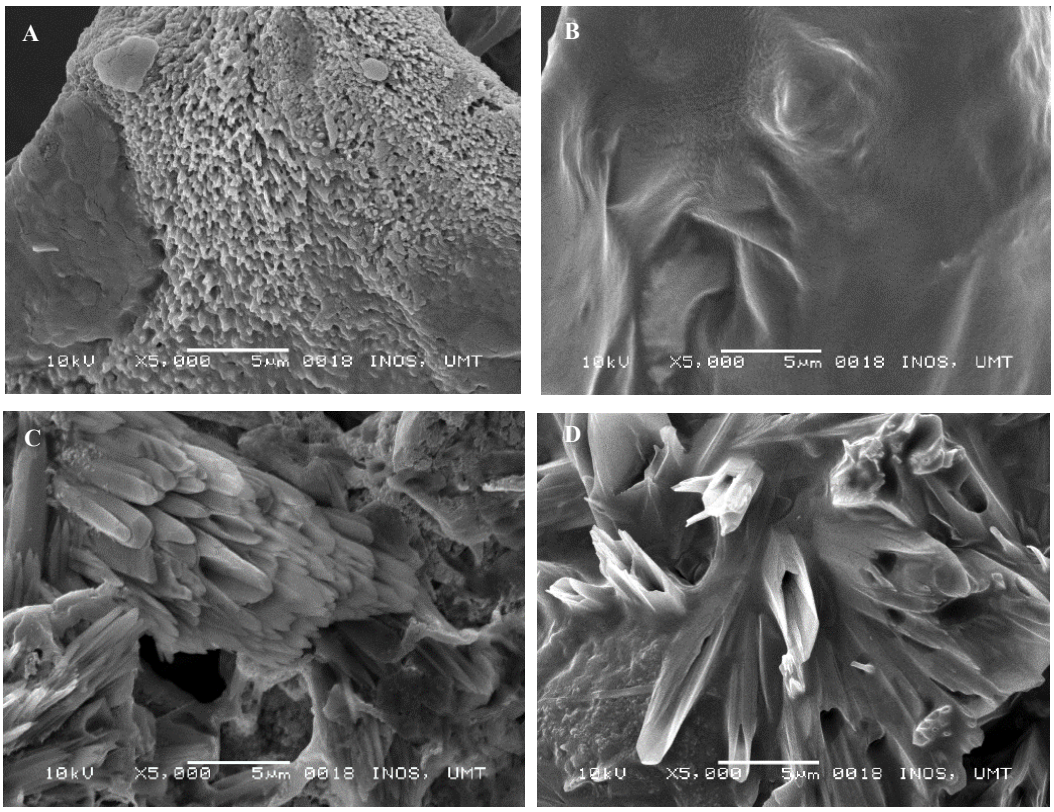

Figure 1. SEM images of powdered protein hydrolysate. A= lime juice, B=in 50% w/v tamarind solution (1:2) for 15 min and rinsed in distilled water, C=3% salt solution (1:3) and 50% (w/v) activated carbon solution (1:2), D= in distilled water (1:2) as control.

Table 2: FTIR region for *C. ensiformis* powdered protein hydrolysate

A	B	C	D	Functional group	Mode of vibration
3385.54	3415.99	3455.33	3451.98	Hydroxyl compound, alcohol, phenols	H-bonded, O-H stretch
1654.28	1601.66	1636.69	1636.89	Ketone compound amide (I)	C=O stretching
-	1399.09	-	-	Phenol or tertiary alcohol	O-H bend, Alcoholic group
1077.25	1072.58	1073.06	1080.85		C-O
					Ref: (Santosh et al., 2013)

Note:
A= lime juice, B=in 50% w/v tamarind solution (1:2) for 15 min and rinsed in distilled water,
C=3% salt solution (1:3) and 50% (w/v) activated carbon solution (1:2), D= in distilled water (1:2) as control.

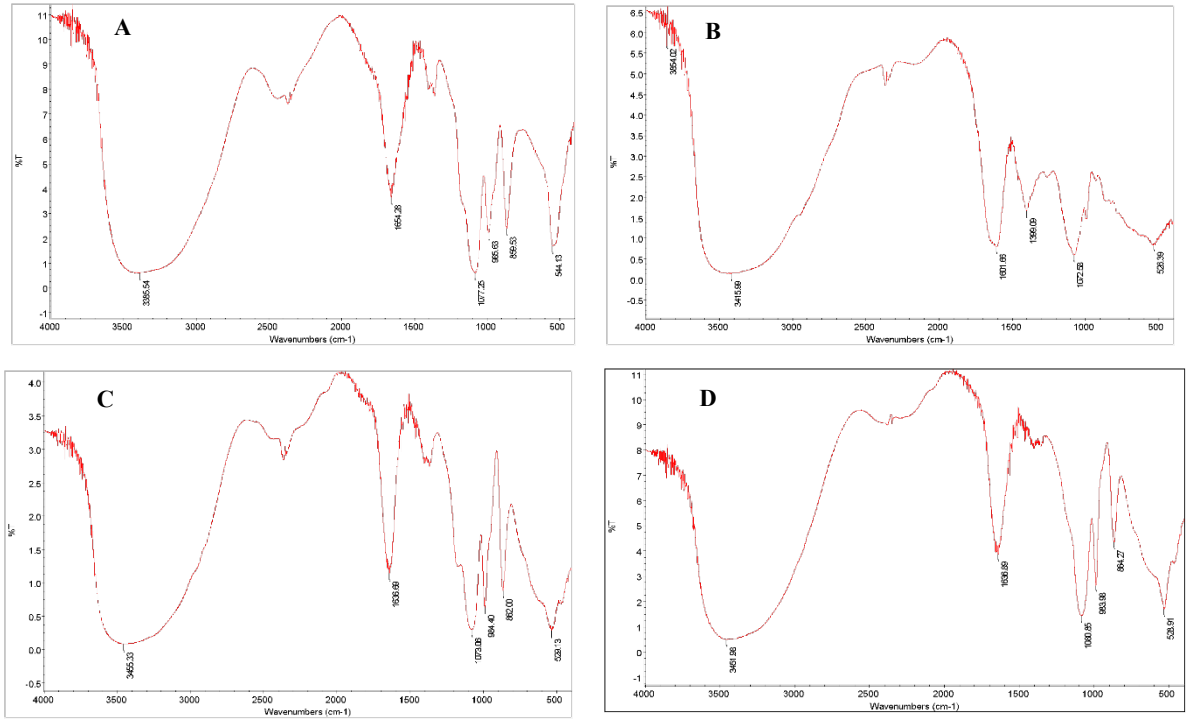

Figure 2. FTIR of *C. ensiformis* powdered protein hydrolysate
Note:
A= lime juice, B=in 50% w/v tamarind solution (1:2) for 15 min and rinsed in distilled water,
C=3% salt solution (1:3) and 50% (w/v) activated carbon solution (1:2), D= in distilled water (1:2) as control.

Table 3: Water holding capacity, oil holding capacity, foaming stability, foaming capacity, solubility and pH value of *C. ensiformis* protein hydrolysate

Sample	WHC (%)	OHC(%)	Foaming capacity	Foaming Stability	Solubility	pH
A	39.74±1.76[b]	0.46±0.01[d]	7.00±1.41[ab]	82.29±1.47[a]	0.89±0.01[b]	7.10±0.05[b]
B	40.18±0.86[b]	1.49±0.01[a]	5.75±1.06[b]	73.46±4.91[a]	1.02±0.02[b]	5.65±0.07[d]
C	71.19±0.48[a]	0.51±0.01[c]	4.00±0.71[b]	74.61±4.49[a]	5.15±0.01[a]	7.55±0.11[a]
D	44.22±0.07[b]	0.96±0.01[b]	9.25±1.06[a]	83.24±9.57[a]	1.07±0.02[b]	6.91±0.12[c]

All values given are means of triplicate analysis express as mean ± SD is included for each average. Means with different letter are significantly different ($p < 0.05$) within the same column.
Note:
A= lime juice, B=in 50% w/v tamarind solution (1:2) for 15 min and rinsed in distilled water,
C=3% salt solution (1:3) and 50% (w/v) activated carbon solution (1:2), D= in distilled water (1:2) as control.

Foaming capacity and foam stability of powdered protein hydrolysate

Protein foaming properties are typically characterized as the ability to foam (FC) and the ability to foam (FS). When whipped, FC is expressed in volume and the volume of the foam over time (normally 0-30 min) gives FS to the protein [41]. Table 3 also showed that the foaming capacity and foam stability of *C. ensiformis* protein hydrolysate in different treatment solutions exhibited a significant difference ($p < 0.05$) among all of the treatments. Treatment D showed the highest foaming capacity (9.3%), followed by treatment A (7%). This might be because a reduction in molecular size causes the inability to form or retain foam [12]. Again, treatment D exhibited the highest foaming stability value (83%) followed by treatment A (82%). This could be explained by the presence of interaction between the protein of the sample within the matrix in different soaking treatments [42]. Solubility was positively correlated with foaming capacity, which indicates that as more protein can migrate to the air-water interface, more foam will be formed [43].

Solubility of *C. ensiformis* protein hydrolysate

Treatment C (5.2%) was found to possess significantly the highest solubility followed with treatment D (1.1%), treatment B (1.0%) and treatment A (0.9%) (Table 3). This might be due to the difference in solubility which was related to the difference in protein surface charges by the exposure / forming of charged residues following the structural transformations of proteins due to deprotonation [44]. Similarly, Rapeseed hydrolysates has been found to exhibit more than 90% solubility at pH 5–9 [45]. The production of hydrolysates that are soluble at acidic pH is essential for the supplementation of fruit juices and acidic drinks. Protein solubility depends on the pH and ionic strengths, whereas protein processing history has a major influence on this property [41].

pH of *C. ensiformis* protein hydrolysate

Generally, a low pH was observed for the samples, which could be attributed to the acidic agent used for the pre-treatments. The pH values were observed in the range 5 to 7 (Table 3). Treatment C was observed as the highest pH (7), even though the treatment solutions were set at pH 5.52 after treating the salt solution. This result is in contrast with the work carried out by Claver et al. [29]. They found that the solubility of wheat protein hydrolysates was strongly influenced by pH, with the lowest and highest solubility at pH 4 and 6, respectively.

Colour profile of *C. ensiformis* protein hydrolysate

In order to evaluate how hydrolysis influences the colour of the hydrolysates, lightness (L*), redness (a*) and yellowness (b*) of the powders were measured. The experimental L*, a* and b* mean values have been shown in Table 4. Treatment B exhibited higher lightness value than other treatments ($p < 0.05$), with lower a* and b* values ($p < 0.05$). Colour data showed that protein hydrolysate in treatment B has a more yellowish colour and is darker than treatment A. While for treatment C and D showed that the protein hydrolysate has the same light yellowish colour.

Table 4: Colour profile of *C. ensiformis* powdered protein hydrolysate

Sample	L*	a*	b*
A	33.19±0.23[b]	0.96±0.04[b]	0.74±0.03 [c]
B	47.00±0.26[a]	-0.35±0.06[d]	4.03±0.03 [b]
C	30.98±0.10[c]	0.68±0.04[c]	-0.27±0.02 [d]
D	30.73±0.16[c]	10.52±0.01[a]	23.63±0.16 [a]

Mean value with same letter in a same column are not significantly different at p>0.05
Note: A= lime juice,
B=in 50% w/v tamarind solution (1:2) for 15 min and rinsed in distilled water,
C=3% salt solution (1:3) and 50% (w/v) activated carbon solution (1:2), D= in distilled water (1:2) as control.

CONCLUSION

The best pre-treatment (soaking solution) of protein hydrolysate from *C. ensiformis* with the best physical properties, water and oil holding capacity, emulsifying stability index (ESI), solubility and antioxidant properties (DPPH) were found to be in treatment C (salt and activated carbon). Different properties of *C. ensiformis* protein hydrolysate was observed under scanning electron microscopes. FTIR analysis confirmed that the size and structure of the hydrolysate of all treatments were irregular in shape, like "collapse building". This study shows that pre-treatment in salt and activated carbon was the best technique to extract a good quality *C. ensiformis* protein hydrolysate that can be used in food or pharmaceutical products, thereby encouraging its use of this underexploited legume.

ACKNOWLEDGMENTS

This work was funded by Talent and Publication Enhancement Research Grant (TAPE-RG) (UMT/CRIM/2-2/2/14 Jld.4) from UMT, as well as CW Agro Sdn. Bhd. for providing the *C. ensiformis* seeds.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in conducting this study.

REFERENCES

1. Tiwari, B., Henchion, M., Mullen, A., Fenelon, M. and Hayes, M. (2017). Future Protein Supply and Demand: Strategies and Factors Influencing a Sustainable Equilibrium. *Foods*. **6**(7), 53.
2. Maphosa, Y. and Jideani, V. A. (2017). The role of legumes in human nutrition. IntechOpen. https://doi.org/10.5772/intechopen69127.
3. Sridhar, K. R. and Seena, S. (2006). Nutritional and antinutritional significance of four unconventional legumes of the genus Canavalia – A comparative study. *Food Chemistry*. **99**(2), 267–288.
4. Tantan, W., Kastaman, R., Setiasih, I. S. and Muhaemin, M. (2014). Reduction model of cyanide and protein content on the Jackbeans using CMS Method (Circulation Mixing System). Conference: International Conference "Food for a good life, Jakarta, Indonesia.
5. Rao, Q., Klaassen Kamdar, A. and Labuza, T. P. (2016). Storage stability of food protein hydrolysates-A review. *Critical Reviews in Food Science and Nutrition*. **56**(7), 1169–1192.
6. Kumar, A. K., and Sharma, S. (2017). Recent updates on different methods of pretreatment of lignocellulosic feedstocks: A review. *Bioresources and Bioprocessing*. **4**(1).
7. Wyman, C. E. (1999). Biomass ethanol: Technical progress, opportunities, and commercial challenges *Annual Review of Energy and the Environment*. **24**, 189–226.
8. Khattab, R.Y. and Arntfield, S.D. (2009). Nutritional quality of legume seeds as affected by some physical treatments 2. Antinutritional factors. *LWT - Food Science and Technology*. **42(**6), 1113–1118.
9. Wang, N., Hatcher, D. W., Tyler, R. T., Toews, R. and Gawalko, E. J. (2010). Effect of cooking on the composition of beans (*Phaseolus vulgaris* L.) and chickpeas (*Cicer arietinum* L.). *Food Research International*. **43**(2), 589–594.
10. Gowen, A., Abu-Ghannam, N., Frias, J. and Oliveira, J. (2007). Modelling the water absorption process in chickpeas (*Cicer arietinum* L.)-The effect of blanching pre-treatmenton water intake

and texture kinetics. *Journal of Food Engineering*. **78**, 810–819

11. Messina, V. (2014). Nutritional and health benefits of dried beans. *The American Journal of Clinical Nutrition*. **100**, 437S-442S.
12. Zainol, M. K., Surianarayanan, S., Abdullah, M.A.A., Hasmadi, M. and Mohd Zin, Z. (2020). Effect of hydrolysis time on antioxidant and antimicrobial properties of Jack Bean (*Canavalia ensiformis*) protein hydrolysate. *Malaysian Journal of Biochemistry and Molecular Biology (MJBMB)*. **1**, 5-11.
13. Herpandi, H., Huda, N., Rosma, A., Wan Nadiah, W. (2012). Degree of hydrolysis and free tryptophan content of skipjack tuna (*Katsuwonus pelamis*) protein hydrolysates produced with different types of industrial proteases. *International Food Research Journal*. **19**(3), 863–867.
14. Chew, R.M., Mohd Zin, Z., Ahmad, A., Mohtar, N.F., Rusli, N.D. and Zainol, M.K. (2020). Physicochemical and sensory properties of deep fried battered squid containing Brownstripe red snapper (*Lutjanus vitta*) protein hydrolysate. *Food Research*. **4** (4), 1245 – 1253.
15. Hau, E. H., Mohd Zin, Z., Nasution, Z., Shaharudin, N. A. and Zainol, M. K. (2018). Physicochemical properties of powdered protein hydrolysate from Yellowstripe scad (*Selaroides leptolepis*) fish. *International Food Research Journal*. **25** (6), 2555-2561.
16. Robinson, H. W. and Hodgen, C. G. (1940). The biuret reaction in the determination of serum protein I A study of the condition necessary for the production of the stable color which bears a quantitative relationship to the protein concentration. *Journal of Biological Chemistry*. **135**, 707–725.
17. Liew, S.Y., Mohd Zin, Z., Mohd Maidin, N.M., Mamat, H. and Zainol, M.K. (2020). Effect of the different encapsulation methods on the physicochemical and biological properties of *Clitoria ternatea* flowers microencapsulated in gelatine. *Food Research*. **4** (4), 1098-1108.
18. AOAC International. (2007). Official Methods of Analysis of AOAC International. AOAC Official Method 990.26. 17th ed. Gaithersburg, MD. USA: AOAC International.
19. Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry*. **102**(4), 1317–1327.
20. Malik, N.H., Mohd Zin, Z., Abd Razak, S.B., Ibrahim, K. and Zainol, M.K. (2017). Antioxidative activities and flavonoids contents in leaves of selected mangrove species in Setiu Wetland extracted using different solvents. *Journal of Sustainability Science and Management. Special Issue*, **3**, 24-34.
21. Mamat, H., Akanda, J.M.H., Zainol, M.K. and Yu, A.I. (2018). The influence of seaweed composite flour on the physicochemical properties of muffin. *Journal of Aquatic Food Product Technology*. **27**(5), 635-642.
22. Ahmad, M. and Benjakul, S. (2011). Characteristics of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) as influenced by acid pretreatment and extraction time. *Food Hydrocolloids*. **25**(3), 381–388.
23. Binsi, P. K., Shamasundar, B. A., Dileep, A. O., Badii, F., & Howell, N. K. (2009). Rheological and functional properties of gelatin from the skin of Bigeye snapper (*Priacanthus hamrur*) fish: Influence of gelatin on the gel-forming ability of fish mince. *Food Hydrocolloids.* **23**(1), 132-145.
24. Leon, A., Angulo, I., Picard, M., Carré, B., Derouet, L. and Harscoat, J. P. (1989). Proximate and amino acid composition of seeds of Canavalia ensiformis . Toxicity of the kernel fraction for chicks. *Annales de zootechnie.* **38** (4), 209-218.
25. Stone, M. T. and Kozlov, M. (2014). Separating proteins with activated carbon. *Langmuir*. **30**(27), 8046–8055.
26. Naqash, S. Y. and Nazeer, R. A. (2011). Antioxidant and functional properties of protein hydrolysates from pink perch *(Nemipterus japonicus*) muscle. *Journal of Food Science and Technology.* **50**(5), 972–978.
27. Wouters, F. C., Blanchette, B., Gershenzon, J. and Vassão, D. G. (2016). Plant defence and herbivore counter-defense: benzoxazinoids and insect herbivores. *Phytochemistry Reviews.* 15(6), 1127–1151.
28. Thiansilakul, Y., Benjakul, S. and Shahidi, F. (2007). Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). *Food Chemistry*. **103** (4), 1385-1394.
29. Claver, I. P. and Zhou, H. (2005). Enzymatic hydrolysis of defatted wheat germ by proteases and the effect on the functional properties of resulting protein hydrolysates. *Journal of Food Biochemistry*. **29**(1), 13–26.
30. Vadivel, V., Cheong, J. N. and Biesalski, H. K. (2012). Antioxidant and type II diabetes related enzyme inhibition properties of methanolic extract of an underutilized food legume, *Canavalia ensiformis* (L.) DC: Effect of traditional processing methods. *LWT - Food Science and Technology*. **47**(2), 255–260.

31. Qiang, M. R., Song, L., Chen, F. H., Li, M. Z., Liu, X. X. and Wang, Q., 2013. A 16-ka lake-level record inferred from macrofossils in a sediment core from Genggahai Lake, northeastern Qinghai-Tibetan Plateau (China). *Journal of Paleolimnology*. **49**, 575-590.
32. Joshi, M., Adhikari, B., Aldred, P., Panozzo, J. F. and Kasapis, S. (2011). Physicochemical and functional properties of lentil protein isolates prepared by different drying methods. *Food Chemistry.* **129**(4), 1513–1522.
33. Nieto-Nieto, T. V., Wang, Y. X., Ozimek, L. and Chen, L. (2014). Effects of partial hydrolysis on structure and gelling properties of oat globular proteins. *Food Research International.* **55***,* 418–425.
34. Adebowale, K. O., Afolabi, T. A. and Olu-Owolabi, B. I. (2006). Functional, physicochemical and retrogradation properties of sword bean (*Canavalia gladiata*) acetylated and oxidized starches. *Carbohydrate Polymers*. ***65***(1), 93–101.
35. Zhao, F., Zhang, D., Li, X. and Dong, H. (2018). High-pressure homogenization pretreatment before enzymolysis of soy protein isolate: the effect of pressure level on aggregation and structural conformations of the protein. *Molecules*. **23**(7), 1775.
36. Kong, J. and Yu, S. (2007). Fourier Transform Infrared Spectroscopic Analysis of Protein Secondary Structures. *Acta Biochimica et Biophysica Sinica.* **39**(8)*,* 549–559.
37. Taheri, A., Anar, S. A. A., Ahari, H. and Fogliano, V. (2012). Comparison the functional properties of protein hydrolysate from poultry byproducts and rainbow trout. *Iranian Journal of Fisheries Sciences*. **12**(1), 154–169.
38. Tang, L., Sun, J., Zhang, H.C., Zhang, C.S., Yu, L.N., Bi, J., Zhu, F., Liu, S.F. and Yang, Q.L. (2012). Evaluation of physicochemical and antioxidant properties of peanut protein hydrolysate. *PLoS One* **7**. e37863.
39. Zhang, S.B., Wang, Z., Xu, S.Y. and Gao, F.X. (2009). Purification and characterization of a radical scavenging peptides from rapeseed protein hydrolysates. *Journal of AOCS*. **86**, 959–966.
40. He, S., Franco, C. and Zhang, W. (2013). Review: Functions, applications and production of protein hydrolysates from fish processing co-products (FPCP). *Food Research International.* **50**, 289-297.
41. Boye, J. I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E. and Rajamo- hamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International.* **43**, 537-546
42. Kempka, A.P., Honaiser, T.C., Fagundes, E. and Prestes, R.C. (2014). Functional properties of soy protein isolate of crude and enzymatically hydrolysed at different times. *International Food Research Journal*. **216**, 2229–2236.
43. Stone, A.K, Karalash, A, Tyler, R.T., Warkentin, T.D. and Nickerson, M.T. (2015) Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. *Food Research International*. **76**, 31–38.
44. Tang, C. H. and Sun, X. (2011). A comparative study of physicochemical and conformational properties in three vicilins from Phaseolus legumes: implications for the structure-function relationship. *Food Hydrocolloids*. **25**, 315-324.
45. Yoshie-Stark, Y., Wada, Y. and Wäsche, A. (2008). Chemical composition, functional properties, and bioactivities of rapeseed protein isolates. *Food Chemistry*. **107**, 32–39.