THE CHOLESTEROL-LOWERING EFFECT OF INDIGESTIBLE PROTEINS ISOLATED FROM PULSES

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Abstract

A total of 12 pulses were utilized to produce indigestible proteins following different processing treatments. Black-eye bean after gelation (slow-cooling) had the highest yield (67.84%) and dry heated (24 h) moong dal washed had the least (0.49%). The indigestible proteins had *in vitro* bile acid-binding property that was dependent on treatment but not pulse variety. Surface hydrophobicity (S_o) of the indigestible proteins was significantly different but there was no correlation between S_o and bile acid-binding ability. Native and indigestible black-eye bean proteins were selected for rats feeding experiment based on yield and bile acid-binding activity. Addition of 5% of indigestible protein reduced plasma triglyceride and total cholesterol levels when compared to casein diet 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 cholesterol-lowering reagent for certain segments of the human population.

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2 CHAPTER ONE

1. Introduction

The incidence of high blood cholesterol is on the rise and continues to attract a wide concern from scientists, especially in developed countries. According to the World Health Organization (WHO, 2012) statistics, among adults (over 25 years old), global prevalence rate of high cholesterol was 38.9% for ≥5.0 mmol/L and 9.8% for ≥6.2 mmol/L, while that of Canada was 53.4% and 16.2% respectively. That means almost one-fifth of the population has hypercholesterolemia, and one in two people has cholesterol level over the borderline. High cholesterol can cause diseases of the heart (coronary artery disease), high blood pressure (BP), stroke (brain attack or paralysis) and pancreatitis (WHO, 2012). Regarding the epidemiological aspect, it is clear that high blood cholesterol level is a significant risk factor of cardiovascular disease. Three large cohorts conducted among young people indicated that mortality risks of coronary heart disease (CHD) and cardiovascular disease (CVD) grew progressively with elevated serum cholesterol level (Stamler et al., 2000). The overall aim of this work was to determine the potential cholesterol-lowering effect of pulse indigestible proteins. Meanwhile, the possible influences of food processing treatments on the formation of indigestible proteins that could function as cholesterol-reducing agents were also examined.

CVD is considered as one of the major causes of death, which can be classified into two categories in terms of pathogenesis: CVD due to atherosclerosis and CVD due to other factors with the former type being the major factor (Mendis, 2011). The WHO statistics showed that

17.5 million people died from CVDs in 2012, accounting for 31% of all-cause deaths worldwide, including 7.4 million that were attributed to coronary heart disease and 6.7 million that were due to stroke (WHO, 2017). The CVD mortality rate was 88.6 per 100,000 persons by 2012 in Canada, contributing 27% of non-communicable diseases (WHO, n.d.). The data was lower than that of 12 years ago, which probably could be attributed to the development of better medical cares and services as well as changes in dietary habits. Moreover, several preventive efforts including reduction of saturated and trans fat in foods combined with increased consumption of plant-based diets have been used to gradually focus on reducing the prevalence and mortality rate of CVD in recent years.

CVD from atherosclerosis is one of the most important threats to human health. Cholesterol deposition happens in the human body during early life time. The first stage of lesion is called 'fatty streak' where lipid-rich macrophages and T-lymphocytes aggregate in the inner layer of artery wall and turns into intermediate lesion when a layer of macrophages and smooth muscle cells form. Fibrous plaques, which is a more severe and advanced lesion then project into artery lumen to block blood flow (Ross, 1993). Ross (1999) indicated that large and medium-sized elastic and muscular arteries are the most common places where lipids, particularly cholesterol, accumulate in long-term periods. Therefore, haemorrhage, thrombosis and arterial occlusion resulting from fibrous plaques, ruptures or fissures will lead to a more severe prognosis, such as sudden ischemic death (Davies et al., 1984; Russell Ross, 1993). Briefly, as for arteries of healthy individuals, cholesterol removal and deposition is balanced unless some factors, namely high fat diet, hypertension, hormone imbalances and nicotine, lead to quantitative advantage of cholesterol deposition resulting in lesion and disease (Ross et al., 1973). In fact, different

fractions of lipoprotein cholesterol show different effects on CVDs. In the cohort from LUdwigshafen RIsk and Cardiovascular health (LURIC) study, high-density lipoprotein (HDL) cholesterol had inverse association with CVD mortality rate (Silbernagel et al., 2013). Moreover, non-HDL-cholesterol level (LDL and VLDL) and low-density lipoprotein (LDL) cholesterol had been proven to be predictors of CVD risk among the Chinese population (Gu et al., 2015). LDL is recognized as a risk factor of the formation of atherosclerosis. On the one hand, when LDL particles become trapped in an artery, they can undergo progressive oxidation and be internalized by macrophages, stimulating the formation of lipid peroxides and accumulation of cholesterol esters. Finally, foam cells form, leading to endothelial dysfunction. On the other hand, a vicious cycle of inflammation, modified LDL and further inflammation can worsen the endothelial lesion. Firstly, up-regulation of the expression of genes for macrophage colonystimulating factor and monocyte chemotactic protein derived from endothelial cells by modified LDL contributed to the inflammation reaction (Ross, 1999). Then, mediators of inflammation such as tumor necrosis factor- α and interleukin-1, in turn, promoted the binding of LDL to endothelium and smooth muscle and increase the transcription of the LDL-receptor gene. Lastly, modified LDL binding to scavenger receptors initiates a series of intracellular reactions that induce inflammatory responses again. As a consequence, migration and proliferation of smooth-muscle cells were stimulated in the area of inflammation where an intermediate lesion was formed (Ross, 1999).

On the contrary, the protective effect of HDL had been shown in cholesterol diet-fed rabbits (Badimon et al., 1990). In this study, 3 groups of animals were given an atherogenic diet (contains 0.5% cholesterol). After 60 days, rabbits in group 1 were killed for recording the

baseline of the aortic fatty streak formation and cholesterol deposition. During the following 30 days, groups 2 (placebo) and 3 (treated group) continued on the same diet and were subjected to 5 mL of saline solution once a week during 4 weeks. The solution of group 3 contained 50 mg HDL-VHDL protein extracted from normal rabbit plasma. The varieties of total blood cholesterol levels between groups were not statistically significant. However, the percentage of the total aortic surface covered by fatty lesion of group 3 (17.8 \pm 4. 1%) was significantly lower than group 1 ($34 \pm 4\%$) and 2 ($38.8 \pm 5\%$). The accumulation of total cholesterol, esterified cholesterol and phospholipid on the aortic wall of HDL-VLDL treated group were less than placebo and baseline as well. The results indicate that 60-day high cholesterol feed induced the formation of fatty streak, but HDL-VLDL treatment reversed this progress showing the protective potential of HDL-VLDL administration. Edward et al. (1991) reported that this protective effect was realized by apop-I, the major protein constituent in HDL. In their experiment, transgenic C57BL/6 mice expressing human apoA-I and control C57BL/6 mice were utilized to evaluate the effect of human apoA-I. The C57BL/6 animals are susceptible to fatty streak lesion due to atherogenic diet. After being fed with high-fat diet for 14 weeks, no difference was found in plasma VLDL and LDL levels between two groups, while the level of HDL was significantly different. In addition, there was less fatty streak lesion in mice with human apoA-I (551 μ m² vs. 3890 μ m²). This research indicates that human apoA-I was probably responsible for the suppression of atherosclerosis progress.

HDLs are likely to be acceptors of excess free cholesterol from peripheric cells via scavenger receptor class B type I (SR-BI) (Ji et al., 1997), so may reverse or prevent the formation of macrophage foam cells (Annema et al., 2013). It had been described in mice that HDL molecules

facilitate reverse cholesterol transport, namely, transporting cholesterol away from extrahepatic tissues and carrying it back to the liver where it can be eliminated through bile to feces. This pathway was called trans-intestinal cholesterol excretion (Reichl et al., 1982). In addition, ATP-binding cassette transporters ABCA1 increase cholesterol efflux to apoA-I while ABCG1 played a dominant role in mediating 7-ketocholesterol and related oxysterols to HDL, inhibiting the oxidized LDL-induced apoptosis (Terasaka et al., 2008).

As a typical non-communicable disease, risk factors such as tobacco use, unhealthy diet, sedentary lifestyle and so forth, stimulate the formation of CVD to a large extent. In other words, modification of unhealthy lifestyle and risky behaviors are likely to reduce the prevalence of CVD. By controlling hypercholesterolemia via modifying diets and medical therapy, reduced CHD mortality rates could be achieved (EUROPEAN ATHEROSCLEROSIS SOCIETY, 1988; Oei et al., 1995). In Lipid Research Clinics Prevalence Mortality Follow-up Study, women with lower HDL-cholesterol had four-fold or higher incidence rate of CHD and mortality rates of CVD and CHD (Gordon et al., 1989) than counterparts with high HDL-cholesterol. All these studies proved that CVD is a preventable disease.

Pulses are essential farm products in the whole world. The total production of pulses worldwide was 77,599,253 tonnes and 5,828,000 tonnes in Canada by 2014 (FAO). FAO defined pulse as 'annual leguminous crops yielding from one to 12 grains or seeds of variable size, shape and colour within a pod'. But crops harvested green for food or used for oil extraction and sowing are excluded (FAO, 1994). In Canada, pulses are indispensable agricultural products and show great economical value. In 2010, 32% of world pea production and 38.5% of world lentil production were accounted to Canada (PulseCanada). In 2012, Canada produced 5,086,400

tonnes, 1,537,900 tonnes, and 3,340,800 tonnes of soybean, lentils and dry peas, ranking 7th, 10th, 11th of the 20 most important food and agricultural commodities, respectively. The protein compositions of pulses are usually over 20%, which is much higher than those of other vegetables. Besides, pulses are highly available and accessible due to the low price and wide distribution.

In contemporary world, people in low income regions probably suffer from starvation, which results in malnutrition. Protein-malnutrition is defined as insufficient protein intake along with or without adequate calories. Obviously, pulse is a wise choice of high protein foods due to availability, low price and high production. Actually, pulses have high nutritional values not only because of high protein content (18%-32%) despite low levels of methionine, cysteine and tryptophan (Boye et al., 2010), but also due to high levels of dietary fibre, vitamins (especially vitamin B) and minerals (such as iron, zinc, phosphorous) and low fat (Curran, 2012). Besides, many studies indicate that pulse proteins, especially indigestible protein, have cholesterol-reducing potential based on animal and human trials (Higaki et al., 2006; Lin et al., 2004; Tonstad et al., 2002). Therefore, the extraction of indigestible protein can provide a safe and effective product for reducing blood cholesterol, thus assisting in the prevention and treatment of CVDs.

1.1. Hypothesis

1. Indigestible proteins can be isolated after treatment of pulse proteins with pepsin and pancreatin digestion.

2. The isolated indigestible proteins will bind to bile acid in vitro and reduce plasma levels

of cholesterol when incorporated into rat feed.

1.2. Objectives

Although a large quantity of work has been done about the cholesterol-lowering effects of resistant proteins, the following points still need to be investigated.

a. How different pulse processing treatments (heat, high pressure, freeze and thaw, etc.) affect digestibility of pulse proteins to produce cholesterol-binding proteins is unknown.

b. Existing *in vitro* and *in vivo* works mostly focus on soybean while studies on peas, lentils and other pulses are scarce.

Therefore, the aims of the project are as follows:

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 bile acid 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.

3 CHAPTER TWO

2. Literature review

2.1. Metabolism of cholesterol and cholesterol-reducing compounds

The metabolism of cholesterol should be clarified. In the human body, about 1100 mg cholesterol enter the body pool per day, including 800 mg from de novo synthesis and 300 mg from diet. In the hepatocyte, bile acids are synthesized from cholesterol, and secreted from canalicular membrane to the gall bladder. Bile is excreted into the gastrointestinal tract (GIT), from where about 95% of the bile acids are taken up from end of ileum (which is reabsorbed and transported back to liver forming the enterohepatic circulation), while the remaining goes into the colon. A portion of the remaining 5% is passively reabsorbed after deconjugation and oxidative reaction of hydroxyl groups while the unabsorbed bile acids are excreted in feces (Cai et al., 2014). Enterohepatic bile acids circulation is of great importance due to contributions to cholesterol balance in the whole body, prevention of gallstones diseases and other health benefits (Cai et al., 2014; Shepherd, 2001). Currently, there are several types of cholesterollowering medicines in the market, with different underlining mechanisms. For example, statins inhibit β-hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase so that cholesterol synthesis in the liver is limited (Shepherd, 2001). However, the poor adherence of patients to prescribed drug doses coupled with negative side effects contributes to the limitation of pharmaceutical agents.

Apart from pharmaceutical agents, some phytochemicals have also been proven to have the ability of reducing blood cholesterol through the similar metabolic pathways as drugs. For

instance, 4-week consumption of 3.0 g/d of soluble fibre significantly lowered LDL-cholesterol and non-HDL cholesterol in hypercholesterolemic participants (Ho et al., 2017). In an animal study, rats fed pectin or psyllium had significantly lower liver total cholesterol level when compared with counterparts fed cellulose (Arjmandi et al., 1992). Furthermore, serum total cholesterol was reduced by pectin as well. On the other hand, short chain fatty acids, namely, butyrate and propionate were higher in fibre-fed rats than the cellulose-fed group. In addition, ingestion of soluble fiber was related with increased excretion of neutral sterols and bile acids (Arjmandi et al., 1992). It is likely that soluble fibre attenuated absorption of cholesterol in the GIT due to the high viscosity and prevented bile acid reabsorption to some extent such that fecal excretion was increased (Topping, 1991). It is worth noting that similar cholesterolreducing function had been found in pea protein (Parolini et al., 2013). Pea protein reduced plasma cholesterol by about 1700 mg/L, which was close to the data of oat fibre and apple pectin diet (by approximately 1600 mg/L and 1600 mg/L, respectively). Interestingly, the combination of pea protein and soluble fibre reduced cholesterol 50% more than those ingredients applied alone. The results indicate the potential of reducing blood cholesterol through consumption of legume protein isolate.

2.2. Plant proteins and cholesterol

Plant proteins have been used to reduce cholesterol in previous research works. For example, total cholesterol levels of male Sprague-Dawley rats fed with high-cholesterol diet containing buckwheat protein was significantly lower than that of rats fed casein (4.66 \pm 0.22 mmol/L versus 3.16 \pm 0.19 mmol/L). Besides, fecal excretion of cholesterol was greater (by 60

μmol/3 d) (Tomotake et al., 2007). Similar results could be seen in studies of Iwami et al., (1986) and Um et al. (2013) where gluten and rice proteins were used. In addition, in a human trial, participants with hypercholesterolemia were provided with a diet that contained about 40% of soy protein (equal to 25 g protein per day) for 8 weeks. A significant 9.8% and 12.0% reductions in total cholesterol and LDL-cholesterol, respectively were observed, while there was no change in HDL-cholesterol (Hoie et al., 2007). In summary, previous works indicate a promising cholesterol-lowering function of plant proteins. Although the underlying mechanism is not clearly known, there is a hypothesis that low digestibility is one of the reasons regarding the evidence that fecal weight was increased along with elevated fecal nitrogen and cholesterol when animals consumed buckwheat (Tomotake et al., 2007). And this is consistent with study of rice protein, which also enhanced fecal bile acid excretion (Um et al., 2013).

2.3. Pulse proteins and blood cholesterol level

Several kinds of protein from pulses, namely, pinto bean, black eye bean, lentil, were verified as having bile acid-binding capacity (Barbana et al., 2011; Kahlon et al., 2002; Kahlon et al., 2004), and these facts indicate potential cholesterol-reducing abilities (Table. 1). In a previous work, the *in vitro* bile acid-binding abilities of soy bean, black eye bean, garbanzo, lima bean, pinto beans and black beans were determined. Cholestyramine was used as standard reference which is a cholesterol-lowering drug. The results showed that cholestyramine bound significantly more bile acids than other beans. When samples were tested based on equal total dietary fibre (TDF) and insoluble dietary fibre (IDF) and protein contents, the significant variety of ability between different beans suggested that bile acid-binding ability did not depend on

TDF and IDF content. However, soybean, black eye bean and lima bean presented similar values when based on an equal dry mass, which indicate that bile acid-binding ability was associated with dry mass content (Kahlon, 2002). Besides, the bile acid-binding in soybeans, black beans and pinto beans were also independent of the protein content (Kahlon, 2002). In summary, studies have shown that the pulse seeds are likely to have promising cholesterol-lowering ability although the specific components or the factors that influence the bile acid-binding capacity were not clarified. In the study of Barbana et al. (2011) where red and green lentil flour and protein were used, although they all presented bile acid-binding ability, no significant difference was found between flour and protein. Moreover, protein hydrolysis also failed to increase the bile acid-binding ability. Lupin protein could also bind different bile acids (such as sodium cholate, sodium chenodeoxycholate, sodium deoxycholate, etc) in degrees of 50% to 60%, which are higher than that of de-oiled soybean protein (Yoshie-Stark et al., 2004). In addition, pepsin and pancreatin hydrolysis of the protein failed to make a significant improvement on the bile acid-binding activity. The authors suggested that conglutin y may have been responsible for the high bile acid-binding capacity of lupin protein.

Reference	Pulse	Bile acid binding (μmol/100 mg)
Kahlon & Woodruff	Pinto bean	2.56 ± 0.08
(2002)	Black bean	3.30 ± 0.08
	Soy bean	0.21 ± 0.02
Kahlon (2004)	Black eye bean	1.59 ± 0.09
	Lima bean	1.92 ± 0.09

Table 1: The bile acid-binding capacity of some pulses protein

2.4. Indigestible protein and cholesterol

However, proteins have been shown to possess greater cholesterol-binding effect after being subjected to the enzymatic treatment. Previous studies demonstrated that the indigestible pulse proteins (resistant to digestive enzymes such as pepsin and pancreatin), which are the residual fraction of the enzymatic digests, have a greater hypocholesterolemic function than native undigested protein (M Sugano et al., 1990). Table 2 illustrates some relevant studies that show activities of indigestible proteins. For instance, indigestible soybean proteins that were isolated after pepsin and microbial protease digestion, had a higher bile acid-binding capacity than soybean protein isolate. The results showed taurocholate-binding capacities of $36.1 \pm 0.2\%$ and $36.4 \pm 0.1\%$, for microbial protease and pepsin indigestible proteins, respectively in comparison to 28.9 ± 0.1% for the undigested soybean protein. Similarly, the glycocholate-binding capacities were $24.6 \pm 0.3\%$ and $23.6 \pm 0.4\%$ for, microbial protease and pepsin indigestible proteins, respectively in comparison to $19.0 \pm 0.3\%$ for and the undigested soybean protein. In a rat experiment, the diets consisted of soybean protein and indigestible proteins as the only nitrogen source in control and test groups, respectively while both diets contained 0.5% cholesterol. Protein source in both diets were at an equivalent nitrogen level. Both microbial protease and pepsin indigestible proteins decreased serum and liver cholesterol level significantly when compared to the diet formulated with undigested soybean protein (M Sugano et al., 1990). These results are consistent with data reported for the indigestible fraction of buckwheat protein (Kayashita et al., 1997). Besides, it was also concluded that lower protein

digestibility enhanced serum cholesterol level reduction. Momma (2006) reported that in red kidney bean, a 20-kDa polypeptide that contained rigid disulfide bonds was resistant to pepsin hydrolysis, and this peptide was identified as a subunit of legumin. Although the hypocholesterolemic effect of this resistant red kidney bean protein is yet to be investigated, it may contribute to the cholesterol-reducing function of pulse seeds.

The underlying mechanism of cholesterol-decreasing effect of indigestible proteins is not yet clarified but some scientists considered it can be attributed to interruption of synthesis and turnover of apo-lipoprotein, which can influence composition and size distribution of HDL (Edward et al., 1991). There is another hypothesis that hydrophobic reaction is the major mechanism of downregulation of cholesterol by the indigestible proteins, which will be discussed in the following paragraphs. Similar results were reported by Higaki et al. (2006). Two in vivo experiments were conducted using indigestible protein fractions obtained after peptic and pancreatic treatments. In the first experiment, 4 rats were given a casein diet for 1 week and then indigestible soybean protein diet for the next 1 week. In another experiment, after a one-week casein diet, rats were fed with the casein as control, 5% indigestible protein fraction + casein and 10% indigestible protein fraction + casein in the next week. Feces of each rat were collected daily. Then the feces were subjected to extraction with 5 mM phosphate buffer followed by 75% EtOH. Results showed that rats fed with indigestible proteins presented 3times higher bile acid-binding capacity than those fed casein. Besides, most fecal bile acids of indigestible protein-fed rats were soluble in EtOH rather than in water, which means indigestible protein binding bile acid had relationship with hydrophobicity of the soybean protein. Another research report by Iwami et al. (1986) indicated similar results. The hydrophobicity and bile

acid-binding capacity of soybean protein, wheat gluten, ovalbumin and casein and their enzyme-indigestible residues were tested by fluorescence measurement and radioactivity measurements. The results showed a direct relationship between fluorescence intensity and radioactivity. Indigestible casein showed the lowest bile acid-binding capacity while the indigestible soybean protein had the highest and those of ovalbumin and wheat gluten were in the 2nd and 3rd place, respectively. In addition, a reverse situation of plasma cholesterol levels of rats fed on these 4 source-based diets could be found, where the data for casein, ovalbumin, wheat gluten and soy protein presented a descending trend. It can be concluded that indigestible fraction of proteins after enzymatic hydrolysis are hydrophobic and are able to decrease blood cholesterol. In addition, after hydrolysis, the contents of hydrophilic amino acids (for example: aspartic, glutamic and serine) decreased, while hydrophobic amino acids such as valine, leucine and isoleucine increased (Higaki et al., 2006). The results indicate that the increase of hydrophobic amino acids contributed to the hydrophobicity of indigestible proteins, which facilitated the cholesterol-lowering effect. On the other hand, compared with rats fed with casein, rats fed with high-molecular weight fraction (HMF) excreted 27.7 \pm 5.7 μ mol/day fecal bile acids, almost 3-fold as much as casein group ($10.3 \pm 0.6 \mu$ mol/day) (Kanamoto et al., 2001). Moreover, the excretion of fecal bile acids in the ileum-resected group was significantly higher than in the jejunum-resected group (Kanamoto et al., 1999). According to the mechanism pathway of bile acids, it is rational to reason that hydrophobic binding interferes with enterohepatic circulation, elevating the excretion of fecal bile acids and resulting in decreased serum cholesterol level.

In vitro micellar solubility of cholesterol was significantly lower in the presence of soy

protein hydrolysates (SPH) (0.19 mmol/L) than soy protein (0.39 mmol/L) and casein hydrolysate (0.45 mmol/L). Moreover, in Caco-2 cells, cholesterol uptake from micelles containing SPH was approximately 1.5-fold lower than that from casein hydrolysate or soy protein containing cholesterol micelles $(5.70 \pm 0.34, 9.50 \pm 0.87, 8.12 \pm 0.82 \text{ pmol/well, respectively})$ (Nagaoka et al., 1999). The experiments indicate that the suppressed micellar solubility of cholesterol was associated with decreased reabsorption in the intestine, and the inhibited absorption of cholesterol in Caco-2 cells may be due to SPH interacting with micelles, which are likely to be the mechanisms of cholesterol-reducing function. This is similar to the indigestible protein and residual peptides produced from rice protein, in which the capacity of bile acid-binding and inhibition of micellar solubility of cholesterol were due to digestion resistance (Um et al., 2013). Besides, hydrophobic peptides can reduce the micellar lipid-carrying capacity by binding bile acids (Um et al., 2013). It is worth noting that a bile acid-binding peptide has been found in soy glycinin (Choi et al., 2002). Based on the findings, it is reasonable to conclude that the bile acidbinding peptides, which are resistant to peptic and pancreatic hydrolysis, play a vital role in binding bile acids through hydrophobic interaction and excretion in the feces.

In summary, indigestible proteins from pulses possess the ability to bind bile acids through hydrophobic interaction so that less bile acids are reabsorbed and delivered back to the liver. Eventually more blood cholesterol need to be transferred to the liver for de novo bile acid synthesis resulting in decrease of blood cholesterol level. Based on the previous works, the structural and functional properties, namely, digestibility, composition of amino acid and hydrophobicity of the indigestible protein are the essential factors contributing to the cholesterol-lowering effect.

Reference	Subject	Sample	Results		Possible
			In vitro	In vivo	mechanism
(M Sugano	male	-Microbial	-Bile acid-	TC (mg/dL):	The bile acid-
et al.,	Sprague-	protease	binding (%):	microbial	binding
1990)	Dawley	precipitate	microbial	protease digest:	ability and
	rats	fraction of	protease digest:	125 ± 5; pepsin	the increase
		soy protein	taurocholate:	digest: 109 ± 5;	of fecal
		and pepsin	36.1 ± 0.2;	TG (mg/dL):	steroid is
		digested-soy	glycocholate:	microbial	likely to be
		protein	24.6 ± 0.3;	protease digest:	the reason
		-20% of	Pepsin:	233 ±39(NS)	for
		undigested	taurocholate:	pepsin digest:	cholesterol-
		sample in	36.4 ± 0.1;	295 ± 46	lowering
		high	glycocholate:		effect
		cholesterol	23.6 ± 0.4	(Significant	
		diet;		when compared	
		3-week		to soy protein)	
		consumption			
(Kayashita	Male	-Precipitate	N/A	Plasma	-Cholesterol-
et al.,	Sprague-	fraction of		(mmol/L):	reducing
1997)	Dawley	buckwheat		TC: 2.80 (NS)	effect was
	rats	protein after		HDL-C: 1.03	attributed to
		trypsin		Feces:	high fecal
		digestion		Neutral sterols	excretion of
		(HMF), 160		(mmol/4 d):	neutral

 Table 2: The bile acid-binding activity and hypocholesterolemic effect of pulse proteins

		g/kg in high		131(NS)	sterols.
		cholesterol		Cholesterol	-The evidence
		diet;		(mmol/4 d): 105	of increased
		-3 weeks		Nitrogen (mg/4	fecal nitrogen
		feeding		d): 369	indicated the
				Fecal protein	effect is
				(g/4 d): 0.52	related to the
				(Significance:	low
				HMF compared	digestibility
				to buckwheat	of buckwheat
				protein)	proteins
(Iwami et	Male	- casein,	- A plot showed	- Rats fed with	- Increased
al. <i>,</i> 1986)	Wistar	ovalbumin,	a possible	soy digestion-	fecal
	rats	soy protein	relationship	resistant protein	excretion of
		isolated (SPI)	between	had the lowest	cholesterol
		or wheat	hydrophobicity	cholesterol level	and bile acids
		gluten in the	and bile acid-	(around 52	led to
		diet	binding capacity	mg/dL),	lowered
		- rats were	of peptic	followed by	blood
		fed with	digests; soy	gluten (55	cholesterol.
		different	protein	mg/dL),	- It is possible
		diets	digestion-	ovalbumin (66	that
		containing	resistant	mg/dL) and	indigestible
		20% of	fraction was the	casein (70	proteins and
		samples for 5	highest	mg/dL)	residual
		weeks			peptides
					influence
					hormones
					secretion,

					such as
					hormones
					from
					pancreas or
					gut that
					regulate
					cholesterol
					metabolism
(Nagaoka	Male	- casein, soy	- Taurocholate-	- Serum	- SPH
et al.,	Wistar	protein (SP),	binding of SPH	(mmol/L):	interacts with
1999)	rats	soy protein	was significantly	Casein vs. SPH:	micelles
		peptic	higher than	TC: -1.41	cholesterol in
		hydrolysate	casein	HDL-C: +0.48	the intestinal
		(SPH)	hydrolysate, but	LDL+VLDL-C: -	epithelia
		- Rats were	both were lower	1.89	interrupting
		fed with diet	than	SP vs. SPH:	cholesterol
		containing	cholestyramine	TC: -0.48	reabsorption
		20% of		HDL-C: +0.28	- The
		sample for 10		LDL+VLDL-C: -	presence of
		days		0.76	SPH suppress
				(changes were	micellar
				significant)	solubility of
				- Feces (µmol/3	cholesterol so
				d):	that
				Casein vs. SPH:	absorption is
				Total steroids:	reduced
				+167.9	
				SP vs. SPH: Total	
				steroids:	

				+76.1 (NS)	
(Higaki et	Male	- Residual	N/A	- Fecal nitrogen,	- Bile acids
al. <i>,</i> 2006)	Fischer-	fraction of		and bile acid	were bound
	344 rats	soy protein		excretion	via
		after pepsin		increased	hydrophobic
		and		sharply after	interaction by
		pancreatin		HMF feeding	HMF
		digestion		(increased by 50	- The HMF
		(HMF) <i>,</i>		mg/g of feces	and feces
		- casein		and 11 µmol/g	contained a
		- rats were		of feces	high level of
		fed with 20%		respectively)	hydrophobic
		of sample in		- The apparent	amino acids,
		the diet for 1		hydrophilicity of	which
		week		peptides in	indicates that
				feces was lower	presence of
				in HMF-fed rats	hydrophobic
				than casein-fed	peptides
				rats	induced the
				- Casein: 80% of	bile acid-
				fecal bile acid	binding
				was extracted	ability and
				by phosphate	these
				buffer	peptides are
				HMF: 70% of	resistant to
				fecal bile acid	digestive
				was extracted	enzymes
				by 75% EtOH	

2.5. Food processing treatments and the protein structure

Various processing treatments could influence the structure and physiochemical properties of proteins, which may change the functional properties. In this section, treatments such as high pressure (HP), wet heat, dry heat, freeze-thaw cycle and gelation treatments will be discussed. He et al. (2013) proved that rapeseed protein had significant increases of 27, 22, and 21 µM/g in free sulfhydryls (SH) content after treatment at pressures of 200, 400, and 600 MPa, respectively when compared to 2 μ M/g for the native protein isolate. However, surface hydrophobicity (So) presented a constant increase from approximately 600 to 2700 as the pressure increased. The results are consistent with values reported for HP-treated soy protein (Wang et al., 2008). In addition, HP also led to higher α -helix and β -sheet contents when the secondary structure of the protein was analyzed. Fourier transform infrared (FTIR) spectroscopy, which is used for characterizing protein aggregation behavior and molar mass and hydrodynamic radius of soluble aggregates in solution indicated that protein unfolding occurred at <400 MPa but there was molecular re-association at 600 MPa. Therefore, lower pressures produced unfolded protein conformation while higher pressures induced protein aggregation (Tang et al., 2009a).

On the other hand, wet heat treatment revealed different results (He et al., 2013). Free SH content of heated rapeseed protein was decreased constantly from 26 μ M/g for 60°C to 15 μ M/g for 100°C while *So* was increased to 700 and 1600 when heated at 60°C and 80°C, respectively but reduced to 1200 at 100°C. Besides, increased β -sheet content and decreased α -

helix content could be detected as a result of wet heat treatment. In another experiment conducted by Tang et al. (2009b), the vicilin-rich protein isolate from kidney bean was heated at 95°C for a series of time (15, 30, 60 and 120 min). Results showed that total free SH content was significantly lowered to $1.27 \pm 0.02 \mu$ mol/g of protein with the increased in heating time from 0 to 30 min and stayed unchanged, while that of exposed SH content showed the same trend until it dropped to $0.33 \pm 1.05 \mu$ mol/g of protein after 120 min of heating. As for the *So*, the values increased in the beginning up to 30 min after heating before it reduced in the following 1.5 h. In summary, protein denaturation-dependent unfolding of protein structure is likely to increase the free SH content and expose hydrophobic groups at relatively reduced high pressure or low temperature treatment. Meanwhile, increased high pressure or high temperature treatment resulted in protein aggregation, which was indicated by the decrease in free SH content and *So*. The aggregation behavior is probably attributable to the formation of intensive structures that are induced by disulfide bonds, which led to reduced exposure of hydrophobic groups.

A previous study reported that freeze-thaw treatment could modify the protein structure, depending on the number of cycles. For example, total SH groups in soybean protein reduced by about 5 μ mol/g from the initial 15.18 ± 0.16 μ mol/g in the control after 5 freeze–thaw cycles. As for the exposed SH, it increased from 2.54 ± 0.07 μ mol/g before treatment to the highest at the second cycle, followed by a decrease to 4.21 ± 0.11 μ mol/g at the fifth cycle. Similarly, surface hydrophobicity presented an ascending change up to 56.63 ± 1.08 after 3 cycles, before dropping to 39.79 ± 0.26 at the last cycle (Zhao et al., 2015). It is likely that the formation of ice crystals during freezing opened protein structure, which was evident at the first freeze-thaw cycles such that the hidden SH groups and hydrophobic groups become exposed and hence the

observed significant increases in SH and *So.* However, as the freeze-thaw cycle increased, there were rearrangements that led to protein aggregation, which resulted in decreased SH and surface hydrophobicity. In addition, the reduction in total SH during extensive freeze-thaw cycles is probably attributable to the formation of disulfide bonds.

In a study about myofibrillar proteins (MP) (Zhang et al., 2015), samples were subjected to different high pressure treatments prior to inducing gelation. The original protein and MP gel samples showed a descending change (from 13 mol/10⁴ g to 11 mol/10⁴ g and from 9.5 mol/10⁴ g to 7.1 mol/10⁴ g respectively) in total SH content with the increasing pressure from 0.1 MPa to 500 MPa. Besides, reactive SH content increased by 2.3 mol/10⁴ g in original protein while that of MP gel increased to 7.9 mol/10⁴ g at 200 MPa before dropping to less than 6 mol/10⁴ g at higher pressures. This was probably attributed to the fact that heat initially enhanced protein unfolding, which exposed the SH groups. However, the cross-link structure was formed in gel. The gel formation and protein aggregation buried the SH groups and made them less detectable. In addition, *So* also increased for both samples, which is consistent with the results of He et al. (2013). The results indicate that the heat treatment was likely to accelerate exposure of hydrophobic groups at lower pressures. Moreover, under higher pressure, thermal treatment contributed to greater hydrophobic interaction, resulting in protein aggregation (Zhang, 2015).

Albumin (classified as "water-soluble" according to the Osborne classification of legume seed protein nitrogen), which is the major protein in isolated bean protein, constitutes 29.3% to 73.7% of total protein nitrogen of 15 legumes (Shpande et al., 1987). Heated albumin was reported to have a lower extent of hydrolysis (20%) than that of native albumins (32%)

(Genovese et al., 1996). According to the gel electrophoresis pattern, the digest of heat-treated albumin revealed a high-molecule fraction of proteins that were unable to enter the resolving gel, but they disappeared with the presence of β -mercaptoethanol. It is likely that high-molecular weight protein was formed by protein-protein aggregation through disulfide bond formation, and may have been responsible for the reduced digestibility of heated albumin (Genovese et al., 1996). The aggregated protein structures would have reduced accessibility of susceptible peptide bonds to enzyme digestion, which was responsible for the low digestibility.

Although protein structures can be altered by dry heat treatment, the changes are different than for wet heat. For dry-heated wheat gluten, the *So* changed from 56.7 to 74.5. SH content increased from 4.74 µmol/g to 16.18 µmol/g but disulfide content decreased from 22.48 µmol/g to 16.76 µmol/g (Zhang et al., 2012). In another study of egg white (Mine, 1997), *So* and surface SH increased during dry heat treatment without a decrease as previously described for wet heat treatments while total SH decreased with heating time. The decrease in sulfhydryl/disulfide content, which meant a loss of disulfide bonds, suggested that there was little aggregation during the dry heat process and the protein stayed unfolded (Zhang, 2012). It can be rationalized from these results that dry heat increased the exposure of hydrophobic groups and cleavage of disulfide bonds but caused limited protein aggregate formation because of reduced hydrophobic interactions, which made peptide bonds more accessible to enzymatic hydrolysis.

To summarize, food processing treatments such as heat, high pressure, freeze-thaw, etc. have different impacts on the structural and functional properties of proteins, and this is also dependent on the duration and level of treatment. Taking together with the findings that reported cholesterol-lowering effect of indigestible proteins, the increased indigestibility that

results from processing treatments can improve yield of enzyme-resistant proteins, and there is possibility that these treatments can be used to enhance the function of cholesterol-binding proteins.

Reference	Sample	Treatments	Results	Mechanism
(He et al.,	Rapeseed	High pressure	Thermal	- formation of
2013)	protein isolate	(HP): 200 <i>,</i> 400	treatment:	disulfide bonds,
		and 600 MPa	- free	which
		for 15 min	sulfhydryls (SH)	contributed to
		Heat: 60, 80	increased under	protein
		100°C for 15	200 MPa but	aggregation and
		min	progressively	decreased
			decreased at	exposure of
			400 and 600	hydrophobic
			MPa treatment	groups
			- heat treatment	- increased
			reduced free SH	interactions
			content	between
			- So increased in	aromatic amino
			both HP and	acid residues
			heat treatments	
			- secondary	
			structure:	
			HP: α -helix and	
			β-sheet	
			contents	

Table 3	3: The	effect	of food	processina	treatments	on prote	ein structure
10010 0			0,,,000	processing		o p. o.c	in stractare

			increased	
			Heat: - tertiary	
			structure:	
			higher ellipticity	
			after treatments	
(Wang et al.,	Soy protein	Treated at 200,	- So increased	- High pressure
2008)	isolate	400 and 600	with increasing	made protein
		MPa,	pressure from 0	structure
		respectively for	to 600 MPa	unfolded and
		15 min.	- free SH	then aggregated
			content	- Pressure
			increased from	disrupted
			0 to 200 MPa	disulfide bonds
			before	but the
			decreased for	reformation
			higher pressure	occurred during
				the protein
				rearrangement.
(Tang & Ma,	Vicilin-rich	- incubated at	- lower total and	- Formation of
2009)	protein isolate	95°C for 15, 30,	exposed free SH	disulfide bonds
	from kidney	60 and 120 min	after heat	contributed to
	bean		treatment, no	the change of
			change found	free SH contents
			during further	- Protein
			treatment but	denaturation
			there was a	initially resulted
			decrease after	in the increase in
			120-min heating	So and the
			- So: increased	subsequent

			in the first 30	decrease is
			min and	because of
			reduced	protein
			thereafter	aggregation via
				hydrophobic
				interactions
(Zhao et al.,	Soy protein	Frozen at	- total SH stayed	- F-T cycles
2015)	isolate (SPI)	-18°C for 2	unchanged in	unfolded protein
		days and	the first two	chains, which
		thawed at 25°C	cycles, and	contributed to
		for 12 h. The	decreased with	surface exposure
		freeze-thaw (F-	increasing	of the sulfhydryl
		T) cycle was	cycles.	groups and
		repeated 1-5	- exposed-SH	fluorescent
		times.	increased from	aromatic amino
			0 to 2 then	acids. With
			decreased	increasing cycles,
			- the highest So	protein might
			was found after	aggregate and
			three F-T cycles	disulfide bonds
			- circular	form.
			dichroism	- tryptophan
			spectra analysis:	exposed to a
			F-T cycles	hydrophobic
			increasing from	environment
			0 to 4 resulted	- high molecular
			in a decrease in	weight (MW)
			β -sheet and	proteins were
			increase in α -	broken into small

		helix	MW molecules
		- tryptophan	by water
		maximum	redistribution,
		fluorescence	ice
		emission	recrystallization
		wavelength	and protein
		shifted to	mechanical
		shorter	damage
		wavelength and	
		the emission	
		intensity peak	
		also slightly	
		shift to blue	
		wavelengths	
Wheat gluten	Heating in oven	- SH content	- Hydrophobicity
	at 120°C for 20	increased from	increased due to
	min	4.74 μmol/g to	the lack of water,
		16.18 µmol/g	which provided
		and disulfide	polar media for
		content	hydrophobic
		decreased from	interactions and
		22.48 µmol/g to	heat delivery
		16.76 µmol/g.	- sulfhydryl
		Contangod	content
		from 56.7 to 74.5	increased due to
			cleavage of
			disulfide bonds
		- post-dry	but protein chain
	Wheat gluten	Wheat glutenHeating in oven at 120°C for 20 min	helix- tryptophanmaximumfluorescenceemissionwavelengthshifted toshorterwavelengthshorterwavelengthalso slightlyshift to bluewavelengthsWheat glutenHeating in oven at 120°C for 20 minMeat glutenSH content increased from at 120°C for 20 increased from 22.48 µmol/g to 16.76 µmol/g to 16.76 µmol/g toSo changed from 56.7 to 74.5

			had a lower α -	did not occur
			helix/ β -sheet	
			ratio	
(Zhang et al.,	Myofibrillar	Original	Original protein:	- Thermal
2015)	protein (MP)	protein:	- decrease in	treatment
		treated at 100,	total SH content	strengthened
		200, 300, 400,	and increase in	unfolding of
		500 MPa (± 10	reactive SH	protein
		MPa) for 10	content with	molecules
		min,	increase of	- Lower pressure
		Gel: heated at	pressure;	unfolded the
		a rate of	- So: increased	protein structure
		1°C/min from	with increasing	while heating
		20°C to 65°C	pressure	makes
		and kept at	MP Gel: -	hydrophobic
		65°C for 20 min	decrease in	residues interact
		after HP	total SH but	to form protein
		treatment.	exposed SH	aggregates
		Then all	increased	during gelation
		samples were	before dropping	
		kept overnight	at pressure of	
		at 4°C.	200 MPa; total	
			SH content was	
			lower than	
			original protein;	
			- So increased	
	sharply before			
---	-----------------------	--		
	keeping			
	unchanged at			
	pressure of 300			
	MPa; <i>So</i> higher			
	than untreated			
	protein			
	- more Trp			
	hydrophobic			
	residues were			
	buried in a			
	hydrophobic			
	environment			
1				

4 CHAPTER THREE

3. Materials and methods

3.1. Materials

Soybean and pulse seeds (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 -20°C before the experiment.

3.2. Protein isolation

Protein isolates from the pulses were produced using isoelectric point precipitation method described by Adebiyi et al. (2011). 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 4°C). 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. The pH of extraction and precipitation were based on the pH-dependent solubility of pulse proteins (Boye et al., 2010; Carbanaro et al., 1997; Tan et al., 2014; Yin et al., 2010).

3.3. Protein pre-treatments

Each protein isolate was subjected to the following treatments:

1) Wet heat: 10% (w/v) aqueous slurry of each protein isolate was prepared in beaker, placed in boiling water for 30 min, and cooled.

2) Autoclave: 10% (w/v) aqueous slurry of each protein isolate was prepared in reagent a bottle, placed in autoclave at 120° C for 20 min, and cooled.

3) Dry heat: 5 g of the protein isolate was spread evenly in a glass petri-dish, covered and placed in an oven at 100°C for 12 h or 24 h; samples were then cooled at room temperature and stored in the freezer.

4) Freeze-thaw: 10% (w/v) aqueous slurry was prepared in sealed plastic bottle. After being frozen for 24 h, sample was placed at room temperature until it thawed completely. Then, the thawed sample was placed back in the freezer for 24 h, thawed again and then repeated for a 3rd cycle.

5) 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 95°C for 1 h, the mixtures were cooled and kept in the refrigerator (4°C) for 14 h. The lowest concentration at which the gel did not flow when the tube was inverted was considered the LGC (He et al., 2013), 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 -20°C).

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

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3.4. Preparation of indigestible proteins

The pretreated samples were subjected to simulated GIT digestion as described by Higaki et al. (2006). Briefly, a suspension of the protein isolates (10%, w/v) was prepared and hydrolyzed with pepsin (1%, w/w) at 37°C, pH 2.0 for 2 h, and was followed by pancreatin (1%, w/w) hydrolysis at 37°C, pH 7.5 for 4 h. The mixtures were centrifuged and the residues containing indigested proteins were washed twice with water and centrifuged again. Then, they were freeze-dried and stored at -20°C. The protein digestion was done in duplicate.

3.5. Determination of percent gross yield and protein content

Gross yield (%) of the protein isolates was calculated as the percentage ratio of the weight of the freeze-dried sample to the weight of the starting material. Gross yield (%) of the indigestible proteins was calculated as the percentage ratio of the freeze-dried indigestible protein to the original weight of the starting material (Adebiyi et al., 2011). Protein content of the protein isolates, pre-treated, and indigestible proteins was measured using the modified Lowry method (Markwell et al., 1978).

3.6. Determination of surface hydrophobicity (So)

Surface hydrophobicity of the indigestible proteins was determined using the method reported by He et al. (2013). 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 (Fl₀) 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 μ L 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 (FI_A) of the mixture was measured immediately at the same condition described above. The initial slope of the plot of net FI (FI_A-FI₀) versus protein concentration calculated by linear regression analysis was used as an index of surface hydrophobicity.

3.7. Determination of *in vitro* bile acid-binding capacity

The ability of the indigestible proteins to bind bile acids was determined as previously reported (Kahlon, 2004; Yoshie-Stark, 2004). The bile acid mixture (2 mM) consisted of glycocholic acid, glycochenocholic acid, glycodeoxycholic acid, taurocholic acid, taurochenocholic acid and taurodeoxycholic acid (purchased from Sigma-Aldrich, USA) 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 μ L of bile acid mixture were mixed and incubated in a water bath at 37°C 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 (Geneway Biotech Inc., USA). Cholestyramine resin (from Sigma-Aldrich), 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 (%) = $((A_{mixture} - A_{supernatant})/A_{mixture}) \times 100\%$, where $A_{mixture}$ refers to the bile acid concentration in the mixture and $A_{supernatant}$ is the bile acid concentration in the supernatant.

4.1.1

3.8. Proximate and Amino acid composition analysis

Based on the cholesterol-binding ability and yield of indigestible proteins, the black-eye bean was selected for further experiments. The proximate composition was analyzed using the AOAC method (AOAC, 1990). The amino acid composition was also analyzed according to the method described by Bidlingmeyer et al., after acidic hydrolysis using 6 M HCl (Bidlingmeyer et al., 1984). The cysteine and methionine contents were analyzed after performic acid oxidation (Gehrke et al., 1985) and the tryptophan content was determined after alkaline hydrolysis (Landry et al., 1992).

3.9. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out on the native and indigestible black eye bean proteins under reducing and non-reducing conditions according to Malomo et al. (2015). The non-reducing separating buffer contained 0.01 M Tris/HCl buffer (pH 8.0), 2.5% SDS, 0.001 M EDTA and 0.01% Bromophenol blue, while the reducing buffer contained 5% (v/v) b-mercaptoethanol (β -ME), in addition to the non-reducing buffer components. Sample (2.5 mg) was dissolved in 1.8 mL of SDS or SDS- β -ME buffer, vortexed, and heated at 95°C for 1 h in a block heater, then cooled and centrifuged for 15 min (10,000 g). The supernatant was collected for the electrophoresis. The samples (5 μ L) were loaded on 8-25% gradient gels (GE Healthcare Bio-Science AB, Sweden) and

electrophoresis was performed with Phastsystem Separation and Development following the instructions from manufacturer (GE Healthcare, USA). Standard proteins (10-200 kDa) were used as the molecular weight marker.

4.1.2

3.10. Molecular weight determination by fast protein liquid chromatography (FPLC)

Black eye bean native protein isolate and indigestible protein samples were subjected to molecular weight analysis by FPLC (GE Healthcare Life Sciences, Montreal, PQ, Canada). The Superdex 75 10/300 GL column (10 × 300 mm, 1,000–300,000 fractionation range) and a UV detector (λ =214 nm) was installed. Column was calibrated with bovine serum albumin (66 kDa), cytochrome c (12 kDa), aprotinin (6.5 kDa), and vitamin B12 (1.8 kDa). Native protein (10 mg/mL) or indigestible protein (20 mg/mL) suspensions were each prepared with buffer (0.05 M phosphate buffer + 0.15 M NaCl, pH 7.0). A 100 µL aliquot of sample was injected and loaded onto the column and then eluted with the phosphate buffer at a flow rate of 0.5 mL/min (He et al., 2013).

3.11. Rat feeding experiment

Male and female Sprague-Dawley Rats (4 wks old) were fed with standard AIN-93G diet as reported by Girgih et al. (2016) with addition of cholesterol and sodium cholate. The ingredients of the diet were purchased from Dyets Inc. (Canada). Rats were provided with normal diet (without addition of cholesterol and sodium cholate) 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 4) was: group 1, standard balanced diet with 20% casein; group 2, 19% casein + 1% isolated indigestible protein; group 3, 15% casein + 5% isolated indigestible 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 SDS-PAGE, sterols (neutral & acidic). Total triglycerides and total cholesterol assay of blood (plasma) samples were measured using commercial kits (Cayman Chemical Company, USA). All rat experiments were approved by the University of Manitoba Animal Ethics Committee.

	Diet group				
		1%	5%	5%	
		indigestible	indigestible	native	
Ingredients	Control	group	group	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	

Table 4: The composition of diets for rat feeding

3.12. Plasma preparation

Blood samples were preserved in blood collection tube with EDTA (Becton, Dickinson and Company, USA). After centrifugation (1000 g, 4° C for 10 min), aliquot of the clear supernatant (yellow plasma layer) was transferred to a centrifuge tube and stored at -80° C.

3.13. SDS-PAGE of feces

SDS-PAGE was analyzed for the feces samples collected at baseline and the sixth week using the method described above.

3.14. Statistical analysis

Data are presented as means \pm standard deviation (SD). Two-way ANOVA and Duncan's multiple range tests 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, USA).

5 CHAPTER FOUR

4. Results and discussions

4.1. Yield of the protein isolates

As shown in Figure 1, the yields from the different pulses were about 10% to 20%, with lima bean and soybean having the lowest (9.34%) and highest (19.83%) yields, respectively. Soybean is well-known as a high-protein food; soybean and soy products, such as soy milk and tofu, are traditionally consumed by Asians and are widely used as protein sources for vegetarians. Previously reported yield of pea protein (alkaline extraction) was 15.3–16.0% (Stone et al., 2015) and that of soybean isolate (alkaline extraction) was 56.6% (Adebowale et al., 2011), which are higher than values obtained in this study.



Figure 1: Yield of pulse protein isolate (native protein without any treatment, mean \pm SD, n=3)

One-way ANOVA was conducted and bars with different letters have mean values that are significantly different (P<0.05).

4.2. 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. 2). 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.

The 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 -20°C and under tap water, respectively, before being kept to gel overnight at 4°C. The different cooling methods had

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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%).

The digestibility of proteins can change after protein pre-treatments. For instance, low digestibility of globulins and red kidney bean protein was observed after heat and high pressure treatments, respectively (Carbonaro et al., 2005; Yin et al., 2008). This is because of rearrangements involving protein aggregation through hydrophobic interaction or disulfide bonds occur during a long treatment time (He et al., 2013; Tanget al., 2009b; Wang et al., 2008; Zhao et al., 2015). The aggregated protein could lower accessibility of cleavage sites to the proteases (Yin et al., 2008), leading to high yields of the indigestible proteins after autoclave, wet heat and gelation treatments in this study. On the other hand, the yield after dry heating was lowest among treatments. Zhang et al. (2012) reported continuous increase in surface hydrophobicity from 56.7 to 74.5 and SH content from 4.74 µmol/g to 16.18 µmol/g after dry heat treatment, indicating less formation of hydrophobic interaction and disulfide bonds, resulting in limited protein aggregation probably due the lack of moisture. Therefore, peptide bonds were more exposed to the surface and more accessible to enzymes after dry heat treatment than wet heat.



Figure 2: Yield of indigestible proteins (Means \pm SD, n=3)

Pulse protein isolates were subjected to food processing treatments followed by pepsin and pancreatin hydrolysis. The residue was collected as indigestible protein.

Two-way ANOVA was conducted between samples and treatments. The lowercase and uppercase letters showed the significance of samples and treatments respectively. Bars with different letters have mean values that are significantly different (P<0.05).

4.3. Protein content of protein isolates

The native protein isolates had protein contents ranging from 50% to 90% (Fig.3); pinto bean had the highest protein content of 87.96%, followed by green lentil and black bean proteins, while chickpea had the least protein (55.90%). Moong dal washed and mung bean had almost same protein content of approximately 69%. Previous works showed that the protein content of soybean protein isolate was about 92.6% to 96.4% (Wally-Vallim et al., 2014), while that of pea and mung bean protein was 83.3% to 86.9% (Stone et al., 2015) and 81.0% (Rahma et al., 2000) which are higher than the results obtained in current study.





One-way ANOVA was conducted and bars with different letters have mean values that are significantly different (P<0.05).

4.4. Soluble protein content of pre-treated protein

The protein contents of the pre-treated samples were about 13.31% for dry heated chick pea to 81.82 % for green split pea (Fig.4). For the majority of the samples, the lowest protein contents were recorded from autoclaved proteins in comparison to other treatments, ranging from 32.08% (black eye bean) to 57.76% (lima bean). On the contrary, freeze-thawed and dry heated samples showed relatively higher percentages, excluding pinto bean, mung bean, small white bean and soybean proteins, which had highest values after wet heat or gelation treatment. The highest protein content among all pulses varied from 64.47% (mung bean) to 81.82% (green split pea). Comparable protein content could be seen in moong dal washed and mung bean protein after most of processing procedures, ranging from 35% (autoclave) to 67% (dry heat 24 hr). However, there were significant differences between two beans in the results of freeze-thaw and gelation. The protein content of freeze-thawed and gelation (rapid cooling) mung bean (55.47% and 51.05%) was 12% and 9% lower than its moong dal washed counterparts. Surprisingly, the protein values for wet heat were the highest in pinto bean and small white bean (83.30% and 79.28% respectively), which were the only two pulses that had high levels after wet heating. Furthermore, red kidney bean was the only pulse that had a high protein content due to autoclave (81.61%). Gelation treatments with different cooling rates resulted in comparable protein content among each pulse. According to statistical analysis, the differences between samples and treatments were significant (p<0.001). But there was no significant difference between soybean and black-eye bean, black-eye bean and mung bean, black bean and moong dal washed. Compared to native protein, the soluble protein contents of pre-treated samples were lower, which was partly attributed to the decrease in solubility. The

solubility of kidney bean protein increased after heating but reduced during the further treatment (Tang et al., 2009b). Similar change was found in high pressure treated lupin protein (Chapleau et al., 2003). This is because the treatments unfolded protein chain, changing hydrophobicity/hydrophilicity ratio of the surface (Moure et al., 2006), thus affecting the solubility. In this study, samples obtained from autoclave, gelation and wet heat treatments presented relatively lower soluble protein contents probably because of increased exposure of more hydrophobic residue than other treatments.



Figure 4: Soluble protein content of pulse protein after 7 kinds of treatment (mean \pm SD, n=3) Protein isolates were subjected to treatments listed respectively: autoclave, dry heat for 12 hr, dry heat for 24 hr, freeze-thaw (3 cycles), gelation with cooling in freezer (rapid cooling), gelation with cooling under tap water (slow cooling) and wet heat. Two-way ANOVA was

conducted between samples and treatments. The lowercase and uppercase letters showed the significance of samples and treatments respectively. Bars with different letters have mean values that are significantly different (P<0.05).

4.5. Soluble protein content of indigestible proteins

The soluble protein content of all indigestible protein samples was less than 50%. The protein content was highest for the dry-heated moong dal washed (47.92%) and lowest for dry-heated indigestible chickpea protein (6.91%). The freeze-thaw and dry heat treatments resulted in the highest indigestible protein content for most of the pulses, compared to gelation and wet heat, which led to lower soluble protein content. Furthermore, for black bean, black-eye bean, pinto bean, small white bean and soybean samples, the values for gelation treatments with different cooling methods were quite close. According to statistical analysis, the differences between samples and treatments were significant (p<0.001).

Comparing the soluble protein contents of native, pre-treated and indigestible proteins, a reduction in levels was observed after all treatments and hydrolysis. One possible reason for the decrease is the alteration of protein structure that led to exposed hydrophobic groups in the indigestible proteins (Moure et al., 2006). In particular, autoclave, gelation and wet heat treatments led to more decreases for all samples, while the changes after dry heat and freeze-thaw treatments were small. The most remarkable decrease in protein content was 64.75% for green lentil after gelation with rapid cooling, whereas only 1% decrease was observed after slow-cooling gelation. The cooling rates during gelation influenced the gel structure and this can affect the digestibility of protein. Compared to rapid cooling, slow cooling allows more time for

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intra- and inter-molecular protein interactions via hydrophobic, hydrogen and disulphide bonds, thereby forming more cross-links, and hence stronger gel structures (Sun et al., 2011; Zhong et al., 2004). Such compact structure obtained after slow cooling can decrease interaction with water, therefore, lower protein content was observed.



Figure 5: Soluble protein content of indigestible protein after different treatments (mean \pm SD, n=3).

Protein isolates were subjected to treatments listed respectively: autoclave, dry heat for 12 hr, dry heat for 24 hr, freeze-thaw (3 cycles), gelation with cooling in freezer (rapid cooling), gelation with cooling under tap water (slow cooling) and wet heat, followed by pepsin and pancreatin hydrolysis. The residue was collected as indigestible protein. Two-way ANOVA was conducted between samples and treatments. The lowercase and uppercase letters showed the significance of samples and treatments respectively. Bars with different letters have mean values that are significantly different (P<0.05).

4.6. Surface Hydrophobicity (S_o)

The surface hydrophobicity of the indigestible proteins is shown in Fig. 6. Among the pulses, lima bean had the highest S_o 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 S_o 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 S_o 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.

He et al., (2013) compared the S_o of rapeseed protein after high pressure and heat treatment, and found that high pressure was more effective than heating, and this can be found in chickpea, green split pea, lima bean, mung bean and red kidney bean in current study. Additionally, dry heated samples showed higher S_o than wet heated counterparts, which is different from previous study (Khan et al., 2011) probably due to the differences in treatment time and condition.

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Figure 6: Surface hydrophobicity (S_o) of indigestible protein from different pulses after treatment (mean \pm SD, n=3).

Protein isolates were subjected to treatments listed respectively: autoclave, dry heat for 12 hr, dry heat for 24 hr, freeze-thaw (3 cycles), gelation with cooling in freezer (rapid cooling), gelation with cooling under tap water (slow cooling) and wet heat, followed by pepsin and pancreatin hydrolysis. The residue was collected as indigestible protein. Surface hydrophobicity was measured spectrofluorimetrically by using 8-Anilino-1-naphthalenesulfonic acid (ANS) as fluorescence probe. Two-way ANOVA was conducted between samples and treatments. The lowercase and uppercase letters showed the significance of samples and treatments respectively. Bars with different letters have mean values that are significantly different (P<0.05).

4.7. Bile acid-binding activity

The bile acid-binding activity of the indigestible proteins is presented in Figure 7. 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 activity did not differ significantly (p=0.44) on the basis of pulse variety. On the other hand, the bile acidbinding capability for black bean, green split pea and small white bean were statistically different (p<0.05) based on pre-treatment type.



Figure 7: Bile acid-binding activity of indigestible protein from different pulses after treatment (mean \pm SD, n=2).

Protein isolates were subjected to treatments listed respectively: autoclave, dry heat for 12 hr, dry heat for 24 hr, freeze-thaw (3 cycles), gelation with cooling in freezer (rapid cooling), gelation with cooling under tap water (slow cooling) and wet heat, followed by pepsin and pancreatin hydrolysis. The residue was collected as indigestible protein. Cholestyramine was used as standard reference. Two-way ANOVA was conducted between samples and treatments. The lowercase and uppercase letters showed the significance of samples and treatments respectively. Bars with different letters have mean values that are significantly different (P<0.05).

The indigestible protein, which is the non-hydrolyzed fraction when subjected to enzymatic hydrolysis, is also called high-molecular-weight fraction (HMF) of protein or resistant protein. For instance, HMFs of soy protein after digestion with pepsin and microbial protease bound approx. 36.1% taurocholate and 24% glycocholate acid, which were significantly higher than the native soybean (28.9% and 19.0% respectively) (Sugano et al., 1990). Similarly, peptic hydrolysate of soybean was also found to have ability of binding taurocholate and it was stronger than native soy protein (Nagaoka et al., 1999). Many studies reported a correlation between the bile acid-binding ability of indigestible proteins and their hydrophobicity. For example, Iwami et al. (1986) compared the binding activity of several peptic digests of proteins, including casein, ovalbumin, gluten and soybean, and found that soybean, which had the highest surface hydrophobicity exhibited the best bile acid-binding ability (Iwami et al., 1986). In addition, dietary fibers, such as alfalfa, bran and so forth, bound approximately 1% to 50% of bile acids in vitro according to their hydrophobic properties (Story et al., 1976). In addition, the hydrophobic peptide fragment of 129–134 residues (VAWWMY) in A1a subunit from indigestible soy protein largely contributed to the bile acid-binding ability (Choi et al., 2002), which could probably interact with the hydrophobic region of bile acids. However, S_o of the indigestible proteins in this study seemed not a major factor for their bile acid-binding capacity, as there was no correlation between both parameters. The bile acid-binding activity of the samples across treatments was significantly different, and thus suggests that the pre-treatments probably affected other structural properties of the proteins that made them function differently. These previous studies reported that the bile acid binding did not depend on the protein content or total dietary fiber but on weight of dry mass (Kahlon, 2002; Kahlon, 2004) This is likely to be the reason for the similar binding activity between some of the samples used in this study.

4.8. Amino acid composition

The amino acid composition of black-eye bean protein isolate (BPI) and its indigestible counterpart (BIP) obtained after gelation pretreatment followed by pepsin and pancreatin digestion was measured (Table 5). There was no obvious change in amino acid profile after gelation treatment and hydrolysis. For both samples, the major amino acid was glutamic acid + glutamine, consisting of 17.62% for BPI and 16.72% for BIP; this was followed by aspartic acid + asparagine (11.59% and 11.99% respectively). The least proportion was cysteine, which was only 0.67% for BPI and 0.62% for BIP. Specifically, the percentage for hydrophobic amino acids (Leucine (Leu), Isoleucine (Ile), Valine (Val), Alanine (Ala) and Methionine (Met)), except Met, Leu (which increased by 0.24% and 0.36% from 1.42% and 8.12%, respectively) decreased by 0.07% for Ile, 0.37% for Val and 0.11% for Val after hydrolysis. The hydrophobic aromatic amino acids, tryptophan remained similar at 1.18% and 1.27% but phenylalanine content was reduced to 5.66% after hydrolysis. Yang et al. (2012) reported that the ratio of Arginine (Arg)/Lysine (Lys) was associated with the more bile acid bound by indigestible fraction of rice protein, which can be attributed to the increased activity of hepatic 7a-hydroxylase (a rate-limiting enzyme for conversion of cholesterol to bile acids). The ratio of the present study was 0.96 for BPI and 1.01 for BIP, suggesting that Arg/Lys ratio was not changed by gelation pretreatment and enzyme hydrolysis.

BPI ¹	BIP ¹
11.59	11.99
3.82	3.58
5.55	5.66
17.62	16.72
4.71	4.63
3.62	3.54
4.24	4.13
0.67	0.62
4.97	4.60
1.42	1.66
4.24	4.17
8.12	8.48
3.45	3.55
5.93	5.66
3.48	4.40
7.11	6.90
1.44	1.46
6.83	6.99
1.18	1.27
	BPI ¹ 11.59 3.82 5.55 17.62 4.71 3.62 4.24 0.67 4.97 1.42 4.24 8.12 3.45 5.93 3.48 7.11 1.44 6.83 1.18

Table 5: composition of amino acid content of black eye bean protein isolate and

indigestible protein

1. BPI: black-eye bean protein isolate; BIP: black-eye bean indigestible protein after gelation treatment (slow cooling)

2. Hydrophobic amino acids: Gly, Ala, Val, Leu, Ile, Pro, Phe, Met, Trp

3. Aromatic amino acids: Phe; Tyr; Try

4.9. Proximate composition and mineral content

The proximate compositions of BPI and BIP show that the products consist mainly of proteins (>80%) as shown in Table 6. The remaining part consisted of ash, fat, moisture carbohydrate and minerals. The protein content of BPI was 83.95% and it was 0.36% higher than BIP. Moisture and fat contents for two samples were comparable, which were 2.1% and 1.89% for moisture and 4.17% and 4.76% for fat (for BPI and BIP respectively). Notably, the content of ash for BPI was 3-fold greater than BIP (9.84% vs. 3.38%). The percentage of fibre for BIP was triple the value for BPI, although both were lower than 1%. In addition, BIP contained 5.87% of carbohydrate while that of BPI was not detectable. It has been proven that the fiber could promote the cholesterol-lowering ability of pea protein (Parolini et al., 2013), which may have contributed to the better cholesterol-reducing ability of the indigestible protein.

In BPI, the non-fiber carbohydrate was not detected but there was 5.87% in BIP sample. This is because the value of non-fiber carbohydrate was calculated by subtraction of other content from 100 percent, which means the content of compounds except non-fiber carbohydrate in BPI was 100% but in BIP was 94.13%.

	Black-eye bean protein isolate		Black-eye bean indigestible	
			protein	
Analysis	As Received	Dry Matter	As Received	Dry Matter
Moisture (%)	2.1	N/A	1.89	N/A
Dry Matter (%)	97.9	N/A	98.11	N/A
Crude Protein (%)	83.95	85.75	83.59	85.2
Crude Fibre (%)	0.16	0.16	0.5	0.51
Fat (%)	4.17	4.26	4.76	4.85
Ash (%)	9.84	10.05	3.38	3.44
Calcium (%)	0.05	0.05	0.03	0.03
Phosphorus (%)	0.85	0.87	0.43	0.44
Magnesium (%)	0.06	0.06	0.02	0.02
Potassium (%)	0.23	0.24	0.08	0.08
Sodium (%)	1.75	1.79	0.99	1.01
Copper (mg/kg)	1.75	1.79	18.95	19.31
Iron (mg/kg)	206.47	210.9	263.69	268.78
Manganese (mg/kg)	9.28	9.48	4.99	5.08
Zinc (mg/kg)	23.12	23.62	36.38	37.08
Non Fibre Carbohydrates (%)	N/A	N/A	5.87	5.02
Total Digestible Nutrients (%)	79.9	80.81	86.23	87.07

Table 6: Proximate composition and mineral content of black-eye bean protein isolate and black-eye bean indigestible protein

1. BPI: black eye bean protein isolate; BIP: black eye bean indigestible protein after gelation treatment (slow cooling)

4.10. Gel electrophoresis

SDS-PAGE was used to analyze the polypeptide composition of black-eye bean protein isolate (native protein) and indigestible black-eye bean protein (indigestible protein); the results are shown in Figures 8. On non-reduced gel, the molecular weights of two major proteins of native protein were around 50 and 53 kDa, which were previously shown to be vicilin (Barac et al., 2010; Carbanaro et al., 1997). Bands at molecular weight between 20 to 40 kDa could also be seen. On the other hand, the indigestible protein predominantly consisted of protein subunits with sizes <15 kDa, which confirms that these are digested protein fragments. By adding β -mercaptoethanol, no obvious substantial effect on both samples were observed, except the increase in intensity of the 100 to 120 kDa polypeptides. The molecular weight distribution obtained in this work is consistent with previously reported data (Grg et al., 1982). Besides, the proteins in the region of 50 to 53 kDa were digested after heating and hydrolysis, which was reflected by the disappearance of two bands in native protein lane. In addition, there were some high-molecular mass molecules that did not enter the stacking under non-reducing condition (pointed by arrow), which could be protein aggregates (Genovese et al., 1996). Through addition of β -mercaptoethanol, intensity of bands was lower, along with the presence of bands at 100 to 120 kDa, which were probably from cleavage of disulfide bonds in aggregated proteins. The results confirmed that the indigestible proteins are low molecular weight polypeptides that were produced from digestion of the high molecular weight proteins.

Due to the low solubility of indigestible protein, sodium dodecyl sulfate was hard to bind amino acids, making bands of indigestible protein lane were blurry.

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Figure 8: SDS-PAGE analysis of black eye bean protein isolate (native protein), indigestible protein (gelation treatment with slow cooling)

4.11. Fast protein liquid chromatography (FPLC)

The molecular weight distribution of native and indigestible black-eye bean is presented in Figure 9. The native protein contained only one major peak, and the estimated molecular weight ranged from 10.34 to 27.64 kDa. On the other hand, indigestible black eye bean protein was composed of 4 fractions with molecular weight of 3.39, 0.98, 0.69, 0.11 kDa respectively, which are 10-times smaller than the native protein. Combined with the SDS-PAGE results, the FPLC confirms that the indigestible protein consisted of mainly low molecular weight proteins when compared to the high molecular weight proteins in the native protein.





Figure 9:Molecular weight distribution of native and indigestible protein of black eye bean.

BPI: Black-eye bean protein isolate

BIP: Black-eye bean indigestible protein

4.12. Rat study

The *in vitro* bile acid-binding was assayed for the different pulses to select the one that has the best activity for further evaluation of its cholesterol-lowering effect in rats. But since the samples had comparable bile acid-binding ability, the indigestible black-eye bean protein was chosen. This is because, among the samples, it had the highest yield after the hydrolysis. Therefore, both the indigestible and native black-eye bean proteins were used for the rat feeding experiments to compare their effects on the rat cholesterol level.

4.12.1. Food intake and body weight gain

Figure 8 illustrates food consumption of rats that were provided with 4 diets with different protein sources. There was an increasing trend with the feeding weeks but fluctuation could be observed. For female rats, group 1 consumed 109.98 g food in total at the first week, and the number increased to 173.30 g at week 4, followed by a decrease to 152.61 g at the end. The intakes for rats in group 2 and 3 were comparable, which were 118.93 g and 117.6 g, and went up to 160.74 g and 164.39 g at the sixth week. As for group 4, rats ate 116.56 g in the first week, and approximately 63 g more food was consumed in the last week. On the other hand, male rats in group 1 consumed 174.50 g diet at the beginning and the consumption increased to 236.17 g in the last week. The food intake for group 2 at the week 1 was 156.92 g and elevated to 225.41 g after 6 weeks. Rats in group 3 ate 150.16 g food and finally increased by more than 100 g (261.79g) at week 6. The number of group 4 at the first week was 155.56 g, and went up to 263.43 g by week 6. The food consumptions did not differ significantly (p>0.05) between the

groups, which suggest that the addition of native or indigestible black-eye bean proteins did not change the food intake of rats. Male rats ate more than female counterparts and the difference was significant (p<0.001). This was attributed to the present of sexual hormones in male and female rats. The administration of testosterone increased meal size of male rats and decreased the number of meals in female rats (Madrid et al., 1993). On the contrary, estrogen and progesterone led to a reduction in food intake (Tarttelin et al., 1971).





Rats in group 1 to 4 were fed with diet with different protein sources. Group 1: 20% of casein; group 2: 1% of indigestible protein + 19% of casein; group 3: 5% of indigestible protein + 15% of casein; group 4: 5% of native protein + 15% casein.

The body weight data for each group of rats are presented in Figure 9. In general, all rats showed an increasing body weight during the feed period. But the weight gain rate was

descending with the feeding process. Rats gained highest weight at the beginning week (approximately 115 g for female and 200 g for male), which was much more than the following weeks. For female rats, the body weight gain of group 1 was 113.19 g, and that of other 3 groups were 3 g, 6 g, and 7 g higher (116.17 g, 119.11 g and 130.10 g respectively). For the next few weeks, the average weight gain of group 1 gradually decreased from 42.19 g to 20.45 g, while that of group 4 also dropped from 34.48 g to 20.90 g. As for group 2, weight gain for week 2 was 41.20 g, and increased slightly to 44.70 g at the next week before it went down to lowest value of 27.63 g at the fifth week, then climbed to 35.57 g at the end. The body weight gain in group 3 changed sharply, in which rats gained 40.46 g at the second week and decreased to 20.56 g at week 4. A surprising weight gain of 42.79 g was observed at week 5 in group 3, before decreasing to 12.10 g at the last week. Male rats showed a higher increase in body weight. The body weight of group 1 and 2 increased by around 81 g at the second week, and the weight gain descended to 48.13 g and 44.75 g respectively. The rats in group 3 grew by 78.03 g after 2-week feeding and weight gain increased to 80.85 g before a decrease to 41.12 g. And for the last group, after gaining a total of 201 g at the first week, total body weight of rats ascended by 88.58 g at week 3, and increased the least by 45.13 g during the last week. According to the result of statistical analysis, no significant difference between groups was found at each week (p>0.05), while gender had significant effect on body weight gain (p<0.05). Rats gained weight fast at the beginning of the experiment because they were growing since they were 4-week old and the weight gain rate decreased when rats were matured during later phase of the study. Besides, the difference of weight gain among genders was attributed to different food intake which was observed in the current study and postnatal action of testicular and ovarian

secretions (Slob et al., 1975).



Figure 11: Body weight of rats fed with different diets (mean \pm SD, n=3)

Rats in group 1 to 4 were fed with diet with different protein sources. Group 1: 20% of casein; group 2: 1% of indigestible protein + 19% of casein; group 3: 5% of indigestible protein + 15% of casein; group 4: 5% of native protein + 15% casein.

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 was consistent to present work. In addition, the tartary buckwheat protein trials carried out on mice (Tomotake et al., 2007), buckwheat protein on hamster (Zhang et al., 2017) and rice protein on Sprague-Dawley rats (Um et al., 2013) also reported similar results on food intake and body weight gain as current work. Besides, there was no difference in body weight at beginning and end of the experiment
by Higaki et al. (2006), while in this work body weight gain could be seen through feeding time. This may be due to the use of matured rats by Higaki et al. (2006) while young (growing) rats were utilized in this work. In summary, it is likely that the protein source in the animal diet has no influence on food intake and body weight gain of animals.

5.1.1.1

4.12.2. Plasma triglyceride (TG)

Figures 10 and 11 present the information on plasma triglyceride levels for female and male rats before and after consuming different diets for 6 weeks. For female rats, plasma TG value of rats fed with casein (group 1) was 57.30 mg/dL at the baseline and slightly dropped to 53.78 mg/dL after 1-week feeding, which was followed by an increase to 116.81 mg/dL. Then, TG level decreased to 64.64 mg/dL before sharply increasing to 153.61 mg/dL. The baseline plasma TG of group 2 was 46.93 mg/dL and then constantly went up to the highest value of 165.03 mg/dL during week 4. After that, it reduced by 74.01 mg/dL to 94.02 mg/dL and the value in the last week was approx. half that of week 5 (191.03 mg/dL). As for group 3, the baseline data was little lower than other groups (55.04 mg/dL), but it increased sharply to the top 207.88 mg/dL after rats were fed for 4 weeks, which was followed by a decrease to 63.76 mg/dL at the end of experiment. The last group, where rats were fed with native black-eye protein, showed relatively higher baseline TG level (78.27 mg/dL). The data reduced for 2 weeks to 51.54 mg/dL, and then increased to the highest level in the final week (486.84 mg/dL). As for male rats, the baseline levels were 62.39, 104.94, 65.40 and 73.55 mg/dL for each group, respectively. The pattern for group 1 was that the TG level went up to 165.03 mg/dL at week 4, before dropping to 78.56 mg/dL. The plasma TG value of group 2 also fluctuated because it increased to the

highest level of 149.48 mg/dL in the second week and then fell to 110.00 mg/dL in fifth week after a slight increase at week 4. Then, the value increased by approx. 22 mg/dL to 132.14 mg/dL in the last week. After consuming diet of group 3 for 3 weeks, there was a sharp increase to 298.37 mg/dL but reduced to 261.03 mg/dL in the last week. In group 4, there was a continuous increase in the first 4 weeks of feeding (to 212.30 mg/dL), before dropping to 136.84 mg/dL in the final week. According to the statistical analysis, there were significant differences between groups among female rats (p<0.05) at week 2, 3, 5 and 6. And significant differences could also be observed in male rats at baseline, week 3, 5 and 6. The plasma TG gain at the last week is presented in Table 5. Since all groups of diets contained high cholesterol, native blackeye bean protein in the diet contributed least to the decrease in plasma TG level (gained 408.57 mg/dL) and 5% of indigestible protein had the best effect (gained 8.36 mg/dL in plasma TG) when compared to other diets. On the other hand, male rats fed with 5% of indigestible protein gained the most TG (195.63 mg/dL), while the group fed with casein caused the least increase (16.17 mg/dL), which was different from female.

In present study, rats were fed high cholesterol diets, and the plasma TG levels were all elevated in both genders. In comparison to casein, the inclusion of 5% indigestible protein decreased blood TG by 91% in females while 1% of indigestible protein and native protein increased the level by approximately 1.5 and 4-fold at the end of the experiment. However, the plasma TG levels were approx. 2 to 4 times higher in male rats fed with all diets that contained the pulse protein than the casein diet. Zhang et al. (2017) reported that buckwheat proteins could effectively lower plasma TG level of hamster after 6-week feeding when compared to casein (117 mg/dL vs. 160 mg/dL), which is consistent with data from present study. In this

study, incorporation of native protein into the diet led to higher plasma triglyceride than when indigestible protein was in the diet. The results obtained in this work are in contrast to those reported for soybean protein, which indicated that rats consuming high-molecular weight fraction prepared after pepsin digestion had a higher serum triglyceride level (295 mg/100 mL) than the 174 mg/100 mL for rats on the native soybean protein diet (Sugano et al., 1990). On the contrary, liver TG was lower in high-molecular weight fraction group than native soybean protein group (Sugano et al., 1990).









	Female					
Group	1	2	3	4		
Baseline	57.30±3.27 ^a	46.93±18.46 ^ª	55.40±10.02 ^ª	78.27±7.14 ^ª		
Week1	53.78±16.37 ^a	62.55±0.83 ^{ab}	82.49±6.85 ^b	67.61 ± 1.70^{ab}		
Week2	75.71±11.99 ^b	77.67±0.49 ^b	107.62±2.63 ^c	51.54±3.60 ^a		
Week3	101.12±7.42 ^a	126.61±17.82 ^a	118.23±24.96 ^a	184.89±0.14 ^b		
Week4	116.81±13.34ª	168.68±13.30 ^{ab}	207.88±22.14 ^b	155.86±30.71 ^{ab}		
Week5	64.63±4.43 ^a	94.02±8.28 ^a	109.47±13.68 ^a	258.82±52.20 ^b		
Week6	153.61±48.50 ^a	191.03±7.71 ^ª	63.76±11.71 ^ª	486.84±105.21 ^b		
$TG \operatorname{gain}^1$	96.31 ^ª	144.10 ^a	8.36 ^ª	408.57 ^b		
	Male					
	1	2	3	4		
Baseline	62.39±0.26 ^a	104.94±2.68 ^c	65.40±3.34 ^ª	73.55±2.55 ^b		
Week1	76.65±13.53 ^a	82.09±11.00 ^a	77.18±2.14 ^ª	105.57±25.62ª		
Week2	102.42±3.47 ^a	149.48±9.01 ^b	108.84±20.59 ^a	132.81±15.81 ^{ab}		
Week3	123.47±15.88 ^a	134.35±10.19 ^a	298.37±18.93 ^b	143.03±4.85 ^a		
Week4	165.03±4.71 ^ª	147.45±40.28 ^a	183.82±49.71 ^a	212.30±15.00 ^a		
Week5	96.46±2.76 ^a	110.00±5.00 ^b	177.20±7.13 ^c	95.24±1.15 ^ª		
Week6	78.56±11.93 ^a	132.14±2.71 ^ª	261.03±45.57 ^b	136.84±6.36 ^a		
TG gain ¹	16.17 ^ª	27.21 ^{ab}	195.63 ^b	63.29 ^{ab}		
Data show	red as mean±SD	. Mean values	within a row	having different		

Table 7: Plasma TG (mg/dL) level of rats fed with different diets for each week

superscript letters (a,b,c) were significantly different (p < 0.05).

¹The subtraction = week 6 minus baseline

4.12.3. Plasma total cholesterol (TC)

Generally, an increasing trend of total cholesterol level in plasma of rats could be observed in both gender (Figures 12 and 13). As for females, the baseline levels of all groups were comparable, which were from 2.46 to 3.51 mmol/L. With the beginning of diets, the TC level started increasing. The value for group 1 increased to 9.70 mmol/L after 3 weeks, and then to 10.33 mmol/L in the last week. The plasma TC level for group 2 increased to 9.08 mmol/L in the first week, which was similar to group 1, followed by a decrease of about 5 to 7 mM during week 2 to 4 but increased in the last two weeks to 13.95 mmol/L. Noticeably, the plasma TC level fluctuated mildly in group 3 throughout whole experiment when compared to other groups. This is evident from the 4.35 mmol/L increase in the first week, a decrease to 2.82 mmol/L in week 3, which was followed by a slight increase to 4.95 mmol/L in the 6th week. On the contrary, the changes of plasma TC in group 4 was the most dramatic, where it increased to 12.47 mmol/L at the beginning and reduced sharply to 3.16 mmol/L two weeks after. After that, the number increased constantly to 19.88 mmol/L at week 6. The male rats showed a generally lower total cholesterol levels than female counterpart, which were about 0.81 to 5.83 mmol/L. For group 1, the TC level increased to 4.46 mmol/L after the feeding started and there was a fluctuation during the experiment and finally reached 5.22 mmol/L at the end. The alteration of group 2 was similar as that of group 1, where the TC value increased to 4.46 at week 1 and then descended to 2.55 mmol/L at week 4 after a slight increase at week 3 (5.36 mmol/L). After the TC increased to 3.79 mmol/L in the first week, it gradually went down to the lowest 0.81 mmol/L at 4th week and then increased by 3.01 mmol/L reaching the top (3.82 mmol/L) in the 6th week for group 3. The baseline TC level of group 4 was 2.38 mmol/L and it increased by 1.95

mmol/L to 4.33 mmol/L after consumption of experimental diet. The lowest level (1.64 mmol/L) was seen at week 4 after which the value increased to 4.34 mmol/L in the 6th week.



Figure 14: Plasma total cholesterol (TC) levels of female rats fed with different diets





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 6week consumption are shown in Table 6. 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 6. 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.

	Female					
Group	1	2	3	4		
baseline	3.51±0.21 ^b	2.46±0.10 ^a	2.51±0.06 ^a	3.16±0.05 ^b		
week1	9.15±1.95 ^{ab}	9.08 ± 0.64^{ab}	4.35±0.60 ^a	12.47±2.02 ^b		
week2	6.28±0.91 ^b	5.23±0.47 ^{ab}	2.97±0.70 ^a	6.88±0.46 ^b		
week3	9.70±2.03 ^a	7.33±0.08 ^ª	2.82±0.03 ^b	3.16±0.35 ^b		
week4	5.58±0.20 ^b	6.23±0.18 ^b	3.16±0.64 ^ª	8.56±0.02 ^c		
week5	5.60±0.16 ^b	11.50±0.37 ^c	3.50±0.24 ^a	13.69±0.37 ^d		
week6	10.33±1.01 ^b	13.95±0.16 ^c	4.95±0.42 ^a	19.87±1.20 ^d		
$TC\operatorname{gain}^1$	6.82 ^b	11.49 ^c	2.43 ^a	16.71 ^d		
-	Male					
Group	1	2	3	4		
baseline	3.75±0.10 ^b	3.49±0.08 ^b	2.63±0.10 ^a	2.38±0.00 ^a		
week1	4.45±0.12 ^a	4.46±0.02 ^a	3.79±0.38 ^ª	4.33±0.29 ^a		
week2	3.32±0.50 ^b	2.99 ± 0.04^{ab}	2.13±0.03 ^a	2.46 ± 0.04^{ab}		
week3	5.83±0.59 ^b	5.36±0.49 ^b	2.24±0.07 ^a	3.62±0.10 ^a		
week4	2.73±0.04 ^c	2.55±0.08 ^c	0.81±0.18 ^a	1.64±0.02 ^b		
week5	4.29±0.27 ^b	5.37±0.12 ^c	2.64±0.12 ^a	3.13±0.08 ^ª		
week6	5.22±0.09 ^c	5.27±0.01 ^c	3.82±0.00 ^a	4.34±0.08 ^b		
TC gain ¹	1.47 ^a	1.79 ^b	1.19 ^ª	1.96 ^b		

Table 8: Plasma TC (mmol/L) level of rats fed with different diets

Data showed as mean \pm 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

In the study of Sugano et al. (1990), rats fed with indigestible fraction of soybean protein had serum cholesterol of 109 mg/100 mL, which was about 180 mg/100 mL lower than that fed with soybean protein, indicating that indigestible protein had better cholesterol-lowering effect than native protein. Meanwhile, Wistar rats provided with the sediment of soy protein hydrolysate was found to have lower serum TC level compared to native soy protein, but the difference was not significant (Nagaoka et al., 1999). Similarly, the HMF of buckwheat protein did not have any significant plasma cholesterol-lowering effect when compared to the native protein (Kayashita et al., 1997). Nonetheless, when compared to casein, soy protein hydrolysis could effectively lower serum cholesterol (2.21 vs. 3.62 mmol/L) (Nagaoka et al., 1999). Furthermore, the lowering effects of other plant proteins have been reported. For example, the experiment carried on hamster showed that in comparison to casein (292 mg/dL), buckwheat protein resulted in TC reductions to 130 mg/dL to 162 mg/dL. But the TC levels of rice and wheat protein-fed groups were comparable (rice protein: 262 mg/dL; wheat protein: 253 mg/dL). Although the lowering effects of rice and wheat proteins were also significant, the plasma TC level were only 30 and 40 mg/dL lower than the casein diet (Zhang, 2017). The results are consistent with the work of Um et al. (2013).

The difference between genders is probably attributed to the sexual steroids. For example, testosterone therapy on men significantly reduced total cholesterol and LDL cholesterol (Permpongkosol et al., 2010; Traish et al., 2009) while androgen deficiency led to an elevated lipid profile (TG, TC) (Traish et al., 2009). In addition, male-to-female (man received estradiol) cross-sex hormone therapy resulted in increases in TG and TC, but the reversed consequences were found in female-to-male transsexuals receiving androgen treatments (Ott et al., 2011). In

this term, androgen presented a protective effect of hypercholesterolemia while estrogen was likely to be a risk factor of increased blood cholesterol. Therefore, it is likely to rationale that androgen has reducing effect on blood cholesterol level, which can be the reason for the lower cholesterol levels found in male rats in current study. However, Hewitt et al. (2003) reported an induced blood cholesterol value in aromatase knockout (ArKO) mouse that couldn't synthesize estrogen using high cholesterol diet. And mice were also found to had supressed expression of Cyp7a, which encode enzyme (7 α -hydroxylase) that convert cholesterol to bile acid. As a result, estrogen on blood cholesterol was not consistent in previous researches and the relationship between sexual hormone and cholesterol metabolism was not clearly known. And the physical activity may also contribute to the difference in cholesterol levels (Philippou et al., 2018).

To summarize, addition of 5% of indigestible protein contributed to a lower blood cholesterol level in female rats, while diets with 1% of indigestible protein and 5% of native protein led to a contrary result. In male rats, plasma cholesterol level was stable when fed with 5% of indigestible protein but the numbers slightly increased after consumption of 1% of indigestible protein and 5% of native protein.

4.12.4. Gel electrophoresis of fecal samples

The SDS-PAGE polypeptide composition of proteins in rat feces is presented in Figures 14 to 17. Figures 14 and 15 are results of male rats in non-reducing and reducing conditions, respectively. Baseline was the feces from rats that were provided with normal diet. The remaining 4 lanes (group 1 to 4) demonstrated protein profile of feces at the sixth week (final

week of the feeding trial) from different feeding groups. Group 1: 20% of casein; group 2: 1% of indigestible protein + 19% of casein; group 3: 5% of indigestible protein + 15% of casein; group 4: 5% of native protein + 15% casein. In non-reduced gels, there was a band at 55 kDa, and three bands in the region of 20 to 30 kDa for the rats at baseline. Besides, two bands were seen at around 15 and 13 kDa. On the other hand, after 6-week feeding, the proteins in rat stool were changed slightly. No band was observed in high molecular weight area (more than 40 kDa) in all groups. Meanwhile, an intensive band at 15 kDa was shown but no band at 13 kDa compared to baseline. And there were also proteins of 10 to 13 kDa dominating in all groups. However, except for group 4 showing bands at 25 to 27 kDa, there were several bands distributed at the region of 20 to 30 kDa, which was similar to the baseline lane. It is worth noting that there was a band at 40 kDa in lane of group 3, which could not be found in other groups. After the addition of β -mercaptoethanol (Fig. 17), all lanes presented bands at 100 to 120 kDa. Moreover, in lane of group 4, the intensity of the band at 17 and 27 kDa was increased. Figure 16 and 17 show the results from female rats fed with the same diet as male counterparts and the result were similar.

In comparison between baseline and different groups at week six, protein molecular weight composition was not much changed. Moreover, there were no variations found between genders. When compared to the native protein lane, the molecular weight of proteins in group 4 (rats fed with native protein) were lower, which was probably attributed to protein digestion in the gut. As for group 2 and 3, which were from fecal sample collected from rat provided with 1% and 5% of indigestible protein, the gel patterns were almost the same as native protein lane. Particularly, all 4 lanes of the feces collected at 6th week were comparable in both male and

female rats with few different bands, and the reason is likely to be that protein source in diet mainly consisted of casein, accounting for 15% to 20%, while samples (native and indigestible black eye bean protein) only made up 1% or 5%. Therefore, native or indigestible protein can hardly change the molecular weight composition of protein in the feces.

By comparing the non-reducing and reducing gels, some bands that did not exist in nonreduced gel were found in reducing one. This may be attributed to the breakage of disulfide bonds of high-molecular weight proteins that could not enter stacking (pointed by arrow), thus increasing the content of lower molecular weight proteins (Genovese et al., 1996).



Figure 16: SDS-PAGE analysis of black eye bean protein isolate and indigestible protein and feces from male rats under non-reducing condition

Marker: composed of standard proteins; Native protein: black eye bean protein isolate; Indigestible protein: indigestible black eye bean protein after gelation treatment with slow cooling; Lane 1: feces from male rats at baseline (one week before experiment); Lane 2 to 5: feces from male rats fed diets of group 1 to 4 at week six.



Figure 17: SDS-PAGE analysis of black eye bean protein isolate and indigestible protein and feces from male rats under reducing condition

Marker: composed of standard proteins; Native protein: black eye bean protein isolate; Indigestible protein: indigestible black eye bean protein after gelation treatment with slow cooling; Lane 1: feces from male rats at baseline (one week before experiment); Lane 2 to 5: feces from male rats fed diets of group 1 to 4 at week six.



Figure 18: SDS-PAGE analysis of black eye bean protein isolate and indigestible protein and feces from female rats under non-reducing condition

Marker: composed of standard proteins; Native protein: black eye bean protein isolate; Indigestible protein: indigestible black eye bean protein after gelation treatment with slow cooling; Lane 1: feces from male rats at baseline (one week before experiment); Lane 2 to 5: feces from male rats fed diets of group 1 to 4 at week six.



Figure 19: SDS-PAGE analysis of black eye bean protein isolate and indigestible protein and feces from female rats under reducing condition

Marker: composed of standard proteins; Native protein: black eye bean protein isolate; Indigestible protein: indigestible black eye bean protein after gelation treatment with slow cooling; Lane 1: feces from male rats at baseline (one week before experiment); Lane 2 to 5: feces from male rats fed diets of group 1 to 4 at week six.

6 CHAPTER FIVE

5. 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-reducing agents have many side effects, a safe and effective substitute is highly needed. Plant proteins have been showed 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 the 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 acidbinding 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 TG and TC contents were reduced by adding 5% of indigestible protein when compared to the casein-only diet. In contrast, plasma TG and TC were increased by the 1% indigestible protein and 5% of native protein diets. However, in male rats, elevation of TG levels was observed in all groups and 5% of indigestible protein did not significantly decreased plasma TC. Additionally, male rats gained less cholesterol than female counterparts.

The results of gel electrophoresis revealed that native black-eye bean protein has major proteins of around 50 and 53 kDa while the indigestible protein contained predominantly proteins sizes that are <15 kDa. Besides, there were some aggregates containing disulfide bonds

in both protein samples that can be cleaved β -mercaptoethanol. In addition, the FPLC confirms that the indigestible protein consisted of mainly low molecular weight proteins when compared to the high molecular weight proteins in the native protein.

The indigestible proteins should be used instead of native for the following reasons. Firstly, the indigestible protein is produced under the certain condition 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 the protease, which can guarantee that they are stable 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.

7 CHAPTER SIX

6. Future research

The present study proves that rats blood cholesterol and triglyceride levels are reduced by the addition 5% of indigestible black eye bean protein in high-cholesterol diet; this effect was not observed with the native protein. Future work will require human trial to determine the actual effect on human beings. Both men and women will be included to identify the difference between genders. Besides, there are two aspects that need to be considered. First is the use of indigestible proteins as a diet component in healthy human participants to determine the preventive effect. Secondly, adding indigestible proteins into diet for hypercholesterolemia patients to determine therapeutic benefits. During the human feeding trials, side effects of the product will also be recorded as well. If findings from human trials are positive, indigestible proteins could become suitable ingredients to formulate hypocholesterolemic functional foods and nutraceuticals.

8 CHAPTER SEVEN

7. Reference

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