Amino acid composition, antioxidant and functional properties of protein hydrolysates from Cucurbitaceae seeds

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Abstract In this study, the effect of enzymatic hydrolysis of globulin fraction of *C. moschata* (CMH), *C. lanatus* (CLH) and *L. siceraria* (LSH) on antioxidant capacity, functional properties, structural and micro-structural properties, as well as amino acid compositions were evaluated. All the hydrolysates exhibited significant antioxidant properties. The essential amino acids content in LSH (92.7 mg/g) was higher than CMH (79.9 mg/g) and CLH (70.5 mg/g). Water absorption capacity (5 g/g), heat stability (89%), emulsifying activity index (98.3 m²/g) and emulsifying stability index (45.1 min) were statistically more significant for LSH as compared to CMH and CLH. In addition, LSH had significantly higher FS and FC at pH 3–9. Among all hydrolysates, LSH showed highest solubility (87.3%) as compared to other hydrolysates. The results suggested that enzymatic hydrolysis improve the antioxidant and functional properties. Thus, the globulin hydrolysates might be served as an innovative source with promising nutritive values, good antioxidant and functional properties. Moreover, these could be used in food and pharmaceutical industries for the development of novel functional foods.

Keywords Trypsin hydrolysate · Functional property · Cucurbitaceae · *Lagenaria siceraria*

Introduction

Over last few decades, epidemiological studies have shown that diets rich in fruits and vegetables lower the risk of development of oxidative stress induced diseases in human beings. Therefore, researchers are giving their best efforts to find out natural antioxidants from the commonly available fruits and vegetables. Among all of the vegetable families, Cucurbitaceae plant species provides highest nutritive values (Yasir et al. 2016). India ranks second in vegetables production in the world, after China. In the world trade, the capacity of vegetables production of India is about 12%, where as Cucurbitaceae family, itself contributes 5 to 6% of the total vegetables produced. Mostly, all the vegetables belonging to this family, are rich in vitamins, minerals, proteins, as well as soluble fibers (Palamthodi and Lele 2014). The juice and pulp of these fruits are commonly known to have nutritional values, while the seeds are generally discarded as waste materials, and a little attention is being paid for their therapeutic and functional uses. Hence, these seeds may be used in “trash- to-treasure” scheme.

In India, Cucurbitaceae seeds are abundantly available, which could replace costly proteins for formulation of nutritional products. Awareness to the consumers, concerning importance of these proteins has been raised up during last few years. Currently, seed proteins have been ranked in world's top 10 established markets for production of functional foods. Proteins were believed as a source of essential amino acids and functional ingredients for health promotional benefits (Thamnarathip et al. 2016). These days, the attention of the researchers has been inclined towards enzymatic hydrolysis of proteins for the production of bioactive peptides. The bioactive peptides are found inactive within the sequences of parent proteins and can be liberated during digestion of proteins in gastrointestinal system or

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during processing of food. They play a vital role in regulation of metabolic pathways along with their potential use as nutraceuticals, functional food ingredients and food supplements for promotion of health. Generally, peptides are considered to be safe for promotion of good health due to their low toxicity and easy absorption (Korhonen 2009). In addition to various biological activities, protein hydrolysates were found to have many functional properties due to their high solubility (de Castro and Sato 2014a, b). Solubility, as well as functional property of proteins is enhanced during enzymatic hydrolysis, which can stabilize emulsion and foam. Therefore, enzymatic hydrolysis can influence the structure, texture and bioactive properties of proteins (Wouters et al. 2016). Besides nutritional values and therapeutic potentials, seed protein isolates are also used as functional food components in the food industry. The functional properties of proteins, such as emulsification, solubility, foaming and water/oil binding capacity are also important for food product development (Ajibola et al. 2016). Functional properties of protein isolates of *Cucurbit moschata*, *Citrullus lanatus* and *Lagenaria siceraria* have previously been studied (Wani et al. 2011; Muhamyankaka et al. 2013; Ogundele and Oshodi 2010). However, to the authors' knowledge, no any study is carried out on antioxidant and functional properties of globulin hydrolysates of these plant seed proteins. The authors' previous research work pointed out that globulin was found most potent than other protein fractions, such as albumin, prolamin and glutelin (Dash and Ghosh 2016). Therefore, the present work was undertaken to investigate the enzymatic hydrolysis of globulin fractions by trypsin, and to study their *in vitro* antioxidant capacity, amino acid composition and functional properties, as well as structural properties.

Materials and methods

Chemicals

Bovine serum albumin, phenylisothiocyanate, trimethylamine, ammonium acetate, DL-2-aminobutyric acid and L-tyrosine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, thiobarbituric acid, trichloroacetic acid, sulphanilamide and naphthylethylene diamine dihydrochloride were procured from Merck Millipore (Darmstadt, Germany).

Extraction and content analysis

The seeds of *C. moschata*, *C. lanatus* and *L. siceraria* were purchased from local market of Bhubaneswar, Odisha,

packaged by Durga seed farm (Chandigarh, India). Further, the seeds were authenticated by Prof. Bijay Kumar Sahoo, Institute of agricultural Sciences, Siksha 'O' Anusandhan University, Bhubaneswar. The kernels (5 g) were dried at 50 °C and subsequently ground to powder using an electric grinder (Bajaj helix mixture grinder, Mumbai, India). Prior to protein extraction, seeds were defatted using hexane and the residue obtained was air dried at room temperature for 18 h. Four gram of residue was homogenized for 30 min with 30 mL of Tris HCl (100 mM) in 0.5 M NaCl at pH 8.1, and then centrifuged at 10,000×g for 30 min. The supernatant containing globulins was collected. The globulins thus obtained, were lyophilized and stored at −20 °C until further use (Labconco Corporation, MO, USA) (Teugwa et al. 2013). The total protein content of seed flour and globulin content was determined using a Bio-Rad protein assay reagent described by Bradford (1976).

Preparation of hydrolysates

Globulin Hydrolysates (CMH, CLH and LSH) were prepared according to the method of Memarpour-Yazdi et al. (2013). Globulin fractions (5 mg/mL) in Tris-HCl buffer (50 mM, pH 7.5) was mixed with trypsin (0.08 mg/ml) at the ratio of 50:1. The solution was incubated for 4 h at 37 °C, and heated in water bath for 20 min. to stop enzymatic hydrolysis. It was centrifuged at 10,000×g for 5 min and the supernatant was collected, and stored at −20 °C.

Amino acid composition

A Pico-Tag Column Amino Acid Analyzer (Waters India Pvt. Ltd., Bangalore, India) was used to determine amino acid compositions of globulin hydrolysate (CMH, CLH and LSH) according to the method described by Girjal et al. (2012). All the globulin hydrolysates were hydrolysed by 6 N HCl at 110 °C for 24 h. Each sample (50 µL) was allowed to be dried at room temperature under vacuum and 10 µL of methanol:water:trimethylamine (2:2:1) was mixed to it, and dried. To this sample, 15 µL of methanol:phenylisothiocyanate:triethylamine:water (7:1:1:1) was added and allowed to be incubated for 30 min. Then, 50 µL of ammonium acetate (0.1 M) was added to the above solution and injected into HPLC (JASCO MD 2015, Tokyo, Japan), using buffer solution A (0.1 M ammonium acetate) and B (0.1 M ammonium acetate:acetonitrile:methanol, 44:46:10) as solvent system. DL-2-aminobutyric acid used as reference compound. The amino acid composition was reported as mg/g protein. The peaks were quantified using following equation:

$$Cs = As \times R \times (Cst/As)$$

where

Cs = amino acid concentration of sample
 As = area of amino acid of sample
 R = response factor
 Cst = amino acid concentration of standard
 Ast = area of amino acid of standard.

In vitro antioxidant activity assay

Scavenging assays

The antioxidant activity of globulin hydrolysates was evaluated by DPPH (Binsan et al. 2008), ABTS (Binsan et al. 2008), hydrogen peroxide (Duh et al. 1999), nitric oxide (Shukla et al. 2009) and ex-vivo lipid peroxidation inhibition (Duh et al. 1999).

Scavenging activity was calculated using the following equation:

$$\frac{(A_c - A_s)}{A_c} \times 100$$

where A_s is the absorbance of globulin hydrolysates at different concentration, A_c is the absorbance of control.

The 50% scavenging (IC_{50}) was the concentration required to scavenge free radicals, which was calculated by plotting the percentage of scavenging activity against the different concentration of globulin hydrolysates (25–400 $\mu\text{g/mL}$).

Reducing capability assays

Reducing capability of globulin hydrolysates were evaluated by the reported method of Oyaizu (1986). High reducing power ability was associated with high absorbance value.

Analysis of functional property

Water/oil absorption capacity (WAC/OAC)

WAC/OAC of the samples was determined as per the method of Elsohaimy et al. (2015). Globulin hydrolysates (2 g) was mixed with de-ionized distilled water (25 mL) and incubated for 30 min at room temperature. Then, the samples were centrifuged at $7000 \times g$ for 30 min and the residue was weighed. The same procedure was repeated following the use of refined soybean oil for determination of OAC. Results were expressed on a dry weight basis.

Foaming capacity (FC) and foaming stability (FS)

FC and FS of globulin hydrolysates were determined according to the method of Muhamyankaka et al. (2013).

15 mL of sample solution (0.5%) was homogenized at $5000 \times g$ for 5 min. The sample was transferred into a 25 mL measuring cylinder and the total volume was measured after 30 s. The effect of FC and FS at different pH (3–9) was also determined.

The FC was calculated according to the following equation:

$$FC(\%) = (A_0 - B) \times 100/B$$

where A_0 is the volume after whipping (mL), B is the volume before whipping (mL).

Foam stability was calculated as follows:

$$FS(\%) = (A_t - B) \times 100/B$$

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

Heat stability (HS)

To 500 mg of globulin hydrolysates, 50 mL of distilled water (50 mL) was added and heated for 2 min at 100 °C. Then the mixture was cooled and centrifuged at $15,000 \times g$ for 10 min. The protein content was determined using Bradford method (de Castro and Sato 2014a, b).

The heat stability was calculated as follows:

$HS(\%) = (\text{protein content in supernatant after heat treatment} / \text{total protein content before the heat treatment}) \times 100$.

Swelling index (SI)

Five hundred milligram of samples was taken in a centrifuge tube and mixed with 10 mL of distilled water, and incubated for 10 min at room temperature. Then it was centrifuged at $2000 \times g$ for 10 min and the volume of sediment obtained was measured (Gao et al. 2015).

The swelling index was calculated using the following equation:

$$S = (V_2 - V_1) / V_1 \times 100$$

where S is the % swelling capacity, V_2 is the volume of the swollen material and V_1 is the volume of the material.

Emulsifying activity index (EAI) and emulsion stability index (ESI)

As per the method described previously by Wani et al. (2015) emulsifying properties of the sample was determined. 7 mL of globulin hydrolysates (0.5%) was mixed with 3 mL refined soybean oil and allowed to be homogenized at $2000 \times g$ for 1 min. Immediately 50 μL of emulsion was taken from the bottom of the tube and mixed

with SDS solution (0.1% w/v). Then, the absorbance was measured at 500 nm at 0 and 10 min.

$$\text{EAI}(\text{m}^2/\text{g}) = \frac{2 \times 2.303 \times A_0 \times DF}{C \times \varphi \times \theta \times 10000}$$

$$\text{ESI}(\text{min}) = \frac{A_0}{A_0 - A_{10} \times 10}$$

where DF is the dilution factor (100), c is the initial concentration of protein (g/mL), φ is the optical path (0.01), θ is the oil volume fraction of the emulsion (0.25) and A_0 and A_{10} the absorbance of the emulsion at 0 and 10 min, respectively.

Protein solubility

Protein solubility of samples was determined according to reported method as described previously (Zou et al. 2016). Ten milligram of the sample was mixed with 7 mL of distilled water and stirred for 30 min, then the mixture was adjusted to 10 mL by adding distilled water and centrifuged at $5000 \times g$ for 15 min. Then, the supernatant was subjected for determination of protein content using Bradford reagent. In this study bovine serum albumin was used as standard. The solubility of the globulin hydrolysates was measured at wide range of pH (2–11).

Peptides solubility was calculated as follows:

Solubility (%) = Protein content in supernatant / Total protein content in sample $\times 100$

FT-IR analysis

The structural characteristics of globulin hydrolysates were analyzed using the method described by Elavarasan et al. (2016). The pellet was prepared by using dried sample and KBr at the ratio of 1:100. FTIR spectra of globulin hydrolysates were recorded using KBr pellet.

Scanning electron microscope (SEM) analysis

The microstructures of globulin hydrolysates were analyzed using scanning electron microscope method described by Jain et al. (2015). The samples were dissolved in acetic acid (0.5 M, 5% (w/v)), dialyzed against distilled water and lyophilized. The resulting samples thus obtained were coated with gold and the microstructure was observed using a Scanning Electron Microscope.

Statistical analysis

Results were expressed as mean values $\pm$ standard deviation of three independent determinations. Statistical analysis was done using SPSS 11.0 (SPSS Inc., Chicago, IL,

USA). The data were analyzed using analysis of variance (ANOVA), followed by Duncan's test ($p < 0.05$).

Results and discussion

Protein content

The total protein content of the seed flour of *C. moschata*, *C. lanatus* and *L. siceraria* was found to be 12 ± 0.2 , 9 ± 0.1 , 11.6 ± 0.4 and globulin content was 46 ± 0.3 , 39 ± 0.1 , $49 \pm 0.3\%$ respectively.

Amino acid composition

Table 1 showed the amino acid compositions of CMH, CLH and LSH. This result suggested that the globulin hydrolysates were rich in amino acids, which might be served as good source of nutrition. Of 22 amino acids, 16 amino acids were present in the globulin hydrolysates. But, some of the amino acids were present in lower concentration compared to FAO pattern (FAO 2007). Among all of the hydrolysates, LSH contained the highest amount of essential amino acids. The amino acid compositions of

Table 1 Amino acid composition of CMH, CLH and LSH (mg/g)

Amino acids	CMH	CLH	LSH	WHO/FAO ^a
Alanine	11.8	9.3	12.5	–
Arginine	14.8	8.5	13.6	–
Aspartic acid	20.6	23.1	22.7	6.45
Glutamic acid	41.9	33.4	49.3	9.91
Glycine	19.7	17.8	29.6	5.49
Histidine	28.6	23.8	29.4	16
Isoleucine	7.4	5.9	8.9	13
Leucine	6.9	9.4	8.5	19
Lysine	8.5	4.1	10.7	16
Methionine	6.2	5.7	7.2	17
Phenylalanine	12.9	11.8	15.7	19
Proline	14.4	11.9	18.5	5.5
Serine	11.6	13.2	7.8	5.5
Threonine	6.3	5.1	7.2	9
Tyrosine	6.2	7.3	14.7	3.26
Valine	3.1	4.7	5.1	13
TAA	220.9	195	258.2	–
TEAA	79.9	70.5	92.7	–
TNEAA	141	124.5	165.5	–

TAA Total amino acid, TEAA total essential amino acid, TNEAA total non essential amino acid

^aFAO (2007)

LSH and CMH revealed that they were rich in histidine, isoleucine, leucine, lysine, methionine and phenylalanine, which were similar to the study of Mokni Ghribi et al. (2015). It has also been reported that globulins from both the pulses viz. lentil and horse gram were rich in hydrophobic amino acids, which was consistent with our study (Ghumman et al. 2016).

In vitro antioxidant activity assay

A comparison of inhibition of free radicals versus concentrations of hydrolysates is shown in Fig. 1. In DPPH radical scavenging assay, IC_{50} values of CMH, CLH and LSH were found to be 49.3 ± 2 , 80.3 ± 1.5 and 46 ± 1.7 $\mu\text{g/mL}$ at highest concentration (400 $\mu\text{g/mL}$) respectively, where as IC_{50} values of LSH and CMH were not significantly ($P < 0.05$) different. In this experiment, these globulin hydrolysates were found to be comparatively higher than hydrolysate of chickpea protein ($IC_{50} = 1000$ $\mu\text{g/mL}$) (Torres-Fuentes et al. 2015). Alkaline amino acids, including histidine, arginine and lysine were reported to have a strong antioxidant activity (Guo et al. 2009). Hence, lower IC_{50} values of LSH might be attributed to high content of alkaline amino acids. The ABTS radical scavenging of CMH, CLH and LSH at highest concentration (400 $\mu\text{g/mL}$) showed 88.6 ± 2.49 , 84.83 ± 2.4 and $94.1 \pm 1.7\%$ of inhibition with IC_{50} values of 142.3 ± 0.57 , 179 ± 2.6 and 108 ± 1 $\mu\text{g/mL}$, respectively. Li et al. (2013) reported that ABTS was more sensitive than DPPH assay as ABTS radical can react with hydroxylated aromatic compound along with the amino acids in peptides. The percentages of nitric oxide scavenging activity of CMH, CLH and LSH were found to be 85.6 ± 3.6 , 83.5 ± 1.5 and 92.4 ± 2.6 at higher concentration (400 $\mu\text{g/mL}$), where as the IC_{50} values were found to be 59.6 ± 1.5 , 102 ± 1.5 , 32.6 ± 1.1 $\mu\text{g/mL}$, respectively. In this study, percentage inhibition of OH radicals by CMH, CLH and LSH at higher concentration (400 $\mu\text{g/mL}$) were found to be 66.3 ± 3.3 , 60.2 ± 2.6 , 91.62% , where as IC_{50} values were obtained as 154.3 ± 2 , 243.3 ± 4.9 , 141.6 ± 1.5 $\mu\text{g/mL}$, respectively. Phenylalanine may also be responsible to enhance the antioxidant activity of peptides as aromatic ring of phenylalanine reacts with OH of free radical to form stable compound (Li et al. 2003). Lipid peroxidation leads to various oxidative diseases and may change the nutritive value of foods. LSH showed significantly ($p < 0.05$) stronger inhibitory activity (20 ± 1.7 $\mu\text{g/mL}$), followed by CMH (43.6 ± 2.1 $\mu\text{g/mL}$) and CLH (49.6 ± 1.5 $\mu\text{g/mL}$) in lipid peroxidation assay. The absorbance of CMH, CLH and LSH were found to be 2.847 ± 0.127 , 2.372 ± 0.15 and 3.754 ± 0.13 at highest concentration (400 $\mu\text{g/mL}$), respectively. Reducing power

is concentration dependent and increased steadily with an increasing concentration of all hydrolysates (Fig. 1f).

Functional property

Water absorption capacity (WAC)

The WAC of globulin hydrolysates is presented in Table 2. The WAC of CMH, CLH and LSH were found to be 4.6 ± 0.05 , 4.8 ± 0.05 and 5 ± 0.11 g/g, respectively. The WAC of globulin hydrolysates was comparable to globulin fractions isolated from African yam bean seed (4.25 g/g) and quinoa protein (3.94 g/g) (Ajibola et al. 2016; Elsohaimy et al. 2015). Among all of the globulin hydrolysates, LSH showed highest water absorption capacity ($p < 0.05$). WAC is the most popular functional property in food processing that is associated with the consistency of compounds. Enzymatic hydrolysis had a very good effect on WAC, as more polar ionizable group was liberated from protein during hydrolysis (Wouters et al. 2016). It was previously reported that WAC of proteins was the function of several parameters, such as number of charged amino acid residues, hydrophilic-hydrophobic balance of amino acids, pH, temperature and concentration of proteins (Chavan et al. 2001). The high value of WAC of globulin hydrolysates may enhance binding capacity, which could be helpful in the formulation of meat substitute, ground meat, baked food and soup.

Oil absorption capacity (OAC)

OAC of globulin hydrolysates is presented in Table 2. The results of the study revealed that the OAC of CMH, CLH and LSH were found 1.73 ± 0.05 , 1.2 ± 0.2 and 1.6 ± 0.15 respectively. The results showed that the OAC of globulin hydrolysates was lower than that of WAC. Among all globulin hydrolysates, CMH and LSH showed significantly higher ($p < 0.05$) OAC than CLH. The OAC was found to be higher in CMH due to presence of hydrophobic proteins. The OAC is the ability of oil to bind with non-polar side chain of proteins. Moreover, the finding of this study was supported by Al-Farga et al. (2016).

Protein solubility (PS)

The solubility is considered as the important parameter, which has significant impact in food systems. Some other functional properties like foaming capacity and emulsifying activity index are generally required for solubilization of protein in relevant medium. In view of this, insoluble proteins were rarely used in food formulation (Wouters

Fig. 1 Antioxidant activity of CMH, CLH and LSH. Results are mean ($\pm$) standard deviations of triplicate determinations. Different letters above the bars indicate significant differences by Duncan's test ($p \leq 0.05$). **a** DPPH radical scavenging assay; **b** ABTs radical

scavenging assay; **c** H_2O_2 scavenging assay; **d** NO radical scavenging assay; **e** Lipid peroxidation scavenging assay; **f** Total ferric reducing power

et al., 2016). Protein solubility was expressed in terms of percentage (%) and carried out over the pH range of 2–11, which is shown in Fig. 3. The LSH, CMH and CLH showed highest solubility, i.e., 89.1, 69 and 75.8%, respectively at pH 11. A significant difference ($p < 0.05$) in PS of the samples at different pH values was observed.

Adzuki bean was previously reported to have highest solubility at pH 11.0 and the higher solubility may also be attributed due to high content of 7S globulin fraction (Chen et al. 2017). The finding of our study was also found to be consistent with this report. The solubility of all the globulin hydrolysates were found to be lower at pH 5.0–6.0 and

Table 2 Physicochemical and functional property of CMH, CLH and LSH

Parameter	CMH	CLH	LSH
Moisture (%)	4.7 ± 0.4 ^c	5.8 ± 0.1 ^b	7.3 ± 0.2 ^a
Ash value	5 ± 0.1 ^a	2.9 ± 0.6 ^c	3.2 ± 0.2 ^b
Water absorption capacity (g/g)	4.6 ± 0.05 ^b	4.8 ± 0.05 ^b	5 ± 0.11 ^a
Oil absorption capacity (g/g)	1.6 ± 0.15 ^a	1.2 ± 0.2 ^b	1.7 ± 0.05 ^a
Heat stability (%)	85.6 ± 4 ^b	77.3 ± 4.5 ^c	89 ± 2.6 ^a
Swelling index	29 ± 0.5 ^a	18 ± 1.3 ^c	23 ± 0.8 ^b
Emulsion ability index (m ² /g)	91.47 ± 0.8 ^b	72.1 ± 0.1 ^c	98.3 ± 0.3 ^a
Emulsion stability index (min)	38.4 ± 0.4 ^b	25.5 ± 0.3 ^c	45.1 ± 0.5 ^a

Values expressed are mean ± SD; Data are the mean ± SD from three individual experiments

Different superscript letters indicate significant differences at $p < 0.05$

increased gradually below pH 6 and above pH 7. The solubility was also increased at alkaline and acidic pH. Adebowale et al. (2009) reported that AYB seed protein isolate showed higher solubility at alkaline and acidic pH values. All hydrolysates were soluble over a wide range of pH, and LSH had the highest solubility (87.3%) as compared to other hydrolysates. Comparing these findings, it was observed that hydrolysates are more soluble in alkaline pH than acidic pH. Enzymatic hydrolysis plays an important role to reduce molecular mass of proteins and results in liberation of ionizable groups. Solubility of plant protein was found to be enhanced during enzymatic hydrolysis, which is in agreement with the previous report of Wouters et al. (2016). Similar findings on whey protein hydrolysates were previously reported by Jeewanthi et al. (2015). This finding suggested that globulin hydrolysates might have been applied in formulation of food due to their good solubility. Good solubility is also responsible for smooth consistency and attractive appearance of food product. Interestingly, the solubility of CMH and LSH at a low pH might have been used in beverages and acidic foods.

Heat stability (HS)

The heat stability of globulin hydrolysates is shown in Table 2. The results of this study showed that heat stability of CMH, CLH and LSH were found to be 85.6 ± 4 , 77.3 ± 4.5 and 89 ± 2.6 , respectively. The thermal effect was particularly important as all protein hydrolysates need to be processed through thermal treatment in real food systems. However, significant differences in heat stability ($p < 0.05$) were observed in all of these globulin hydrolysates. Protein hydrolysates with low molecular weight are generally found to be stable on heating. Higher heat stability of the protein might be beneficial in manufacturing of nutritional supplements, which was invulnerable to malodorant during thermal processing (Zhou et al. 2016).

Swelling index (SI)

CMH showed highest swelling index (29 ± 0.5), followed by LSH (23 ± 0.8) and CLH (18 ± 1.3). Similar types of result have been reported by Li et al. (2016), who found that polysaccharide of *Hohenbuehelia serotina* has good swelling index.

Emulsifying activity index (EAI)

The emulsifying capacity of protein depends on its ability to absorb on the interface. Protein-solubility and its hydrophobicity generally determine initial adsorption of proteins on the interface and thus affecting their emulsifying properties (Shevkani et al. 2015). In this study, EAI of CMH, CLH and LSH were presented in Table 2. EAI values of CMH, CLH and LSH were 91.47 ± 0.8 , 72.1 ± 0.1 and 98.3 ± 0.3 , respectively. However, in the present study, globulin hydrolysates of CMH and LSH showed same emulsifying property ($p \leq 0.05$). Moreover, it was found that emulsifying ability of globulin hydrolysates was more than that of red kidney bean protein ($28.0 \text{ m}^2/\text{g}$) (Yin et al. 2008). Hydrolysis improved EAI and ES by promoting adsorption at the interface of water in oil. Additionally, hydrolysis also improved the accessibility of hydrophobic groups in peptides, which results in higher EAI (Wouters et al. 2016). These finding were supported by Raikos et al. (2014), who reported that presence of hydrophobic amino acid residues in hydrolysates enhance the surface activity and adsorption at the interface of oil and water. This result was found in consistent with previous study reported by Naqash and Nazeer (2013).

Foaming capacity (FC) and foaming stability (FS)

FC was assessed for the CMH, CLH and LSH over a wide range of pH (3–9). CMH, CLH and LSH showed maximum

FC (93.3 ± 1.2), (89.1 ± 1.8) and (100 ± 1.4), respectively, at pH 9. The FC of these protein hydrolysates showed an influence of pH variation. Both the acidic and alkaline pH CMH, CLH and LSH showed better foaming properties. Adebiyi and Aluko (2011) reported that foaming capacities of *Pisum sativum* seed protein isolates were found to be better in acidic and alkaline pH and the findings of this study were found to be correlated with this report. The FS of CMH, CLH and LSH were found to be 79.5 (pH 8), 62.5 (pH 9) and 80.6 (pH 8), respectively. The FC and FS of CMH, CLH and LSH were depicted in Fig. 2. Among all of the globulin hydrolysates, LSH showed highest FC and FS. The foaming properties of protein hydrolysates differed significantly ($p < 0.05$) due to the presence of protein types. Enzymatic hydrolysis is very important for those proteins which are available in native forms with tertiary structures. The native proteins can neither diffuse to nor adsorb at the interface, due to unavailability of hydrophobic groups. The hydrolytic treatment accelerated diffusion or adsorption and increased the availability of hydrophobic regions, which resulted in

higher adsorption at the interface and foaming capacity of protein (Wouters et al. 2016). In this study, the FC of the globulin hydrolysates was in agreement with the work previously reported by Benelhadj et al. (2016). The above findings showed evidence that the good capacity and stability of foaming properties of globulin hydrolysates could be used in bakery industry for formulation of cakes, chocolates, mousses and whipped creams.

FT-IR analysis

FT-IR spectroscopy is generally considered as an important tool and authenticated technique for analysis of secondary structure of proteins. Figure 3 shows the infrared (FT-IR) spectra of globulin hydrolysates of CMH, CLH and LSH. In this study, FT-IR analysis was used to investigate the secondary structures of globulin hydrolysates of these samples. Infrared (IR) absorption bands like amide A, B, and I–VII were frequently observed in protein molecules (Kong and Yu 2007). Amide I band (1640–1690) was

Fig. 2 Foaming stability (a), foaming capacity (b) and solubility profile (c) of CMH, CLH and LSH as influenced by pH. Results are mean ($\pm$) standard deviations of triplicate determinations. Different letters above the bars indicate significant differences by Duncan's test ($p \leq 0.05$)

Fig. 3 Fourier transforms infrared spectra of CMH (a), CLH (b) and LSH (c)

found to be most sensitive and widely used in analysis of protein secondary structure of proteins.

In the IR spectra, the amide-I region ($1700\text{--}1600\text{ cm}^{-1}$) of proteins is found to be most sensitive for determination of secondary structure (β -sheet, α -helix, coils and turn) (Shevkani et al. 2015). This band appeared due to coupling of C=O stretching vibration of the amide group with in-plane NH bending (Kong and Yu 2007). The amide I absorption band in CMH, CLH and LSH samples were observed at 1650.77 , 1650.77 and 1649.80 cm^{-1} respectively, which correspond to the previous study reported by Wang et al. (2013), who carried out IR study of casein hydrolysates. The results were consistent with those reported for globulins isolated from kidney bean and field pea protein isolates (Shevkani et al. 2015). Amide II bands in CMH, CLH and LSH appeared at the wave numbers of 1540.86 , 1539.88 and 1539.88 cm^{-1} , respectively. Presence of Amide II at the range of $1480\text{--}1575\text{ cm}^{-1}$, is mainly due to NH bending and CN stretching vibration. Protein hydrolysate from *Cirrhinus mrigala* revealed the presence of amide II band at 1549 cm^{-1} due to NH bending and CN stretching vibration (Elavarasan et al. 2016). The amide band III ($1400\text{--}1200\text{ cm}^{-1}$) was associated with N–H bending (in-plane) and C–N stretching of amide linkage, as well as $\text{--CH}_2\text{--}$ wagging vibrations in proline side chain. LSH showed Amide III band at wave number of 1235.18 cm^{-1} , whereas at this wave number, no any amide III band was present in CMH and CLH. In this study, CLH and LSH have shown N–H stretching band at 3290.93 and 3287.97 cm^{-1} , respectively. Generally, it is found that N–H stretching vibration of amide A occurs at wave number of $3440\text{--}3400\text{ cm}^{-1}$. The test samples showed IR absorption band at lower frequencies compared to normal range of frequency due to involvement of N–H group of shorter peptides of these samples in hydrogen

bonding. CMH, CLH and LSH showed absorption band at 2922.59 , 2925.48 , and 2925.48 cm^{-1} respectively, which were near to 2930 cm^{-1} , correspond to C–H stretching vibration of CH_2 from amino acids.

Scanning electron microscope (SEM) analysis

In this study, SEM was used to examine the micro-structural changes of protein hydrolysates. Figure 4 depicts the scanning electron micrographs of the protein hydrolysates. The micro-structural analysis is generally used to establish the relationships between protein structure and functional property. All tested protein hydrolysates showed porous type morphological characters. The changes in morphological characters may affect functional properties of protein hydrolysates (Zhao et al. 2015). Since the microstructure of LSH contained loose network with large number of pores as compared to the other test samples, it showed higher solubility and emulsifying properties. Therefore, the difference in microstructure of LSH, CMH and CLH might be attributed to their functional properties.

Conclusion

The enzymatic hydrolysis carried out using trypsin resulted formation of CMH, CLH, LSH and the resultant hydrolysates exhibited good antioxidant, functional and structural properties. LSH showed effective antioxidant and functional properties, whereas CMH and CLH also exhibited remarkable antioxidant and functional properties, as well. Therefore, globulin hydrolysates prepared from the seeds of these plants might be served as a potent source of antioxidant peptides, which are rich in essential amino acid. Hence, these hydrolysates could be utilized in food

Fig. 4 SEM micrographs of CMH (a), CLH (b) and LSH (c)

and pharmaceutical industries for the development of functional foods with potent antioxidant properties.

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