

The Effect of Thermal and Ultrasonic Treatment on Amino Acid Composition, Radical Scavenging and Reducing Potential of Hydrolysates Obtained from Simulated Gastrointestinal Digestion of Cowpea Proteins

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Abstract The effect of thermal and ultrasonic treatment of cowpea proteins (CP) on amino acid composition, radical scavenging and reducing potential of hydrolysates (CPH) obtained from *in vitro* simulated gastrointestinal digestion of CP was evaluated. Hydrolysis of native and treated CP with gastrointestinal pepsin and pancreatin yielded CPH that displayed antioxidant activities based on oxygen radical scavenging capacity (ORAC), ferric reducing antioxidant power (FRAP) and superoxide radical scavenging activity (SRSA). CPH derived from the treated CP yielded higher ORAC values than CPH from untreated proteins. However, lower significant FRAP and SRSA values were observed for these samples compared to untreated CPH ($p < 0.05$). Amino acid analysis indicated that CP processing decreased total sulphur-containing amino acids in the hydrolysates, particularly cysteine. The amount of cysteine appeared to be positively related to FRAP and SRSA values of CPH samples, but not ORAC. The results indicated that thermal and ultrasonic processing of CP can reduce the radical scavenging and reducing potential of the enzymatic hydrolysates possibly due to the decreased amounts of cysteine. Since the hydrolysates were generated with gastrointestinal

enzymes, it is possible that the resulting compounds are produced to exert some health functions during normal consumption of cowpea.

Keywords Thermal · Ultrasonic · Cowpea · Protein hydrolysates · Radical scavenging · Reducing potential

Introduction

There is growing interest in value-added use of nutritious traditional foods as nutraceuticals and functional foods [1, 2]. Current evidence has indicated that food protein-derived bioactive peptides exhibit physiological functions relevant in human health sustenance such as antihypertensive [3, 4], antioxidant, anti-inflammatory, anticancer, cholesterol-lowering and immunomodulatory activities [2, 5]. These peptides can be produced by enzymatic hydrolysis or microbial fermentation, and their release from food proteins depends on various factors including protein treatment by heat [6, 7], hydrostatic pressure [8] and sonication [9]. It is thought that treatment of proteins prior to enzymatic hydrolysis can lead to protein unfolding and increased accessibility of gastrointestinal enzymes to peptide bonds, which can result in the liberation of bioactive peptides after consumption of the proteins [2]. However, there is a scarcity of information on the impact of protein treatment on amino acid profiles of hydrolysates in relation to their antioxidant properties. The radical scavenging and reducing potential of protein hydrolysates are dependent on the presence of large amounts of antioxidant amino acid residues within the hydrolysates, such as cysteine, histidine, tryptophan, tyrosine and phenylalanine [10]. Antioxidants contribute to human health promotion by scavenging free radicals, retarding their cellular formation and associated oxidative damages to

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physiological macromolecules, and can be used for intervention against cardiovascular and inflammatory diseases, cancer and aging-induced degenerative diseases [11, 12].

Cowpea (*Vigna unguiculata*) is a leguminous seed that serves as an inexpensive dietary protein source (contains up to 35% proteins) in many developing countries [13]. The consumption of legumes has been associated with lower incidences of coronary heart diseases and diabetes possibly due to their antioxidant polyphenol contents [14]. Previous studies have demonstrated that phenolic extracts from cowpea seeds exhibited *in vitro* bioactivities relevant to human health sustenance [15–17]. Moreover, cowpea protein hydrolysate fractions were recently reported to inhibit angiotensin converting enzyme *in vitro*, and their activity was dependent on molecular weight (MW) and hydrophobicity of the peptide fractions [18]. The purpose of this study was to evaluate the effect of thermal and ultrasonic treatment of cowpea proteins on amino acid composition, radical scavenging and reducing properties of the enzymatic hydrolysates after *in vitro* gastrointestinal digestion.

Materials and Methods

Cowpea Protein Extraction and SDS-PAGE Analysis

Dried cowpea seeds purchased from Madina market, Accra, Ghana were milled and sieved through a 425 μm mesh and the resulting flour was stored at $-20\text{ }^{\circ}\text{C}$ until further use. The cowpea proteins were extracted by alkaline solubilisation. A 10% aqueous suspension of the flour (w/v) was adjusted to pH 10 using 0.5 M NaOH and stirred for 30 min. The suspension was then centrifuged at $15,000\times g$ for 30 min and the supernatant decanted and stored at $-20\text{ }^{\circ}\text{C}$ until used. The extracted cowpea proteins (CP) were quantified using the BioRad protein assay kit, and profiled by SDS-PAGE as follows: 10 μl of crude protein solution was mixed with sample buffer, SDS and β -mercaptoethanol. The mixture was heated for 10 min at $50\text{ }^{\circ}\text{C}$, centrifuged at $14,000\times g$ for 5 min, and 10 μl (containing approximately 25 μg protein) loaded onto a 12% polyacrylamide SDS gel and subjected to electrophoresis at 200 V for 50 min in a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA, USA). Thereafter, the gel was washed three times with water each for 5 min and the separated proteins stained with GelCode Blue Stain (Pierce, Rockford, IL, USA) and visualized using Chemi Genius 2 Syngene Bio imaging system.

Thermal and Ultrasonic Treatment of Cowpea Proteins

For thermal treatment, 100 ml of 1.25% (w/v) CP was continuously stirred in a beaker and then heated in a water bath to $90\text{ }^{\circ}\text{C}$ for 30 min. The resulting heated sample was

cooled to room temperature and used for further work. Sonication of CP was conducted following the method described by Lei et al. [9] with modifications. Briefly, a 100 ml solution of 1.25% (w/v) cowpea proteins in a beaker was placed on ice and the ultrasonic processor probe was immersed to a depth of 70% of the height of the solution in the centre of the beaker. Sonication of the samples was performed at 25% amplitude for 10 min using a Vibra-Cell VC 505 sonicator (Sonics & Material Inc., Newtown, CT, USA). The sonicator probe was then carefully removed and the treated CP samples were used for further studies. The thermal and ultrasonic CP treatments were conducted in duplicate.

Peptic and Pancreatic Hydrolysis of Cowpea Proteins

Each replicate of the untreated and treated CP samples were subjected to hydrolysis using gastrointestinal proteases as previously reported [19]. Aqueous suspensions (1.25%, w/v) of the untreated and treated CP (100 mL) were each adjusted to $37\text{ }^{\circ}\text{C}$ and pH 2 using 1 M HCl, followed by the addition of 12.5 mg (475 units) pepsin from porcine stomach mucosa (E/S 1:100) to initiate proteolysis. After 90 min, the solution was adjusted to pH 7.5 with 1 M NaOH to inactivate pepsin, followed by the addition of 12.5 mg porcine pancreatin. After 2 h, the reaction mixture was heated to $90\text{ }^{\circ}\text{C}$ for 15 min to denature the proteases. The CP hydrolysates (CPH) were cooled to room temperature, centrifuged at $15,000\times g$ to remove undigested materials and further processed by ultrafiltration.

Ultrafiltration of Cowpea Protein Hydrolysates

Crude CPH was subjected to ultrafiltration for 2 h using a 1,000 DaMW cut-off membrane and 40 psi pressure. The ultrafiltration permeates (MW < 1,000 Da) were collected in flasks on ice and all the fractions (permeates and retentates) were frozen at $-80\text{ }^{\circ}\text{C}$, freeze dried and stored at $-20\text{ }^{\circ}\text{C}$ for further studies.

Determination of Amino Acid Profile

Amino acid analysis of the crude CPH (untreated, thermal, ultrasonic and thermal-ultrasonic treated) samples was performed by the Advanced Protein Technology Centre, The Sick Kids Hospital, Toronto, Canada using a Waters Pico-Tag System after hydrolysis with 6N HCl, pre-column derivatization with phenylisothiocyanate and reverse-phase HPLC.

Oxygen Radical Absorbance Capacity (ORAC) Assay

ORAC values of the CPH samples were determined as earlier reported [20] with modifications. The CPH samples

(50 μ l crude CPH solution or 50 μ l of 0.1 mg/mL CPH ultrafiltration fraction) were mixed with 75 mM sodium phosphate buffer (pH 7.4) and 300 nM fluorescein in a 96-well microplate; the mixture was incubated at 37 °C for 15 min. Thereafter, 50 μ l of 80 mM of 2,2'-azobis(2-methylpropionamide)dihydrochloride (AAPH, a radical initiator) was added and the change in fluorescence was measured at 1 min intervals for 90 min at excitation wavelength of 485 nm and emission wavelength of 528 nm. ORAC values were calculated as previously reported [20]; 5–80 μ M of the antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used to prepare a standard curve and ORAC values expressed as μ M Trolox equivalent. The ORAC assay was conducted in triplicate.

Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP was measured according to a method by Girgih et al. [19]. Briefly, 50 μ l crude CPH solution or 50 μ l CPH ultrafiltration fraction (1 mg/mL) were vortex mixed with 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide ($K_3[Fe(CN)_6]$) solution and incubated at 50 °C for 20 min. Thereafter, 50 μ l of 10% trichloroacetic acid was added to the mixture. A 100 μ l aliquot of the solution was transferred into a microfuge tube, mixed with 20 μ l of 0.1% $FeCl_3$ and the mixture was allowed to stand at room temperature for 10 min. The mixture was then centrifuged at $1,000\times g$ for 5 min and absorbance of the supernatant was measured at 700 nm in a 96-well microplate. The FRAP assay was conducted in triplicate and data expressed as Trolox equivalent as reported in the ORAC assay.

Superoxide Radical Scavenging Assay

Superoxide radical scavenging assay was conducted according to the pyrogallol autoxidation method [21]. Briefly, 80 μ l of crude CPH solution or 80 μ l CPH ultrafiltration fraction (1 mg/mL) in 50 mM Tris–HCl buffer with 1 mM EDTA (pH 8.3) was mixed with 80 μ l of the Tris–HCl buffer and 40 μ l of pyrogallol (prepared in 10 mM HCl) in a 96-well microplate. The rate of superoxide radical-induced pyrogallol polymerization was measured at 420 nm for 4 min at room temperature. The blank and positive control assays contained Tris–HCl buffer and glutathione, respectively, in place of the sample. The superoxide scavenging activity was calculated as: $[(\Delta R_b - \Delta R_s)/\Delta R_b] \times 100$; R_b and R_s are the rates in the absence and presence of samples, respectively. The assay was conducted in triplicate.

Statistical Analysis

Hydrolysis was conducted once for each replicate of untreated and treated CP, and the assays were conducted in triplicate.

Data were combined ($n=6$) and analyzed by one-way analysis of variance and differences among treatment means were determined by Duncan's multiple range test using the Statistical Analysis Software version 9.1.3 (Carey, NC, USA).

Results and Discussion

Extraction and SDS-PAGE Profile of Cowpea Proteins

The protein content of the milled cowpea flour was $16.9\pm 0.8\%$, which is lower than reported values of 20–35% [13]. The variation can be due to differences in variety, agronomic practices, and methods of extraction and analysis. CP was treated by thermal and ultrasonic methods with the intent to unfold the proteins for easier access to the peptide bonds during enzymatic hydrolysis [22]. According to the SDS-PAGE analysis, the protein subunit patterns were similar in the native and treated CP except for the presence of high-MW bands in the heated CP, which could be residual products of aggregation of thermally unfolded proteins formed due to exposed hydrophobic regions [23]. A major protein band was observed between 43 and 55 kDa. MW of 25–105 kDa have been reported for cowpea polypeptides [13, 24] and the discrepancy in the case of heated CP could be attributed to the presence of protein-carbohydrate complexes or aggregation of protein monomers [24].

Amino Acid Profiles of CP Hydrolysates

The amino acid profile of protein hydrolysates determines their potential antioxidant properties [10, 25]. The amino acid profile of the hydrolysates from the same protein in native or denatured forms may vary due to the changes at the structural level; in general, the denaturation of proteins unfolds them thereby increasing their susceptibility to enzymatic proteolysis and can lead to different peptide profiles [7–9, 22]. Table 1 shows the complete amino acid profiles of CPH derived from untreated, thermal, ultrasonic and thermal-ultrasonic treated CP. Compared to native CP, heat-induced protein unfolding led to a 38% decrease in the amount of Cys in CPH whereas combined thermal-ultrasonic treatment increased Cys content of the hydrolysates by 41%. The total hydrophobic, aromatic and cationic amino acids were not affected, and the difference in total sulphur-containing amino acids was primarily due to changes in the amount of Cys as explained above. Apart from Cys, individual antioxidant amino acid residues (*e.g.*, His, Phe, Tyr, Pro) did not change as a result of the treatment.

Effects of the Cowpea Protein Treatment on Radical Scavenging and Reducing Activities

Treatment of proteins by heating, sonication or hydrostatic pressure can contribute to protein unfolding, enhanced

Table 1 Amino acid composition (g/100 g) of enzymatic hydrolysates produced from untreated, thermal and ultrasonic-treated cowpea proteins

Amino acid	CPH	CPH-H	CPH-S	CPH-HS	Amino acid	CPH	CPH-H	CPH-S	CPH-HS
Asx	13.1	12.8	13.0	12.5	Met	1.31	1.4	1.28	1.33
Glx	20.1	20.6	20.1	20.2	Ile	4.15	4.23	4.14	4.04
Ser	4.64	4.80	4.66	4.68	Leu	7.26	7.37	7.26	7.07
Gly	3.55	3.77	3.57	3.68	Phe	5.91	6.14	5.99	6.01
His	3.31	3.18	3.40	3.32	Lys	6.54	6.28	6.85	6.98
Arg	8.53	7.83	8.23	8.14	Cys	1.08	0.66	0.93	1.53
Thr	3.51	3.57	3.50	3.45	Trp	0.09	0.07	0.07	0.12
Ala	4.21	4.34	4.18	4.14	HAA	36.6	37.1	36.6	36.9
Pro	4.21	4.44	4.16	4.24	CAA	18.3	17.2	18.4	18.4
Tyr	3.22	3.17	3.46	3.37	SCAA	2.39	2.06	2.21	2.86
Val	5.21	5.26	5.13	5.11	AAA	9.22	9.38	9.52	9.50

CPH-H, CPH-S and CPH-HS represent cowpea protein hydrolysates derived from heated, sonicated and heated + sonicated proteins, respectively. *Asx* Asp + Asn, *Glx* Glu + Gln, *HAA* hydrophobic amino acids (Ala, Val, Ile, Leu, Tyr, Phe, Trp, Pro, Met, Cys), *CAA* cationic amino acids (Arg, Lys, His), *SCAA*, sulphur-containing amino acids (Cys, Met), *AAA* aromatic amino acids (Phe, Trp, Tyr)

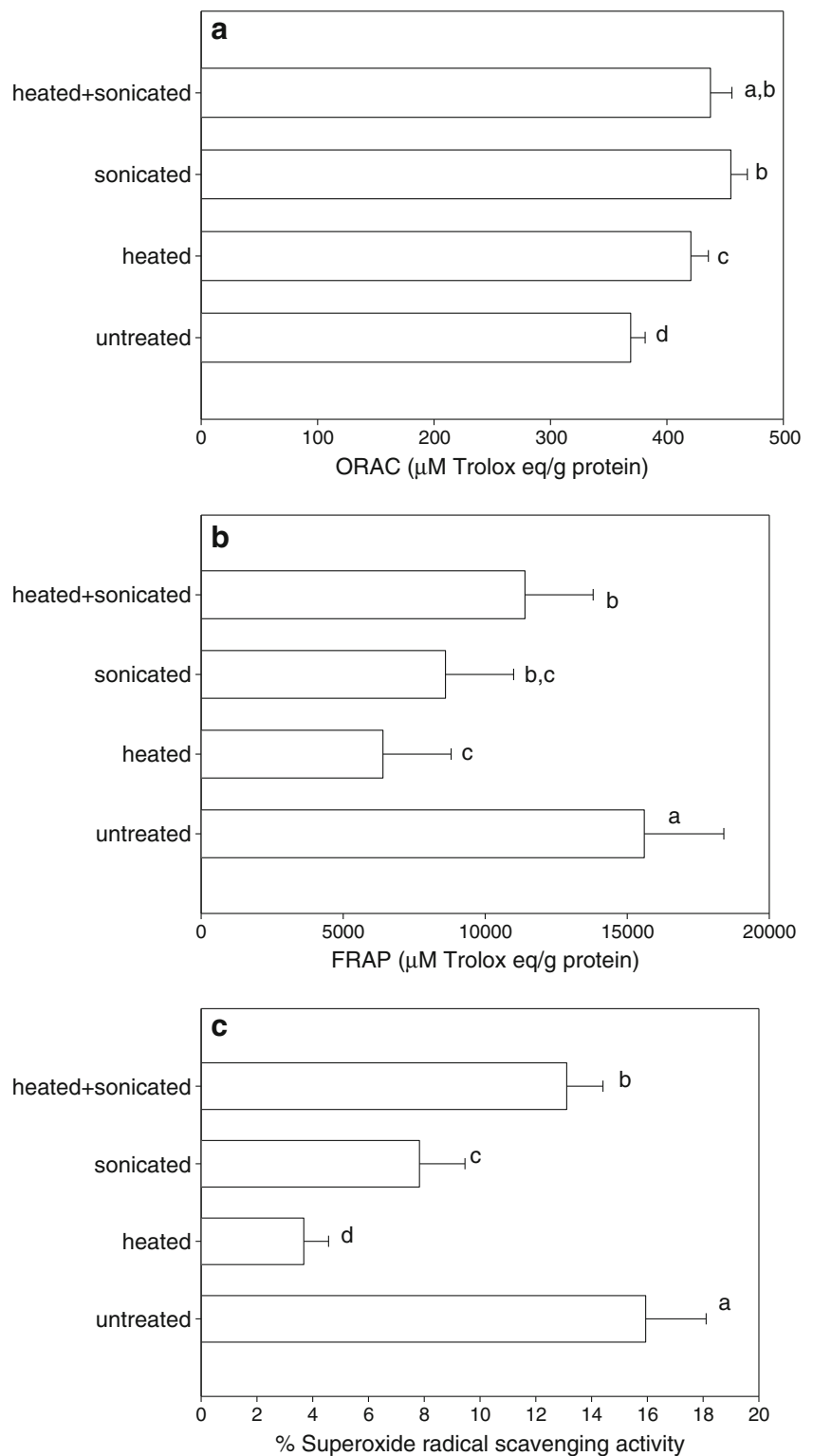
enzymatic hydrolysis and subsequent release of bioactive peptide sequences as has been shown for ovalbumin, ovotransferrin, whey and some legume proteins [7–9, 26]. As shown in Fig. 1a, thermal, ultrasonic and combined CP treatment resulted in CPH with higher ORAC values compared to untreated CPH. This observation can be attributed to the liberation of bioactive peptides from CP proteolysis due to increased accessibility of proteases to their specific amino acid residues in the unfolded proteins. The ultrasonic treated CP yielded a hydrolysate with significantly ($p < 0.05$) higher ORAC value than thermal treated CP. These results are contrary to previous reports where protein treatments by sonication did not influence the antioxidant activity of ovotransferrin hydrolysates, although this treatment enhanced the release of angiotensin converting enzyme-inhibiting peptides [9]. Thermal treatment could improve the release of other bioactive peptide activities. For example, thermal treatment enhanced the antihypertensive properties of enzymatic hydrolysates derived from whey and legume proteins [7, 26]. Conversely, Fig. 1b and c showed that thermal and ultrasonic treatments decreased the FRAP values and superoxide radical scavenging activity (SRSA) of the resulting CPH. The combined thermal-ultrasonic treatment resulted in additive SRSA values, although these were lower than SRSA value for CPH derived from native proteins and glutathione standard (21% SRSA at 0.04 mg/mL). The antioxidant patterns appeared to be related to the total amount of sulphur-containing amino acids in the hydrolysates, particularly Cys (Table 1), which possesses an antioxidant sulfhydryl group that has a redox role during endogenous oxidative stress [27]. These data indicated that sonication yielded CPH with better antioxidant activities than thermal treatment, and that native cowpea proteins can serve as better precursor of antioxidant hydrolysates

than treated proteins. Thermal-induced aggregation of proteins has been reported [23] and the resulting protein aggregates can be resistant to enzymatic proteolysis possibly leading to the release of fewer active peptides. Moreover, the susceptibility of the different CP samples to proteolysis may have played a role in the release of peptides with different amino acid profiles and antioxidant activities. In addition, molecular requirements for potent antioxidant activities differ for peroxy radical scavenging (ORAC), reducing potential (FRAP) and SRSA as suggested in a chemometric study, which demonstrated that different amino acid functionalities affect the antioxidant activities of protein hydrolysates [10].

Effect of Cowpea Protein Treatment and MW of Antioxidant Activities

Post-hydrolysis processing of food protein hydrolysates based on important peptide structural features such as hydrophobicity and molecular size can lead to enhancement of bioactivity due to increased amount of active principles [2]. In this study, ultrafiltration of CPH derived from treated and untreated CP yielded low-MW (<1,000 Da) and high-MW (>1,000 Da) fractions, which were evaluated for antioxidant activities. Figure 2a shows a substantial increase in the ORAC value of high-MW CPH (untreated) compared to corresponding low-MW fraction and unfractionated hydrolysates. The observed antioxidant trend was different in CPH derived from treated CP; no significant difference was found in the heated or combined treatment samples and their ORAC values were lower than the original untreated hydrolysate. Moreover, the ORAC values observed for all the freeze-dried CPH (Fig. 2a) were higher ($p < 0.05$) compared to freshly prepared hydrolysate solutions (Fig. 1a). This can

Fig. 1 **a** Oxygen radical absorbance capacity (ORAC), **b** Ferric reducing antioxidant power (FRAP) and **c** Superoxide radical scavenging activities of untreated or treated (thermal and ultrasonic) cowpea protein (1.25% protein, w/v) after *in vitro* gastrointestinal digestion; Bars with different letters represent means that are significantly different ($p < 0.05$)

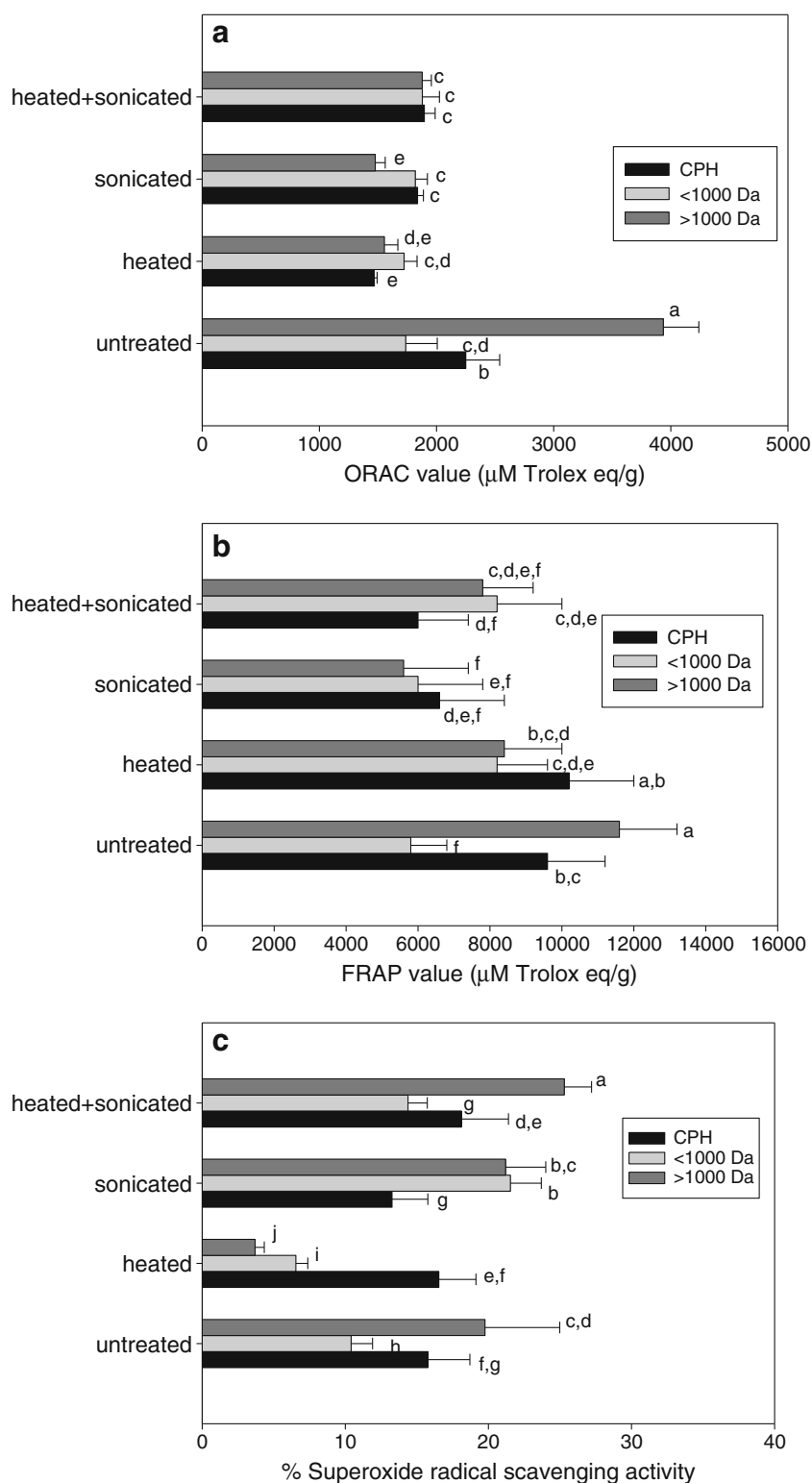


be due to alteration of the molecular properties of the constituent peptides due to aggregation during freeze-drying near neutral pH, which can influence the ORAC values of peptides [28].

As shown in Fig. 2b and c, FRAP and SRSA values were also enhanced in the high-MW fraction for untreated CPH

compared to unfractionated CPH and low-MW fraction. MW can influence FRAP values of protein hydrolysates since more reducing (electron-donating) amino acid functionality can be potentially more abundant in high-MW fractions [19]. The FRAP values of the >1,000 Da fraction from untreated hydrolysates were statistically higher ($p < 0.05$) than the FRAP

Fig. 2 **a** Oxygen radical absorbance capacity (ORAC), **b** Ferric reducing antioxidant power (FRAP) and **(c)** Superoxide radical scavenging activities of freeze dried cowpea protein hydrolysates (CPH) and its ultrafiltration fractions of MW <1,000 Da and >1,000 Da. Bars with different letters represent means that are significantly different ($p < 0.05$)



values of the same fraction from other treatments. However, the FRAP values of the <1,000 Da fraction was statistically lower ($p < 0.05$) for untreated CPH compared to other treatments except the CPH from ultrasonic treatment (Fig. 2b). However, the SRSA of the high-MW fractions were enhanced

for the sonicated CPH and substantially reduced by thermal treatment compared to other fractions (Fig. 2c). In addition to molecular size, other factors which may influence antioxidant properties of protein hydrolysates include specificity of enzymes used in hydrolysis [29], peptide net charge [30],

hydrophobicity [21] and the preponderance of antioxidant amino acid residues [10, 25]. As demonstrated in this study, thermal and ultrasonic protein treatments were generally not effective in enhancing the antioxidant values of CPH even after ultrafiltration.

Conclusion

The present study showed that cowpea proteins can serve as a renewable source of radical scavenging and reducing peptides with potential applications in the functional foods industry. Based on this study, treatment of cowpea proteins by heating and sonication prior to *in vitro* hydrolysis with gastrointestinal enzymes resulted in statistically significant enhancement of the ORAC values and reduction of FRAP and SRSA of the resulting hydrolysates. Moreover, the FRAP and SRSA values appeared to be positively related to the amount of Cys. Therefore, the native proteins were better precursors of antioxidant peptides based on the assays conducted. Ultrafiltration shows that peptides with MW > 1,000 Da possess higher antioxidant activities for untreated CP. However, in general, the fractions of the other treatments had similar or even lower antioxidant activity than the CPH. Since the hydrolysates were generated with gastrointestinal enzymes, it is possible that the resulting compounds from hydrolysis are produced to exert some health functions during normal consumption of cowpea, if efficiently absorbed and bioavailable. Moreover, the presence of antinutritional factors may discourage the direct use of the legume proteins. Based on these results, the cowpea protein hydrolysates can be further explored as functional ingredients for effects against oxidative stress-related health conditions using physiologically relevant models. Further work is needed to identify the peptides with MW > 1,000 Da for the untreated CPH in order to obtain a better understanding of structure-function relationships of antioxidant peptides.

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