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INDONESIAN FOOD AND NUTRITION PROGRESS

Indonesian Food and Nutrition Progress, 2014, Vol. 13, Issue 1

Isolation and Characterization of The Functional Properties of The Major Protein Fraction from *Nyamplung* (*Calophyllum inophyllum*)

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Received 26 May 2014; Accepted 15 August 2014; Published Online 10 September 2014

Abstract

Defatted *nyamplung* (*Calophyllum inophyllum*) seeds as by-products of oil extraction is a rich source of protein. In order to evaluate its potential as value-added of *nyamplung* seeds, *nyamplung* proteins were isolated by solubilization-precipitation method at pH 3 and 5. The obtaining protein isolates were characterized with respect to their functional properties, including water binding capacity, oil binding capacity, foaming capacity, foaming stability, emulsifying activity, emulsifying stability, gelation capacity, and amino acid composition. The results show that *nyamplung* protein could be considered as high protein quality because essential amino acids leucine (4.39 %), proline (4.22 %), valine (3.34 %), aspartic acid (3.23 %) and lysine (3.34 %) were found to be the major amino acids. Polar amino acids were higher than non-polar amino acid (1.7 times). With the consequence in higher ratio of water binding capacity to oil binding capacity (2.7 times) and high value of hydrophile-lypophile balance. In general, the isolated protein from precipitation at pH 3 (IP3) was found to have better functional properties than that being precipitated at pH (IP5), and showed excellent in water binding, emulsifying, gelation and foaming properties. In conclusion, IP3 can be utilized as high quality proteins and emulsifier in oil in water emulsion system.

Keywords: *nyamplung* (*Calophyllum inophyllum*), protein isolates, functional properties, water binding capacity, emulsifier

Introduction

Recently, *nyamplung* (*Calophyllum inophyllum*) is an oil seed, gains a big attention as a source of bio-fuel (Sudradjat, 2009; Venkanna and Reddy, 2009; Asralian, 2009). The oil content of *nyamplung* is 61-75% db (Crane et al., 2005; Bustomi et al., 2008; Asralian, 2009). In Indonesia, *nyamplung* can be found almost in all islands cover 480,000 ha with an annual production over 500,000 tons per year (Bustomi et al., 2008; Sudradjat, 2009). It grows in areas with annual rainfall ranging from about 1000 to 5000 mm.

The cakes from oil extraction, as a by-product, still have high concentration of protein (30%, unpublished data). It is very potential source for protein isolate, animal feed, fertilizer and chemical-based materials. Many studies on the characteristics and functional properties of proteins have been reported from various oilseeds, such as crambe seed (Massoura et al., 1998), soybean and lupin seed (Rodriguez-Ambris et al., 2005), sesame seed (Gandhi and Srivastava, 2007), rapeseeds (Yoshie-Stark et al., 2008), bayberry kernel (Cheng et al., 2009), sunflower seeds (Pickardt et al., 2009), and sweet lupin seed (Jayasena et al., 2010). However, the characteristics and functional properties of *nyamplung* protein isolate have not been explored yet. The process of isolation and fractionation of proteins might affect the functional properties of the proteins. In order to explore its potential as protein resources for industrial applications, a study was done to isolate the protein by solubilization of protein at pH 10 and further precipitation at pH 3 (IP3) or pH 5 (IP5). Protein isolates were evaluated with respect to the

chemical compositions and its functional properties including, water- and oil-binding capacity, foaming capacity, foaming stability, emulsifying capacity, emulsifying stability and gelation capacity.

Materials and Methods

Materials

Nyamplung seed and palm oil were provided from local suppliers in Yogyakarta, Indonesia. NaOH, HCl, methanol, triethylamine, sodium acetate, TRIZMA, and 2-mercaptoethanol were purchased from Merck KGaA (Darmstadt, Germany). Acetonitrile was purchased from Sigma-Aldrich Ltd (St. Louis, MO, USA).

Preparation of Sample

The seeds were shelled and the kernels were separated and crushed. The oil was extracted by hydraulic press, followed by solvent extraction using hexane. The defatted material was air-dried at ambient temperature and crushed again followed by sieving (80 mesh). The defatted flour was stored in a refrigerator at 7°C prior to analysis.

Isolation of Protein

Defatted *nyamplung* flour was dispersed in distilled water (1 : 20, w/v). The pH was adjusted to 11.0 using 1 N NaOH at 30 °C. After 2 h, it was centrifuged at 4000 g for 30 min. The supernatant was decanted. The residue was extracted again to obtain high yield of proteins. The supernatants were combined and separated into 2 parts. The proteins were precipitated by adjusting pH to 3.0 (IP3) and 5.0 (IP5), respectively. The precipitated proteins were recovered by centrifugation at 4000 g for 30 min. Protein curd was washed twice with distilled water and freeze-dried.

Composition Analysis

Concentration of water, ash, fat and crude protein were determined according to the method of standard Association of Official Analytical Chemists (AOAC, 1990).

Water and Oil Binding Capacity

Water and oil binding capacity were determined as described by Manak et al., (1980). One g of protein isolate was added into 10 mL of distilled water and palm oil in a weighted centrifuge tube for determination of binding capacity of water and oil, respectively. The mixture was homogenized for 30 s every 5 min using a Vortex stirrer. After 30 min, the tubes were centrifuged at 4000 rpm for 20 min. The free water or palm oil was decanted. The amount of bound water or oil was measured by weighing. The binding capacity of water or oil was expressed as the amount of water or oil retained per 100 g of proteins.

Foaming Capacity and Foam Stability

The foaming capacity (FC) and foam stability (FS) of the protein isolates were determined as described by Sathe et al., (1982). For FC determination, protein solution was prepared by adding protein isolate into distilled water (1% w/v). The pH was adjusted to 7 by 1 N NaOH. It was further homogenized using Nissei AM 10 homogenizer at 10,000 rpm for 5 min. The foam volume was determined. FC values were calculated using Eq. (1):

$$FC = \frac{foam\ volume\,(ml)}{total\ \ volume\ of\ \ suspension\,(ml)} \times 100 \quad (1)$$

On the other hand, FS was evaluated over a period of 2 h and determined base on the remained foam volume at 15, 30, 60, 90 and 120 min. It was calculated using Eq. (2):

$$FS = \frac{foam\ volume\,(ml)\ at\ time\ t}{initial\ \ \ foam\ volume\,(ml)} \times 100 \quad (2)$$

Emulsifying Activity and Emulsion Stability

Emulsifying capacity (EA) and emulsion stability (ES) were determined as described by Naczk et al., (1985) with slightly modification. Protein isolate was added into distilled water (1 % w/v). The pH was adjusted to 7 by 1 N NaOH. The solution was homogenized at 10,000 rpm using Nissei AM 10 homogenizer. Five mL of palm oil was added gradually to the solution with continuous stirring. Another 5 mL of oil was added. Volume of the emulsion layers was determined. EA was calculated using Eq. (3):

$$EA = \frac{volume\ of\ \ emulsion\ layer\ \ (ml)}{total\ \ volume\ of\ \ suspension\,(ml)} \times 100 \quad (3)$$

The ES was determined by heating the emulsion, as prepare before, for 15 min at 85 °C, followed by cooling and centrifugation at 3,000 g for 5 min. The emulsion stability was expressed as the percentage of remained emulsifying activity after heating at pH 7.

Least Gelation Concentration

The least gelation concentration (LGC) was determined according to the method of Sathe et al*.,* (1982). Test tube containing of 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20% (w/v) protein isolate in distilled water (5 mL) were heated for 1 h in the boiling water followed by cooling in ice. Temperature was maintained at 4 °C for 2 h. LGC was defined as the minimum protein concentration, in which the formed gel did not flow when the test tube was inverted.

Amino Acid Analyses

Protein isolate (250 mg) was hydrolyzed using 5 ml 6 N HCl at 110 °C for 24 h and further derivatized with solution containing of methanol, Na-acetate and triethylamine for 20 min at 25 °C. The hydrolyzed protein was then analyzed by HPLC at ambient temperature using PICO TAG 3.9 x 150 nm column using a gradient system with 1 M sodium acetate pH 6.0 and 60% acetonitrile. Detector was set at 254 nm. Chart speed and run time were 2 cm/min and 32 min., respectively. Amino acid composition was expressed as g of amino acid per 100 g of protein.

Colour Evaluation

Colour of protein isolate was determined using a Minolta Chroma Meter CR-300 (Minolta Camera Co., Osaka, Japan). Measured values were expressed as L; a; b colour units, where L = lightness, + a = redness, - a = greenness, + b = yellowness, -b = blueness.

Results and Discussion

Isolation of Protein

The compositions of *nyamplung* seed and defatted flour are shown in **Table 1**. De-oil process of *nyamplung* seeds caused an increase in all concentrations of flour components due to a decrease in cake oil concentration. Protein and carbohydrate concentrations increased 3.9 and 2.8 times, respectively.nHigh protein concentration of flour (30.4%) is suggested that the defatted flour was very potential as protein resource for food and non-food application. It was comparable with the defatted *Lesquerella fendleri* flour (31.8%) (Hojilla-Evangelista and Evangelista, 2009). However, it was slightly lower compared with the defatted of *jatropha* flour, bayberry flour, rapeseed flour, *Lupinus compestris* and soybean flour, in which the values were 56.4%, 60.5%, 48.2%, 55.3% and 52.4%, respectively (Rodriguez-Ambris et al., 2005; Makkar et al., 1997; Cheng et al., 2009; Yoshie-Stark et al., 2008).

Isolation of protein using solubilization-precipitation technique resulted in protein recovery of 54.88 ± 7.37% and 44.27 ± 5.27% for IP3 and IP5, respectively. Protein concentration of protein isolates were 91.25 ± 0.04 % and 87.42 ± 1.15% for IP3 and IP5, respectively. Low recovery of protein might be due to their retention in the residue and the forming of protein complex with other molecules such as lipid and carbohydrate. The protein yield was comparable with the result of beach pea protein isolate (59.4-67%) (Chavan et al., 2001), but it was 2 times higher than cotton seed protein isolate (Tsaliki et al., 2003).

Table 1. Composition of *nyamplung* seed and defatted flour

	Seed	Defatted flour
Moisture (%)	6.37 ± 1.02	20.97 ± 3.87*
Ash (% db)	1.86 ± 0.05	4.91 ± 0.67
Protein (% db)	7.67 ± 0.96	30.43 ± 3.83
Lipid (% db)	69.39 ± 6.90	0.89 ± 0.14
Carbohydrate (% db)	14.69 ± 6.83	41.78 ± 4.81

*Moisture content including solvent.

Amino Acid Composition

Amino acid composition is one of the factors that affect the functional properties of protein. The results demonstrated that protein isolates from *nyamplung* were rich in lysine, leucine, proline, aspartic acid and glutamic acid but they limited in tryptophan, methionine and cysteine (**Table 2**). Since IP3 and IP5 had most of the essential amino acids, they could be considered as a high quality protein.

Polar amino acids are the primary site of protein-water interaction, and non-polar amino acids affect protein-lipid interaction by hydrophobic interaction. IP3 had polar amino acids such as glycine, proline, tyrosine, threonine, lysine, arginine, histidine, aspartic acid and glutamic acid. There were not significantly different on the total polar amino acids in IP3 (23.2%) and in IP5 (22.7%). Total non-polar amino

acids in IP3 (13.28%) were also not significant difference comparing with IP5 (13.42%). However, it was suggested that amino acid compositions affected the capacity of water binding, oil binding, gelation, emulsion and foaming due to their interaction and conformational formation as shown on **Table 3.**

Table 2. Amino acid composition of *nyamplung* protein isolates

Amino acid	IP3 (g/100g)	IP5 (g/100g)
Glycine	0.67	0.65
Alanine	1.16	1.17
Valine	3.34	2.98
Isoleucine	1.34	1.42
Leucine	4.39	4.68
Prolin	4.22	4.29
Phenylalanine	1.18	1.21
Tyrosine	1.88	1.89
Serine	1.83	2.22
Threonine	1.47	1.32
Methionine	1.18	1.27
Cystine	0.69	0.68
Aspartic acid	3.23	3.10
Glutamic acid	6.28	5.98
Histidine	0.98	1.06
Arginine	1.12	1.08
Lysine	3.34	3.42

*IP3:Isoelectric point pH 3, IP5: Isoelectric point pH5

Functional Properties

The functional properties of *nyamplung* protein isolate are shown in **Table 3**. IP3 had higher capacity of both water binding and foaming than IP5, but it did not have significantly difference in the capacity of oil binding, gelation and emulsifying.

Water Binding Capacity

The degree of water-protein interaction determines the water binding capacity of protein. Results showed that water binding capacity of IP3 was 1.7 times higher than IP5 (**Table 3**). It may be due to the difference in the average of protein charge at pH 7. IP3 had lower average pI (3) than IP5 (5). It may result in more negative charge at pH 7 than IP5. Therefore, water-protein interaction was more effective in IP3.

Water binding capacity also depended on polar amino acids availability on the primary sites of protein for protein-water interactions (Zayas, 1997). However, the calculated amino acid polar side chain of IP3 (23.2%) and IP5 (22.7%) were not significantly different. The results suggested that protein conformation and the presents of protein complex with other molecules such as, carbohydrate, tannin and lipid may also have important role in water binding capacity. Water binding capacity of IP3 was comparable with *Lupinus angustifolius* seed protein isolate, bayberry protein isolate, cotton seed protein isolate, in which the values were 446.7%, 300% and 470%, respectively (Lqari et al., 2002; Cheng et al., 2009; Tsaliki et al., 2003).

Oil Binding Capacity

Oil binding capacity is one of the important functional properties of food product. It has an important role in mouth feel and flavour retention. Oil binding capacity of IP3 and IP5 were not significantly different (**Table 3**). The results were consistent with the calculated non-polar amino acids, in which they were also not significantly different with non-polar amino acids. It indicated that oil binding capacity has a correlation with the lipophilic amino acid contents (Zayas, 1997).

Oil binding capacity of isolates was lower comparing with their water binding capacity is. This result was also consistent with a high ratio of polar amino acids to non-polar amino acids of proteins (1.7 times). The oil binding capacities were comparable with the protein isolates of bayberry, *Lupinus campestris*, soy bean and *Lupinus angustifolius*, in which the values were 180%, 170%, 150%, and 195%, respectively (Cheng et al., 2009; Rodriguez-Ambriz et al., 2005; Lqari et al., 2002).

Gelation Capacity

Heating of protein solution at certain concentration will induce gel formation. LGC is defined as the lowest protein concentration at which gel remain in the inverted tube. It is used as an index of gelation capacity. The lower LGC means that the better of the gelating ability of the protein ingredient. Results showed that protein concentration of 8% was required to form a protein gel at pH 7.0. LGC was not significantly different with of IP3 and IP5 (**Table 3**). LGC of *nyamplung* protein isolates were lower comparing

with *Lupinus angustifolius* protein isolate, chickpea protein isolate, indian chickpea and *mucuna* bean protein concentrates, in which the values were 10%, 14 %, 14 % and 12 %, respectively (Lqari et al., 2002; Zhang et al., 2007; Kaur and Singh, 2007; Adebowalea and Lawal, 2003). Thus results indicated that *nyamplung* protein isolates had a better gelating capacity than these other proteins and they may be used as gelating agent.

Table 3. Functional Properties of *Nyamplung* Protein Isolate

Protein Properties	IP3	IP5
Water binding capacity (% w/w)	431.03 ± 8.31	252.44 ± 3.07
Oil binding capacity (% w/w)	155.92 ± 12.44	171.74 ± 13.88
Gelation capacity (% w/v)	8	8
Emulsifying capacity (% v/v)	59.15 ± 0.37	56.23 ± 0.4
Emulsifying stability (% v/v)	55.07 ± 2.28	10.06 ± 0.21
Hydrophilic Lypophilic Balance	>16.7	>16.7
Foaming capacity (% v/v)	145.33 ± 1.15	49.83 ± 0.76

Emulsifying Properties

Nyamplung protein isolates contained both hydrophilic and hydrophobic amino acid fractions. Interaction of protein with both water and oil in a water-oil system has an important role in stabilization of emulsion. **Table 3** showed that EA of IP3 was not significantly different with IP5. Both protein isolates had high HLB, indicated that they could stabilize oil in water emulsion system. The finding was consistent with high ratio of water to oil binding capacity of IP3 and IP5, namely 2.7 and 1.5, respectively. EA was slightly higher comparing with *Lequerella fendleri* seed of 32.3% (Hojilla-Evangelista and Evangelista, 2009) and bayberry protein isolate of 48.7% (Cheng et al., 2009).

The values of ES were affected by various factors including pH, droplet size, net charge, interfacial tension, viscosity, and protein conformation (Hung and Zayas, 1991). The results showed that ES of IP3 was 5.5 times higher than IP5. The finding was consistent with higher ratio of water to oil binding capacity of IP3 (2.7) than IP5 (1.5). Since IP5 had lower net charge due to higher pI of IP5 comparing with IP3, the results suggested that low ES of IP5 may be attributed to low net charge of protein. Thus, the high ES may be attributed to the dissociation of some proteins, and the forming of the resulting subunits had more hydrophobic groups which interacted more strongly with the lipid phase (Mahajan and Dua, 1995).

Foaming Capacity and Stability

The results show that FC of IP3 was 2.9 times higher than IP5 (**Table 3**). It may be due to higher net charge of IP3 at pH 7 than that of IP5 because of the deprotonated carboxyl groups of proteins at higher pH than that of their pI. High net charge will weakened the hydrophobic interactions, and it increases the flexibility of the protein. As a result, it allows the protein to diffuse more rapidly into the air-water interface to encapsulate air particles and then enhances the foam formation (Aluko and Yada, 1995). The finding was consistent with the fact that IP3 had high ratio of water to oil binding capacity. Comparing with an other reported data, FC of IP3 was comparable with the protein isolates of beach pea and *Lupinus angustifolius*, in which the values of FC were 128-143% and 116-119%, respectively (Chavan et al., 2001; Lqari et al., 2002).

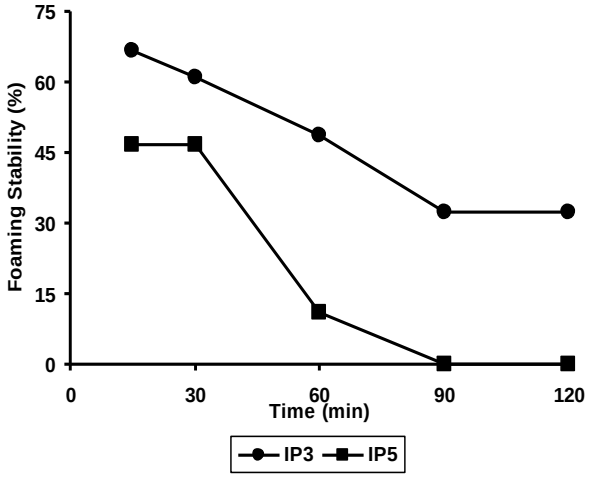

Fig. 1 Foaming stability of IP3 (●) and IP5 (■) disperse in distilled water as function of time at pH 7

As shown in **Fig. 1**, FS of IP3 was found to be higher than IP5, and it remained 32.23% after 120 min, but the foam of IP5 was completely lost after 60 min. Better FS of IP3 may be due to the difference in the electrostatic repulsion. It is suggested that electrostatic repulsion increases with an increase in pH due to the deprotonated of carboxyl groups of proteins. As a result, the

ability of protein to interact with water to encapsulate air particles becomes better. FS of *nyamplung* protein isolates was lower than the protein isolate of beach pea and *Lupinus angustifolius*, in which the foam still remained 90.1% after 60 min and 94.8% after 120 min, respectively (Chavan et al., 2001; Lqari et al., 2002).

Colour of Protein Isolates

The colour of protein isolates may limit the use of protein in foods. Colour determination of *nyamplung* proteins showed dark brown with the values of L, a and b as shown in **Table 4**. The results indicated that the covalent binding between phenolic compounds and reactive groups of the proteins, such as cysteine and lysine, occurred during alkaline protein isolation process (Sosulski, 1979; Sahidi and Naczk, 2004). The results were similar to that of Adebowale et al*.,* (2007) for *Mucana* bean protein isolate.

Table 4. Colour evaluation of protein isolate using a Minolta Chroma Meter CR-300

Protein Isolate	L-value	a-value	b-value
IP3	33.48±0.35	1.56±0.33	15.6±0.45
IP5	33.24±0.22	1.08±0.11	15.02±0.22

Conclusion

Nyamplung protein isolates that were obtained from the defatted *nyamplung* seeds cake by solubilisation-precipitation method at pH 3 (IP3) and 5 (IP5), can be considered as a high quality protein since they have most of the essential amino acid. IP3 protein isolate differed significantly from IP5 with respect to both the capacity of water binding and foaming. Both isolates had excellent in emulsifying capacity. Since total polar amino acid was about 1.7 times higher than non-polar amino acid, it might result in high ratio of water-oil binding capacity and HLB. *Nyamplung* protein isolates also had a better gelation capacity. In conclusion, *nyamplung* protein isolates are better source of protein and may stabilize oil in water emulsion system.

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