Project Title: **Extraction and functional characterization of cholesterol-binding indigestible proteins from Manitoba-grown pulses**

Principal Investigator: Dr. Rotimi Aluko, Department of Food and Human Nutritional Sciences, University of Manitoba, Winnipeg.

**Abstract:** A total of 12 pulses were utilized to produce indigestible proteins following different processing treatments (wet heat, dry heat, freeze-thaw, gelation and autoclave). Black-eye bean after gelation (with slow cooling) had the highest yield (67.84%) and dry heated (24 h) moong dal washed had the least (0.49%). Soluble protein content ranged from 47.92% for dry heated moong dal washed to 6.91% of dry heated chick pea. The indigestible proteins had *in vitro* bile acid-binding property that was dependent on processing treatment but not pulse variety. Surface hydrophobicity (*So*) of the indigestible proteins was significantly different but there was no correlation between *So* and bile acid-binding ability. According to amino acid composition analysis, 4 hydrophobic amino acids were higher in indigestible protein than native protein. Native and indigestible black-eye bean proteins were selected for rats feeding experiment based on yield and *in vitro* bile acid-binding activity. Addition of 5% of indigestible protein reduced plasma triglyceride and total cholesterol levels when compared to casein diet only in female rats but not in males. We conclude that indigestible black eye bean protein may be effective for lowering plasma cholesterol, indicating a potential function as a cholesterol-lowering agent for certain segments of the human population.

**Introduction:** There is growing interest in the development of functional foods that can prevent or slow down the progression of cardiovascular diseases (CVD). High blood cholesterol level is a well-known risk factor for CVD and several drugs are available to reduce cholesterol synthesis. Similarly, phytosterols have been developed to reduce cholesterol absorption during food digestion. However, a potentially safer and effective method involves the use of protein materials that are resistant to gastrointestinal tract (GIT) digestion. These resistant proteins have a highly hydrophobic character that enables cholesterol binding and elimination through the fecal route. Previous works have shown that certain fractions of legume proteins are resistant to enzymatic digestion even after removal or inactivation of protease inhibitors and lectins. Therefore, oral consumption of legume proteins usually leads to residual protein waste, but the amounts generated from most relevant legume seeds remain unknown. In this work, commonly available food processing methods such as heating (wet and dry), gelation and freeze-thaw were used as pretreatment tools to reduce protein digestibility and enhance the level of undigested proteins in legume flours. The indigestible protein were isolated and tested for in vitro cholesterol-binding properties followed by a rat feeding study to determine actual reduction in blood cholesterol.

**Objectives:**

1. Optimize the production of indigestible proteins from pulse seeds using various food processing methods.

2. Isolate and determine the structural properties of indigestible proteins that maximize cholesterol binding.

3. Determine *in vitro* bile acid-binding ability of isolated indigestible proteins.

4. Determine actual blood cholesterol-reducing effect and possible mechanism of the indigestible proteins using a rat model.

**Materials:** Twelve pulse seeds (soybean, pinto bean, lima bean, black bean, red kidney beans, mung bean, moong dal washed, green split bean, black eye bean, small white bean, green lentil, chick peas) were purchased from a local grocery store in Winnipeg and ground into flours. All samples were stored at -20oC before the experiment.

**Methods**

### **Protein isolation:** Protein isolates from the pulses were produced using isoelectric point precipitation method. Each flour was mixed with water to form a slurry (5%, w/v) and adjusted to pH 10 using 0.1 M NaOH to solubilize the proteins. The mixture was stirred for 1 h at room temperature and then centrifuged (10,000*g* for 30 min at 4oC). The supernatant (containing the proteins) was acidified to pH 4.3 using 0.1 M HCl, which precipitated the proteins. This was followed by centrifugation, washing of the precipitate with water, and freeze-drying to obtain the dried protein isolate.

### **Protein pre-treatments:** Each protein isolate was subjected to the following treatments:

* Wet heat: 10% (w/v) aqueous slurry of each protein isolate was prepared in beaker, placed in boiling water for 30 min, and cooled.
* Autoclave: 10% (w/v) aqueous slurry each protein isolate was prepared in reagent a bottle, placed in autoclave at 120oC for 20 min, and cooled.
* Dry heat: 5 g of the protein isolate was spread evenly in a glass petri-dish, covered and placed in an oven at 100oC for 12 h or 24 h; samples were then cooled at room temperature and stored in the freezer.
* Freeze-thaw: 10% (w/v) aqueous slurry was prepared in a sealed plastic bottle. After being frozen for 24 h, samples were placed at room temperature to thaw rapidly. The thawed samples were then placed in the freezer for 24 h, thawed again and repeated for a 3rd cycle.
* Gelation: The least gelation concentration (LGC) of the samples was determined to be used for the gelation treatment. The protein isolates were suspended in water at concentrations of 8%-20%, w/v dry weight in glass test tubes. Then, the sealed test tubes were heated in water bath at 95oC for 1 h, the mixtures were cooled and kept in the refrigerator (4oC) for 14 h. The lowest concentration at which the gel did not flow when the tube was inverted was considered the LGC, and was used for the gelation treatment. The treatment was done as described above, but the cooling process involved slow cooling (under tap water) and rapid cooling (at -20oC).

Every treatment for each protein sample was duplicated and the pretreated samples were freeze-dried and stored in the freezer for further use.

**Preparation of indigestible proteins:** The pretreated samples were subjected to simulated gastrointestinal tract (GIT) digestion as follows. Briefly, a suspension of the protein isolates (10%, w/v) was prepared and hydrolyzed with pepsin (1%, w/w) at 37oC, pH 2.0 for 2 h, and was followed by pancreatin (1%, w/w) hydrolysis at 37oC, pH 7.5 for 4 h. The mixtures were centrifuged and the residues containing undigested proteins were washed twice with water and centrifuged again. Then, they were freeze-dried and stored at -20oC as the indigestible proteins. The protein digestion was done in duplicate.

**Determination of surface hydrophobicity (So):** Surface hydrophobicity of the indigestible proteins was determined as follows. Indigestible protein stock solutions (1%, w/w) were prepared and diluted to 0.005% to 0.025% (w/v) protein concentration, using 0.01 M phosphate buffer (pH 7.0). Fluorescence intensity (FIo) of the diluted samples was recorded using a Jasco FP-6300 spectrofluorometer (Jasco Inc., Tokyo, Japan) at excitation wavelength of 390 nm and emission wavelength of 470 nm. Then, 20 µL of 8-Anilinonaphthalene-1-sulfonic acid (ANS) solution (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was added to 4 mL of the diluted samples and vortexed. Fluorescence intensity (FIA) of the mixture was measured immediately at the same condition described above. The initial slope of the plot of net FI (FIA-FIo) versus protein concentration calculated by linear regression analysis was used as an index of So.

**Determination of *in vitro* bile acid-binding capacity:** The ability of the indigestible proteins to bind bile acids was determined as follows. The bile acid mixture (2 mM) consisted of glycocholic acid, glycochenocholic acid, glycodeoxycholic acid, taurocholic acid, taurochenocholic acid and taurodeoxycholic acid in 0.1 M phosphate buffer (pH 7). The mixture contained glycine- and taurine-conjugated bile acids at the ratio of 3:1, based on the composition of human bile acids. Sample suspension (20 mg/ml) was prepared by mixing indigestible protein with the phosphate buffer. Sample (100 µL) and 900 µL of bile acid mixture were mixed and incubated in a water bath at 37oC for 2 h, followed by centrifugation. The supernatant was transferred into a 5-mL volumetric flask. Then, 1 mL of phosphate buffer was added to the residue, washed, mixed, and centrifuged. The supernatant was combined with the previous one in the flask. The concentration of bile acids was measured at 540 nm using commercial bile acid assay kit. Cholestyramine resin, a drug that binds bile acid and lowers blood cholesterol was used as a reference. All analyses were performed in duplicate. The bile acid-binding activity was calculated as: bile acid binding activity (%) = (Amixture - Asupernatant)/Amixture×100, where Amixture refers to the bile acid concentration in the mixture and Asupernatant is the bile acid concentration in the supernatant.

**Rat feeding experiment:** Male and female Sprague-Dawley Rats (6 wks old) were fed with standard AIN-93G diet. Rats were provided with normal diet for 1 week before given the experimental diet and housed individually at 23°C with a 12-h light−dark cycle in an animal room. There were four groups with 6 rats (3 male + 3 female) per group. The diet of each group (Table 1) was: group 1, standard balanced diet with 20% casein; group 2, 19% casein + 1% isolated resistant protein; group 3, 15% casein + 5% isolated resistant protein; group 4, 15% casein + 5% (w/w) native protein isolate (Morita et al., 2004). All the diets contained 0.125% sodium cholate and 0.5% cholesterol (to induce hypercholesterolemia). *Ad libitum* feed and water were provided. Experiment was carried out for 6 weeks during which feces were collected every week and blood was collected weekly from the jugular vein under light anesthesia using isoflurane. Body weight and feed consumption were recorded weekly as well. At end of the experiment, the blood was collected *via* cardiac puncture under deep anesthesia and rats were subjected to euthanasia. Fecal samples were freeze-dried and analyzed for further analysis. Total triglycerides and total cholesterol assay of blood (plasma) samples were measured using commercial kits. All rat experiments were approved by the University of Manitoba Animal Ethics Committee. Blood samples were preserved in blood collection tubes with EDTA. After centrifugation (1000 g, 4oC for 10 min), aliquot of the clear supernatant (yellow plasma layer) was transferred to a centrifuge tube and stored at -80oC.

**Table 1: The composition of diets for rat feeding**

|  |  |
| --- | --- |
| Ingredients | Diet group |
| Control | 1% indigestible group | 5% indigestible group | native isolate |
| Cornstarch | 39.605 | 39.605 | 39.605 | 39.605 |
| casein | 20 | 19 | 15 | 15 |
| indigestible protein | 0 | 1 | 5 | 0 |
| native protein isolate | 0 | 0 | 0 | 5 |
| maltodextrin | 13.22 | 13.22 | 13.22 | 13.22 |
| sucrose | 10 | 10 | 10 | 10 |
| soy oil + TBHQ | 6.5 | 6.5 | 6.5 | 6.5 |
| Fiber | 5 | 5 | 5 | 5 |
| Mineral mix | 3.5 | 3.5 | 3.5 | 3.5 |
| Vitamin mix | 1 | 1 | 1 | 1 |
| L-cysteine | 0.3 | 0.3 | 0.3 | 0.3 |
| Choline | 0.25 | 0.25 | 0.25 | 0.25 |
| sodium cholate | 0.125 | 0.125 | 0.125 | 0.125 |
| Cholesterol | 0.5 | 0.5 | 0.5 | 0.5 |
| total | 100 | 100 | 100 | 100 |

### **Statistical analysis:** Data are presented as means ± standard deviation (SD). Two-way ANOVA and Duncan`s multiple range test was 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, USA).

**Results**

**Yield of indigestible proteins:** The protein isolates were subjected to different treatments before being hydrolyzed. The residual fraction of the hydrolysate was collected as indigestible proteins. The pre-treatments of the protein isolates resulted in significant differences (p<0.001) in the yield of the indigestible proteins (Fig. 1). Generally, for all the protein samples, the highest yield was obtained with both rapid and slow cooling gelation treatments, while the dry-heated and freeze-thawed treatments had lower protein yields. Also, the yields after autoclave were comparable with gelation treatments. Specifically, as with autoclave, similar results (around 40% to 60%) were found in all pulses for other treatments. For the autoclaved samples, the indigestible protein yield was highest for black-eye bean (61.21%) and lowest for lima bean (38.84%). After 12 h of dry heating, chickpea had the highest indigestible protein yield (21.73%), and was followed by pinto bean (20.32%) and green split pea (18.98%), whereas other samples, such as green lentil, soybean, black bean, were less than 5%. The yields of the indigestible proteins after longer heating for 24 h were found to either increase or decrease. For instance, the yield from chickpea was three-fold higher, while green split pea decreased to 7.74%. In comparison, chickpea had the highest yield and moong dal washed was the lowest (0.49%). After the wet heat, red kidney bean yielded the lowest indigestible protein of 14.28%, while the yield from chickpea was the highest, which is similar to the result obtained with 12 h dry heating. Results of the freeze-thawed treatment varied from 0.51% (black bean and small white bean) to 30.90% (green split pea). For the gelation treatment, there were two cooling procedures- rapid and slow cooling, which refer to cooling the samples at -20oC and under tap water, respectively, before being kept to gel overnight at 4oC. The different cooling methods had no significant impact on the yield of the indigestible proteins. For instance, the yield from green lentil after rapid cooling was only 0.77% more than the slowly-cooled sample. Similarly, rapid and slow cooling treatments for black eye bean yielded 67.49% and 67.84%, respectively. Among the samples, the green split pea had the least yield for both rapid cooling (41.41%) and slow cooling (37.52%).

***Fig. 1:* *Yield of indigestible proteins (Means ± SD, n=3)***

**Surface hydrophobicity:** The surface hydrophobicity of the indigestible proteins is shown in Fig. 2. Among the pulses, lima bean had the highest *So* of 4877.67 after dry heating, while values of green lentil, green split pea, mung bean and soybean were relatively lower than 1000. The highest *So* for most samples was observed after dry heat and freeze-thaw treatments, while autoclave, wet heat and gelation produced the least values. For example, the *So* for the 24 h dry heated chickpea was 3955.03 while freeze-thawed black bean was 2791.57, and the autoclaved green lentil had the lowest value of 37.36. According to statistical analysis, the differences between samples and treatments were significant (p<0.001), and there was a significant interaction between sample and treatment (p<0.001). However, no significant difference was found between black-eye bean and mung bean, while gelation (slow cooling) did not differ from wet heat treatment.

**Bile acid-binding activity:** The bile acid-binding activity of the indigestible proteins is presented in Figure 3. Cholestyramine resin, a drug used to treat hypercholesterolemia, was used as a standard reference. Cholestyramine is a bile acid sequestrant that has positively charged anion that can bind negatively charged anion in bile acids, forming insoluble complexes in gastrointestinal tract, which prevents the reabsorption of bile acids and stimulates their excretion in the feces (United States National Institutes of Health, 2018). In this study, cholestyramine bound 67.46% of bile acids, while the indigestible proteins bound 20% to 37%. Samples obtained after 12h heating and freeze-thaw pre-treatments were found to bind more bile acids compared to other treatments, while the samples after wet heat and gelation with slow cooling were relatively weak. Except black bean, black-eye bean, green lentil and green split pea that had the lowest activities for approximately 22% after gelation treatment (slow cooling), the least percentages of all other pulses were produced by wet heat (20% to 24%). The bile acid-binding

**Fig. 2: Surface hydrophobicity (So) of indigestible protein from different pulses after treatment (mean ± SD, n=3).**



activity did not differ significantly (p=0.44) on the basis of pulse variety. On the other hand, the bile acid-binding capability for black bean, green split pea and small white bean were statistically different (p<0.05) based on pre-treatment type.

**Fig. 3: Bile acid-binding activity of indigestible protein from different pulses after treatment (mean ± SD, n=2)**

Except baseline and the first week, different protein sources in diet had significant effects on blood total cholesterol (p<0.05) among male rats, while for female, there was significant difference between groups found at all weeks except week 1 (p<0.05). The TC gained after 6-week consumption are shown in Table 5. Male rats gained less TC for all types of diet and the difference between genders was significant (p<0.001). In addition, for both gender, TC gain was significantly different from each group. Among female rats, diet with native black-eye bean protein contributed to the highest increase in plasma TC (16.71 mmol/L) while that of 5% of indigestible protein decreased the TC gain when compared to control group where casein was used (2.43 mmol/L vs. 6.82 mmol/L). High cholesterol diet increased blood cholesterol in rats in comparison to the normal diet at baseline. The increases in total cholesterol after 6-week consumption of 4 diets are shown in Table 5. Among female rats, the TC gain for casein group was 6.82 mmol/L, while 5% indigestible protein in replacement of casein in the diet of group 3 effectively reduced plasma by 4.39 mmol/L. On the contrary, the addition of 1% indigestible protein and 5% native protein significantly induced increase in plasma cholesterol (gained 11.49 and 16.71 mmol/L). However, similar trend could not be seen in male rats. The TC gain in 5% indigestible protein group was 1.19 mmol/L, which was lower than control although the difference was not significant. Conversely, rats fed with diets containing 1% indigestible (group 2) and 5% native proteins (group 4) gained significantly more plasma cholesterol (1.79 and 1.96 mmol/L, respectively) than the control group, though this was less pronounced in the male than the female rats.

**Table 2: Plasma total cholesterol (mmol/L) level of rats fed with different diets with or without black-eye bean indigestible proteins**

|  |  |
| --- | --- |
| Group | Female |
| 1(Control diet) | 2(1% indigestible proteins) | 3(5% indigestible proteins) | 4(5% protein isolate) |
| baseline | 3.51±0.21b | 2.46±0.10a | 2.51±0.06a | 3.16±0.05b |
| week1 | 9.15±1.95ab | 9.08±0.64ab | 4.35±0.60a | 12.47±2.02b |
| week2 | 6.28±0.91b | 5.23±0.47ab | 2.97±0.70a | 6.88±0.46b |
| week3 | 9.70±2.03a | 7.33±0.08a | 2.82±0.03b | 3.16±0.35b |
| week4 | 5.58±0.20b | 6.23±0.18b | 3.16±0.64a | 8.56±0.02c |
| week5 | 5.60±0.16b | 11.50±0.37c | 3.50±0.24a | 13.69±0.37d |
| week6 | 10.33±1.01b | 13.95±0.16c | 4.95±0.42a | 19.87±1.20d |
| TC gain1 | 6.82b | 11.49c | 2.43a | 16.71d |
|  | Male |
| Group | 1 | 2 | 3 | 4 |
| baseline | 3.75±0.10b | 3.49±0.08b | 2.63±0.10a | 2.38±0.00a |
| week1 | 4.45±0.12a | 4.46±0.02a | 3.79±0.38a | 4.33±0.29a |
| week2 | 3.32±0.50b | 2.99±0.04ab | 2.13±0.03a | 2.46±0.04ab |
| week3 | 5.83±0.59b | 5.36±0.49b | 2.24±0.07a | 3.62±0.10a |
| week4 | 2.73±0.04c | 2.55±0.08c | 0.81±0.18a | 1.64±0.02b |
| week5 | 4.29±0.27b | 5.37±0.12c | 2.64±0.12a | 3.13±0.08a |
| week6 | 5.22±0.09c | 5.27±0.01c | 3.82±0.00a | 4.34±0.08b |
| TC gain1 | 1.47a | 1.79b | 1.19a | 1.96b |
| Data showed as mean±SD. Mean values within a row having different superscript letters (a,b,c) were significantly different (p < 0.05).1The TC gain = week 6 minus baseline |

**Conclusions:** With the increasing prevalence of hypercholesterolemia, it becomes a major health concern in the world, because it is the major risk factor of many diseases, such as coronary artery disease, hypertension and pancreatitis. Especially, high blood cholesterol level is a well-known promoter of the progression of cardiovascular diseases, which is the top cause of death in the world. Since currently available cholesterol cholesterol-reducing agents have many side effects, a safe and effective substitute is highly needed. Plant proteins have been approved to be able to reduce blood cholesterol according to existing studies. Pulses were used in this research because they have high protein content (more than 20%), and high availability (Canada is a major pulse producer in the world). Indigestible protein, a fraction that is resistant to proteases in gastrointestinal tract, has a highly hydrophobic character that enables bile acid binding and elimination through the fecal route. Food processing treatments can change the structural and physiochemical properties of protein, which are likely to affect the bile acid-binding ability. In the present study, indigestible proteins were isolated from pulse protein (soybean, pinto bean, lima bean, black bean, red kidney beans, mung bean, moong dal washed, green split bean, black-eye bean, small white bean, green lentil, chick peas) after autoclave, dry heat, gelation, freeze-thaw or wet heat treatment. Food processing treatments significantly affected yield and protein content of the treated products. Autoclave, gelation and wet heat contributed to higher yields of indigestible proteins due to protein aggregation, which made the peptide bonds less accessible to enzymes, thus lowering the digestibility. On the other hand, products from dry heat or freeze-thaw treatment had higher soluble protein content, which indicates formation of less proteins aggregates. Surface hydrophobicity values were also changed after the pre-treatments. Denaturation of protein increases exposure of hydrophobic groups, which was observed after dry heat or freeze-thaw treatment. However, protein aggregation through hydrophobic interaction hides the hydrophobic ends, resulting in lower surface hydrophobicity.

Bile acid-binding activities of samples were not affected by pulse variety but the treatments led to variations in binding ability. There was no correlation between surface hydrophobicity and bile acid-binding ability of indigestible proteins even though previous works suggested that they were highly associated, indicating that treatments affected other structural properties of the proteins. Black-eye bean protein was chosen as the sample for rat feeding experiments due to the high yield of indigestible protein. High-cholesterol diet induced weight gain in all rat groups. Noticeably, native and indigestible protein did not change the food intake and weight gain, but the difference between genders was significant. Moreover, for the female groups, increases in plasma total cholesterol contents were reduced by adding 5% of indigestible protein when compared to the casein-only diet. In contrast, plasma total cholesterol was not reduced by the 1% indigestible protein and 5% of native protein diets. Therefore, the cholesterol-reducing ability of the indigestible proteins was dependent on dose. However, in male rats, the 5% indigestible protein diet did not significantly decrease plasma cholesterol. Additionally, male rats gained less cholesterol than female counterparts. The indigestible proteins should be used instead of native for the following reasons. Firstly, the indigestible protein is produced under the certain conditions where temperature, pH and the usage of protease were well controlled to make sure the active compounds are created. Secondly, indigestible proteins are resistant to digestive enzymes, which guarantee their stability in the gastrointestinal tract.

In conclusion, indigestible proteins can bind bile acids *in vitro*,although surface hydrophobicity and amino acid composition are not correlated to this ability. As for the rat study, indigestible proteins were better than the native protein and casein-only diet in reducing blood cholesterol. These findings reveal a potential role for cholesterol-lowering indigestible proteins in the therapeutic management of hypercholesterolemia. However, the results suggest better benefits to the female population than the males. Therefore, the specific role of gender in the response to indigestible proteins needs further elucidation. Moreover, future studies should investigate higher doses of the indigestible proteins to determine the maximum level of cholesterol-reducing ability.