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Isolation and characterization of protein isolated from defatted cashew nut shell: Influence of pH and NaCl on solubility and functional properties

Maria Yuliana [a], Chi Thanh Truong [b], Lien Huong Huynh [b], Quoc Phong Ho [b], Yi-Hsu Ju [a,*]

[a] Department of Chemical Engineering, National Taiwan University of Science and Technology, 43, Keelung Road, Section 4, Taipei 10607, Taiwan
[b] Department of Chemical Engineering, Cantho University, 3-2 Street, Cantho City, Viet Nam

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ABSTRACT

This work reports the isolation of protein from defatted cashew nut shell (CNS), with the crude protein product containing 91.07% protein. Under its natural conditions, the solubility of this protein isolate is comparable (74.02%) to that of mustard green meal protein. The solubility of the protein isolate decreases with decreasing pH, with the minimum solubility observed at its isoelectric point (pH 3). The water holding capacity, oil holding capacity, foaming capacity, foam stability, emulsifying capacity and emulsion stability were found to be 2.56 cm^3 H_2O/g protein, 4.28 cm^3 oil/g protein, 76.88%, 70.98%, 62.0% and 79.0%, respectively. The profiles of these functional properties were determined with varying pH values and NaCl concentrations, and improved properties were observed in the alkaline pH range and in the presence of NaCl. Electrophoretic analysis showed that the high molecular weight protein globulin was the major protein in the protein isolate.

1. Introduction

Production of high protein food from under-exploited sources is one response to the growing protein malnutrition in developing countries. Several new protein sources have been identified, such as cashew nut protein isolate (Deng et al., 2011; Ogunwolu, Henshaw, Mock, Santros, & Awonorin, 2009) and milkweed seed protein isolate (Mila, Roque, & Wuc, 2009). The annual production of cashew nuts (with shells) is the highest of all tree nuts, with a value of more than 3.5 million tons (FAOSTAT, 2009). Cashew nut shell (CNS) is a by-product of cashew nut production and is a source of unsaturated long-chain phenols such as anacardic acids, anacardols and cardols. After removing lipids and phenols, the residue (60% of CNS) has a protein content of 26.17% (Yuliana, Huynh, Ho, Truong, & Ju, 2012). The use of CNS as a protein source not only helps to reduce waste from cashew nut production but also represents a low-cost protein source for human consumption.

Plant protein should ideally possess several desirable physicochemical and functional characteristics such as solubility, foaming and emulsification, water and oil binding capacity and gelation (Wang & Kinsella, 1976). These intrinsic properties affect the behavior of proteins in foods during processing, manufacturing and storage (Kinsella, 1979). Therefore, it is important to study the relationships between functional properties and the environment of the protein such as pH and ionic strength to enable the effective utilization of low-cost proteins (Aluko & Yada, 1995; Myers, 1988).

The isolation and characterization of proteins from defatted CNS have not previously been studied. The aim of this study was to isolate protein from defatted CNS, characterize the protein isolate by studying its chemical composition, and monitor the effects of environmental conditions such as pH and NaCl concentration on its functional properties.

2. Materials and methods

2.1. Materials and chemicals

CNS was obtained from the waste of cashew nut (variety *Venguria-4*) production in a factory in Solo, Indonesia. The nuts were ground, sieved, and stored at −4 °C. Defatted CNS was obtained by subjecting CNS to soxhlet extraction using methanol for 10 h followed by *n*-hexane for another 10 h.

Abbreviations: CNS, cashew nut shell; WAC, water absorption capacity; OAC, oil absorption capacity; FC, foaming capacity; FS, foam stability; EC, emulsifying capacity; ES, emulsion stability; LGC, lowest gelation concentration; pI, isoelectric point; MW, molecular weight.

* Corresponding author. Tel.: +886 2 2737 6612; fax: +886 2 2737 6644.
E-mail address: yhju@mail.ntust.edu.tw (Y.-H. Ju).

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Methanol (99.5% purity) and ethanol (95% purity) were purchased from Echo Chemical (Miao Li, Taiwan) while *n*-hexane (95% purity) was obtained from Tedia (OH, USA). Hydrochloric acid (37% purity) was provided by Thermo Fisher Scientific (MA, USA). Deionized water was supplied from a mixed bed deionizer, model MB18-PVN/m9060 (Pure Aqua Inc., Santa Ana, CA, USA).

The chemicals used in protein functional analysis were obtained from Sigma Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), protein assay dye reagent concentrate and SDS-PAGE analysis kits were purchased from Bio-Rad (CA, USA).

2.2. Protein isolation from defatted CNS

Protein was isolated from defatted CNS following the method of Chavan, McKenzie, and Shahidi (2001). CNS (10 g) was soaked in water (90 g) and stirred for 1 h at 30 °C. The mixture was then centrifuged (1500×*g*, 30 min) and the supernatant was decanted. The residue was extracted consecutively by a 0.15 mol dm^{-3} NaCl solution, ethanol solution ($\varphi_{ethanol} = 0.7$) and 0.2 mol dm^{-3} NaOH solution under the same conditions. Extraction and centrifugation were repeated twice. All supernatants were combined, adjusted to pH 4.4, and left to stand for 1 h. The precipitate was recovered by centrifugation (1500×*g*, 30 min) and freeze dried (Labconco Free Zone 2.5 Benchtop freeze dryer model 7670520, Kansas City, MO). The dried protein was ground and stored at −20 °C.

2.3. Chemical composition of protein isolate

Protein and ash contents were determined by AOCS Official Methods Ba 4a-38 (1997) and Ba 52-49 (1997), respectively. Total dietary fiber (TDF) was analyzed using the modified method described by Fabian, Ayucitra, Ismadji, and Ju (2011). TDF content was measured as the residue weight minus the protein and ash weight. Retained starch, expressed as soluble sugar, was analyzed by the modified AOAC Official Method 996.11 (1996) using 3,5-dinitrosalicylic acid (DNS) as the reagent.

2.4. Protein solubility

Protein solubility was analyzed according to the method of Cepeda (1998). The protein sample (125 mg) was dissolved in NaOH solution (20 cm^3, 0.1 mol dm^{-3}) and stirred at 30 °C for 1 h. The pH of the mixture (10 cm^3) was adjusted using 1.0 mol dm^{-3} HCl solution or 1.0 mol dm^{-3} NaOH solution. The mixture was then centrifuged (1500×*g*, 15 min). The protein content of the supernatant was analyzed using the Bradford method. The protein solubility was also analyzed at its pI at various NaCl concentrations (0, 0.5, 1.0, 1.5, 2.0 mol dm^{-3}).

2.5. Functional properties of the protein isolate

2.5.1. Water absorption capacity (WAC)

The WAC of the protein was measured using a modified version of the method of Lopez, Falomir, and Olivares-Vasquez (1991). Protein isolate was vigorously mixed with water (1:10, w/w) for 5 min. The slurry was then centrifuged (1500×*g*, 30 min). The supernatant was weighed and WAC was determined.

2.5.2. Oil absorption capacity (OAC)

The OAC of the protein isolate was determined using the method of Chavan et al. (2001). Protein isolate (0.5 g) was mixed with castor oil (5 cm^3) for 1 h at 30 °C. The mixture was centrifuged (1500×*g*, 30 min) and the oil was decanted. The oil trapped mixture was weighed. OAC was expressed as cm^3 of oil trapped per g protein isolate.

2.5.3. Foaming capacity (FC) and foam stability (FS)

FC and FS were studied using a method adopted from Sze-Tao and Sathe (2000). Protein isolate (250 mg) was mixed with water (250 cm^3) and the mixture was adjusted to the desired pH (2–11) and NaCl concentration (0, 0.5, 1.0, 1.5, 2.0 mol dm^{-3}). The mixture was blended for 5 min. FC was determined as the percentage volume increase due to blending. In measuring FS, the change in foam volume was monitored for 60 min and the FS was expressed according to the following equation.

$$\mathrm{FS}(\%) = \frac{\text{volume after 60 min standing} - \text{volume before blending}}{\text{volume after blending} - \text{volume before blending}} \times 100\%$$

2.5.4. Emulsifying capacity (EC) and emulsion stability (ES)

EC and ES were analyzed by the method of Chau and Cheung (1998) with minor modification. Protein isolate in water (1%, 5 cm^3) was vigorously blended with 5 cm^3 castor oil for 5 min. The mixture was then centrifuged (1500×*g*, 5 min). EC was determined by dividing the height of the emulsifying layer by the total height. The mixture was then heated at 80 °C for 30 min, followed by centrifugation (1500×*g*, 5 min). ES was expressed as the ratio of the height of the emulsifying layer after heating to that before heating.

2.6. Bulk density

The bulk density of the protein was measured following the method of Wang and Kinsella (1976). A graduated cylinder was weighed and the isolated protein was added until it reached 10 cm^3. The cylinder was tapped and the total weight was measured. Bulk density was calculated as the weight of 10 cm^3 of protein isolate.

2.7. Gelation properties

The gelation properties of protein isolates were determined using the method described by Coffman and Garcia (1977). Sample suspensions with a protein fraction ($x_{protein}$) of 0.02–0.2 were prepared with an interval of 0.02. The samples were heated for 1 h in boiling water and abruptly cooled in an ice bath, and then cooled further at 4 °C for 2 h. The lowest gelation concentration (LGC) was taken as the lowest concentration of the suspension when the sample from the inverted tube did not fall or slip.

The effect of pH on the sample gelation was investigated. The suspension was adjusted to the desired pH before heating. The effect of the NaCl concentration on gelation was also studied. The LSDs were determined as described above.

2.8. SDS-PAGE analysis

SDS-PAGE analysis was carried out using a modified version of the method of Shiu, Ju, Chen, and Lee (2013). The sample was prepared by dissolving the protein isolate in water (10 mg cm^{-3}) and adding 2-mercaptoethanol. The mixture was heated in boiling water for 10 min. SDS-PAGE electrophoresis was performed on a 0.12 g cm^{-3} acrylamide resolving gel and a 0.05 g cm^{-3} acrylamide stacking gel. Approximately 30 μl of the protein solution was loaded onto the gel. The pre-electrophoresis was conducted at 70 kV for 30 min, followed by 110 kV for 75 min. After the gel was soaked in ethanol-based Coomassie blue, protein bands were visualized by de-staining the background color of the gel using acetic acid in ethanol. Native-PAGE was carried out using the non-denatured protein solution.

3. Results and discussion

3.1. Chemical composition of the protein isolate

Four extraction agents (water, 0.15 mol dm^{-3} NaCl, a $\varphi_{ethanol} = 0.7$ ethanol solution and 0.1 mol dm^{-3} NaOH) were used to separate protein from the defatted CNS. Acid precipitation was used to separate proteins from other components such as carbohydrates and fine fiber. Approximately 86 g of crude protein product was recovered from 100 g of defatted CNS. The isolated crude protein product contained 91.07% protein, 2.34% fiber, 4.72% ash and 1.38% soluble sugar. The proportions of protein in the water, salt, alcohol and alkaline fractions were 53.12%, 7.65%, 11.43% and 27.80%, respectively. The ash content of this crude protein is considerably higher than that of cashew nut protein isolate, perhaps due to salt formation during precipitation at the pI (Chavan et al., 2001).

3.2. Effect of pH on the solubility and functional properties of the protein

The effect of pH on the solubility and functional properties of isolated protein is shown in Table 1. A minimum solubility of 48.44% was observed at pH 3, and the solubility increased rapidly with both increasing and decreasing pH. At pH 2 and 11, 82.31% and 84.75% protein were soluble, respectively. In general, the dependence of the solubility of this protein product on pH agrees with the observations of previous studies (Chau & Cheung, 1998; Shanmugasundaram & Venkataraman, 1989). Damodaran (1997) stated that the minimal protein solubility occurs at its pI and that the majority of food proteins are acidic, with minimum solubilities at pH 4–5 and maximum solubilities at alkaline pH. At low or high pH, proteins have either net positive or net negative charges, leading to an electrostatic repulsive force that helps to keep protein molecules apart, disrupting the native protein structure, shifting the equilibrium toward the unfolded form and subsequently exposing the buried functional groups in protein molecules, thus leading to an increase in protein solubility. In contrast, near the pI, proteins aggregate due to strong intermolecular interactions, resulting in less interaction with water and thus reducing protein solubility.

At neutral pH, the solubility of the protein isolated from CNS is comparable to that of mustard green meal (77.9%) (Aluko, McIntosh, & Katepa-Mupondwa, 2005) but higher than that of peanut protein isolate (60.5%) (Cherry, 1990) and soya protein isolate (71.7%) (Lin, Humbert, & Sosulski, 1974). The solubility of defatted CNS protein should be adequate for food formulation without requiring pH adjustment.

WAC profiles as a function of pH are shown in Table 1. At pH 3, the protein isolate has a minimum WAC of 1.2 cm^3 H_2O/g protein. Near the pI, proteins tend to aggregate due to strong intermolecular interactions, decreasing their interaction with water, and thus decreasing WAC. Higher WAC values were observed on either side of pI. WAC increased significantly as pH was increased from 3 to 8 and decreased slightly as the pH was increased further.

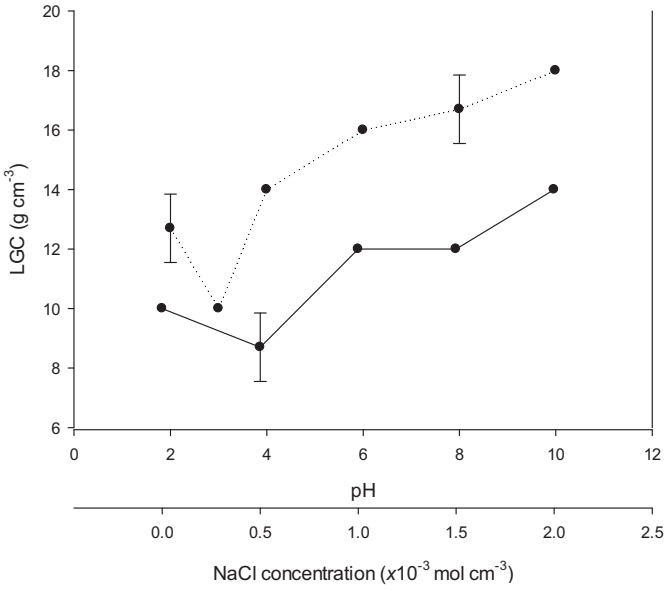

Fig. 1. Effect of pH and NaCl concentration on the lowest gelation concentration (LGC) of protein isolated from defatted CNS. (···●··· LGC (g cm^{-3}) vs. pH, —●— LGC (g cm^{-3}) vs. NaCl concentration (mol dm^{-3})).

The minimum FC occurs at pH 3 (28.65%) because of the behavior of proteins at their pIs, and FC increases with pH above the pI, with FC values ranging from 32.34% to 90.01% (Table 1). The increase in FC with pH is likely due to the increased net charges on the proteins, which weakened the hydrophobic interactions and increased the flexibility of protein. This allowed the protein to diffuse more rapidly to the air–water interface to encapsulate air particles, enhancing foaming. Deng et al. (2011) also mentioned that high protein solubility is required to increase FC and FS.

A similar trend was observed in the dependence of FS on pH. FS is the lowest (38.89%) at the pI (pH 3), while increasing the pH to 10 resulted in the highest FS. The high FS values at pHs above the pI could be attributed to the greater amount of solubilized proteins. This resulted in an increase in viscosity and facilitated the formation of a multilayer cohesive protein film at the interface. The increase in FS at higher pH may have been due to a decreased tendency for foam particles to coalesce as a result of the higher net negative charge of the protein. Deng et al. (2011) mentioned that the decrease in FS at pH > 10 might be due to ionic repulsion among peptides. Makri and Doxastaskis (2006) added that pH actually alters the structure of protein molecules, leading to differences in FC and FS.

Table 1 shows that pH has similar effects on the emulsifying properties of the protein isolate as it does on the foaming

Table 1
Effect of pH on the protein solubility and functional properties (WAC, FC, FS, EC and ES).[a]

Protein properties	pH									
	2	3	4	5	6	7	8	9	10	11
Solubility (%)	82.31	48.44	50.98	59.05	72.23	74.02	75.78	79.33	80.45	84.75
WAC (cm^3 H_2O/g protein)	2.13	1.20	1.50	2.03	2.49	2.56	2.97	2.88	2.78	2.75
FC (%)	60.13	28.65	32.34	48.77	67.91	76.88	83.45	89.43	90.01	86.51
FS (%)	52.39	38.89	40.98	50.57	65.77	70.98	76.43	83.88	84.51	83.75
EC (%)	54.0	40.0	42.0	54.0	58.0	62.0	64.0	68.0	70.0	66.0
ES (%)	44.0	50.0	62.0	68.0	75.0	79.0	80.0	82.0	84.0	85.0

[a] These results represent the means of three determinations. The SD values range from 0 to less than 5%, and thus are regarded as insignificant.

properties. The lowest EC value (40.0%) was found at the pI (pH 3), while the highest (70.0%) was observed at pH 10. The relationship between EC and pH is similar to that between protein solubility and pH. This agrees with the report of Lawal, Adebowale, Ogunsanwo, Sosanwo, and Bankole (2005). pH affects EC primarily by altering the charge distribution of protein molecules (Deng et al., 2011). At pH above 10, hydrophobic forces decrease as a result of the increased protein net charge and the increased flexibility. This enables proteins to rapidly diffuse to the air–water interface, resulting in poor EC.

ES was also observed to increase with increasing pH. The low stability of the emulsion under acidic conditions may be attributed to the increased interaction between emulsified droplets, thus facilitating protein aggregation and reducing ES. When the pH was increased toward alkaline values, coulombic repulsion increased between neighboring droplets and the hydration of the charged protein molecules increased. These factors reduced the interfacial energy and led to the coalescence of emulsion droplets (Chavan et al., 2001).

As shown in Fig. 1, the highest and lowest LGC values were observed at pH 10 and pH 3, respectively. At its natural pH, the LGC of the defatted CNS protein isolate was found to be 10%, comparable to that of lupine protein concentrate (12%) (Lqari, Vioque, Pedroche, & Millán, 2002) and that of wheat protein isolate (7.5%) (Schmidt, 1981). Electrostatic repulsion was minimal at the pI and may enhance the intermolecular forces among protein molecules, facilitating gelation.

3.3. Effect of NaCl concentration on protein solubility and functional properties

Table 2 shows the effect of NaCl concentration on protein solubility and functional properties. Protein solubility increased from 48.44% to 75.37% when the NaCl concentration was increased from 0 to 0.5 mol dm^{-3}. The addition of a small amount of NaCl induced salting-in, increasing protein solubility. However, further increases in NaCl concentration resulted in the interaction of negatively charged chloride ions with positively charged protein molecules, leading to a decrease in electrostatic repulsion, thus enhancing hydrophobic interactions.

WAC increased from 1.23 to 2.04 cm^3/g as the NaCl concentration was increased from 0 to 0.5 mol dm^{-3} and then decreased moderately with further increases in NaCl concentration. Lawal et al. (2005) stated that at low salt concentration, hydrated salt ions neutralize charges on the protein surface, reduce the ordered water around the protein and increase the system entropy. However, as salt concentration increases, much of the existing water is bound to the salt ion, thus enhancing the intermolecular interactions among proteins and leading to the dehydration of the protein and a reduction in WAC.

The effects of NaCl concentration on FC and FS are similar to the effects on other functional properties. The results show that FC, FS and protein solubility increased with increasing NaCl concentration up to 0.5 mol dm^{-3} due to the weakening of hydrophobic interactions. Increasing NaCl also increases the propensity for adhesion between protein molecules and results in the formation of interfacial protein layer with improved rheological properties, maintaining the integrity of the foam during mechanical whipping. Further increases in NaCl concentration had adverse effects on FC due to the salting-out effect. Lawal et al. (2005) explained that the ion screening effect at high salt concentrations improves the hydrophobic interactions of proteins and destroys protein films, promoting flocculation, aggregation and precipitation. On the other hand, further addition of NaCl improves the FS of the protein. This phenomenon is likely due to the increased solubility and surface activity of soluble protein.

As shown in Table 2, EC and ES increase rapidly with increasing NaCl concentration up to 0.5 mol dm^{-3}. This might be attributed to enhanced hydrophobic protein–protein interactions. This condition could favor emulsion by improving the rheological properties of the interfacial protein films that encapsulate the oil droplets. An increase in rheological strength could reduce the mechanical deformation and desorption of the interfacial protein, resulting in more emulsified droplets. The increase in ES may be due to the formation of charged layers around fat globules, resulting in mutual repulsion due to the formation of a hydrated layer around the interfacial material and retarding droplet coalescence (Kinsella, Damodaran, & German, 1985). However, at NaCl concentrations above 0.5 mol dm^{-3}, the salting-out effect resulted in a rapid decrease in EC and ES with increasing NaCl concentration.

LGC decreases with increasing NaCl concentration up to 0.5 mol dm^{-3} (Fig. 1). Protein gels are formed by intermolecular interactions, which produce a continuous three-dimensional network exhibiting structural rigidity. Cross-linking involves the formation of hydrogen bonds (Eldrigde & Ferry, 1954), disulfide bonds (Huggins, Tapley, & Jensen, 1951) and the exchange and formation of peptide groups (Bello, 1965). Low ionic strength enhanced the unfolding of buried functional groups within protein matrices, improving interactions among protein molecules. However, further increases in ionic strength caused salting-out, thus impairing gelation. Schmidt (1981) also stated that for a given type of protein, a critical concentration is required for gelation, and the type of gel varies with protein concentration. Considerably higher protein concentrations are usually required for the gelation of globular proteins.

Table 2
Effect of NaCl concentration on protein solubility and functional properties.[a]

Protein properties	Salt concentration (mol dm^{-3})				
	0	0.5	1	1.5	2
Protein solubility (%)	48.44	75.37	73.33	69.36	66.72
WAC (cm^3 H_2O/g protein)	1.23	2.04	1.92	1.78	1.73
FC (%)	28.65	81.34	80.66	79.15	76.53
FS (%)	56.03	65.94	74.82	75.09	76.91
EC (%)	42.0	72.0	66.0	62.0	56.0
ES (%)	50.0	88.0	72.0	68.0	66.0

[a] The results represent the means of three determinations. The SD values range from 0 to less than 5%, and thus are regarded as insignificant.

3.4. Oil absorption capacity (OAC)

At neutral pH and without NaCl addition, the OAC was found to be 4.28 cm^3 oil/g protein. This value is similar to the OAC of cashew nut protein isolate (4.42 cm^3/g) (Ogunwolu et al., 2009) and soybean protein isolate (4.88 cm^3/g) (Okezie & Bello, 1988). Kinsella (1976) stated that the oil binding mechanism can be explained as the physical entrapment of oil by capillary attraction.

3.5. Bulk density

The bulk density of the protein isolate was found to be 0.148 g cm^{-3}. This is lower than that of commercial soybean protein isolate (0.238–0.47 g cm^{-3}) (Aremu, Olaofe, & Akintayo, 2007; Chau & Cheung, 1998), comparable to that of the protein isolate of processed defatted fluted pumpkin seed flour (0.180–0.380 g cm^{-3}) (Fagbemi, Oshoudi, & Ipinmoroti, 2006), and higher than that of the peanut protein isolate of conarachin I (0.080 g cm^{-3}) and conarachin II (0.084 g cm^{-3}) (Monteiro & Prakash, 1994). Bulk density depends on interrelated factors such

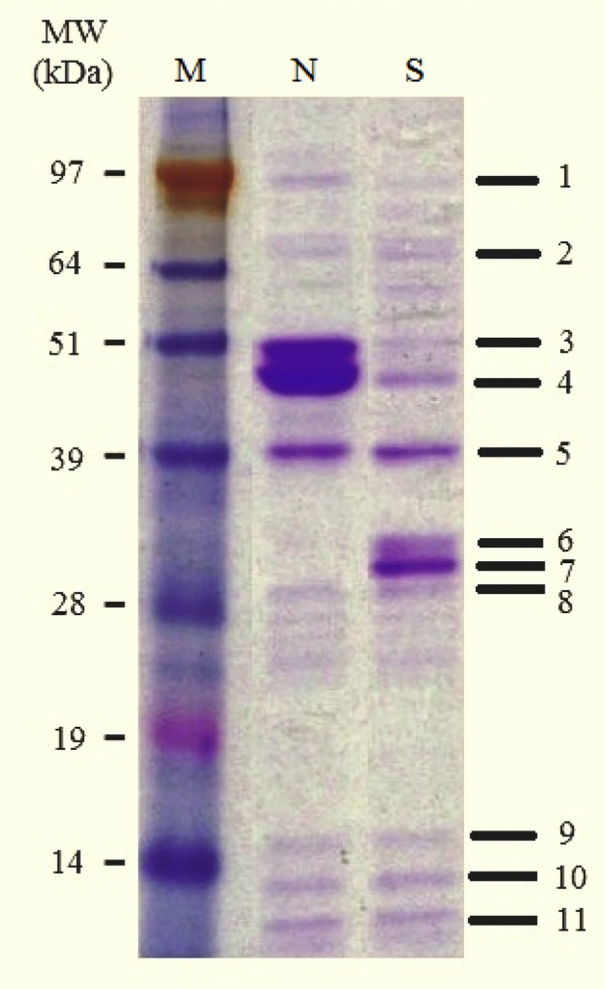

Fig. 2. Coomassie-stained SDS-acrylamide gel (0.12 g cm^{-3} acrylamide) containing protein isolated from defatted CNS. Left–right: Molecular weight (MW), Marker (M), Native-PAGE (N), SDS-PAGE (S).

as the intensity of attractive interparticle forces, particle size and the number of contact points (Peleg & Bagley, 1983).

3.6. SDS-PAGE

The MW distribution of the protein isolate is shown in Fig. 2. The protein bands range from less than 14–97 kDa and can be divided into 11 fractions. Fractions 1–4 are high-MW proteins, and the other seven fractions are low-MW proteins. Based on the Native-PAGE pattern, the high-MW protein fraction has a greater intensity than the low-MW fraction, and fractions 3 and 4 are the major fractions. The absence of fractions 3 and 4 and the presence of fractions 6 and 7 in the SDS-PAGE analysis indicated the dissociation of high-MW proteins to lower MW subunits by heating. According to El-Adawy (2000), the high-MW protein may be mostly globulin, while the other fractions consist of albumin, glutelin and gliadin.

4. Conclusion

A product with 91.07% protein content was successfully isolated from defatted CNS. The product also contains 2.34% fiber, 4.72% ash and 1.38% soluble sugar. The solubility of the protein isolated from defatted CNS was 74.02% at neutral pH and decreased with decreasing pH. The minimum solubility occurred at the pI of the protein isolate (pH 3). Other properties were investigated at the pI including WAC, OAC, FC, FS, EC and ES. Adjusting the pH and NaCl concentration can improve the characteristics of this protein product by affecting its solubility, water holding capacity, foaming and emulsifying properties. The PAGE results demonstrate that this protein product mainly contains high-MW protein.

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