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Indigestible cowpea proteins reduced plasma cholesterol after long-term oral administration to Sprague-Dawley rats

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Abstract

Cowpea protein isolate (CPI) was subjected to various dry and wet heat pretreatments followed by sequential digestion with pepsin and pancreatin; the undigested residues were isolated as the indigestible cowpea proteins (ICPs). All the ICPs exhibited in vitro bile acid-binding capacity but ICP from the slow cooling-induced gelation had the highest yield (68%) and was used for rat feeding experiments to determine effect on plasma total cholesterol (TC). Groups consisting of 3 male and 3 female Sprague-Dawley rats each were fed hypercholesterolemic diets that contained casein only or casein that was partially substituted with ICP of CPI for 6 weeks. Results showed diet that contained 5% (w/w) ICP was more effective in preventing TC increase (1.8 mmol/L) when compared to increases of 9.34 and 4.15 mmol/L for CPI and casein only diets, respectively.

Keywords: Cowpea, Pepsin, Pancreatin, Indigestible proteins, Cholesterol, SDS-PAGE

Introduction

Cardiovascular diseases contribute to high mortality rates worldwide with high blood level of cholesterol as a major risk factor. Therefore, high blood cholesterol especially in developed countries is a cause for epidemiological concern, because the prevalence among adults above 25 years old was 38.9 and 9.8% for ≥5.0 mmol/L and ≥ 6.2 mmol/L, respectively (WHO, 2011). High blood cholesterol especially excessive levels of low density lipoproteins (LDL) or the so called 'bad cholesterol' can be an underlining factor for other non-communicable diseases such as ischemic heart disease, high blood pressure, and paralysis. Several studies have shown a positive effect on reducing hyperlipidemia using dietary proteins and the low rates of coronary heart diseases in Asian countries have been linked to the higher consumption of plant based proteins as compared to animal proteins (Kahlon & Shao, 2004; Nagaoka, 2019). The hypocholesterolemic effect of intact proteins from pulses such as soybean, cowpea and others is believed to be due to ability to bind bile acids, which prevents re-absorption from the intestinal tract into blood circulatory system (Frota et al. 2008; Kahlon & Woodruff, 2002). Therefore, ability to resist digestion in the intestinal tract could increase the amount of dietary pulse proteins available for interactions with bile acids.

Vigna unguiculata [L.] Walp, a subspecies of cowpea, commonly known as black-eyed beans, black-eye peas or goat peas is a pulse domesticated in Africa. Cowpea is adapted to warm climates with adequate rainfall and therefore, mostly cultivated in Africa, Asia, southern United States, Latin America and some Mediterranean counties (Jayathilake et al. 2018). Cowpea is also a good source of protein (22–28%), dietary fiber (11–24%) and minerals (2.9–4.4%), nutrients which are affected by processing methods such as grinding, boiling or sprouting (Gupta et al. 2010; Hall et al. 2017). In addition to their cost effectiveness, high protein contents and availability, pulses are used to replace animal products and substitute cereals in diets because they are high in lysine and

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arginine, which are usually limiting in cereals (López-Barrios et al. 2014). In a previous work, it was shown that the isolated 7S globulin from cowpea when incorporated into a rat diet could reduce the level of cholesterol in the blood. In addition, the blood cholesterol-reducing ability of whole cowpea seeds or the isolated protein has also been demonstrated in hamsters (Frota et al. 2008). Moreover, on a protein weight basis, cowpea has better bile acid-binding capacity than soybean, which suggests the potential for use as a food source of hypocholesterolemic agents (Kahlon & Shao, 2004; Kahlon & Woodruff, 2002).

Enzymatic hydrolysis of intact proteins is typically used to obtain bioactive peptides, some of which have been identified in cowpeas such as those with antihypertensive and antioxidant activities (Campos et al. 2010; Jayathilake et al. 2018; Marques et al. 2015). While most of this work have been reported on low molecular weight peptides or digestible proteins, a few studies have reported the cholesterol lowering ability of indigestible proteins from various foods (Iwami et al. 1986; Sugano et al. 1990). Overall, limited research activities have been carried out on the bioactive properties of cowpeas, especially the cholesterol lowering ability of the indigestible proteins. This is probably because it is typically believed that peptides generally perform better because of their small size when compared to complex proteins (Campos et al. 2010). However, early studies that explored the use of pulses for cholesterol level reductions have indicated promising results with high molecular weight and indigestible proteins (Frota et al. 2008; Higaki et al. 2006; Sugano et al. 1990). Therefore, the aim of this study was to determine the effect of some processing methods such as wet heat, dry heat, freeze-thaw, gelation and autoclaving on the cholesterol-binding properties of indigestible proteins from cowpea, which were produced after simulated gastrointestinal digestion of the pretreated protein isolate with pepsin and pancreatin. The indigestible protein product with highest yield was subsequently tested for ability to reduce plasma cholesterol level in vivo when fed to rats.

Materials and methods

Materials

Cowpea seeds were purchased form a local grocery store in Winnipeg, Manitoba and ground into flour using a coffee blender (Cuisinart, Woodbridge ON, Canada). Pepsin and pancreatin were purchased from Sigma-Aldrich, St Louis, MO, U.S.A. Triglyceride and cholesterol assay kits were purchased form Cayman Chemical Company (Ann Arbor, MI, USA). All other reagents used were of analytical grade and purchased from Fisher Scientific, Oakville, ON, Canada.

Preparation of cowpea protein isolate (CPI)

CPI was produced using the isoelectric point precipitation method as described by He et al. (2014) with slight modifications. Cowpea seeds were ground into flour, mixed with water (5%, w/v) and adjusted to pH 10.0 using 1 M NaOH. The mixture was stirred for 1 h at room temperature and centrifuged (5600 g) for 30 min at 4 °C. The supernatant was acidified to pH 4.5 using 1 M HCl to precipitate the protein. The acidified solution was centrifuged and the precipitate washed with water and freeze dried to obtain the CPI. Protein content was determined using the Kjeldahl method (AOAC, 1990).

CPI pretreatments

CPI was pre-treated using wet heat, dry heat, autoclave, freeze-thaw and gelation processes as follows:

Autoclave: A 10% (w/v) aqueous mixture of CPI was placed in an autoclave safe bottle, followed by heating at 120 °C for 20 min and cooled.
Freeze-thaw: Aqueous CPI mixtures (10%, w/v) were mixed and frozen in sealed tubes for 24 h before they were thawed. This cycle was repeated 3 times to obtain the freeze-thaw sample.
Gelation: Gels were prepared using the method of He et al. (2014), which was modified as follows. An 8% (w/v) CPI mixture in water was heated in a boiling water bath for 30 min, removed and allowed to cool at room temperature (slow cooling) or immediately placed in an ice bath (rapid cooling). After cooling, both types of gels were then stored at 4 °C.
Dry heat treatment: CPI was weighed into a petri dish, covered and placed in an oven at 100 °C for 12 h or 24 h, after which they were cooled to room temperature and stored at − 20 °C.
Wet heat treatment: Aqueous CPI (10%, w/v) mixture was prepared in a beaker and placed in a water bath at 100 °C for 30 min, and then cooled.

All the pretreated samples were prepared in duplicates, freeze dried and stored at − 20 °C.

Preparation of indigestible cowpea proteins (ICPs)

The freeze-dried pretreated CPI samples were subjected to simulated gastrointestinal enzymatic digestion, using pepsin and pancreatin sequentially with each at 1:100 enzyme to substrate ratio (Higaki et al. 2006). An aqueous mixture (10%, w/v) of each pretreated CPI was adjusted to pH 2.0 and 37 °C followed by pepsin digestion for 2 h. The pepsin digest was then adjusted to pH 7.5 and pancreatin added for further 4 h digestion at 37 °C followed by heating in boiling water for 10 min to terminate the enzyme reaction. The pancreatin digest was centrifuged (5600 g, 30 min, 4 °C) and the supernatant

discarded while the precipitate (residue), which contains the ICPs was washed with water, centrifuged again, freeze dried and stored at − 20 °C. Gross yield was estimated as the percentage weight ratio of the freeze-dried material to that of CPI used for hydrolysis.

Determination of amino acid composition

The CPI and ICP were digested with 6 M of HCl for 24 h followed by amino acid analysis using the HPLC Pico-Tag system according to the method previously described by Bidlingmeyer et al. (1984). The cysteine and methionine contents were determined after performic acid oxidation (Gehrke et al. 1985) while the tryptophan content was determined after alkaline hydrolysis (Landry & Delhaye, 1992).

Determination of surface hydrophobicity (So)

So of the ICPs was determined using the method described by He et al. (2014). A stock solution of 10 mg/mL of each sample was used to prepare serial dilutions (50–250 μg/mL) using 0.01 M phosphate buffer (pH 7.0). A fluorescence probe, 8-Anilinonaphthalene-1-sulfonic acid (ANS) solution (8.0 mM in 0.01 M phosphate buffer, pH 7.0, maintained at 37 °C in the dark) was used to perform the assay. The fluorescence intensity (FI) of a 200 μl aliquot of each sample was measured at different concentrations in the absence (FI_A) and presence (FI_P) of 20 μL ANS solution at excitation and emission wavelengths of 390 and 470 nm, respectively using a Jasco FP-6300 spectrofluorometer (Jasco Inc., Tokyo, Japan). The net FI (FI_P- FI_A) was plotted against the sample concentration and the slope calculated to obtain So.

Determination of in vitro bile acid-binding capacity

The bile acid binding activity of each ICP was determined according to a previously reported method (Yoshie-Stark & Wäsche, 2004). The bile acid mixture (2 mM) consisted of glycocholic acid, glycochenocholic acid, glycodeoxycholic acid, taurocholic acid, taurochenocholic acid and taurodeoxycholic acid (Sigma-Aldrich, St Louis, MO, USA) in 0.1 M phosphate buffer (pH 7.0). The mixture contained glycine-and taurine-conjugated bile acids at a ratio of 3:1, based on the composition of human bile acids. Sample suspension (20 mg/mL) was prepared by mixing the ICP with phosphate buffer. A 100 μL aliquot of sample was mixed with 900 μL of bile acid mixture and incubated at 37 °C for 2 h, before centrifugation (14,500 g for 10 min). The first supernatant was transferred into a 5 mL volumetric flask while 1 mL of phosphate buffer was added to the precipitate, washed, mixed and centrifuged again to collect a second supernatant. The two supernatants were pooled and made up to 5 mL with buffer. The absorbance (Abs) of bile acids in each sample was measured at 540 nm using a commercial bile acid assay kit (Geneway Biotech Inc., USA). Cholestyramine resin was used as the assay standard and measured the same way as the sample. The bile acid-binding activity was calculated as:

$$\text{Bile acid binding activity } (\%) = \frac{Abs_{mixture} - Abs_{supernatant}}{Abs_{mixture}} \times 100$$

Molecular weight determination by fast protein liquid chromatography (FPLC)

The CPI and ICP (from slow cooling gelation pretreatment) were subjected to molecular weight analysis using an AKTA FPLC system (GE Healthcare Life Sciences, Montreal, PQ, Canada). The Superdex 75 10/300 GL column (10 × 300 mm, 1–300 kDa fractionation range) and a UV detector (λ = 214 nm) were coupled to the system. The following protein standards were used to calibrate the column: bovine serum albumin (66.5 kDa), cytochrome c (12.38 kDa), aprotinin (6.51 kDa), vitamin B12 (1.35 kDa) and glycine (0.75 kDa). CPI (10 mg/mL) or ICP (20 mg/mL) were solubilized by mixing thoroughly with buffer (50 mM phosphate buffer, containing 0.15 M NaCl, pH 7.0) followed by filtration through a 0.45 μm filter. A 100 μL aliquot of the filtrate was loaded onto the column and eluted with the phosphate buffer at a flow rate of 0.5 mL/min (He et al. 2014).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reducing SDS-PAGE was carried out as previously described (Malomo & Aluko, 2015). The non-reducing buffer (0.01 M Tris/HCl buffer, pH 8.0 containing 2.5% SDS, 0.001 M EDTA and 0.01% Bromophenol blue) was used to dissolve samples (1.25 mg/mL), vortexed, and heated at 95 °C for 1 h in a block heater, cooled and centrifuged for 15 min (10,000 g). The supernatant was collected and 5 μL loaded on 8–25% gradient gels followed by electrophoresis using the Phastsystem Separation and Development Unit (GE Healthcare Life Sciences, Montreal, PQ, Canada). Standard proteins (Fisher BioReagents™ EZ-Run™ Rec Protein Ladder 10–200 kDa) were used as the molecular weight marker.

Animal feeding experiment

Based on highest yield, the ICP (residue) obtained after enzymatic digestion of the gelation (slow cooling) pretreated protein isolate was used for the rat feeding experiments. Four weeks old male and female Sprague-Dawley rats were purchased from Charles River (Montreal, PQ, Canada), housed individually in cages at 23 °C with a 12-h light-dark cycle and fed regular chow AIN-93G purified rodent diet (Dyets Inc., Bethlehem, PA, USA) for 1 week. Rats were then placed in 4 groups with 6 rats (3 males and

3 females) per group and fed diets that contained the following proteins: group 1, 20% casein; group 2, 19% casein + 1% ICP; group 3, 15% casein + 5% ICP; group 4, 15% casein + 5% CPI. All the diets contained 0.125% sodium cholate and 0.5% cholesterol (to induce hypercholesterolemia). Water and feed were provided ad libitum over a 6 weeks period with feed consumption and body weights recorded weekly. Blood and feces were collected before and at the end of the feeding period. The fecal samples were freeze-dried and analyzed for polypeptide composition by SDS-PAGE as described above. The blood was centrifuged (1000 g at 4 °C for 10 min), plasma collected (stored at − 80 °C) and analyzed for total cholesterol (TC) using commercial assay kits.

Statistical analysis

Data are presented as means ± standard deviation (SD). Two-way ANOVA and Duncan's multiple range test were applied for determining the difference between samples and treatments. Differences were considered significant at $P < 0.05$. All analyses were conducted using SPSS 23 (IBM, Armonk, NY, USA).

Results and discussion

Yield and protein contents

The CPI and ICP had protein contents of 85.75 and 85.20% (dry weight basis), respectively, which indicate that the enzymatic hydrolysis did not have any effect on the total amount of recovered proteins. Figure 1 shows that the percentage yields of ICP from the enzymatic digestion were dependent on the pretreatment method. Gelation pretreatment followed by enzyme digestion led to significantly ($P < 0.05$) highest yield with about 68% of the original proteins in CPI recovered as undigested residue (ICP) after pepsin + pancreatin hydrolysis. Wet heat (50.2%) and autoclave (52.0%) pretreatments followed by enzyme digestion also produced high ICP yields. In contrast, dry heat (5.5–8.5%) and freeze-thaw (12.6%) pretreatments followed by enzyme digestion gave the lowest ICP yields. The high indigestibility of gelled cowpea proteins may be due to the formation of complex three-dimensional networks that are difficult for the enzymes to penetrate, hence less hydrolysis occurred. In addition, the protein aggregation that precedes gelation could have reduced enzyme access to susceptible peptide bonds and consequently prevented sufficient level of enzyme digestion (Moure et al. 2006). Wet heat and autoclave pretreatments both transmit heat through water, which may have produced protein aggregates that are resistant to proteolysis (Lam et al. 2018), and could be responsible for the similar ICP yields. Dry heat or freeze-thaw produces physical degradation of proteins, which would have made the smaller fragments even more

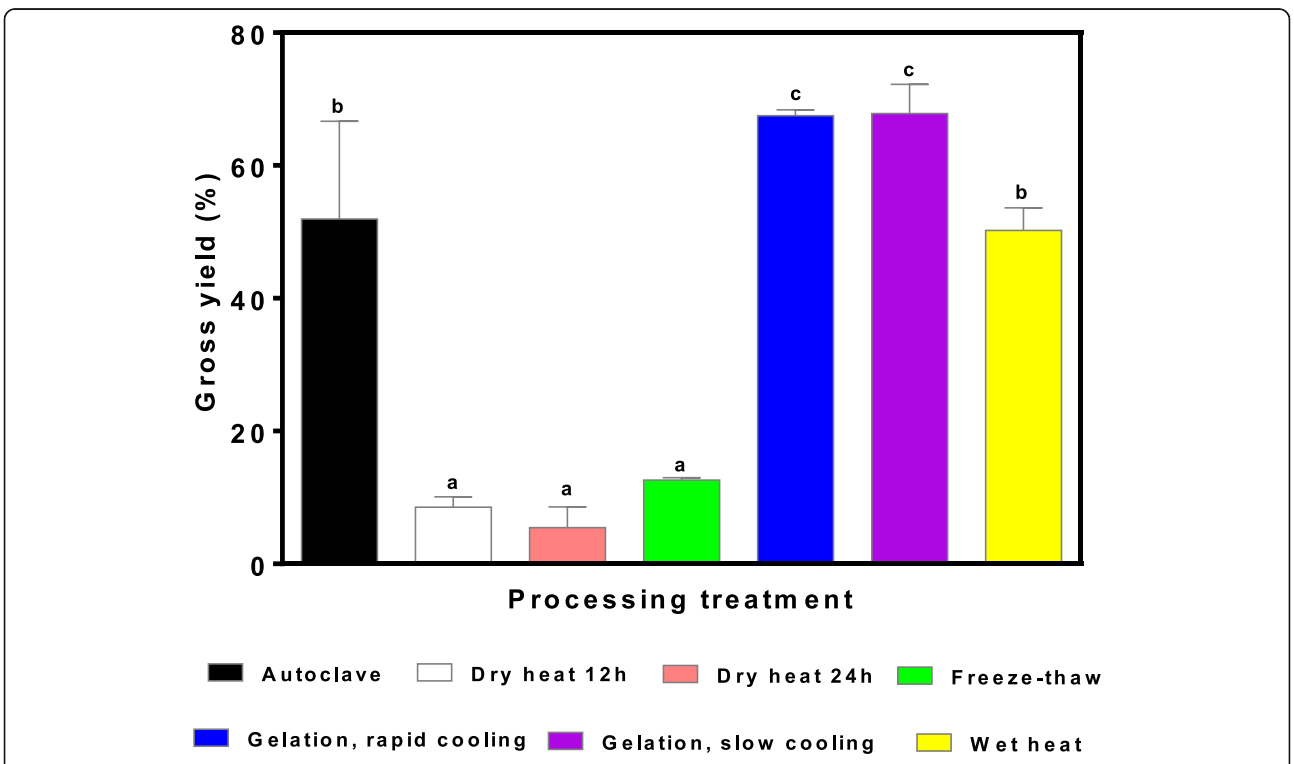

Fig. 1 Percentage gross yields of indigestible cowpea proteins obtained after pepsin + pancreatin digestion of cowpea protein isolate pretreated with autoclave, 12 h dry heat, 24 h dry heat, freeze-thaw (3 cycles), gelation (rapid cooling), gelation (slow cooling) and wet heat

susceptible to proteolysis; hence, the ICP yields were very small.

Amino acid composition, surface hydrophobicity (So) and in vitro bile acid binding activity

The amino acid composition of CPI and ICP used for the rat studies were not different as shown in Table 1. The results suggest that the gelation process and subsequent proteolysis did not alter the amino acid composition of the sample and are consistent with previously reported data for cowpea proteins (Jayathilake et al. 2018). The ratio of arginine to lysine has been associated with activity of 7α-hydroxylase, the rate limiting enzyme that helps in the conversion of cholesterol into bile acids in the liver (Yang et al. 2012). However, the arginine to lysine ratio for CPI and ICP were similar (0.96 and 1.01, respectively), which indicate that this attribute may not be involved in any observed differences in the blood cholesterol-reducing effects of the samples. ANS So values show that dry heat pretreatment led to the production of ICPs with greater exposure of aromatic amino acids when compared to the wet treatments (Fig. 2a). The results are consistent with greater susceptibility of the dry-heated samples to enzyme hydrolysis and low yield of indigestible proteins as already shown in Fig. 1. This is also reflected in the higher So of the ICP from 24 h dry heat treatment in comparison to the 12 h, which indicates that the longer period produced more fragmented proteins. Increased protein fragmentation would lead to greater proteolysis and hence more exposed aromatic residues. As expected, the ICP from the digested freeze-thaw pretreated CPI, which could have also produced physical degradation of proteins had higher So than ICPs from other wet pretreatments. The ICPs from autoclave or wet heat pretreatment had higher So values probably because of the greater proteolysis, which exposed more aromatic residues when compared to ICPs obtained from gelation pretreatment. Overall, results of the So are inversely related to ICP yields, which confirm processing treatments that enhance protein fragmentation ultimately led to greater exposure of aromatic amino acid residues.

Table 1 Amino acid composition of cowpea protein isolate (CPI) and indigestible cowpea protein (ICP)[a]

Amino acid	CPI (%)	ICP (%)
Asx	11.7	12.1
Thr	3.9	3.7
Ser	5.6	5.7
Glx	17.7	16.8
Pro	4.8	4.7
Gly	3.7	3.6
Ala	4.3	4.2
Cys	0.8	0.7
Val	5.1	4.7
Met	1.5	1.7
Ile	4.3	4.3
Leu	8.2	8.6
Tyr	3.5	3.6
Phe	6.0	5.7
His	3.6	4.5
Lys	7.2	7.0
Arg	6.9	7.1
Trp	1.3	1.4

Asx aspartic + asparagine, *Glx* glutamic + glutamine
[a] ICP is the residue obtained after pepsin + pancreatin digestion of CPI pretreated with gelation (slow cooling)

One of the main bioactive effects of indigestible food proteins has been the ability to bind bile acids, which may be exploited to reduce blood cholesterol levels (Ferreira et al. 2015; Sugano et al. 1990). There were significant differences ($P < 0.05$) in the bile acid binding activity of the samples with the ICP from digested freeze-thaw pretreated CPI having the highest value (Fig. 2b). In contrast, the ICP from digested gelation or wet heat pretreated CPI had the lowest binding capacity. However, all the ICPs had lower bile acid binding capacity than cholestyramine, a cholesterol-lowering drug. Bile acid binding capacity may be linked to protein hydrophobicity, which enhances disruption of micelles to reduce lipid (including cholesterol) absorption (Nagaoka, 2019; Nagaoka et al. 1999). There was no correlation between the bile acid binding activity and the surface hydrophobicity in this study, which suggests influence of protein or peptide structure. For example, protein conformation with segregated hydrophobic and polar residues between two faces of the helix has been suggested to enhance adsorption at the polar–apolar interfaces (Zhukovsky et al. 2019), which is similar to lipid micelles.

Molecular weight (MW) distribution

The MW distribution shows that CPI had a major peak with MW of 27–18 kDa while ICP had lower molecular weight in the 18–0.7 kDa range (Fig. 3). The lower MW sizes of ICP can be attributed to the enzymatic digestion, which led to formation of polypeptides with smaller sizes than the native proteins in CPI. Even though ICP consists mainly of digested fragments, the main MW values are greater than 1 kDa and such molecular weight protein fractions have been shown to lower cholesterol levels in animal models by interfering with the bile acid absorption (Higaki et al. 2006). Moreover, low MW peptides such as VAWWMY and VVYP have been shown to possess strong bile acid-binding properties (Nagaoka, 2019). Therefore, the small peptide fragments present in

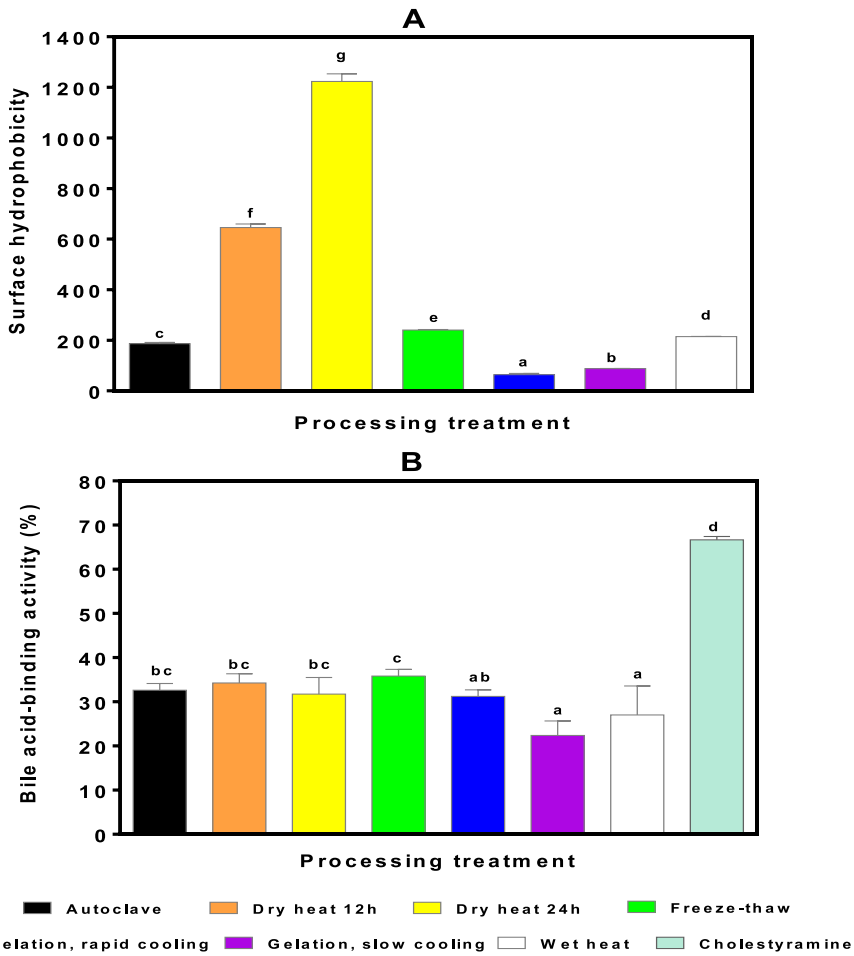

Fig. 2 **a** Surface hydrophobicity and **b** bile acid binding activity (%) of indigestible cowpea proteins obtained after pepsin + pancreatin digestion of cowpea protein isolate pretreated with autoclave, 12 h dry heat, 24 h dry heat, freeze-thaw (3 cycles), gelation (rapid cooling), gelation (slow cooling), or wet heat

the ICP could have also contributed to the observed bile acid-binding effect.

Rat feeding studies

The food consumption and body weight of the rats show that the male rats consumed 35% more food and consequently had higher (50%) weight gains than the females (Table 2). However, there were no significant differences between the groups with respect to feed consumption and body weight gains throughout the 6-week experimental period. The food consumption results are different from the reported decreases in rats fed hypercholesterolaemic and hyperlipidaemic diets that contained 7S fractions of cowpea or adzuki (Ferreira et al. 2015). The gender differences in the body weight gain and food consumption may be linked to physiological (sexual hormones) differences, nutritional requirements and mode of food consumption between the male and female rodents (Bonthuis & Rissman, 2013). For example, administration of testosterone was shown to have increased meal size of male rats (Madrid et al. 1993). On the contrary, estrogen and progesterone led to a reduction in food intake (Tarttelin & Gorski, 1971). Previous works have also shown that the soybean resistant protein did not change the body weight and food consumption of Fischer-344 rats (diet contained 20% of resistant protein), when compared to casein (Higaki et al. 2006), which is consistent with data obtained in present work. In contrast, incorporation of rice protein into male Sprague-Dawley rat diets led to decreases in feed consumption and body weight but without effect on food consumption (Um et al. 2013).

The accumulation of cholesterol in the body over a prolonged period is a major risk factor implicated in the onset of various cardiovascular diseases due to blockages in the artery walls or in severe cases forming fibrous plaques (Ferreira et al. 2015). Therefore, animal models are used to study the effects of proteins and their fractions

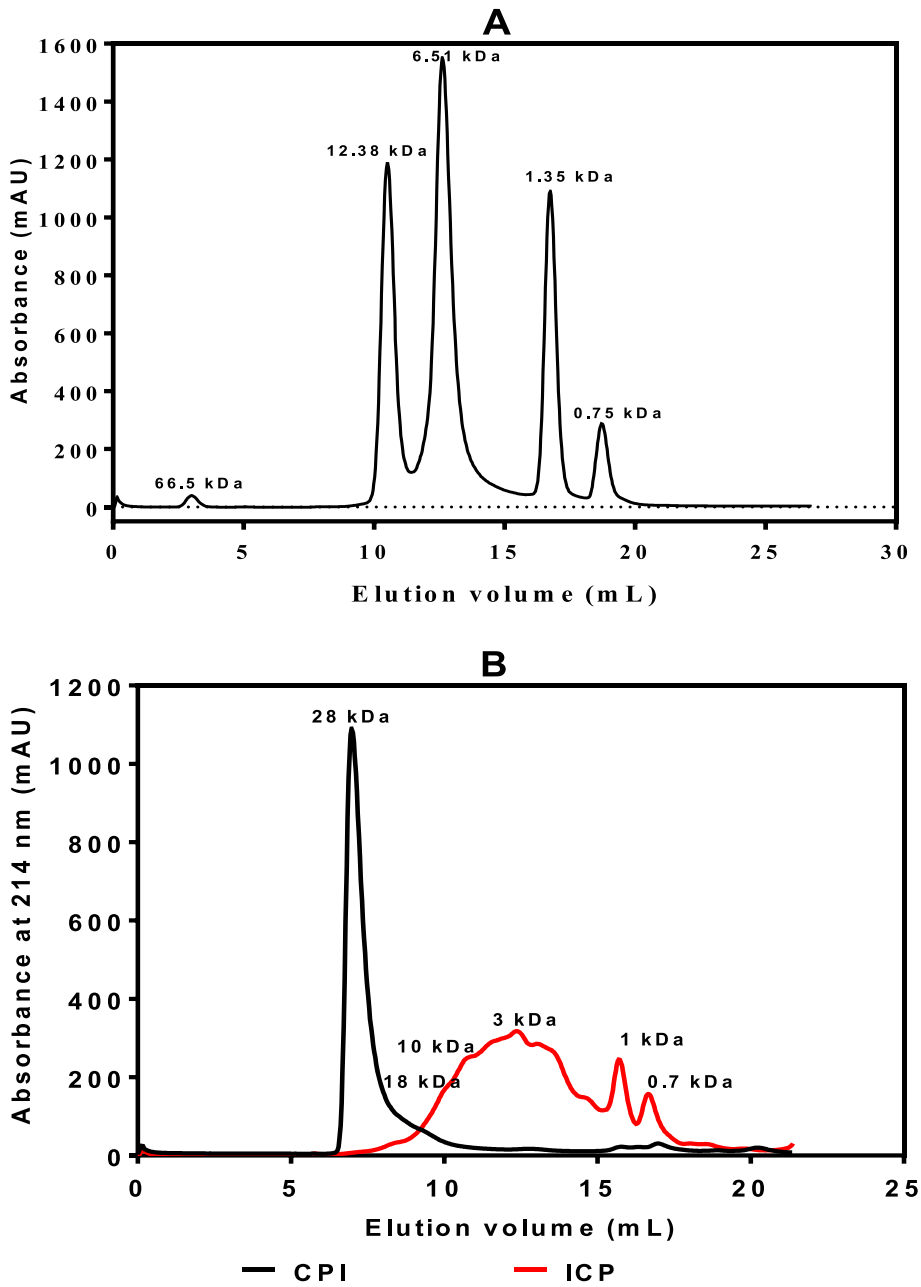

Fig. 3 a Standard proteins used to calibrate the gel-permeation column: bovine serum albumin (66.5 kDa), cytochrome c (12.38 kDa), aprotinin (6.51 kDa), vitamin B12 (1.35 kDa) and glycine (0.75 kDa). **b** Molecular weight distribution of cowpea protein isolate (CPI) and indigestible cowpea protein (ICP) from pepsin + pancreatin digestion of CPI pretreated with gelation (slow cooling)

either individually or in combination with other food components like fiber or phytochemicals in preventing or reducing the accumulation of cholesterol in the organs and body fluids (Ferreira et al., 2015; Parolini et al. 2013; Zhang et al. 2017). Initial and final total plasma total cholesterol (TC) levels in the rats indicate significant changes in the females but not males (Table 2). For the four groups, the mean initial TC was 3.06 and 2.91 mmol/L at week 0 for male and female rats, but increased to 4.66 and 12.78 mmol/L at week 6, respectively. This represents an average increase of 1.60 mmol/L (35%) in male rats when compared to 9.37 mmol/L (69%) for the females after 6 weeks of feeding. The higher TC increases in the female rats could be associated with the higher percentage of body fat than male rats (Bonthuis & Rissman, 2013). The difference between genders may also be due to the sexual steroids. For example, testosterone therapy on men significantly

Table 2 Effect of experimental diets on feed consumption, body weight and plasma cholesterol levels in rats fed cholesterol-enriched diets[a]

	Group 1	Group 2	Group 3	Group 4
Body weight gain (g/week)				
-Male	77 ± 24	72 ± 14	72 ± 15	75 ± 17
-Female	34 ± 10	42 ± 11	35 ± 21	38 ± 16
-Mean	55 ± 21	57 ± 15	54 ± 18	56 ± 19
Feed consumption (g/week)				
-Male	36.5 ± 4.1	36.2 ± 4.7	35.7 ± 6.4	34.3 ± 5.8
-Female	23.8 ± 3.6	24.7 ± 3.5	21.5 ± 3.2	23.4 ± 3.3
-Mean	30.2 ± 7.4	30.4 ± 7.1	28.6 ± 8.7	28.8 ± 7.3
Plasma cholesterol (mmol/L)				
Week 0				
-Male	3.75 ± 0.10	3.49 ± 0.08	2.63 ± 0.10	2.38 ± 0.01
-Female	3.51 ± 0.21	2.46 ± 0.10	2.51 ± 0.06	3.16 ± 0.05
-Mean	3.63 ± 0.12	2.98 ± 0.52	2.57 ± 0.10	2.77 ± 0.39
Week 6				
-Male	5.22 ± 0.09	5.27 ± 0.01	3.82 ± 0.00	4.34 ± 0.08
-Female	10.33 ± 1.01	13.95 ± 0.16	4.95 ± 0.42	19.87 ± 1.20
-Mean	7.78 ± 2.56	9.61 ± 4.31	4.39 ± 0.57	12.11 ± 7.77

CPI cowpea protein isolate, *ICP* indigestible proteins obtained after pepsin + pancreatin digestion of CPI pretreated with slow cooling gelation)
[a] Group 1: 20% casein; group 2: 19% casein + 1% ICP; group 3: 15% casein + 5% ICP; group 4: 15% casein + 5% CPI

reduced total cholesterol and LDL cholesterol while androgen deficiency led to an elevated lipid profile (Permpongkosol et al. 2016; Traish et al. 2009). Therefore, previous works suggest androgen has a protective effect against hypercholesterolemia while estrogen was likely to be a risk factor of increased blood cholesterol. Male rats have higher physical activity than females, which could also contribute to the difference in plasma cholesterol levels (Philippou et al. 2018).

The 2.38–3.75 mmol/L range of initial cholesterol levels for male rats used in this work is similar to the average of 3.5 mmol/L reported for male Wistar rats (Ferreira et al. 2015). However, in the present work, there was an overall 1.6 mmol/L change in TC of male rats compared to < 1.1 mmol/L for the Wistar rats that consumed 7S globulin of cowpea or adzuki beans (Ferreira et al. 2015). The differences suggest that the ICP used in the present work may be more effective in reducing male rat plasma TC when compared to the 7S globulins. The male rats in group 3 (5% ICP) had the lowest (1.19 mmol/L) increase in plasma TC in this study, which suggest better effectiveness of the cowpea indigestible proteins when compared to the whole native protein. The female rats that were fed 5% ICP also had the lowest (2.44 mmol/L) increase in plasma TC when compared to 16.71 mmol/L for the whole cowpea protein (group 4) and 6.82 mmol/L for the casein group. Overall, the mean value for each group confirms that the 5% ICP-containing diet was the most effective in reducing plasma TC of the rats. Similar results were also reported when rats fed with indigestible fraction of soybean protein had lower serum cholesterol than the group that ate soybean protein, indicating that the indigestible protein had better cholesterol-lowering effect than native protein (Sugano et al. 1990). However, the results obtained in this work are in contrast to a previous report that plasma cholesterol was reduced when rats were fed whole pea proteins (Parolini et al. 2013). The differences could be due to variations between the structural properties of pea and cowpea proteins. A study that involved male hamster fed whole cowpea seeds or CPI recorded significant reductions in plasma TC after 28 days; however, the whole seed was more effective than the CPI (Frota et al. 2008). The stronger cholesterol-lowering effect of the whole cowpea seeds could be due to the presence of dietary fiber, which are also able to binding bile acids. The lack of a hypocholesterolemic effect of the CPI used in the present work suggest differences in the physiological response of rats and hamsters to nutritional intervention.

SDS-PAGE

The SDS-PAGE polypeptide profiles of feces from male (Fig. 4a) and female (Fig. 4b) rats show visible differences in the molecular weight of the bands, especially between the native protein (CPI) and indigestible protein (ICP). The CPI had pronounced bands around 40–60 kDa, with less prominent bands appearing between 20 and 40 kDa, which is consistent with a previous report (Ferreira et al. 2015). In contrast, these main 40–60 kDa polypeptides bands were not visible in the ICP (lane 3) or the feces. Therefore, the results indicate that the main cowpea polypeptides were digested during production of ICP and as they passed through the gastrointestinal tract (GIT) of the rats (lane 8). However, all the feces had smaller polypeptides (< 15 kDa) that were not present in the CPI, which also indicates that the main cowpea proteins were digested during in vitro production of ICP and within the GIT. Most importantly, there were bands with > 200 kDa sizes in lanes 2, 3, 6 and 7, which represent CPI, ICP alone, 1% ICP + casein and 5% ICP + casein, respectively. Even though the > 200 kDa polypeptides were present in the CPI, they were absent in the feces of rats that consumed CPI-containing diet, which indicate susceptibility to digestive enzymes and differentiates them from the indigestible proteins. In contrast, the ICP had the > 200 kDa polypeptides, which were detected in the feces of rats that consumed the ICP-containing diets and confirms resistance to GIT proteases. The results indicate that the ICP did not

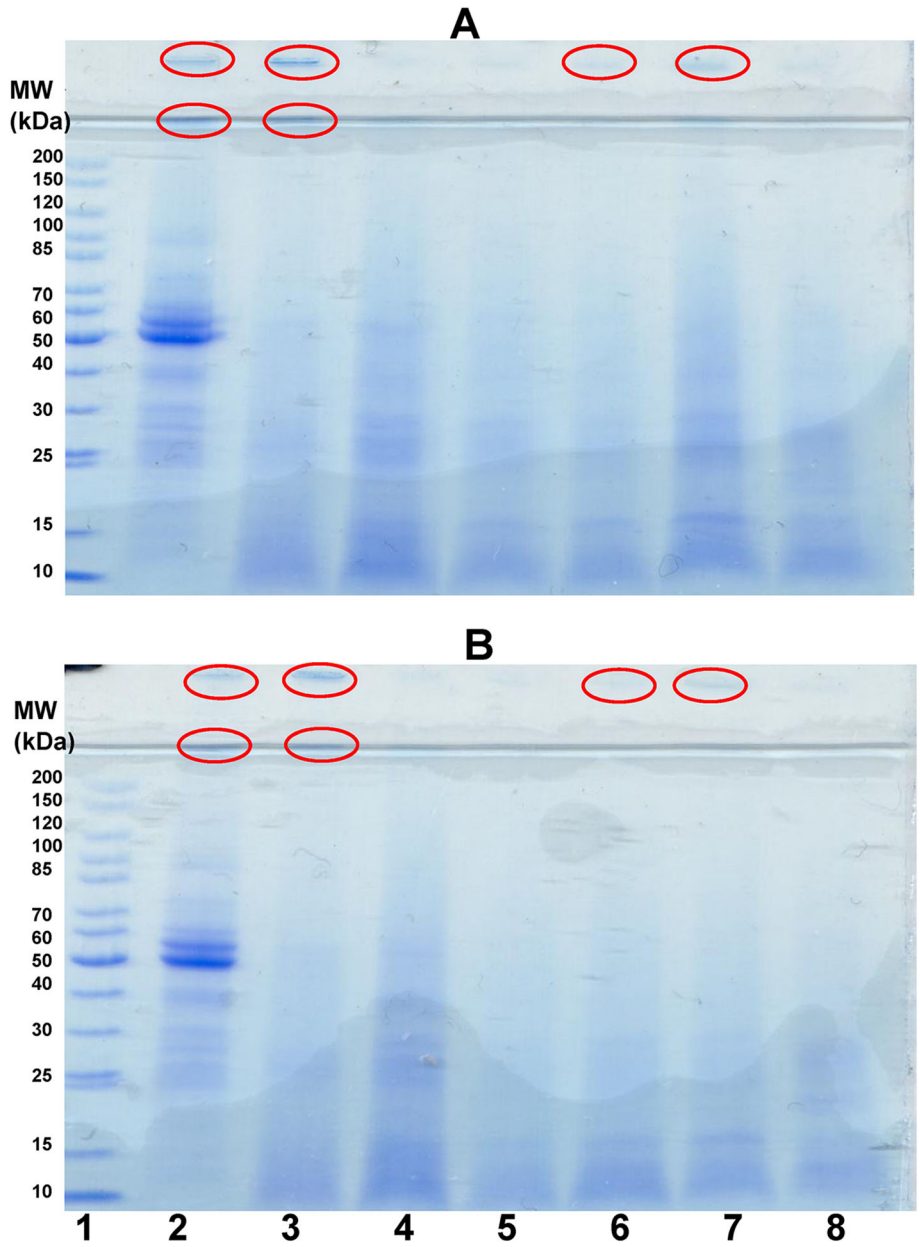

Fig. 4 Non-reducing SDS-PAGE of cowpea protein isolate (CPI), indigestible proteins (ICP) obtained after pepsin + pancreatin digestion of CPI pretreated with gelation (slow cooling), and 6th week feces from **a** male and **b** female rats: lane 1, standard molecular weight markers; lane 2, CPI; lane 3, ICP; lane 4, feces at baseline (before feeding experiment); lane 5, 20% of casein; lane 6, 19% of casein + 1% ICP; lane 7, 15% casein + 5% ICP; lane 8, 15% casein + 5% CPI. The high molecular weight bands are circled

undergo further digestion in vivo and presence in the diet could be responsible for the observed decreases in plasma cholesterol of rats that consumed the 5% ICP containing diets.

Conclusion

Pretreatment of cowpea with various food processing methods identified gelation induced by slow cooling as the most effective in reducing protein susceptibility to pepsin + pancreatin digestion, which facilitated ICP production (higher yields). Surface hydrophobicity of the ICPs was not correlated with bile acid binding capacity, which indicates a role for protein structural conformations. The presence of low molecular weight proteins in the ICP confirmed that the original cowpea proteins were broken down into smaller polypeptides during the in vitro digestion. However, presence of the > 200 kDa in the feces indicates that the ICP also contained bigger

size proteins that were resistant to in vitro and in vivo protease hydrolysis. The diet that contained 5% (w/w) ICP exhibited cholesterol-binding ability as reflected in the lower plasma cholesterol of rats when compared to the diet formulated with native cowpea proteins. The presence of ICP high molecular weight polypeptides in the feces confirm resistance to further proteolysis within the GIT, which could have also contributed to the observed plasma cholesterol-lowering effect. The marked differences in the response of male and female rats to the ICP-containing diets indicate that gender is an important consideration when designing therapeutic nutritional tools. Further studies are required to understand the specific mechanisms involved in the cholesterol-lowering effect of the ICP. In addition, future work is needed to identify and elucidate the amino acid sequence of the ICP polypeptides in order to enable proper understanding of the structure-function relationships of hypocholesterolemic peptides.

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Authors' contributions
Author Aluko R.E. conceived the project idea and designed the experiments while author Wu H. conducted the experiments and analyzed the data. Authors Aluko R.E. and Alashi A.M. supervised the project while Alashi A.M. drafted the manuscript. Author Aluko R.E. reviewed and edited the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials
Please contact Dr. R.E. Aluko for data requests.

Declarations

Ethics approval and consent to participate
All the rat protocols used in this work were approved by the University of Manitoba Animal Ethics Committee.

Consent for publication
All authors have consented to the publication of this work.

Competing interests
Author Dr. Rotimi Aluko is a member of Editorial Board of *Food Production, Processing and Nutrition* and he was not involved in the journal's review of, or decisions related to this manuscript.

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