The Hypolipidemic and Antiatherosclerotic Effect of Fungal Polysaccharides

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B. Sc. (Hons), The Chinese University of Hong Kong

A Thesis Submitted in Partial Fulfillment
of the Requirement for the Degree of
Master of Philosophy
in
Biochemistry

August 2000

Acknowledgment

I would like to show my gratitude towards my supervisor, Prof. Y. M. Choy, for his guidance and supervision throughout the project, only with which all the works were made possible.

I would also like to express my appreciation to Dr. Z. Y. Chen and Dr. W. P. Fong for their insightful suggestions and comments. I would also like to thank my colleagues working in the Department of Biochemistry for their technical supports and encouragement.

I would also like to thank my parents, sister, brother and Miss W. S. Woo for their understanding and encouragement throughout the two hard-working years.

Abbreviations

ALT	Alanine Aminotransferase
AP	*Auricularia polytricha*
APP	*Auricularia polytricha* Polysaccharide
AST	Aspartate Aminotransferase
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CHD	Coronary Heart Disease
DF	Dietary Fiber
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
Fuc	Fucose
G6P	Glucose-6-Phosphate
G6PDH	Glucose-6-Phosphate Dehydrogenase
GalN	Galactosamine

GLC	Gas Liquid Chromatography
Glc	Glucose
GlcN	Glucosamine
HDL-C	High-Density Lipoprotein Cholesterol
HMG-CoA	3-Hydroxy-3-methylglutaryl CoA
HPLC	High Performance Liquid Chromatography
LDL-C	Low-Density Lipoprotein Cholesterol
Man	Mannose
NADP	Nicotinamide Adenine Dinucleotide Phosphate
PMP	3-Methyl-1-phenyl-2-pyrazolin-5-one
RP	Reverse Phase
SBF	Sugar-Beet Fiber
SCFA	Short Chain Fatty Acid
Tal	Talose
TC	Total Cholesterol
TFA	Trifluoroacetic acid

TG	Triglyceride
TLC	Thin Layer Chromatography
Xyl	Xylose

Abstract

Heart disease is the second leading cause of death in Hong Kong. Moreover, hyperlipidemia is a common health problem that strongly associated with cardiovascular diseases such as atherosclerosis and coronary heart disease. Although there are many factors leading to these diseases, a strong relationship between low density lipoprotein cholesterol (LDL-C) concentration and extent of coronary atherosclerosis has been shown in many studies. Moreover, there is an inverse relationship for high density lipoprotein cholesterol (HDL-C).

All fungal walls contain a mixture of fibrillar and matrix components. For the matrix components, it includes various polysaccharides that can serve as dietary fiber and that of water-soluble one are more important which include gums, pectins and psyllium. Dietary fiber has been reported with hypocholesterolemic effects. Water-soluble polysaccharide was extracted from fungus *Auricularia polytricha* and its hypocholesterolemic and antiatherosclerotic effects was studied.

Polysaccharide was extracted by hot water. Then, it was studied by ethanol precipitation and size exclusion chromatography, a heterogeneous mixture of polysaccharide was found in this fungus. By HPLC analysis of its monosaccharide composition, it was predicted to contain mainly glucomannans and galactomannans. The

composition of crude APP, APP-A and APP-B were similar. The results showed that there is no toxic effect of APP to liver by measuring AST and ALT activity.

In our study, Golden Syrian Hamster was used as animal model. In experiment 1, the protective effect of APP from hypercholesterolemia was studied. Normal hamsters was fed with high cholesterol diet with supplementation of APP. Liver cholesterol, plasma total cholesterol and HDL-cholesterol were decreased significantly in the test groups. Therefore, APP can protect hamster from hypercholesterolemia. Moreover, it can increase both neutral and acidic sterols excretion with small difference for neutral sterol. In experiment 2, the therapeutic effect of APP from hypercholesterolemia was studied. Hamsters were boosted into hypercholesterolemic state for four weeks and then it was fed with diet with supplementation of APP. Similar results with experiment 1 were obtained and APP was shown to lower the cholesterol level of hamster with hypercholesterolemia. In experiment 3, the dose response of APP was studied. Increased amount of APP supplementation can lower hepatic cholesterol and LDL-cholesterol level. However, only hepatic cholesterol showed a dose response with APP. Moreover, APP can also increase the steroids excretion but do not show any dose-dependent response. In experiment 4 and 5, short chain fatty acids (SCFA) were shown to have hypocholesterolemic effect by lowering hepatic cholesterol and LDL-cholesterol. By measuring the activity of HMG-CoA Reductase, SCFA and APP were reported to lower the de novo cholesterol synthesis by lowering the activity of enzyme. Polysaccharides may be fermented by colonic bacteria to produce SCFA. Therefore, parts of

hypocholesterolemic effect of APP may due to the action of by-product SCFA. In experiment 6, the antiatherosclerotic effect of APP was studied. Atheroma was stained by Sudan III and the area of atherosclerosis was calculated. APP was reported to decrease the degree of atherosclerosis. In all experiments, APP has no effect on plasma triglyceride level.

Results have shown that the hypocholesterolemic effects of APP were due to decrease in de novo cholesterol synthesis and increase the excretion of sterols in feces. However, further experimental investigations were needed to elucidate the detail mechanisms such as the fermentation of polysaccharide and the binding of polysaccharide to sterols. Moreover, the activity of cholesterol-7-alpha-hydroxylase was also important in the determination of its hypocholesterolemic mechanisms.

摘要

心疾病在香港是第二號殺手，高血脂是一種常見的疾病，而且與心血管疾病有很大的關係。血管硬化和冠心病是其中的例子。雖然心血管病有很多的致病因素，但很多研究指出低密度脂肪蛋白膽固醇 (LDL-C)是引致心血管硬化的主要原素，而高密度脂肪蛋白膽固醇 (HDL-C)與此疾病有反關係的現象。

所有真菌細胞壁都有纖維狀的和基層的混合物質。基層物質包含了多種糖，而多糖可作爲食物纖維。比較重要的水溶性食物纖維有樹膠、果膠和歐車前。研究指出食物纖維有降低膽固醇的作用。因此我們從毛木耳真菌中抽取其水溶性多糖及測試毛木耳多糖的降膽固醇和抗動脈粥樣硬化的能力。

毛木耳多糖經過熱水抽取及乙醇沈澱後，然後用尺寸排阻色譜法進一步純化。結果得出，毛木耳多糖是一種多相混合多糖。經過高效液相色譜法分析毛木耳多糖的單糖成份，估計毛木耳多糖主要包含了葡甘露聚糖及半乳甘露聚糖。粗制毛木耳 (crude APP)多糖及乙醇沉澱多糖 (APP-A & APP-B)的單糖成份是相似的。

透過量度天冬氨酸轉氨酶及丙氨酸轉氨酶的活性，結果顯示了毛木耳多糖對肝臟沒有明顯的毒性。

由實驗一至五中，敘利亞黃金倉鼠會用作動物模型。第一項實驗是測試毛木耳多糖對於避免高膽固醇的功用。混合了毛木耳多糖的高膽固醇糧食是用作餵飼正常的倉鼠。測試組中的肝臟膽固醇、血漿總膽固醇及高密度脂肪蛋白膽固醇的含量相對減少。因此，毛木耳多糖可避免倉鼠進入高密度膽固醇的狀況。再者，毛木耳多糖可增加糞便中的中性及酸性類固醇的排泄，與對比組比較，中性類固醇的排泄量比較少。第二項實驗是測試毛木耳多糖對於治療高膽固醇的功用。在首先的四星期中，高膽固醇的糧食是用作餵飼倉鼠，目的在於提高它體內

膽固醇的含量。在其後的八個星期中，混合了毛木耳多糖的高膽固醇糧食取替了單是高膽固醇的糧食。結果顯示，毛木耳多糖可幫助高膽固醇血症倉鼠降低身體膽固醇的含量。第三項實驗是測試毛木耳多糖的劑量對於降低膽固醇的功效。測試指出只有肝臟膽固醇按著毛木耳多糖劑量的提升而降低，但血漿中各項的膽固醇及糞便中的類固醇並沒有隨著毛木耳的劑量而變更，於實驗四及五中，短鏈脂肪酸 (SCFA)顯示其降膽固醇的功效，它能降低肝臟膽固醇及血漿中低密度脂肪膽固醇的含量。短鏈脂肪酸及毛木耳多糖可降低 β-羥-β-甲戊二酸單酰輔酶A 還原酶 (HMG-CoA Reductase)的活性，代表了它們可降低肝臟膽固醇的從頭合成。多糖可被大腸中的細菌發酵而轉變成短鏈脂肪酸。因此，毛木耳多糖的部份降膽固醇能力可能是出於副產品-短鏈脂肪酸的作用。第六項實驗是主要測試毛木耳多糖對於抗血管粥樣硬化的功用。此項實驗中，紐西蘭白兔是測試的對象。苯偶氮間苯二酚 (Sudan III) 可用作粉瘤 (Atheroma) 染色，而染色的面積代表了血管粥樣硬化。實驗證明毛木耳多糖有減少血管粥樣硬化的程度。於所有實驗中，毛木耳多糖對於血漿三甘油脂沒有固定的影響。

毛木耳多糖的降膽固醇功效是由於它能減少肝臟膽固醇的從頭合成及增加糞便中類固醇的排泄。但詳細的機制還需要更多實驗去闡明，例如多糖的發酵、多糖與類固醇的結合及測試 膽固醇-7α-羥化 酶(C-7α-hydroxylase)的活性等等。

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Chapter one:

Introduction

1.1 Introduction

Many studies were claimed on the possible involvement of dietary fibers in the occurrence of cardiovascular disease (Veldman *et al*, 1997), several epidemiological observations (Cheng *et al*, 1999) have suggested that a low dietary fiber intake is associated with a higher incidence of coronary heart disease (CHD) (Kromhout *et al*, 1982).

In fact, the modern western diet is generally poor in fiber (15-20 g/d) and rich in fat (38-45% energy) and cholesterol (up to 0.5 g/d). On the contrary, the Stone-age diet (35-15,000 y ago) which is well representative of the dietary environment which determined the collective human genome was poor in fat (20-25% energy) and basically rich in dietary fiber (40 g/d and above).

Given the documented association of blood lipid and lipoprotein levels (Chait *et al*, 1993; Cleeman, 1997) with the risk of coronary heart disease, research has mainly been focused on the possible alternations and improvement of lipid metabolism by

dietary fiber (Menon & Kurup, 1976; McIntosh *et al*, 1991). Whether dietary fibers affect lipid metabolism and how dietary fibers can exert such effects has been the matter of several hundreds of studies all over the world during the last three decades.

1.2 Classification of Plant Polysaccharides

Plant polysaccharides may be separated into two broad categories (Hans & Geoffrey, 1996). Starch, a ubiquitous storage polysaccharide, is an α-linked glucan and is the major carbohydrate of dietary staples such as cereal grains and potatoes. The nonstarch polysaccharides (NSP) of plants, such as cellulose, pectin, and hemicellulose, are non- α-glucan polysaccharides. The NSP tend to have a structural function and are the principal components of the plant cell wall. Recently NSP have become the objective in the measurement of dietary fiber.

Because of its α-glucosidic linkages, starch is susceptible to hydrolysis by pancreatic α-amylase. In contrast, NSP completely resist digestion by the enzymes secreted into the small intestine of man.

Before a decade, starch was thought to be entirely digested while NSP entirely resisting digestion in the small intestine. However, it is now clear from a number of studies in man (Englyst & Cummings, 1988a; Englyst & Cummings, 1985) and animals (Millard & Chesson, 1984) that this is not the case and that a significant proportion of

dietary fiber may pass intact into the colon, where it becomes available for fermentation by gut bacteria. In addition, for some animals a considerable amount of NSP (and starch) may be fermented in the small intestine.

1.2.1 Definition of Dietary Fiber

Dietary fiber can be classified into water-soluble DF and water-insoluble DF and it is defined as the "plant polysaccharides and lignin which are resistant to hydrolysis by the digestive enzymes of man". However, some starch may also escape or resist to hydrolysis of digestive enzymes. Therefore, for analytical purposes dietary fiber should be defined as the sum of the nonstarch polysaccharide (Englyst & Cummings, 1988b). Since lignin is not a polysaccharide, it is not included in the definition of dietary fiber.

1.2.2 Types of Soluble Dietary Fiber

Several reviews document the hypocholesterolemic effects of soluble fibers in humans and the lack of effect of insoluble fiber (Story *et al*, 1997). Examples of insoluble fiber included cellulose, hemicellulose, resistant starch and chitin. The following are the examples of water-soluble fiber:

Gums

Guar gum, a glucomannan or galactomannan extracted from the endosperm of the Indian cluster bean, has been used extensively in clinical studies. Glucomannan appears to be linear polymers with both mannose and glucose in the chain. The ratio of mannose to glucose is between 1:1 and 2.4:1. Hardwood glucomannans appear to contain no galactose and are relatively insoluble, but the glucomannans from gymnosperms have galactose side chains and a higher mannose to glucose ratio (3:1). The presence of side chains tends to make these polysaccharides more soluble in water, possibly because the side chains prevent the formation of intermolecular hydrogen bonding. Glucomannans are part of the hemicellulose fraction of the plant cell wall.

Guar gum appears to be a good model with which to examine the effect of increased luminal viscosity on small intestine function. It can also reduce plasma total cholesterol and LDL-cholesterol but with no effect on HDL-cholesterol and triglyceride (Topping, 1994). Guar gum slows the rate of diffusion of cholesterol mixed micelles in vitro at concentration as low as 0.25% (Phillip, 1986). It appears to inhibit convection effects of intestinal movements. In vivo it binds or entraps bile acids (Gallaher & Schneeman, 1986). Several studies were also reported of increased bile acid excretion with guar gum.

Pectins

The most widespread soluble dietary fiber structure in foods is pectin, a polygalacturonic acid found in fruits, vegetables, legumes, and roots (sugar beets and potatoes) as storage polysaccharide. Pectin is the general term designating those water-soluble pectinic acids of varying methylester content and degree of neutralization which are capable of forming gels with water and acid under suitable conditions. Pectin is found in the primary cell wall and intracellular layer. It changes from an insoluble material in the unripe fruits to a much more water-soluble substance in the ripe fruit. Its ability to form gels and its ion binding capacity may be important in human nutrition. Pectins was claimed to have significant hypocholesterolemic effects (Reiser, 1987) with no influence on plasma triglyceride level. It shares many properties with the gums.

Psyllium

Psyllium hydrophilic mucilloid, a highly branched acidic arabinoxylan extracted from the husk of *Plantago ovata* seeds grown in Asia, has been widely used for management of large intestine disorders for more than 50 years. Past studies have been demonstrated that psyllium administration had hypocholesterolemic effects (Anderson *et al*, 1988).

Oat Products

Oatmeal and oat bran are rich in the water-soluble β-glucan or oat gums, which have significant hypocholesterolemic effects. β-glucan is a glucose polymer consisting of β-linked glucose molecules, with a mixed linkage, i.e., some β-1,3-linkages interspersed with the β-1,4-linkages.

Oat products have the characteristic of selectively decreasing serum LDL-cholesterol (Kirby *et al*, 1981) while preserving or actually increasing serum HDL-cholesterol, leading to significant and favorable reduction in the LDL: HDL-cholesterol ratio. Thus, in addition to a low-fat and low-cholesterol diet, regular use of oat products may have an important role in reducing risk for coronary heart disease (Anderson & Gustafson, 1988).

1.3 Physiological Effect of Fiber

Ingestion of a diet rich in dietary fiber may result in slower gastric emptying, delayed absorption in the small intestine, faster colonic transit, and an increased stool output (Anderson, 1985). These effects, however, are dependent on the type of dietary fiber ingested. The plant polysaccharides that make up dietary fiber have a variety of chemical structures and physical properties. The soluble polysaccharides form viscous

solutions in the intestine lumen and delay absorption of nutrients in the small intestine (Rosado & Diaz, 1995). Many of these soluble polysaccharides are extensively fermented in the proximal colon and have minimal effects on stool output. In contrast, the insoluble polysaccharides act as indigestible solids and have little effect on small intestine physiology. However, they are more resistant to bacterial degradation and have a greater influence on stool output and colonic transit.

1.3.1 Reduction in Absorption by Viscous Polysaccharides

The addition of viscous polysaccharides to carbohydrate meals reduces postprandial hyperglycemia (Wursch & Pi-Sunyer, 1997), indicating an impairment of carbohydrate absorption. The rate of absorption was slowed but that the total amount absorbed was not reduced. There is also a malabsorption of lipids after pectin ingestion.

1.3.2 Gastric Emptying

In general, viscous polysaccharides slow the emptying of liquids from the stomach, and this effect was thought to be responsible for the reduced rate of absorption of rapidly absorbed substance (Holt *et al*, 1979). However, there is no correlation between the rate of gastric emptying and postprandial plasma glucose levels after

administration of guar gum, which suggests that the action of viscous polysaccharides on gastric emptying may not be the dominant factor in reducing postprandial glycemia. In fact, at low concentration, some viscous polysaccharides, such as locust bean gum, accelerate gastric emptying but still depress postprandial glycemia.

1.3.3 Effect of Viscous Polysaccharides on Intraluminal Mixing

Two mechanisms bring nutrients into contact with the epithelium where they are absorbed. First, intestinal contractions create turbulence and convective currents, which mix the luminal contents and bring material from the center of the lumen close to the epithelium, and second, nutrients have to diffuse across the thin relatively unstirred layer of fluid lying adjacent to the epithelium (Blackburn *et al*, 1984). Viscous polysaccharides could reduced intestinal absorption by reducing mixing and/or diffusion. Viscous polysaccharides have been shown to increase the apparent thickness of the unstirred layer. The unstirred layer, however, is not an anatomic reality but a functional concept that has been invented to explain the changes in absorption that occur under stirred or less stirred conditions. An increase in unstirred layer thickness could imply a decrease in the diffusion coefficient for the test solute across an unstirred layer of unchanged thickness, or it could imply a reduction in luminal convection causing an actual increase in the unstirred zone.

1.3.4 Effect of Luminal Secretions on Viscosity

Because the reduction in intestine absorption by soluble fibers is related to viscous properties, it is important to consider the effect of intestinal secretions on the viscosity of ingested plant polysaccharides.

1.4 Physicochemical Qualities and Hypocholesterolemic Effects

Many of the physiochemical characteristics of fibers affect their hypocholesterolemic properties. Some of the important physicochemical properties of dietary fibers (Chau & Cheung, 1999) are the following: particle size, preparation and surface area; water-holding capacity; solubility features including viscosity and gelling properties; binding properties including ion exchange and absorption of organic molecules; and degradability including fermentation by bacteria (Dreher, 1987).

Smaller particles may have greater bile-acid-binding capacity but increase fecal bulk less than larger particles. Water-holding capacity may relate to viscosity and gelling characteristics but does not have a defined role in the hypocholesterolemic effects of dietary fiber.

The viscosity and gelling properties of soluble fibers may have important effects on the hydrolysis and absorption of lipids and the absorption of bile acid. Fibers that

increase the viscosity of intestine contents or that produce gels may decrease intestinal mobility, thereby decreasing the mixing of nutrients, digestive enzymes and other intestinal components required to interact for optimal micelle formation and absorption (Schneeman, 1987). Decreases in intestinal mobility may decrease bile acid pool size because of decreasing absorption rate.

The physicochemical properties of pectins are better characterized than most other fibers and correlate with their hypocholesterolemic potential. In general, the large-molecular-weight pectins with more extensive esterification of uronic acid groups with methyl groups decrease serum cholesterol more than lower-molecular-weight, lower-methoxylated pectins

The binding properties of dietary fibers may affect the absorption of cholesterol, fatty acids, and bile acids. Wheat bran and cabbages, for example, have high cation-exchange capacities, whereas psyllium and cellulose have low capacities (Dreher, 1987). Whereas dietary fibers with ion-exchange properties have cation-exchange properties, the therapeutic bile-acid-binding resins such as ©Cholestyramine and colestipol are strong anion-exchange resins. Thus, it seems unlikely that the ion-exchange properties of dietary fibers have an important effect on bile acid binding and excretion. It seems more likely that the organic-binding characteristics and viscosity-gelling properties of fibers are important in altering the absorption and metabolism of bile acids (Selvendran, 1987).

Most dietary fibers are partially fermented by bacteria in the human colon, and some, such as pectin, are almost completely fermented. Fermentation products include

hydrogen, carbon dioxide, methane, water and short chain fatty acid (SCFA) such as acetate, propionate and butyrate. These SCFA are almost completely absorbed from the colon and may affect hepatic cholesterol synthesis (Chen *et al*, 1984). In addition, the presentation of more dietary fiber to the colon stimulates the growth of more bacteria, leading to further conversion of primary to secondary bile acids.

1.5 Gastrointestinal Events and Hypocholesterolemic Effects

Dietary fibers have major effects on gastrointestinal functions, resulting in alterations in absorption of nutrients, altered hormonal responses and production of SCFA (Rosado & Diaz, 1995). These event could act individually or collectively to decrease serum cholesterol. The effect of dietary fiber on different segments of the gastrointestinal tract can be examined to explore potential mechanisms by which dietary fiber may decrease serum cholesterol.

1.5.1 Mouth

High-fiber foods are less energy dense. Although not extensively studied, high-fiber foods appear to produce greater satiety between meals than low-fiber foods

(Blundell & Burley, 1987). These event may lead to lower energy intake and slight reductions in body weight, which could produce small reductions in serum cholesterol.

1.5.2 Stomach

Soluble fibers tend to delay gastric emptying which may contribute to earlier fullness and satiation leading to decreased energy intake.

1.5.3 Small intestine

Through their viscosity and gelling properties, soluble fibers may delay or even decrease the digestion and absorption of nutrients. These effects could be mediated by decreasing the mixing of intestinal contents, decreasing intestinal mobility, and increasing the unstirred water layer, presenting a physical barrier to nutrient absorption. Certain fibers increase small intestinal transit, whereas others may slow small ntestinal transit, providing less or more time for nutrient absorption. Dietary fibers may affect small intestinal cell function through morphological and cell proliferation effects. These various effects could independently affect cholesterol homeostasis or work in combination with other effects.

1.5.4 Large intestine

Dietary fibers have well-documented effects on fecal weight, stool frequency, transit time, and intraluminal pressures. These effects may not be important in cholesterol metabolism, since wheat brans, one the most active effectors of these events, does not lower serum cholesterol. More important, it seems that the proliferation of colonic bacteria and their metabolic processes and produce such as conversion of primary to secondary bile acids and short-chain fatty acid production contribute substantially to the hypocholesterolemic effect of dietary fiber.

1.6 Proposed Mechanisms for Hypocholesterolemic Effects

Although numerous investigators have documented the cholesterol-lowering effects of dietary fiber, the mechanisms responsible for these effects remain unclear. Several hypothesis have emerged regarding the cholesterol-lowering mechanism, including these: (1) fiber may alter bile acid absorption and metabolism; (2) fiber may modify lipid absorption and metabolism; (3) short-chain fatty acid (SCFA) resulting from fiber fermentation may affect cholesterol or lipoprotein metabolism; and (4) fiber may change insulin or other hormone concentrations or tissue sensitivity to hormones.

1.6.1 Altered Bile Acid Absorption and Metabolism

One of the earlier hypotheses concerning the hypocholesterolemic effect of dietary fiber suggested that fiber could bound bile acids and increased their fecal excretion. Thus, more hepatic cholesterol was needed for bile acid synthetic pathways, and there was less cholesterol available for lipoprotein synthesis pathways (Story & Lord, 1987). There was a good relationship between the in vitro bile acid binding by various fibers and their hypocholesterolemic effect (Vahouny, 1985).

Dietary fibers increase fecal bile acid excretion by several mechanisms: binding of bile acid by forming gels or highly viscous solutions in the intestine, and interfering with micelle formation. Pectins and gums may affect bile acid absorption in the small intestine through their viscosity and gelling properties, leading to decreased micelle formation and entrapment of bile acids and lipids to a greater extent than through their physical binding properties (Selvendran, 1987).

In general, fibers that have significant hypocholesterolemic effects also significantly increase fecal bile acid excretion. Fibers that do not have hypocholesterolemic effects usually do not increase the fecal bile acid excretion significantly.

1.6.2 Modified Lipid Absorption and Metabolism

Proposed Mechanisms

Dietary fibers may affect the absorption of lipids as well as bile acids from the small intestine by these and other mechanisms: (1) altered gastric function; (2) decreased availability of bile acids; (3) interference with effective micelle formation; (4) altered digestive enzyme availability or activity; (5) changes in intestinal mobility and mixing of intestinal contents; and (6) alterations in the unstirred water layer or other physiological changes in the absorptive surface.

1.6.3 Effects of SCFA on Lipid Metabolism

In the human colon, polysaccharides such as dietary fibers are fermented by a rich and difference bacterial population. Short-chain fatty acids (SCFA) including acetate, propionate, and butyrate are major end products of this process. Therefore, SCFA was proposed to affect hepatic cholesterol metabolism leading to hypocholesterolemic effects. In animal studies, increased dietary fiber intakes are accompanied by significantly higher portal vein concentration of SCFA than is cellulose. In vitro studies using isolated rat liver cells suggest that physiological concentrations of propionate may significantly decrease hepatic cholesterol synthesis. Although pharmacological doses of propionate and its derivatives inhibit important hepatic lipid

regulatory enzymes both in vitro and in vivo, yet effects of physiological doses are not well documented. Moreover, some studies concluded that acetate rather than propionate was represented for the hypocholesterolemic of effect of dietary fiber. Therefore, this mechanism is still under extensive investigation.

1.6.4 Changed Hormone Concentrations

Increased intake of dietary fiber affects serum insulin values and levels of several other important regulatory hormones, contributing to the regulating of lipid metabolism. Insulin is a key regulatory hormone both for lipid synthesis in many body tissues and for lipid mobilization from adipose tissue and liver. Glucagon antagonizes many of these effects on lipid metabolism. Several studies suggest that serum glucose, insulin and glucagons concentration affect rates of hepatic cholesterol and lipoprotein synthesis, particularly the secretion of very-low-density lipoprotein.

High fibers diets compared to conventional diets lower insulin requirement of diabetic individuals and decrease serum insulin concentrations of normal subjects. This high fiber diets also increase insulin sensitivity of diabetic individuals and normal subjects. These reductions in insulin concentration would be expected to reduce hepatic cholesterol and fatty acid synthesis.

Chapter Two:

Materials and Methods

2.1 Materials

2.1.1 Fungus

A semi-dried fruiting body of *Auricularia polytricha* (AP) was purchased in a local Chinese medicine store. The Fungus was imported from China.

2.1.2 Animals

2.1.2.1 Golden Syrian Hamster

Male Golden Syrian Hamsters of weight 100 ± 10g were housed in polystyrene cage (5 per cage) in a room of 23°C with alternating cycles of 12 h light (7:00 a.m. to 7:00 p.m.) and 12 h darkness (7:00 p.m. to 7:00 a.m.) in Laboratory Animal Service Center (LASC). All hamsters were habituated for one week before the experiments were carried out.

Fresh semi-synthetic diets were given to hamsters daily and uneaten food was discarded. Daily food consumption was recorded and body weight was measured once a week. The fecal sample was collected from each cage. All hamsters were allowed to access food and water *ad libitum*.

2.1.2.2 Rabbit

Male New Zealand Rabbits of weight ~ 3 kg were housed in cages (one per cage) and living conditions were the same as hamster. Food consumption and body weight were recorded and fecal sample was also collected.

2.1.3 Characterization of *Auricularia polytricha*

Dextran

Dextran was purchased from Sigma Chemical Co., USA with molecular weight of 72,200 kD. It was used as a standard for carbohydrate content determination.

D-glucurono-6, 3-lactone

D-glucurono-6, 3-lactone was purchased from Aldrich Chem. Co., USA. It was used as a standard for uronic acid content determination.

Sodium tetraborate-10-hydrate

Sodium tetraborate-10-hydrate was purchased from Riedel-deHaën, Germany. Nine hundred milligrams of sodium tetraborate decahydate was dissolved in 10 ml of dH_2O and 90 ml of ice-cold 98% concentrated sulfuric acid was added carefully to form a layer. It was allowed to leave undisturbed overnight to mix without excessive heat production. The mixture (Reagent A for Carbazole assay for uronic acids determination) was checked to ensure that it was thoroughly mixed and at room temperature before use. This reagent was stable indefinitely if refrigerated.

Carbazole

Carbazole was purchased from Aldrich Chem. Co., USA. One hundred milligrams of carbazole was dissolved in 100 ml absolute ethanol and it was Reagent B for Carbazole assay for uronic acids determination. This reagent was stable indefinitely if refrigerated.

Bovine Serum Albumin (BSA)

BSA was purchased from Sigma Chemical Co., USA. It was used as a standard for BCA protein assay.

Bicinchoninic acid solution (Reagent A)

Bicinchoninic acid solution was purchased from Sigma Chemical Co., USA. It was one of the reagents used for the BCA protein Assay.

Cupric sulphate (Reagent B)

Cupric sulphate was purchased from BDH Chemicals Ltd., England. Two grams of cupric sulphate was dissolved in 50 ml of dH_2O. It was one of the reagents used in the BCA protein Assay.

α-Naphthol

α-Naphthol was purchased from Sigma Chemical Co., USA. It was used as a component for the visualizing reagent of thin layer chromatography.

Sodium bisulfite

Sodium bisulfite was purchased form Sigma Chemical Co., USA. It was used to pretreat the TLC plate prior to spotting.

Blue dextran

Blue dextran was purchased from Sigma Chemical Co., USA with average molecular weight larger than 200 kD. It was used for the estimation of molecular weight of AP Polysaccharide.

Monosaccharide standards

A set of monosaccharide standards including mannose, glucosamine, talose, galactosamine, glucose, galactose, xylose, and fucose were all purchased from Sigma Chemical Co., USA.

Trifluoroacetic acid (TFA)

TFA was purchased from Sigma Chemical Co., USA. It was used in the hydrolysis of polysaccharide prior to HPLC analysis.

3-Methyl-1-phenyl-2-pyrazolin-5-one (PMP)

PMP was purchased from Aldrich, USA. PMP can react with reducing carbohydrates almost quantitatively to yield 2:1 compounds having no stereoisomers, which strongly absorb UV light at 245 nm. PMP must be recrystallized from methanol before use.

Butyl ether

Butyl ether (GC) was purchased from Fluka Chemie GmbH, Germany and acted as an extraction solvent in High Performance Liquid Chromatography (HPLC) analysis of monosaccharides.

2.1.4 Chromatographic materials

Sepharose CL-4B

The gel was purchased from Pharmacia LKB, Sweden and used to fractionate AP Polysaccharide. Sepharose CL-4B has a fractionation range of molecular weight of 30-5000K.

TLC plate

The glass plates are pre-coated with Kiesegel 60 F254 of dimensions of 20cm × 20cm. The plates were purchased from E. Merck, Germany.

C-18 HPLC column

The RP-HPLC column was purchased from Rainin LC & Supplies, USA. C-18 column was used for the separation of monosaccharides from hydrolysate of polysaccharide with particle size of 5 μm and pore size of 100 Å. The dimension of the column is 4.6 × 250.0 mm.

AG® 1-X8 formate resin

AG® 1-X8 formate resin was purchase from Bio Rad, CA and was an anion exchange resin. In the HMG-CoA Reductase assay, the product mevalonolactone formed was retained by this resin.

Silica Capillary GC Column

A fused silica capillary column (SAC™-5, 30 m × 0.25 mm, i.d.) was purchased from Supelco, Inc., Bellefonte, PA. It was used for GLC analysis of TMS-ether derivative of cholesterol, neutral sterols and acidic sterols.

HPLC System

ÄKTA Purifier: P-900, UV-900, pH/C-900, INV-907, PV-908, M-925 & Frac-900, Amersham Pharmacia Biotech

2.1.5 Determination of Plasma TC, HDL-C, LDL-C, TG, AST and ALT

Plasma Total Cholesterol (TC)

Plasma TC level was determined by using Sigma Diagnostic Total Cholesterol Kit purchased from Sigma Chemical Co., St. Louis, MO, USA.

High Density Lipoprotein Cholesterol (HDL-C)

Plasma HDL-C was determined by using a HDL cholesterol reagent purchased from Sigma Chemical Co., St. Louis, MO, USA.

Low Density Lipoprotein Cholesterol (LDL-C)

Plasma LDL-C was determined by using Sigma Diagnostics LDL Cholesterol Kit purchased from Sigma Chemical Co., St. Louis, MO, USA.

Triglyceride (TG)

Plasma TG was determined by using Sigma Diagnostic Triglyceride Kit purchased from Sigma Chemical Co., St. Louis, MO, USA.

Aspartate Aminotransferase (AST)

Plasma AST was determined by using Sigma Diagnostic AST Kit purchased from Sigma Chemical Co., St. Louis, MO, USA.

Alanine Aminotransferase (ALT)

Plasma ALT was determined by using Sigma Diagnostic ALT Kit purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.1.6 HMG-CoA Reductase Activity Assay

Homogenizing Buffer

Homogenizing buffer contains 0.1 M sucrose, 0.05 M KCl, 0.03 M EDTA and 0.04 M KH_2PO_4 at pH 7.2.

Assay Buffer

Assay buffer contains 0.06 M EDTA, 37.8 mM NaCl, 0.14 M sucrose, 0.07 M KCl and 0.056 M KH_2PO_4 at pH 7.2.

Dithiothreitol (DTT) (purchased from Sigma Chemical Co., USA)

Glucose-6-Phosphate Dehydrogenase (G6PDH) (purchased from Sigma Chemical Co., USA)

Glucose-6-Phosphate (G6P) (purchased from Sigma Chemical Co., USA)

Nicotinamide Adenine Dinucleotide Phosphate (NADP) (purchased from Sigma Chemical Co., USA)

HMG-CoA, DL-3-[glutaryl-3-^{14}C]- (purchased from NEN™ Life Science Products, Inc., MA)

Mevalonolactone, RS-[5-3H(N)]- (purchased from NEN™ Life Science Products, Inc., MA)

Scintillation Fluid, (OptiPhase "HiSafe" 3 universal scintillation cocktail)

2.1.7 Quantitative Determination of Liver Cholesterol, Acidic and Neutral Sterol

Stigmastanol

Stigmastanol was purchased from Sigma Chemical Co., USA and used as an internal standard for liver cholesterol determination.

Hyodeoxycholic acid

Hyodeoxycholic acid was purchased from Sigma Chemical Co., USA and used as an internal standard for acidic sterol determination.

Stigmasterol

Stigmasterol was purchased from Sigma Chemical Co., USA and used as an internal standard for neutral sterol determination.

Sil-A reagent

Sil-A reagent is a commercial TMS-reagent which was purchased from Sigma Chemical Co., USA. Sil-A was used for the conversion of cholesterol in the samples into its trimethylsilyl (TMS)-ether derivative.

2.1.8 Animal Diets

2.1.8.1 Hamster Diets

All the semi-synthetic diets were based on a high cholesterol diet with 0.1% of pure cholesterol added except for normal diet. The ingredients included casein, lard, starch, sucrose, mineral mix, vitamin mix, DL-methionine and cholesterol. With 2% of AP Polysaccharide added, 1% of starch and 1% of sucrose were subtracted from the ingredients. For the short chain fatty acid (SCFA) test group, 0.5% of starch and 0.5% of sucrose were replaced with 1% of each SCFA. The composition of diets are shown in Table 2.1.

2.1.8.2 Rabbit Diets

All diets were purchased by Glen Forrest Stockfeeders, West Australia. The normal diet was based on the formula provided by Glen Forrest Stockfeeder. The details of diet preparation were as follow. Two tons of a commercial diet formulated for rabbits was prepared according to the company's standard operating procedures and specifications. One ton of the diet was returned to the mixing chamber before pelleting and 20 kg of cholesterol (1%) (grade: NF (USP), purity: >95% (GLC)) added and it was the control diet. The other ton of diet was pelleted and formed the normal diet without

cholesterol as shown in Table 2.2. The normal pelleting process involve steam injection and heating to 85°C for 10 to 20 minutes. To minimize storage changes both the control and cholesterol diets were stored at 4°C. Because of shortage of APP, the test diet preparation was scaled down with supplementation of 1% cholesterol and with 2% APP added; other conditions remain unchanged.

Table 2.1. Composition of Experimental Diets for Hamsters

	Content (% w/w)					
Ingredients	Control	Test	Ac	Pr	Bu	Ac,Pr
Casein	20.0	20.0	20.0	20.0	20.0	20.0
Lard	20.0	20.0	20.0	20.0	20.0	20.0
Starch	42.8	41.8	42.3	42.3	42.3	42.3
Sucrose	11.0	10.0	10.5	10.5	10.5	10.5
Mineral Mix	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin Mix	2.0	2.0	2.0	2.0	2.0	2.0
DL-Methionine	0.1	0.1	0.1	0.1	0.1	0.1
Cholesterol	0.1	0.1	0.1	0.1	0.1	0.1
AP Polysaccharide	/	2.0	/	/	/	/
Sodium Acetate	/	/	1.0	/	/	0.5
Sodium Propionate	/	/	/	1.0	/	0.5
Sodium Butyrate	/	/	/	/	1.0	/

Table 2.1. Composition of Normal Diet for Rabbits

Ingredients	Content (% w/w)
Oats	5.00
Lupins	20.50
Millmix	25.00
Meat Meal	1.70
Lucerne	32.00
Wheaten Chaff	16.00
Dicalcium phosphate	0.57
Tallow	0.50
Fish oil	0.50
Vegetable oil	3.80
Vitamin and Mineral premix	0.49

2.2 Methods

2.2.1 Extraction of Water-Soluble AP Polysaccharide (APP)

Fungus *Auricularia polytricha* was dried in an oven at 50°C overnight to remove residual water. Two hundred grams of dried fungus were broken down into small pieces and boiled in 3 liters of hot water (80°C) for 8 hours (Kiho *et al*, 1991). The water extraction was subjected to centrifugation (18,480 g, 30 min.) to remove small particles. The extract was then subjected to lyophilization for 5 days and finally dried water-soluble crude AP polysaccharide was obtained.

Crude AP polysaccharide can be semi-purified by ethanol precipitation. One gram of crude AP polysaccharide was first dissolved in 20 ml of dH_2O. The precipitated polysaccharide (APP-A) was obtained by adding 30 ml of absolute ethanol (1:1.5 v/v) after centrifugation. The remaining solution was pooled together and further 30 ml of absolute ethanol was added and precipitated polysaccharide (APP-B) was obtained (1:3 v/v) after centrifugation.

2.2.2 Characterization of *Auricularia polytricha*

2.2.2.1 Determination of carbohydrate content of AP Polysaccharide

The method of (Dubois M. *et al*, 1956) was adopted. In the assay, dextran (MW 72,200) was used as a standard. Phenol reagent (200 µl, 5%) was mixed with 200 µl sample (1 mg/ml dH_2O). To the 400 µl mixture, 1 ml concentrated sulfuric acid was added and allowed to stand for 30 min at room temperature. The absorbance of the reaction mixture at 490 nm was then measured. The amount of carbohydrate present was calculated from the dextran standard curve.

2.2.2.2 Determination of uronic acid content of AP Polysaccharide

The method of (Taylor & Buchanan-Smith, 1992) was adopted. In the assay, samples, standards, and controls (250 µl) were cooled in an ice-bath. 1.5 ml ice-cold Reagent A (see section 2.1.2) was added carefully with mixing and cooling in the ice-bath. The mixture was heated at 100°C for 10 min and then was cooled rapidly in an ice-bath. 50 µl of Reagent B (see section 2.1.2) was added and mixed well. The mixture was reheated at 100°C for 15 min and then cooled rapidly to room temperature. The

absorbance of the reaction mixture at 525 nm was then measured. The amount of uronic acids present was calculated from the D-glucurono-6, 3-lactone standard curve.

2.2.2.3 Determination of protein content of AP Polysaccharide by BCA protein assay

The protein assay reagent was prepared by adding 9.8 ml of reagent A to 200 µl of reagent B (see section 2.1.2). 2 µl and 4 µl of 5 mg/ml of samples were added to 96-well plate and 200 µl of protein assay reagent was added to each well. The plate was incubated for 30 min and absorbance at 540 nm was measured. The average protein content of the samples was calculated from the BSA standard curve (Walker, 1994).

2.2.2.4 Determination of component sugar units of AP Polysaccharide

The method of (Szustkiewicz & Demetriou, 1971) was adopted for chromatographic separation of monosaccharide units from hydrolyzed samples. Hydrolysis was done by adding 5 mg of polysaccharide or standards (Glucose, Mannose, Xylose, Galactose, Fucose, Arabinose) were dissolved in 0.25 ml of 2M HCl and heated at 100°C for periods of 3 hr in a sealed tube. The TLC plate was pretreated by impregnating the layer with 0.1 M sodium bisulfite. The plate was air-dried and

activated it at 100°C for 30 min prior to spotting. The hydrolysate (2 μl) from each sample was spotted on a TLC plate (Kiesegel 60 F_{254}, Merck). The spots were air-dried, and the TLC plate was developed in a fume hood for a distance of 10 cm in a tank saturated with mobile phase, ethylacetate: formic acid: acetic acid: dH_2O (10:1:3:2). The plate was then removed and air-dried. The monosaccharides on the TLC plate were detected by α-Naphthol-Sulfuric acid visualizing reagent rather than alkaline silver staining method (Trevelyan W.E. *et al*, 1950). The visualizing reagent was prepared by adding 5 g of α-Naphthol to 33 ml ethanol, then 13 ml H_2SO_4, 81 ml ethanol and 8 ml H_2O. The TLC plate was sprayed with this visualizing reagent and heated for 5 min at 100°C.

2.2.2.5 Fractionation of AP Polysaccharide

Gel filtration chromatography (Andrews P., 1965) was used to fractionate AP Polysaccharide. Sepharose CL-4B gel was pre-swollen when it was purchased. Sepharose gel was packed in a column of 2.6 cm × 60 cm dimension. The gel was eluted with 0.1 M NaCl (running buffer) for equilibration. Twenty milligrams per milliliter of AP Polysaccharide was dissolved in 0.1 M NaCl and 1 ml of sample solution was applied to the column. The flow rate of the packed column was adjusted to 0.25 ml/min with a peristaltic pump. The eluent of the column was collected by a fraction collector

(FRAC-200, Pharmacia Biotech., Sweden) with each tube collected 3 ml eluent. The void volume of the column was characterized by Dextran (Average MW > 5000kD, 10 mg/ml). The detection of samples and dextran were determined by phenol-sulfuric acid assay at 490 nm.

2.2.2.6 Determination of monosaccharides of AP Polysaccharide by HPLC

Hydrolysis

Samples of polysaccharide (50 μg) were dissolved in 1 ml of 6 M trifluoroacetic acid (TFA) (Kwon & Kim, 1993) in a hydrolysis tube (13 × 100mm) fitted with a Teflon-lined screw cap. Talose (2 nmol) as an internal standard was added to each sample. The hydrolysis tube was sealed and incubated at 121°C for 2 h in a heating block. A set of monosaccharide standards, 10 nmol each of mannose (Man), glucosamine (GlcN), talose (Tal), galactosamine (GalN), glucose (Glc), galactose (Gal), xylose (Xyl), and fucose (Fuc), was treated identically as the samples. After being cooled to room temperature, the hydrolysis tubes were opened, and each reaction mixture was transferred to a microcentrifuge tube and then evaporated to dryness by concentration under reduced pressure. The residue was then dissolved in 0.5 ml of 2-propanol and again evaporated to dryness to remove residual TFA (Honda *et al*, 1989).

Re-crystallization of PMP

100 ml of methanol was added to 50 g of PMP. The solution was mixed by shaking on an orbital mixer at 40-50°C for 5-6 min. An homogeneous yellow solution was formed and allowed to cool at room temperature for 1-2 hr. White PMP crystals will settle at the bottom while a yellow solution remains at the top. The yellow solution was decanted and the crystal was briefly rinsed with methanol. The crystal was dried in desiccators or oven for 1-3 hr. 0.5 M PMP solution was prepared to check purity (Fu & O'Neill, 1995). The absorbance must not grater than 0.025AU at 400 nm, otherwise, a second re-crystallization should be performed.

PMP Labeling

The dried hydrolyzed polysaccharide sample and monosaccharide standards were directly labeled with PMP (Fu & O'Neill, 1995)by adding 20 µl of PMP solution (0.5 M in methanol) and 20 µl of sodium hydroxide solution (0.3M), vortexing, and incubating at 70°C for 2 h (White *et al*, 1979). The mixture was then neutralized by vortexing for at least 5 s. Phase separations were enhanced by brief centrifugation. The organic phase (upper layer) was carefully removed and discarded. This extraction process was repeated two more times. The resulting aqueous phase was mixed with water (150 µl) and subjected to centrifugation to remove particles prior to HPLC analysis.

HPLC Analysis

Analysis of the PMP-labeled monosaccharides was carried out on by a HPLC system (ÄKTA Purifier) provided by Amersham Pharmacia Biotech. A C-18 column, 220 X 2.1 mm, (Rainin LC & Supplies, USA) for the separation of PMP-labeled carbohydrates, was used. The flow rate was set to 200 μl/min. The wavelength for UV detection was 245 nm. For neutral and amino sugar separation, buffers A and B were 100mM ammonium acetate (pH 5.5) with 10 and 40% acetonitrile, respectively. A gradient of 20% to 75% buffer B in 90 min was used for separation.

2.2.3 Determination of plasma TC, HDL-C, LDL-C, TG, AST and ALT

2.2.3.1 Plasma Total Cholesterol

The Diagnostic Kit involves enzymatic reactions in measuring TC content. Cholesterol esters are first hydrolyzed by cholesterol esterase (EC 3.1.1.13) to cholesterol. The cholesterol produced by hydrolysis is oxidized by cholesterol oxidase (EC 1.1.3.6) to cholest-4-en-3-one and hydrogen peroxide (H_2O_2). The hydrogen peroxide produced is then coupled with the chromogen, 4-aminoantipyrine and p-hydroxybenzenesulfonate in the presence of peroxidase (EC 1.11.1.7) to yield a quinoneimine dye which has an absorbance maximum of 500 nm. The intensity of the

color produced is directly proportional to the total cholesterol concentration in the plasma sample.

2.2.3.2 Plasma HDL-Cholesterol

The HDL cholesterol reagent contains phosphotungstic acid in $MgCl_2$, precipitates LDL and VLDL fractions of the plasma, leaving the HDL fraction in solution. Cholesterol concentration in HDL fraction was then assayed by the enzymatic kit that is used in measuring plasma total cholesterol.

2.2.3.3 Plasma LDL-Cholesterol

The LDL cholesterol reagent utilizes latex beads coated with affinity purified goat polyclonal antisera to specific human apolipoproteins which facilitate the removal of HDL and VLDL in the specimen. After a short incubation and centrifugation, the LDL cholesterol remains in the filtrate solution. The LDL cholesterol concentration was determined by the enzymatic kit that is used in measuring plasma total cholesterol.

2.2.3.4 Plasma Triglyceride

Methods for triglycerides determination generally involve enzymatic hydrolysis or alkaline hydrolysis of triglycerides to glycerol and free fatty acid following by either chemical or enzymatic measurement of the glycerol released. Enzymatic hydrolysis following by enzymatic measurement was employed by this Triglyceride Diagnostic Kit.

Triglycerols were hydrolyzed by lipase to glycerol and free fatty acid. The glycerol produced was then measured by coupled enzyme reactions catalyzed by glycerol kinase, glycerol-1-phosphate dehydrogenase and diaphorase.

2.2.3.5 Plasma Aspartate Aminotransferase

AST catalyzes the transfer of the amino group from aspartate to 2-oxoglytarte to yield oxalacetate and glutamate. The oxalacetate was reduced to malate in the presence of malate dehydrogenase with the simultaneous oxidation of reduced nicotinamide adenine dinucleotide. The rate of decrease in absorbance at 340 nm was directly proportional to AST activity.

2.2.3.6 Plasma Alanine Aminotransferase

ALT catalyzes the transfer of the amino group from Alanine to 2-oxoglutarate, to form glutamate and pyruvate. The pyruvate formed was then reduced to lactate in the presence of lactate dehydrogenase with simultaneous oxidation of reduced nicotinamide adenine dinucleotide. The rate of decrease in absorbance at 340 nm was directly proportional to ALT activity.

2.2.4 HMG-CoA Reductase Activity Assay

2.2.4.1 Preparation of Hepatic Microsome

2 g of liver sample was homogenized in 3 volumes of homogenizing buffer using a glass Potter-Elvehjem homogenizer with Telfon pestle (Dietschy & Spady, 1984). The homogenate was centrifuged at 12,000 g for 10 min at 4°C. Fat layer was removed by filtering through a piece of muslin. The filtrate was then centrifuged again at 105,000 g for 60 min. The pellet obtained after centrifugation was resuspended in 1 ml homogenizing buffer and the protein content was determined by BCA protein assay. The microsome was stored at –70°C before use if not use immediately.

2.2.4.2 HMG-CoA Activity Assay

For each assay, 150 µl microsome preparation was mixed with 100 µl assay buffer (pH 7.2), 10 µl 54 mM dithiothreitol (DTT), 10 µl G6PDH (40 units/ml), 10 µl G6P (162 mM), 10 µl NADP (16.2 mM) and 30 µl dH_2O (Vergara-Jimenez *et al*, 1998). The mixture was pre-incubated at 37°C for 20 min with shaking. The reaction mixture was initiated by adding 10 µl of HMG-CoA, DL-3-[glutaryl-3-^{14}C]- with 1 Ci/mol. The reaction mixture was then further incubated at 37°C for 60 min. After incubation, the reaction was stopped by adding 25 µl of 5 N HCl and 10 µl of mevalonolactone, RS-[5-^{3}H(N)]- with 10 µCi/ml was added as internal standard. The total reaction mixture was then incubated at 37°C for 30 min. After incubation, the precipitated protein was removed by brief centrifugation and 200 µl of supernatant was applied to an AG® 1-X8 formate (Edwards *et al*, 1979) column (0.7 × 10 cm, purchased from Bio-Rad, CA), made up in water. The column was then eluted with H_2O, the first 300 µl was discarded and the next 3 ml fraction was collected. The collected eluent was mixed with 19 ml liquid scintillation fluid for scintillation counting. Time zero assay, no substrate assay and no microsome protein background were also performed which was needed for the calculation of the HMG-CoA Reductase activity. One unit of HMG-CoA Reductase activity was defined as one pmol/min/mg protein.

2.2.5 Quantitative Determination of Liver Cholesterol

2.2.5.1 Cholesterol Extraction and its Silylation

Three hundred milligrams of liver were weighed and one milligram of stigmastanol (dissolved in chloroform-methanol (2:1, v/v)) was added as an internal standard. The liver was then homogenized in 15 ml chloroform-methanol (2:1, v/v) and 3 ml saline using a Polytron homogenizer. Phase separation was enhanced by centrifugation at 3,000 rpm for 15 min using a table-top centrifuge. After centrifugation, the bottom organic layer was collected and dried by nitrogen gas. The samples were then subjected to alkaline hydrolysis with 5 ml 1 N NaOH in 90% ethanol at 90°C for 1 hour. Six milliliters of cyclohexane and 1 ml of dH_2O were added for cholesterol extraction. Phase separation was enhanced by brief centrifugation. The upper cyclohexane layer was collected and evaporated under nitrogen gas. Three hundred milliliters of TMS-reagent (dry pyridine-hexamethyldisilazane-tricholrosilane, 9:3:1, v/v/v, Sil-A reagent, Sigma Chemical Co., USA) was added for the conversion of sample cholesterol to its trimethylsilyl (TMS)-ether derivative. It was then incubated at 60°C for 1 h and the mixture was dried under nitrogen gas. The derivative was dissolved in 600 μl of hexane and the hexane was transferred to a GC vial for GLC analysis after centrifugation.

2.2.5.2 GLC Analysis of TMS-Ether Derivative of Cholesterol

The TMS-ether derivative was analyzed on a fused silica capillary column in a Shimadzu GC-14B gas-liquid chromatograph equipped with a Shimadzu flame-ionization detector. Column temperature was programmed at 285°C and maintained for 20 min. Helium was used as the carrier gas at a head pressure of 22 psi.

2.2.6 Quantitative Determination of Neutral and Acidic Sterols

2.2.6.1 Separation of Neutral and Acidic Sterols

Three hundred milligrams of fecal samples were dried by lyophilization and then ground to powder form. One milliliter of 0.3 mg/ml stigmasterol in chloroform and one milliliter of 0.3 mg/ml hyodeoxycholic acid in 1 N NaOH were added to the fecal sample as internal standards for neutral and acidic sterols respectively. Alkaline hydrolysis of samples was carried out by adding 8 ml 1 N NaOH in 90% ethanol at 90°C for 1 h. The sample mixture was then cooled down to room temperature. Extraction was performed by adding 1 ml of dH_2O and 8 ml of cyclohexane. After centrifugation, the lower aqueous layer and the upper organic layer (cyclohexane) were collected for acidic and neutral sterols analysis respectively.

2.2.6.2 Conversion of Neutral Sterols to its TMS-Ether Derivative

The cyclohexane layer was evaporated to dryness under nitrogen gas. Three hundred milliliters of TMS-reagent (Sil-A) was added and heated at 60°C for 1 h for the conversion of neutral sterols into its TMS-ether derivatives. The mixture was then evaporated to dryness under nitrogen gas and the resulting pellet was dissolved in 400 µl of hexane. After centrifugation, the hexane layer was transferred to a GC vial for GLC analysis.

2.2.6.3 Conversion of Acidic Sterols to its TMS-Ether Derivatives

The remaining aqueous phase was left for acidic sterols analysis. One ml of 10 N NaOH was added to the aqueous phase and the mixture was heated at 120°C for 3 h. The tube was then cooled to room temperature. One ml of dH_2O and 3 ml 25% HCl (pH<1) were added for acidification. The mixture was extracted with 7 ml of diethyl ether twice. Phase separation was enhanced by brief centrifugation. The ether phase was then dried down by nitrogen gas. Methylation was performed by adding 2 ml methanol, 2 ml dimethoxypropane and 40 µl concentrated HCl. The mixture was vortexed thoroughly and allowed to stand overnight at room temperature. The solvent was dried down by nitrogen gas and 300 µl TMS-reagent (Sil-A) was added for conversion of acidic sterols

into its TMS-ether derivatives. The mixture was then dried down by nitrogen gas and the resulting residue was dissolved in 300 μl hexane. After centrifugation, the hexane layer was transferred to a GC vial for GLC analysis.

2.2.6.4 GLC Analysis of Neutral and Acidic Sterols

The GLC analysis of fecal neutral and acidic sterols was performed by a fused silica capillary column as described in section 2.2.5.2. For neutral sterols, the column temperature was programmed at 285°C and maintained for 30 min. For acidic sterols, the column temperature was programmed at 230°C to 280°C at a rate of 1°C/min. Helium was used as the carrier gas at a head pressure of 22 psi in both neutral and acidic sterol GLC.

2.2.7 Study of Atherosclerosis of Rabbit

2.2.7.1 Sudan III staining of the thoracic aorta

At the end of the 12th week rabbits were bled and weighed before being sacrificed by CO_2. The rabbits were opened using a mid-line incision from the pubis to the xiphoid process, exposing the abdominal viscera. The rib cage was removed by cutting the ribs down both sides of the thorax, exposing the thoracic viscera. Whilst livers from the rabbits were harvested for cholesterol determination, for the study of atherosclerosis, the thoracic aorta from the bulb of the aorta to the branching of the celiac artery was removed (Popesko *et al*, 1992).

The fresh aortas were obtained and cleaned of all visible adventitial tissue and washed in saline solution until all traces of blood had been removed. The vessels were cut longitudinally and immersed in a solution of Sudan III (4 g in 70% alcohol) (Haines *et al*, 1999) for 45 min to identify atheromatous lesions on the surface of the aorta. This is an established method of staining vessels to identify atheroma formation (Holma *et al*, 1958; Wilson *et al*, 1982; Smith & Burton, 1977). Sudan III is an organotrophic dye which was previously thought to stain lipid on the principle that the dye was more soluble in tissue fat than the dye solvents. However, it is considered that the staining of lipids is due to adsorption of the dye by the lipid. Following preparation and staining, the thoracic aorta was pinned to a corkboard with the intimal surface face down in order to

remove any residual adventitial fat shown up by staining. The features of the intimal surface were then captured by digital scanning and stored as a bitmap using established methodology (Haines *et al*, 1999).

2.2.7.2 Measurement of atheroma formation in the aorta

Atheroma on the intimal surface of the aorta was identified using a self-organizing map neural network to characterize tissue according to selective color staining. A self-organizing map neural network clusters input patterns from the scanned image to a given software output node. The map is initially trained by presented normalized input patterns, in this case the individual color components of each pixel from a test image, to the neural network. Internally the network compares the input pattern to one of its pre-existing patterns associated with each output to see which it is closest to. The difference between the input pattern and the best matching pattern represents the training error. The input patterns are presented repetitively until all input patterns can be matched to all available outputs, to a predefined error level. At this point the network is trained and available to interpret other images of a similar type, in this case the scanned images of the Sudan III stained aortas. The matching patterns initially are set to randomized values and hence the final matching patterns used to interpret other images are empirically derived. This ability to interpret the color pattern of the

scanned images allows the computer program to calculate the area of aortic atheroma and the area of normal aorta. The area of atheroma was expressed as a percentage of the total area of aorta scanned into the computer.

The percentage was calculated as follows:

$$\frac{\textbf{Total area of atheromatous plaques (i.e. area stained with Sudan III)} \times \textbf{100}}{\textbf{Total area of atheromatous plaques + Total area of normal intima (i.e. area stained with Sudan III + area not stained)}}$$

The images were captured as 24 bit true color using a Hewlett Packard Scanjet 6100C desk scanner. All images were captured at a horizontal and vertical resolution of 300 dpi (a pixel to pixel distance of 0.085mm) using a brightness setting of 126 units and a contrast of 112 units. Prior to image capture all specimens were placed adventitial side down on to white tissue and then turned over with the intimal surface placed onto a layer of saline solution on the glass surface of the scanner. The saline solution acted as a transmission medium between the glass surface of the scanner and the tissue, thereby minimizing surface reflections. This method is a simple and reliable method of image quantification according to the degree of stain absorption using a self-organizing map. It was successfully used in this study to identify atheromatous plaques of different sizes and shapes on the surface of the aorta. Patterns with broadly similar shades can be

reduced to one or more generic patterns aiding plaque identification and measurement. All images obtained for analysis in this study were captured using the same scanner with fixed contrast and brightness settings. This was done so as to exclude any difference due to different lighting sources employed by individual manufacturers within their products. The neural map offers the advantages that it is consistent and repeatable.

2.2.8 Animal Experiments

2.2.8.1 Protective Effect of APP in Hyperlipidemic Study (Exp. 1)

Twenty hamsters (100 ± 5 g) were habituated and fed with commercial diet for one week. The hamsters were then divided randomly into two groups (n=10 per group) and fed with different experimental diet for four weeks. The experimental diets were composed of a semi-synthetic and high-cholesterol diet, with supplementation of 2% APP. The semi-synthetic diets were prepared with the method described by Sanders & Sandaradura (Trowell *et al*, 1976) with little modification. The control group was fed with control diet and test group was fed with AP diet (Table 2.1). The powder diet was then mixed with gelatin solution (20 g/L). The diet was made into small pieces and stored at -20°C.

At the end of week 4, all hamsters were fasted for at least 16 h before they were sacrificed. Blood was collected in a heparin tube via the abdominal aorta and centrifuged

at 1,500g for 10 min. The livers were washed with saline and weighed after exsanguinations. Liver was frozen in liquid nitrogen and stored in -70°C for cholesterol analysis.

2.2.8.2 Therapeutic Effect of APP in Hyperlipidemic Study (Exp. 2)

Twenty hamsters (100 ± 5 g) were fed with control diet for 4 weeks in order to boost the hamsters into hyperlipidemic state(Talwinder *et al*, 1997). After 4 weeks, all hamsters were divided into two groups randomly (n=10 per group). Control group was fed with control diet and test group was fed with AP diet (Table 2.1).

Four weeks later (week 8), five hamsters from each group were sacrificed after overnight fasting. Blood and liver were collected as described in section 2.2.8.1. Four weeks later (week 12), the remaining hamsters were sacrificed and treated as previously. The parameters measured were the same as experiment 1.

2.2.8.3 Dose Response of APP in Hyperlipidemic Study (Exp. 3)

Forty hamsters (100 ± 5 g) were divided into four groups randomly (n=10 per group) and fed with different experimental diets diet for 4 weeks. The experimental diets

were composed of a semi-synthetic and high-cholesterol diet, with supplementation of 1%, 2% and 4% APP.

At the end of week 4, all hamsters were sacrificed after overnight fasting and same analysis was performed as previously.

2.2.8.4 Hypolipidemic Effect of Short Chain Fatty Acid (Exp. 4)

Sixty hamsters (100 ± 5 g) were divided into six groups randomly (n=10 per group) and fed with different experimental diets for 4 weeks. The experimental diets were composed of a semi-synthetic and high-cholesterol diet, with supplementation of 1% sodium acetate, sodium propionate sodium butyrate and APP (Chen *et al*, 1984). One group fed with supplementation of 0.5% sodium acetate and 0.5% propionate. (Table 2.1)

At the end of week 4, all hamsters were sacrificed after overnight fasting and same analysis was performed as previously.

2.2.8.5 Effect of APP and SCFA on HMG-CoA Reductase Activity (Exp. 5)

All sixty hamsters were treated identically as described in section 2.2.8.4 except without overnight fasting. Only livers were collected for measuring HMG-CoA

Reductase activity. Liver microsome fractions were obtained immediately and not stored (-70°C) more than one 24 hr to prevent enzyme degradation. All fractions were tested at once without repeating freeze and thaw.

2.2.8.6 Hypolipidemic and Anti-atherosclerotic Effect of APP (Exp. 6)

Twelve New Zealand rabbits were divided into three groups randomly (n=4 per group) and fed with different experimental diets for 12 weeks. The experimental diets were composed of a normal diet, with supplementation of 1% cholesterol (control group) or 1% cholesterol & 2% APP (test group). The third group was fed with a normal diet without cholesterol. The same analysis was performed as previously plus the measurement of atheroma formation in the aorta.

2.3 Statistical analysis

All results were expressed as the arithmetic mean ± standard derivation (S.D.). Student's "t" test was used to determine the confidence limits in population comparisons. $P < 0.05$ was regarded as significant.

Chapter Three:

Fractionation and Characterization of *Auricularia polytricha* Polysaccharide (APP)

3.1 Introduction

The role of dietary fiber (DF) in human nutrition and health has become increasingly topical in recent years as its physiological effects have been more widely appreciate and as its chemistry has been better understood. Polysaccharides, which are the principal constituents of dietary fiber, show a wide spectrum of physical properties, reflecting the nature and extent of intermolecular association. At one extreme, the polymer chains may be packed together into ordered assemblies, such as cellulose fibrils, which are almost totally resistant to hydration and swelling. At the other extreme, polysaccharide chains can exist in solution as fluctuating, disordered coils, interacting with one another only by physical entanglement. Between these extremes lie on the hydrated, swollen networks typical of plant tissue and of many manufactured foods.

Trowell et al. (1976) (Trowell *et al*, 1976) defined DF as all the polysaccharides and lignin in the diet that are not digested by the endogenous secretions of the human digestive tract. For analytical purposes, DF refers mainly to the non-starch polysaccharides and lignin we consume (Southgate, 1976). However, lignin is not polysaccharide and only non-starch polysaccharide is defined as DF.

3.2 Fungal polysaccharides from *Auricularia polytricha*

All fungal walls contain a mixture of fibrillar components and amorphous or matrix components (PCK Cheung, 1997). In Basidiomycetes, the main fibrillar components include chitin which is a straight-chain b-(1-4)-linked polymer of N-acetylglucosamine and the main matrix components include various polysaccharides (such as a- and b-1-3 glucans, and mannans), proteins, and small amounts of lipids. Therefore, most of the edible fungi (mainly from the Basidiomycetes) are rich in nonstarch polysaccharides which can be a good source of dietary fiber for humans

Auricularia, a group of jelly fungi, are often used in Asian cuisine. Both are sold dried in Asian markets and are reasonably priced compared to many wild or cultivated mushrooms. *Auricularia polytricha* is variously called "wood ear," "tree ear," "black fungus," or "muk nge" which was used for investigate its hypocholesterolemic effect (Cheung, 1996) in our project. The dried ear-shaped cap is medium sized, dull in texture,

and dark brown to black. The wavy lower surface has a contrasting powdery gray color. The stem is absent or rudimentary. It has no gills. It is a native of Asia and some Pacific Ocean islands in humid climates. Most major Asian countries successfully cultivate *A. polytricha* today.

These fungi are used for their crisp, snappy texture and their color rather than their taste.

3.3 Results

3.3.1 Extraction and Fractionation of *Auricularia polytricha*

The lyophilized fungal powder makes up around 21% of the dry weight after removal of water in oven at 50°C. This fraction was the crude Auricularia polytricha Polysaccharide. After the first round of ethanol precipitation of 1 g / 20ml of polysaccharide solution by adding 1.5 X volume of absolute ethanol (1:1.5 v/v). The precipitate was subjected to lyophilization and the powder obtained was APP-A and the yield was 69.6%. Further 1.5 X volume of absolute ethanol was added to the remaining solution (1:3 v/v). The precipitate was subjected to lyophilization and the powder obtained was APP-B and the yield was 22.8%. The results are shown in Table 3.1.

3.3.2 Determination of Carbohydrates Content

The carbohydrate content of the extracted fractions were estimated with phenol-sulfuric acid assay method. Dextran (M.W. 72,200) with concentration of 0.5 mg/ml, in dH_2O was used as the standard. Crude APP, APP-A and APP-B were estimated to contain carbohydrate 51.8 ± 5.6%, 39.4 ± 4.2%, and 40.0 ± 6.1% respectively. The results were listed in Table 3.2.

Table 3.1. The yield of different polysaccharide fractions isolated from the fungus *Auricularia polytricha*.

Sample	Mass (g)	Percentage yield (%)	
Dried fungus	200.00	100.00	
Crude APP	42.60	21.30	100.00
APP-A	29.67		69.60
APP-B	9.71		22.80

Dry powder of *Auricularia polytricha* was dissolved in a concentration of 1 g/ 20ml, 1.5 volume of absolute ethanol (1:1.5 v/v) was added to the sample solution. After centrifugation (3,000 × g, 30 min.), the precipitate obtained (APP-A) was dried by freeze-dryer. The remaining solution after centrifugation was poured into 1.5 volume of absolute ethanol (1:3 v/v) to obtained APP-B after centrifugation and freeze-drying.

Table 3.2. The carbohydrate content of different polysaccharide fractions isolated from *Auricularia polytricha*; Crude APP, APP-A and APP-B.

Sample (0.5 mg/ml in dH_2O)	Percentage of carbohydrate (%)
Dextran	100.0
Crude APP	51.8 ± 5.6
APP-A	39.4 ± 6.2
APP-B	40.0 ± 6.1

The carbohydrate content was determined by phenol-sulfuric acid method (Dubois, 1956) and absorbance was read at 490 nm using dextran (M.W. 72,200) as a standard.

3.3.3 Determination of Protein Content

Bicinchoninic acid protein assay (BCA) was adopted instead of the Bradford protein microassay (Bradford, 1976) and the Folin-Lowry method (Lowry *et al*, 1951). It is because the protein value determined by the Folin-Lowry method is affected by the presence of carbohydrate. Furthermore, the BCA method was the most sensitive assay compared to the other protein assay. The percentage of protein content of dextran, crude APP, APP-A and APP-B were 0.496%, 6.284%, 3.73% and 1.874% respectively. The total percentage yield of APP-A and APP-B was 92.4%. The total percentage protein content of APP-A and APP-b was 5.604%. If half of the total percentage protein content (APP-A + APP-B) was divided by the total percentage yield of APP-A and APP-B, the actual percentage protein content of the ethanol precipitated sample (APP-A and APP-B) is equal to 3.032%. When comparing the value to crude APP (6.284%), there is a great difference. Therefore, APP was found to contain extrinsic protein that is not associated with the polysaccharide chain.

3.3.4 Determination of Uronic Acid Content

The method (Chaplin & Kennedy, 1986) adopted was widely used but with disadvantages. Under the experimental conditions, neutral sugars at similar

concentration show 10% interference. In the estimation, D-glucurono-6, 3-lactone was used as standard. The uronic acid content of APP-A (5.92%) was much larger than APP-B (3.46%). There is no loss of uronic acid after ethanol precipitation. The results were showed in Table 3.4.

Table 3.3. Determination of protein content (%) of dextran, crude *Auricularia polytricha* Polysaccharide (APP) and ethanol precipitated sample APP-A and APP-B obtained from crude APP.

	Protein Content (%)
Dextran	0.496 ± 0.05
Crude APP	6.284 ± 0.10
APP-A	3.730 ± 0.07
APP-B	1.874 ± 0.08

The protein content was determined by BCA protein Assay (Walker, 1994) and absorbance was read at 540 nm using BSA as a standard.

Table 3.4. Determination of uronic acid content (%) of dextran, crude *Auricularia polytricha* Polysaccharide (APP) and ethanol precipitated sample APP-A and APP-B obtained form crude APP.

	Uronic Acid Content (%)
Dextran	15.39 ± 1.10
Crude APP	4.03 ± 0.42
APP-A	5.918 ± 0.45
APP-B	3.460 ± 0.32

The uronic acid content was determined by the carbazole reaction (Chaplin, 1986) and absorbance was measured at 525 nm.

3.3.5 Determination of component sugars of AP Polysaccharide

The sugar components of the crude APP were determined by a thin layer chromatographic method. The solvent system adopted was efficient in separating various monosugars such as glucose, mannose, xylose, galactose, arabinose and fucose, as shown in Fig. 3.1. The solvent system employed a mixture of ethylacetate, formic acid, acetic acid and distilled water in the ratio of 10:1:3:2. The monosaccharide standards showed a different Rf value in the thin layer chromatogram. After acid hydrolysis, the sample was tested in duplicate and the chromatogram showed that the crude sample contains mannose component. However, there is a tailing effect due to the residual acid in sample after acid hydrolysis. Tailing can be minimized by adding an equal molar of sodium hydroxide solution to the acid hydrolysate.

As seen in Fig. 3.1, the result was not so satisfactory as only one monosaccharide can be seen. As we do not expect only one monosaccharide in the APP, we performed the monosaccharide analysis by a more sensitive system, HPLC. By HPLC, monosaccharide components of the APP can be tested quantitatively and qualitatively.

Fig. 3.1. The Thin Layer Chromatogram of monosaccharides. Xylose (1), glucose (2), galactose (3), mannose (4), fucose (6) and arabinose (7) were used as standard for locating the sample (5,8) monosaccharide obtained from acidic hydrolysis of polysaccharide (crude APP). The sample was tested in duplicate and the mobile phase was mixture of ethylacetate: formic acid: acetic acid: dH_2O (10:1:3:2).

3.3.6 Fractionation of AP Polysaccharide

The fractionation of AP polysaccharide was performed by size exclusion chromatography. Sepharose CL-4B was used. The results are shown in Fig. 3.2, Fig. 3.3, Fig. 3.4 and Fig. 3.5. Fig. 3.2 shows the elution profile of dextran with average molecular weight of 5,000K to 40,000K. As Sepharose CL-4B has a fractionation range of molecular weight of 30-5,000K, dextran was used to find the void volume of the column. The peak absorbance was at fraction 39, and then the void volume was approximately 117 ml (39 × 3 ml). From Fig. 3.3, the elution profile of crude APP shows two peaks, a broad peak and a sharp peak. The broad peak represents a elution volume of 114 – 255 ml (fraction 35-89) and sharp peak with elution volume of 300 – 351 ml (fraction 100-117). From Fig. 3.4, it shows the elution profile of ethanol precipitated polysaccharide APP-A (See section 2.2.1). The elution volume was 99 – 240 ml (fraction 33-80). The elution profile was similar to the first peak of Fig. 3.3. From Fig. 3.5, it shows the elution profile of ethanol precipitated polysaccharide APP-B (See section 2.2.1). The elution volume was 105 – 321 ml (fraction 35-107). The elution profile was also similar to the first peak of Fig. 3.3. However, the sharp peak in Fig. 3.3 does not appear in either Fig. 3.4 or Fig. 3.5.

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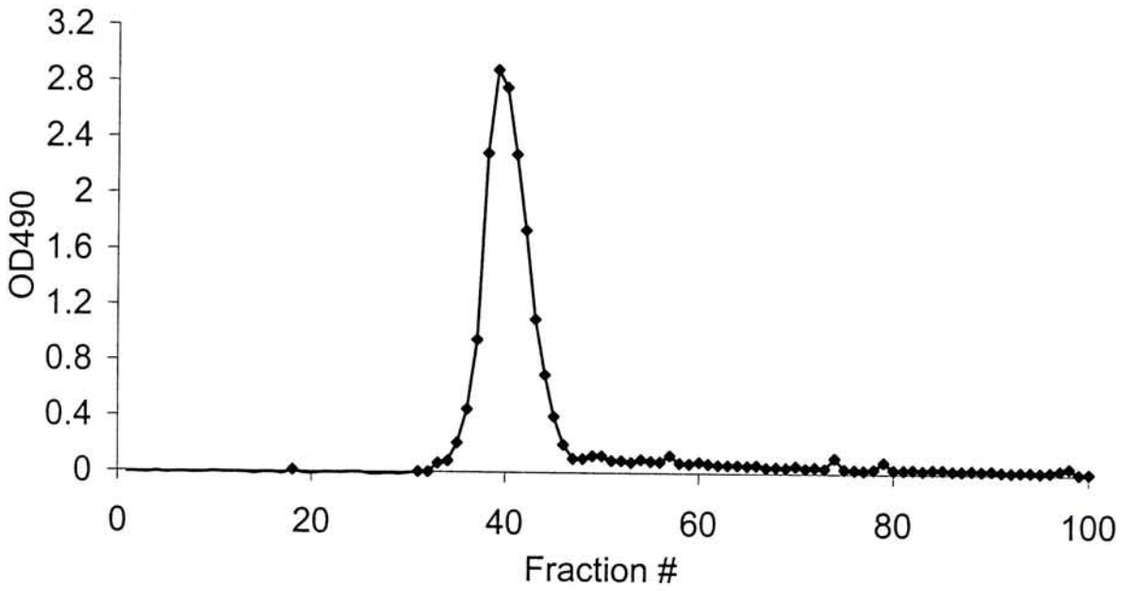


Fig. 3.2. Elution Profile of Dextran (M.W>5000K) by Sepharose CL-4B size exclusion chromatography for estimation of void volume. 10 mg/ml of dextran was prepared and 1ml of dextran solution was added to column. The flow rate was set to 0.25 ml/min and 3 ml fraction was collected.

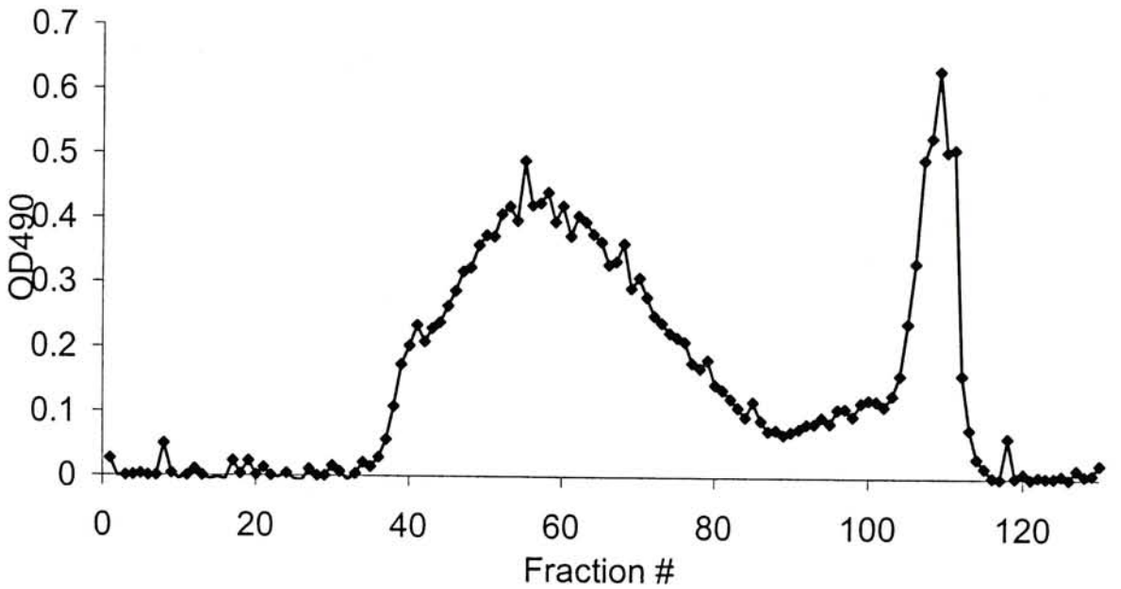


Fig. 3.3. Elution Profile of crude APP by Sepharose CL-4B size exclusion chromatography. 20 mg/ml of crude APP was prepared and 1ml of crude APP solution was added to column. The flow rate was set to 0.25 ml/min and 3 ml fraction was collected.

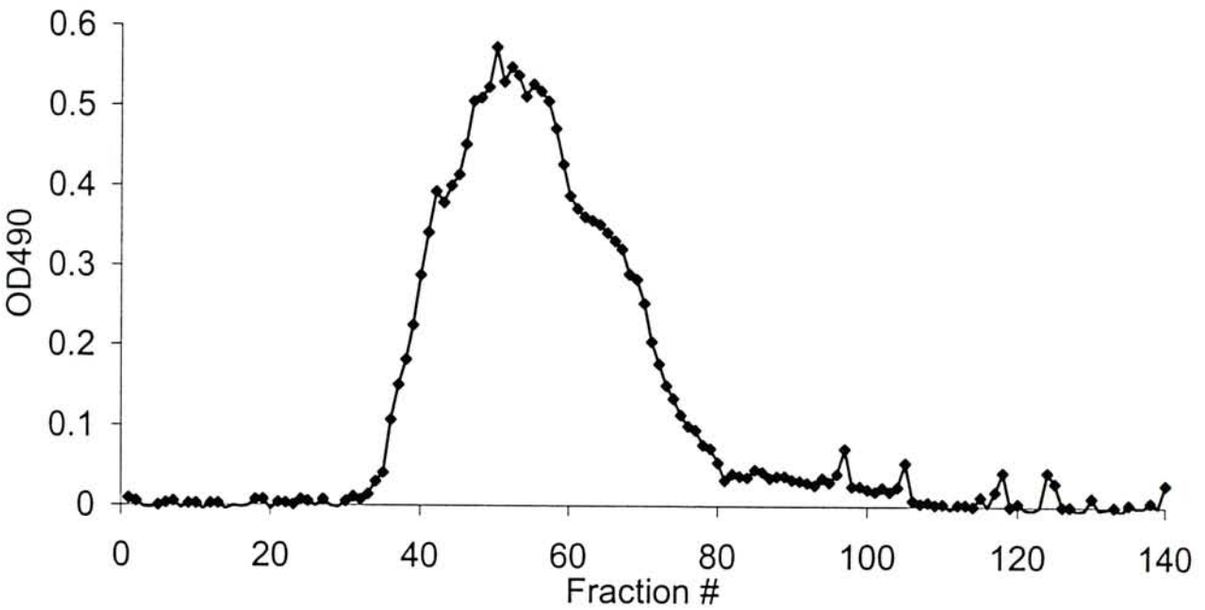


Fig. 3.4. Elution Profile of APP-A by Sepharose CL-4B size exclusion chromatography. 10 mg/ml of APP-A was prepared and 1ml of APP-A solution was added to column. The flow rate was set to 0.25 ml/min and 3 ml fraction was collected.

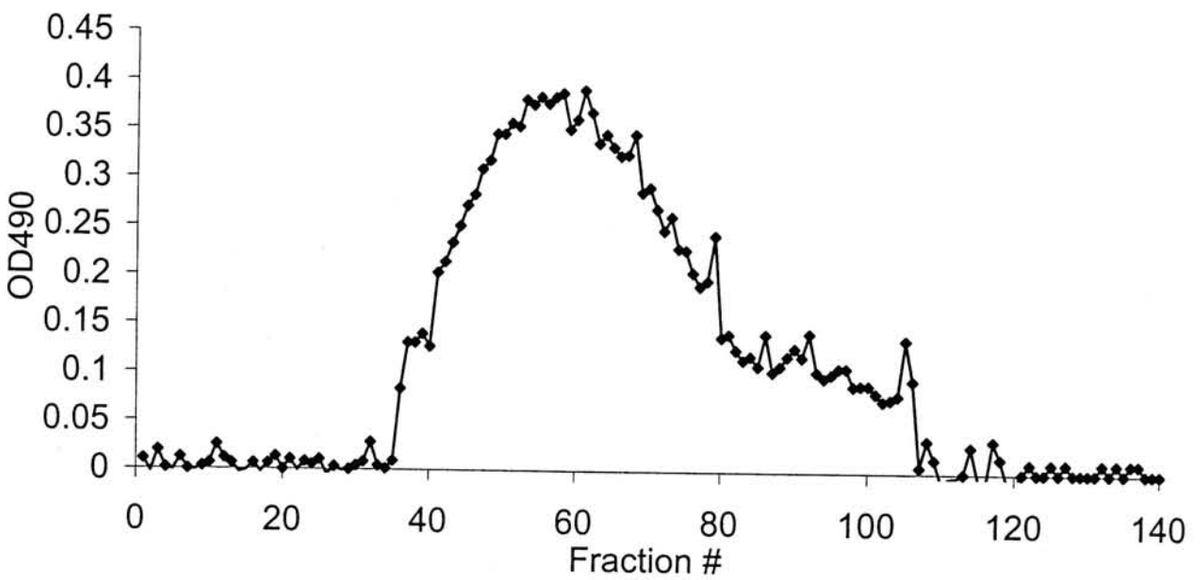


Fig. 3.5. Elution Profile of APP-B by Sepharose CL-4B size exclusion chromatography. 10 mg/ml of APP-B was prepared and 1ml of APP-B solution was added to column. The flow rate was set to 0.25 ml/min and 3 ml fraction was collected.

3.3.7 Determination of monosaccharide components of AP Polysaccharide by HPLC

A simple and sensitive high-performance liquid chromatography (HPLC)-based method for complete monosaccharide composition analysis of polysaccharides is described in section 2.2.2.6. In this method, polysaccharides are first hydrolyzed using an optimized method to give the constituent monosaccharides, which are subsequently labeled with 1-phenyl-3-methyl-5-pjyrazolone (PMP) as previously described by Honda *et al.* (Honda *et al*, 1989). The labeled monosaccharides are separated by reverse-phase HPLC.

Various monosaccharides behave differently during derivatization and hydrolysis. To adjust for these differences, both internal and external standards were used. A known amount of the internal standard, talose, was added to the samples before hydrolysis and its amount was used for monosaccharide quantification. An equal molar mixture of monosaccharides, treated under identical condition as the samples, was used as external standards to adjust for the loss of monosaccharides during acid hydrolysis and for any differences in PMP-labeling efficiency and HPLC analysis. By using both internal and external standards, accuracy of the analyses was greatly improved.

In the experiment, the set of external standards that were included mannose, glucosamine, galactosamine, glucose, galactose, xylose and fucose. Talose was the internal standard. From Fig. 3.6, all the external and internal standards were well

separated excluding galactosamine. Galactosamine was included in HPLC analysis, but it cannot be resolved in the system. Since a large excess of PMP is used for derivatization, removal of the excess reagent by extraction prior to HPLC analysis is necessary. Butyl ether gives the best result for both extraction efficiency and product recovery. However, there is still a small amount of PMP left in the reaction mixture which is represented by the minor peak 1 in Fig. 3.6.

Fig. 3.7, Fig. 3.8 and Fig. 3.9, showed that the monosaccharide composition of crude APP, APP-A and APP-B can be resolved by HPLC respectively. From these three figures, a similar profile was obtained and five monosaccharides have been resolved including the internal standard talose. Other four monosaccharides were mannose, glucose, galactose and xylose. However, there is a minor peak next to mannose and a minor peak between talose and glucose that cannot match any standard in Fig. 3.6. In Table 3.5, the predominant monosaccharide in crude APP, APP-A and APP-B was mannose; all three samples consisted of more than 65% of mannose. The amounts of monosaccharide in three samples were mannose, glucose, galactose and xylose in descending order.

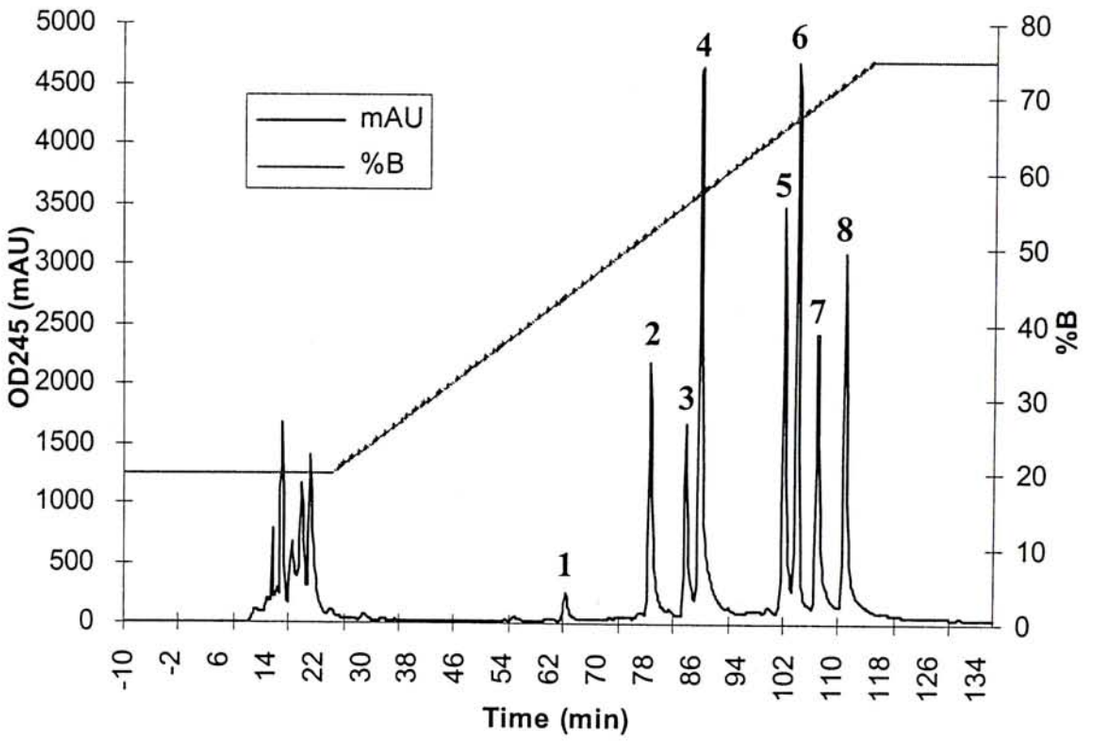


Fig. 3.6. The RP-HPLC profile of standards including mannose (2), Glucōsamine (3), internal standard talose (4), glucose (5), galactose (6), xylose (7), fucose (8). The small peak was PMP (1) left in the reaction mixture. Buffer A was 10% acetonitrile with 100 mM ammonium acetate and buffer B was 40% acetonitrile with 100 mM ammonium acetate with pH 5.5. The C-18 RP-column was equilibrate with 20% buffer B and the gradient was started from 20% buffer B to 75% buffer B within 90 min.

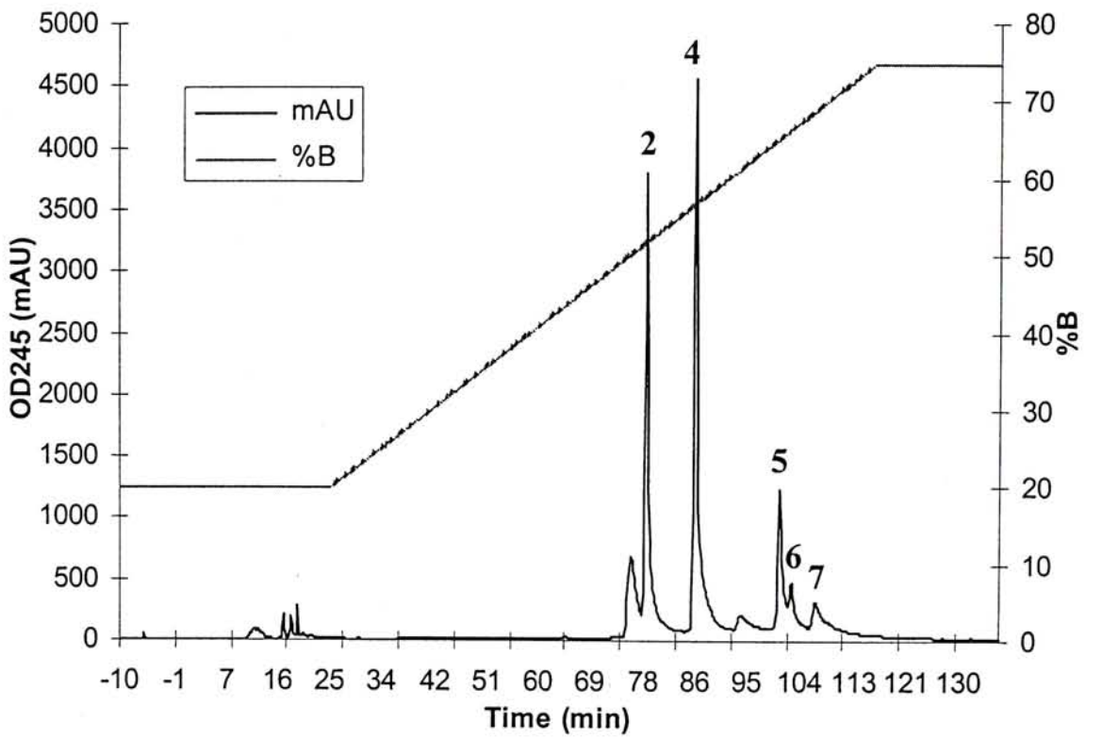


Fig. 3.7. The RP-HPLC profile of crude APP after acidic hydrolysis. Mannose (2), internal standard talose (4), glucose (5), galactose (6) and xylose (7) were resolved. Buffer A was 10% acetonitrile with 100 mM ammonium acetate and buffer B was 40% acetonitrile with 100 mM ammonium acetate with pH 5.5. The C-18 RP-column was equilibrate with 20% buffer B and the gradient was started from 20% buffer B to 75% buffer B within 90 min.

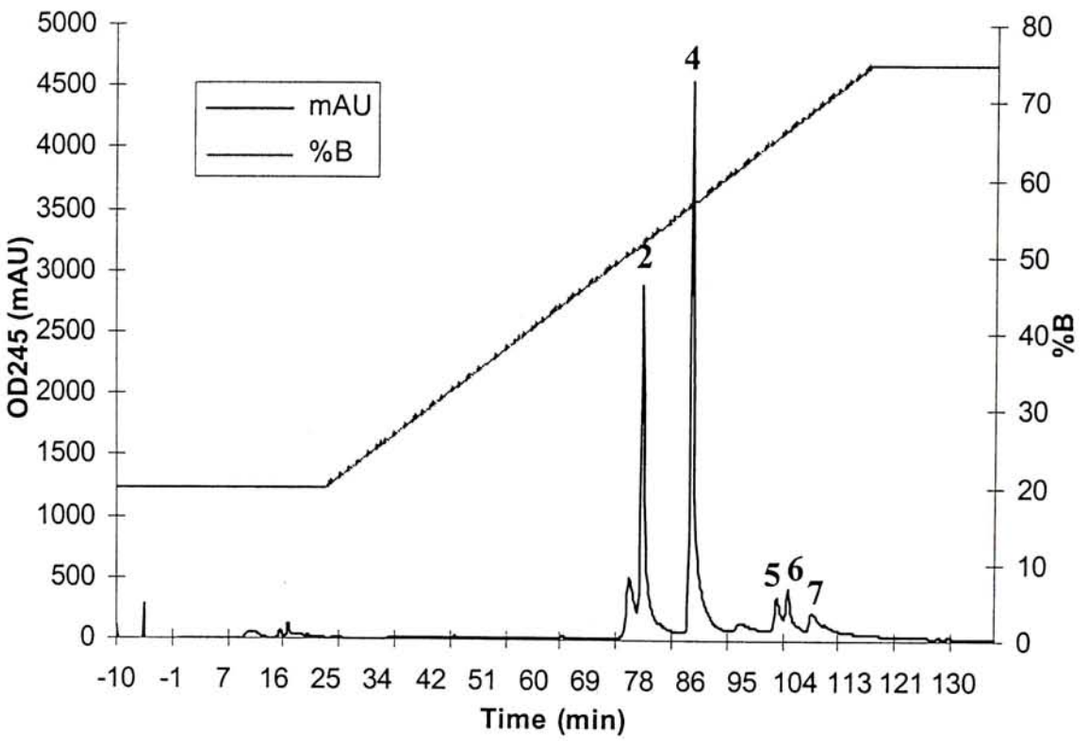


Fig. 3.8. The RP-HPLC profile of APP-A after acidic hydrolysis. Mannose (2), internal standard talose (4), glucose (5), galactose (6) and xylose (7) were resolved. Buffer A was 10% acetonitrile with 100 mM ammonium acetate and buffer B was 40% acetonitrile with 100 mM ammonium acetate with pH 5.5. The C-18 RP-column was equilibrate with 20% buffer B and the gradient was started from 20% buffer B to 75% buffer B within 90 min.

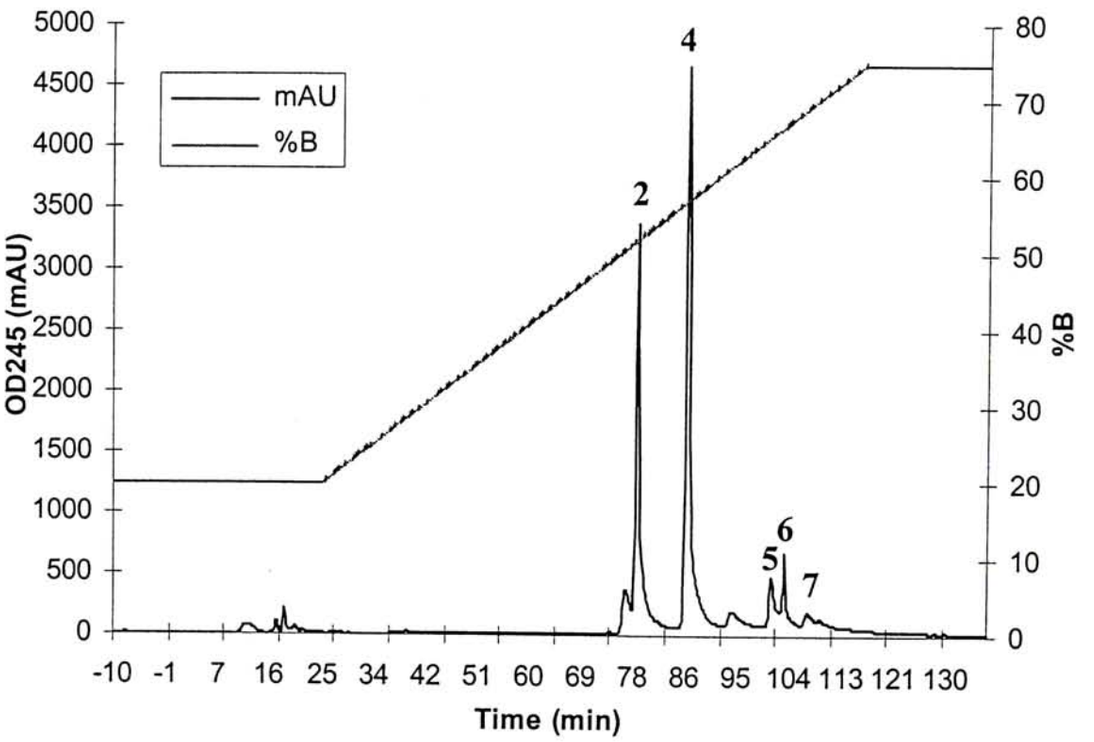


Fig. 3.9. The RP-HPLC profile of APP-B after acidic hydrolysis. Mannose (2), internal standard talose (4), glucose (5), galactose (6) and xylose (7) were resolved. Buffer A was 10% acetonitrile with 100 mM ammonium acetate and buffer B was 40% acetonitrile with 100 mM ammonium acetate with pH 5.5. The C-18 RP-column was equilibrate with 20% buffer B and the gradient was started from 20% buffer B to 75% buffer B within 90 min.

Table 3.5. Monosaccharides Composition analysis of polysaccharide obtained from *Auricularia polytricha*. The value showed the amount (nmole) of monosaccharides in 0.12 mg of crude APP, APP-A and APP-B.

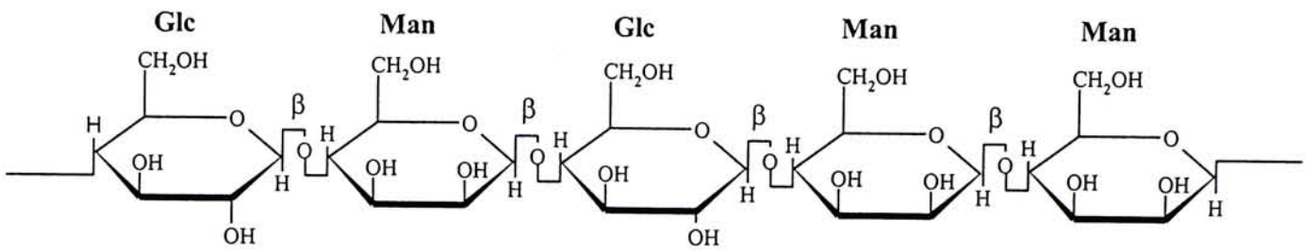
	Amount (nmole)					
Samples (0.12mg)	Man	GlcN	Glc	Gal	Xyl	Fuc
Crude APP	225.14	0.00	70.51	24.15	17.93	0.00
APP-A	197.49	0.00	18.31	24.50	27.00	0.00
APP-B	186.35	0.00	28.22	6.91	11.65	0.00

3.4 Discussion

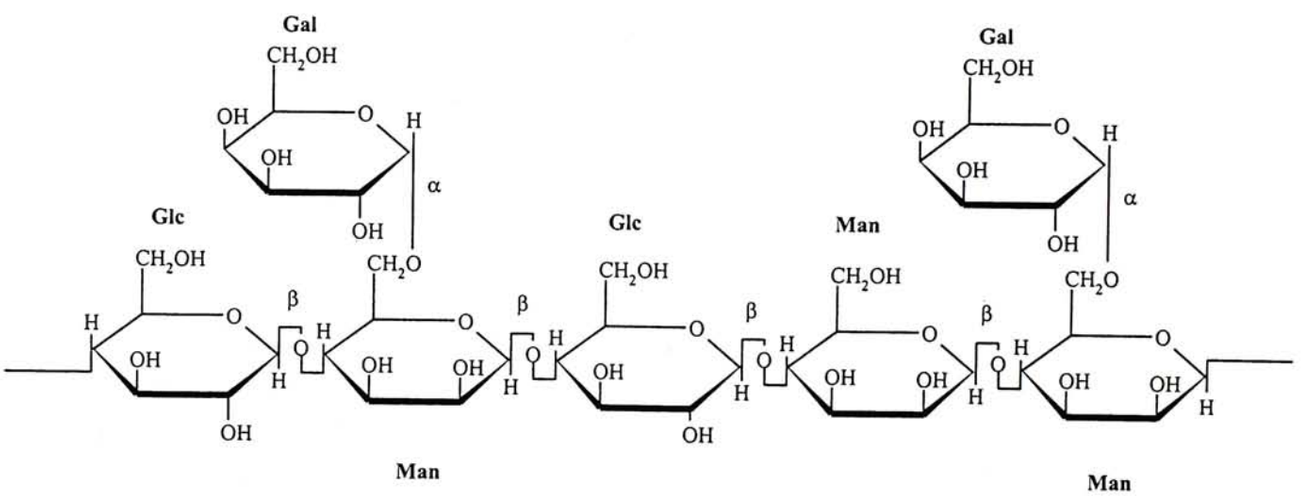
In the extraction of *Auricularia polytricha* polysaccharide, hot water was used. When a set of specified conditions was used in extraction of polysaccharide, the yield of polysaccharide extracted was higher than 20%. However, this value doesn't represent the total polysaccharide in the fungus. There is still a large amount of polysaccharide left in the residue. The carbohydrate content of *Auricularia polytricha* was more than 80% according to a previous study (PCK Cheung, 1997). For ethanol precipitation of crude *Auricularia polytricha* polysaccharide (APP), the total amount of APP-A and APP-B recovered was 92.4% and there is only a small amount of polysaccharide lost during the precipitation process. The efficiency in precipitation of polysaccharides must be due to the large molecular weight of APP. However, it is difficult to determine the molecular weight of the polysaccharide, as results showed that the peak was broad according to the CL-4B size exclusion profile. The molecular weight of APP was not larger than 5,000 K. APP was a heterogeneous mixture of various molecular weight polysaccharides. Moreover, the high viscosity of the APP solution also reflects the high molecular weight of APP. One gram per twenty milliliter of APP solution makes it difficult to flow and because it has a high water-holding capacity. However, APP is very soluble in water. The protein content of polysaccharide after ethanol precipitation decreased by 50%. It showed that some extrinsic protein was extracted together with APP. However, there is

still some intrinsic protein that is associated with the polysaccharide chain to form proteoglycans. In fact, the protein and uronic acid content of the APP was insignificant- no more than 5% of the total mass of the sample. The sugar component of the crude APP, APP-A and APP-B was determined in order to predict its structure. By thin layer chromatography, only mannose can be visualized. However, it does not imply that mannose was the only monosaccharide in APP. Therefore, a much more sensitive method (HPLC) was adopted in determining the monosaccharide composition of APP. In the determination, mannose, glucosamine, galactosamine, glucose, galactose, xylose and fucose were chosen as standards in the determination as these sugars are commonly found in fungal cell wall (Crook & Johnston, 1961). As expected, the results of the method showed that there was not only mannose in the hydrolysate of the APP. There was also glucose, galactose, xylose in the hydrolysate but with much more high percentage of mannose. The second most abundant monosaccharide component was glucose. In the HPLC analysis, the profile of crude APP, APP-A and APP-B did not differ by much. Therefore, all three samples have approximately the same monosaccharide composition. With the composition known, APP was predicted to be glucomannans, galactoglucomannans and galactomannans. Glucomannans is a polysaccharide which appears to be linear polymers with both mannose and glucose in the chain. Galactomannans are polysaccharides that have both galactose and mannose in the chain in varying proportions. Guar gum is a representative example of

galactomannans. All of them are water-soluble. The chemical structures are shown in Fig. 3.10.



Glucomannan



Galactoglucomannan

Fig. 3.10. Chemical structures of Glucomannan and Galactoglucomannan.

Chapter Four:

Protective, Therapeutic and Dose Effect of *Auricularia polytricha* Polysaccharide (APP) on Hyperlipidemia

4.1 Introduction

Water-soluble polysaccharides significantly lower serum or plasma cholesterol in humans and animals, whereas water-insoluble polysaccharides do not (Anderson & Tietyen-Clark, 1986). Soluble polysaccharides, the dietary fibers extracted from plant cell walls by hot water, include pectins, gums and some hemicelluloses. Insoluble polysaccharides include other hemicelluloses, cellulose and lignin (Southgate, 1977). Soluble polysaccharides are important components of most plant foods and comprise, on average, the following percentage of total dietary fibers for these food group: vegetable 32%, cereals 32%, beans 25%, and fruits 38% (Anderson & Bridges, 1988). Soluble polysaccharides constitute about 30% of the total dietary fiber intake for a representative Western diet (Anderson *et al*, 1989).

The mechanisms responsible for the hypocholesterolemic effects of soluble polysaccharides are not well delineated. In this chapter, the effects of water-soluble

Auricularia polytricha polysaccharide on blood cholesterol, triglyceride and liver cholesterol were reported. The first experiment (Experiment 1) is the protective effect of APP on hyperlipidemia. Hamsters were in normal status (not hyperlipidemia) and 2% of APP was added together with the high cholesterol diet in order to see whether APP can prevent elevation of body lipid. The second experiment (Experiment 2) is the therapeutic study of APP on hyperlipidemic status hamsters. Hamsters were boosted into high cholesterol status by feeding them with high cholesterol diet for four weeks. After that, hamsters were fed with high cholesterol diet with supplementation of 2% of APP for eight weeks in order to see whether APP can decrease body lipid level in pre-existing hyperlipidemia status. The third experiment is the dose-dependent response of APP on hyperlipidemia. This experiment aims to investigate the amount of APP on hypolipidemic effect and the toxicity of APP by measuring the AST and ALT enzyme activity.

Golden Syrian hamster is a common rodent widely used as an experimental animal model in studying of cholesterol metabolism (Singhal *et al*, 1983), atherosclerosis (Nistor *et al*, 1987) and hypocholesterolemic agents (Sugiyama *et al*, 1995). Similar to human, its major plasma cholesterol carrier is LDL. By using electrophoresis in agarose gel, it is found that about 50% of hamster plasma cholesterol occurs in the LDL fraction. Moreover, hamster LDL cross-reacts with antibody raised against human LDL (Nistor *et al*, 1987). Other similarities include similar response to

different diets and drugs, and manner in handling biliary sterol secretion (Bocan & Guyton, 1985; Spady & Dietschy, 1985).

The advantage in choosing hamsters is also partially due to their low rate of hepatic cholesterol synthesis. It is because increased hepatic influx of absorbed cholesterol could not be compensated in the species by down-regulation of cholesterol synthesis, but may alter hepatic cholesterol excretion (Berr *et al*, 1993). In this way, the factor from hepatic cholesterol synthesis can be negligible.

4.2 Results (Exp. 1)

4.2.1 Body Weight and Food Intake

The food intake and body weight in test group was just about 6% higher than control group. However, there was no significant difference in food intake and body weight gained among the three groups throughout the study period as showed in Table 4.2.

The present study showed that high dietary cholesterol increased the average liver weight in hamsters both in control and test (with 2% APP supplementation) group, as compared with that from the normal groups as shown in Table 4.1. However, there was no significant difference in average liver weight between test group and control group. Therefore, the increment of average liver weight was due to the high cholesterol and high fat diets.

4.2.2 Effect of APP Supplementation on Hepatic Cholesterol

In the protective study, the control group was fed with high cholesterol and fat diets while the test group was fed with the same diet with supplementation of 2% APP. This experiment was aimed to see whether APP supplementation could prevent rising of

body cholesterol level. The result showed that the hepatic cholesterol of the test group was significantly lower than control group. Liver cholesterol was decreased by 29.03% in test group when compared with control group as shown in Fig. 4.1 and a typical gas-liquid chromatogram of tissue cholesterol was shown in Fig. 4.2. However, the hepatic cholesterol level in both test and control group were significantly higher than the normal group. Therefore, APP supplemented diet can decrease hepatic cholesterol but not reduce it to normal levels.

4.2.3 Effect of APP Supplementation on Plasma TC, HDL-C and TG

Fasting plasma total cholesterol was significantly decreased by 12.7% ($P<0.05$) in hamsters fed with 2% APP-supplemented diet (test group) for 4 weeks, as compared with control group (Fig. 4.3). The plasma HDL-C, in the test group was also significantly decreased by 10.3% ($P<0.01$) as compared with the control group (Fig. 4.3). However, there was no significant difference in plasma TG between control and test group (Fig. 4.4). Moreover, plasma TC, HDL-C and TG of test and control group were significantly higher than that of normal group.

Table 4.1. Effect of APP on the average liver weight of six experiments. Liver weight in the 4th row was the average of experiments 4 and 5. The diets based on the high cholesterol diet (C) with the supplementation of 1% APP (1%), 2% APP (2%), 4% APP (4%), 1% sodium acetate (Ac), 1% sodium propionate (Pr), 1% sodium butyrate (Bu) and 0.5% sodium acetate plus 0.5% sodium propionate (Ac,Pr). The normal diet (N) contains no cholesterol and was provided by Laboratory Animal Service Center (LASC). For experiment 2, the first data was the liver weight at week 8 while the latter one was the one at week 12.

Average Liver Weight (g)

Exp.	N	1%	2%	4%	C	Ac	Pr	Bu	Ac,Pr
1	3.96	/	4.96	/	4.65	/	/	/	/
2	4.13/ 4.80	/	5.16/ 6.07	/	4.82/ 6.14	/	/	/	/
3	3.85	4.60	/	4.10	4.67	/	/	/	/
4,5	3.99	/	6.03	/	4.69	4.72	3.88	4.67	4.51
6	75.47	/	112.35	/	127.23	/	/	/	/

Table 4.2. Effect of APP on body weight and food intake of hamsters. Male hamsters were fed with normal diet, test diet (with 1%, 2% or 4% APP supplementation) and control diet. The experimental period for experiment 1 and 3 were four weeks. For experiment 2, all three groups of hamsters were fed with control diet (high cholesterol diet) for 4 weeks. Then, the hamsters were divided into normal, test and control group and fed with different diet for eight weeks more. The first data of final body weight was corresponding to week 8 and the second was corresponding to week 12.

	Experiment 1		
	Normal	Test (2%)	Control
Initial body weight (g)	101.60 ± 6.87	105.90 ± 7.07	107.04 ± 3.39
Final body weight (g)	154.34 ± 8.49	155.87 ± 9.58	147.44 ± 7.30
Food intake (g/day)	7.34 ± 0.56	8.03 ± 0.63	7.53 ± 0.53

	Experiment 2		
	Normal	Test (2%)	Control
Initial body weight (g)	102.60 ± 6.07	106.85 ± 2.70	106.52 ± 7.77
Final body weight (g)	165.12 ± 13.21/ 166.35 ± 12.32	179.90 ± 12.35/ 181.44 ± 14.36	166.51 ± 16.72 / 163.12 ± 20.05
Food intake (g/day)	7.21 ± 0.43	7.74 ± 1.11	7.37 ± 0.81

	Experiment 3			
	Normal	Control	Test (1%)	Test (4%)
Initial body weight (g)	102.60 ± 6.45	97.10 ± 7.47	102.32 ± 6.25	100.54 ± 5.91
Final body weight (g)	150 ± 5.23	150.12 ± 5.68	152.15 ± 6.35	161.80 ± 4.17
Food intake (g/day)	7.30 ± 0.55	7.02 ± 0.57	7.25 ± 0.46	7.57 ± 0.57

* Data are expressed as means ± SD.

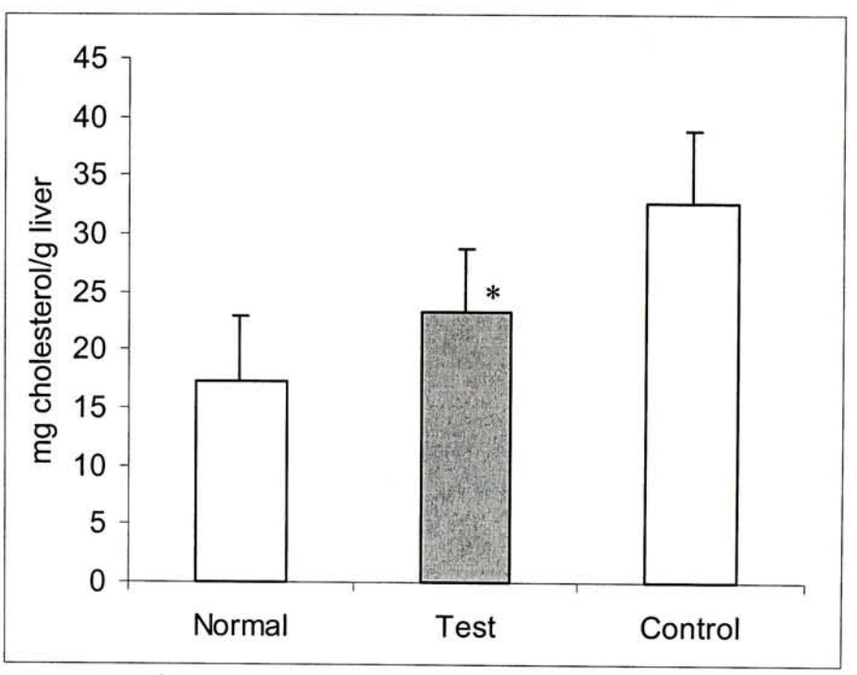


Fig. 4.1. Effect of APP on the liver cholesterol level. Male hamsters were fed with normal diet, test diet (with 2% APP supplementation) and control diet. After 4 weeks, liver cholesterol content was analyzed by GLC.
(* $P<0.05$)

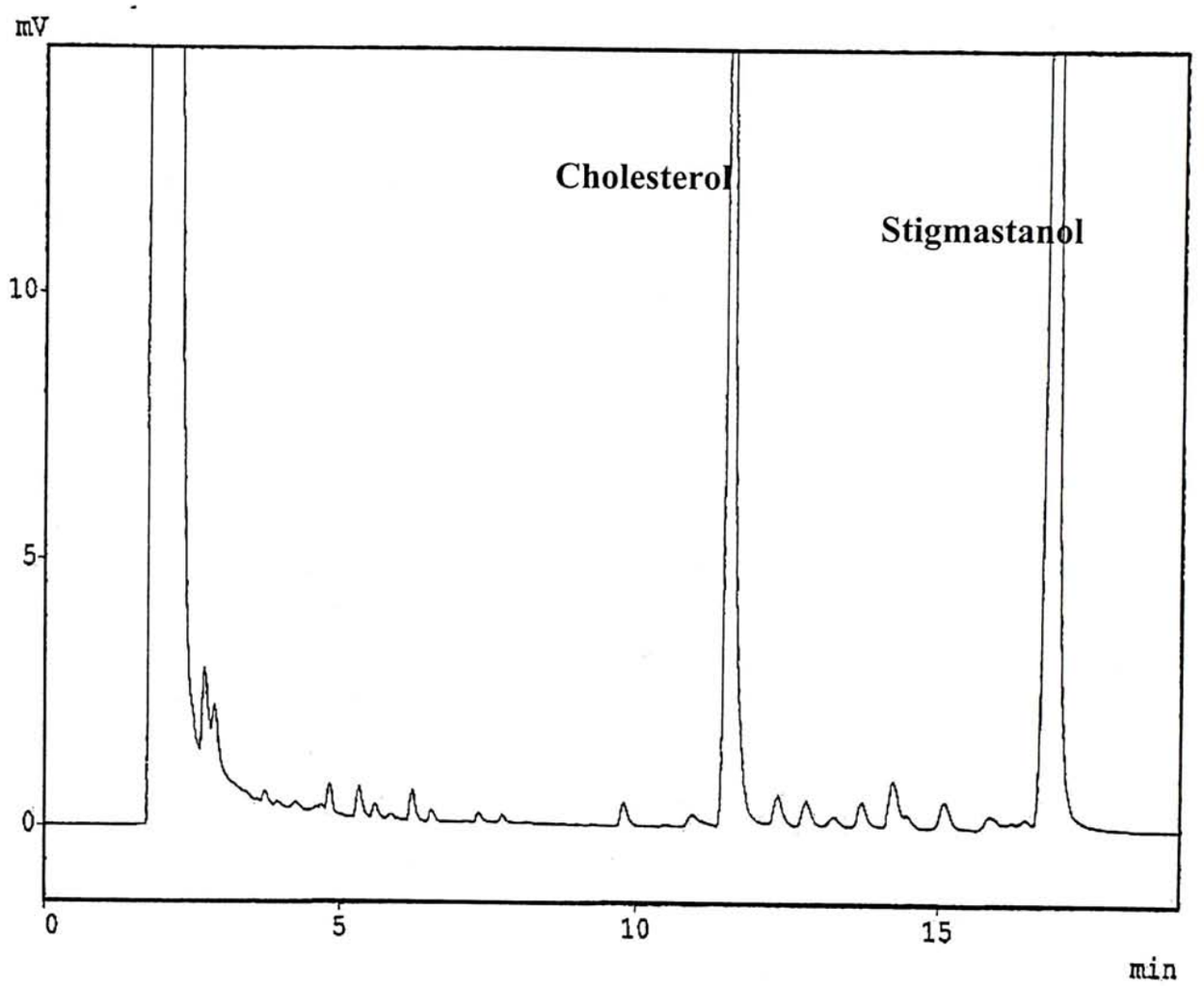


Fig. 4.2. Gas-liquid chromatographic profile of hepatic cholesterol and internal standard (stigmastanol).

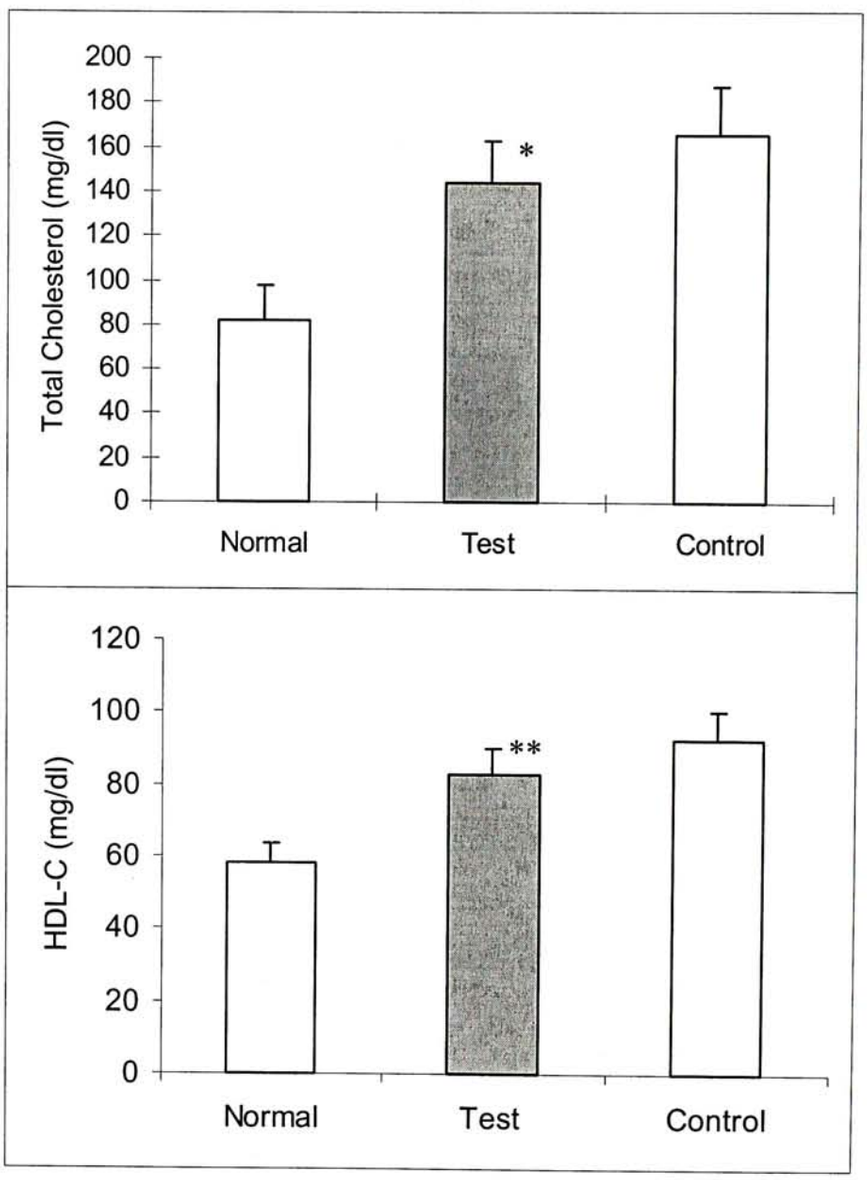


Fig. 4.3. Effect of APP on the plasma total cholesterol and HDL-cholesterol level. Male hamsters were fed with normal diet, test diet (with 2% APP supplementation) and control diet. After 4 weeks, plasma total cholesterol and HDL-cholesterol were measured by enzymatic diagnostic kit. (* $P<0.05$) (** $P<0.01$)

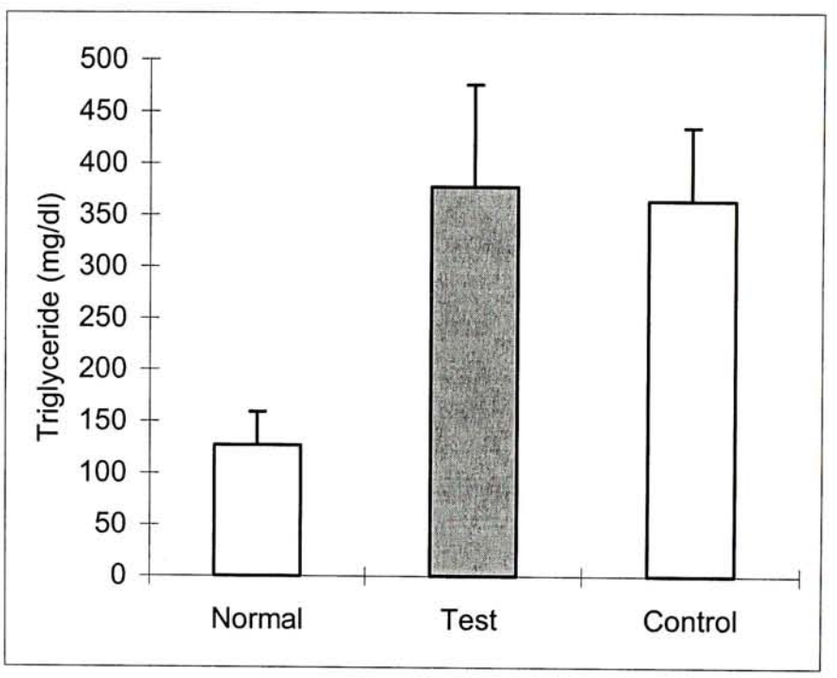


Fig. 4.4. Effect of APP on the plasma triglyceride level. Male hamsters were fed with normal diet, test diet (with 2% APP supplementation) and control diet. After 4 weeks, plasma triglyceride content was measured by enzymatic diagnostic kit.

4.2.4 Effect of APP Supplementation on Fecal Output of Neutral Sterols

The neutral sterols included coprostanol, coprostanone, cholesterol, dihydrocholesterol, campersterol, stigmasterol (internal standard), β-sitosterol and stigmastanol were separated and quantified by GLC (Fig. 4.5).

From Fig. 4.5.1, the fecal neutral sterol at week 1 is much higher than at week 2,3 and 4. The percentage increase in fecal neutral sterol was 4.33% and 11.91% at week 2 and 3 respectively. However, there is no difference between the test and control groups at week 4.

4.2.5 Effect of APP Supplementation on Fecal Output of Acidic Sterols

The acidic sterols included lithocholic acid, deoxycholic acid, cholic acid, hyodeoxycholic acid (internal standard) and ursodeoxycholic acid were resolved and quantified by GLC (Fig. 4.6).

In this study, from Fig. 4.6.1, the fecal acidic sterol at week 1 is also much higher than at week 2, 3 and 4. The percentage increase in fecal acidic sterol was 38.77%, 42.17% and 46.52% at week 2,3 and 4 respectively.

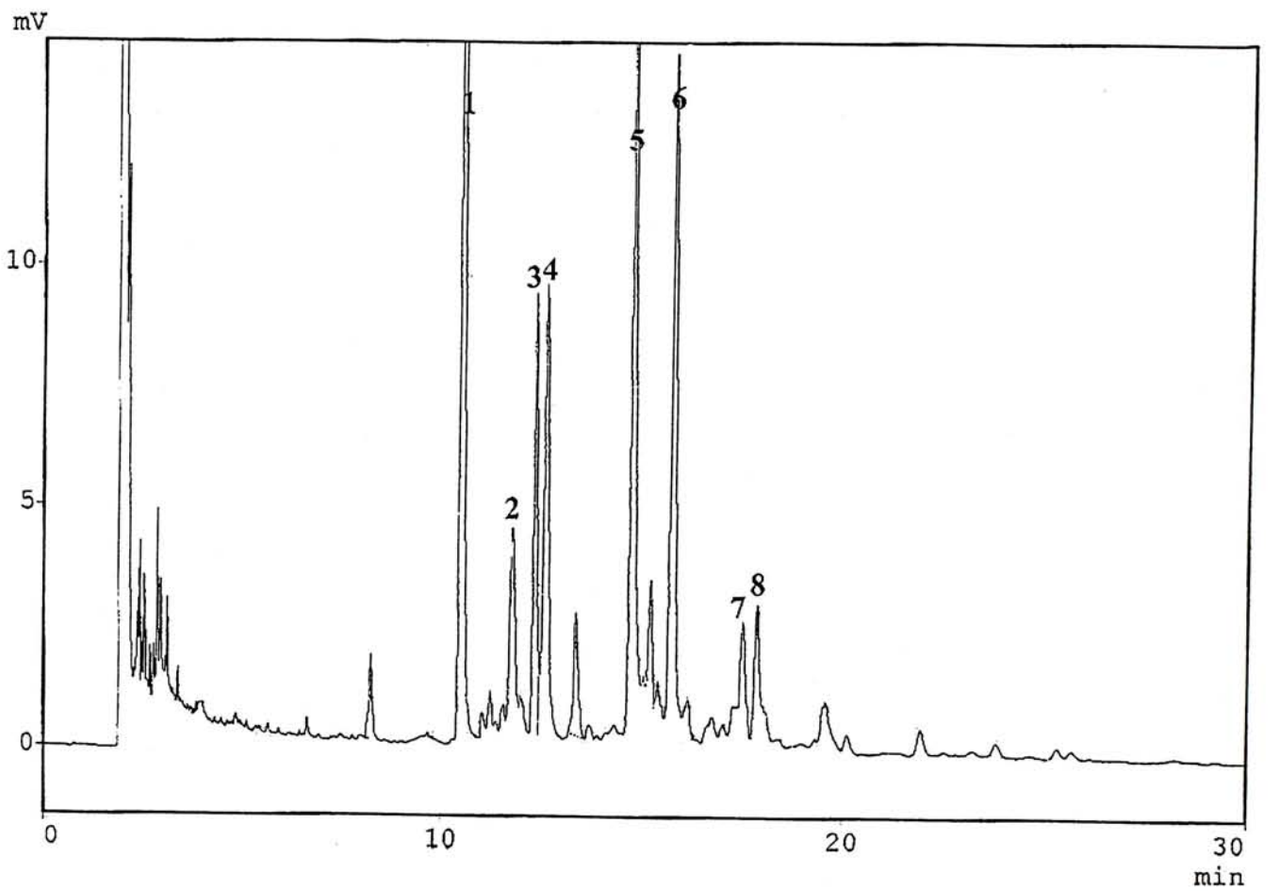


Fig. 4.5. Gas liquid chromatographic profile of fecal neutral sterols. Identification of peaks: 1- coprostanol, 2- coprostanone, 3- cholesterol, 4- dihydrocholesterol, 5- campersterol, 6- stigmasterol (internal standard), 7- β-sitosterol, 8- stigmastanol. See section 2.2.6 for the conditions of separation.

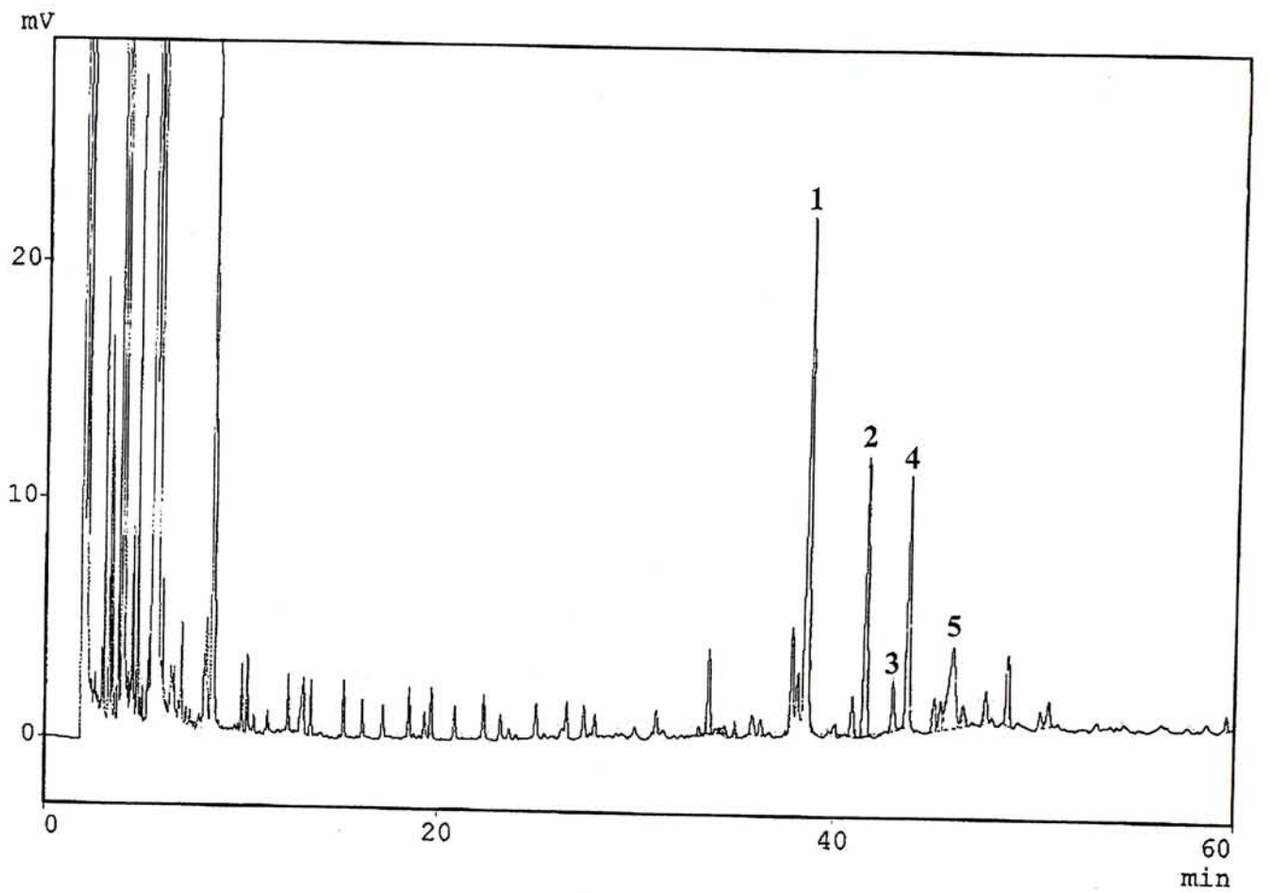


Fig. 4.6. Gas liquid chromatographic profile of fecal acidic sterols. Identification of peaks: 1- lithocholic acid, 2- deoxycholic acid, 3- cholic acid, 4- hyodeoxycholic acid (internal standard), 5- ursodeoxycholic acid. See section 2.2.6 for the conditions of separation.

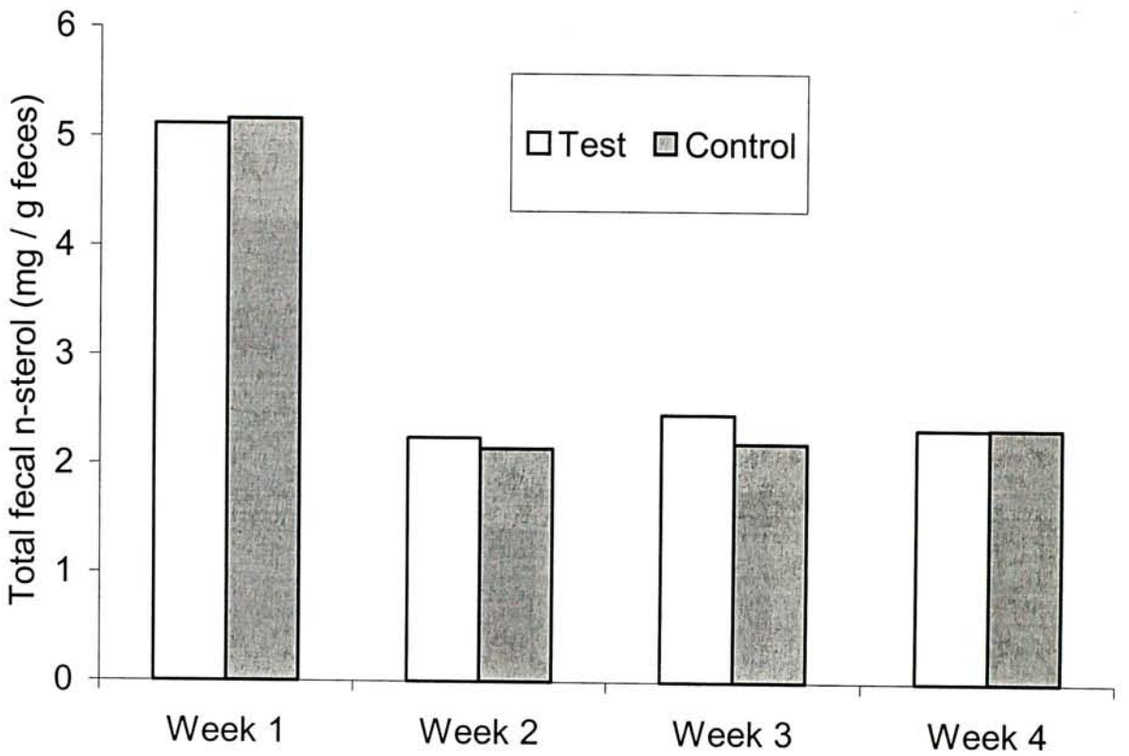


Fig. 4.5.1. Total fecal neutral sterol in the control and test group. Test group diet was supplemented with 2% APP.

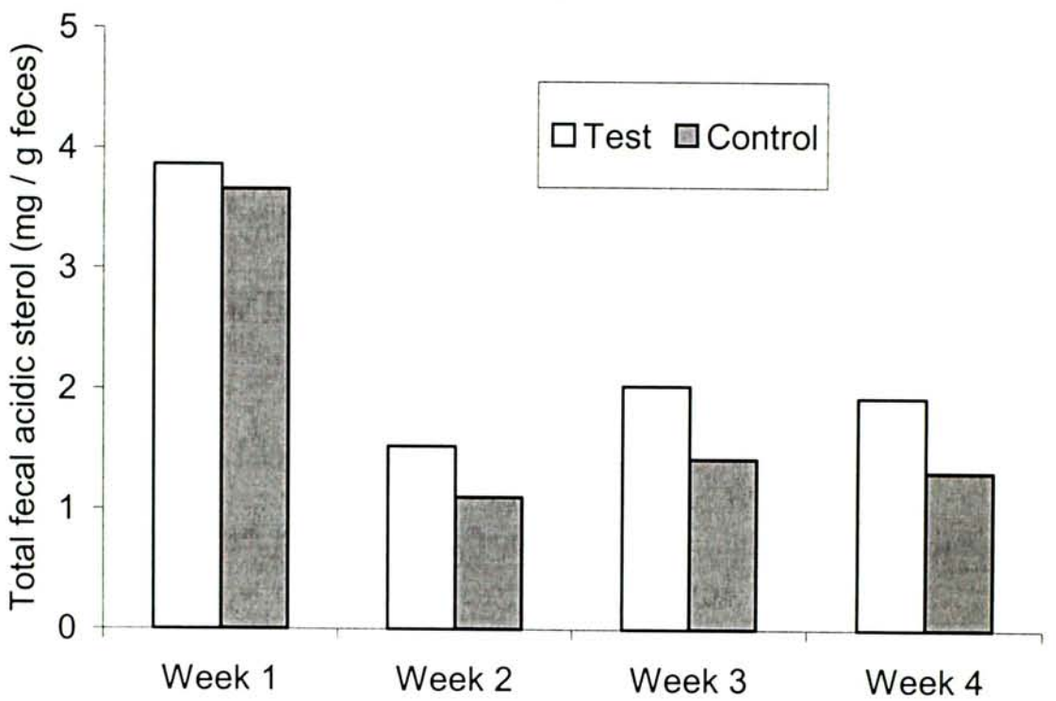


Fig. 4.6.1. Total fecal acidic sterol in the control and test group. Test group diet was supplemented with 2% APP.

4.3 Discussion (Exp. 1)

From the results, there was no significant difference in food intake and final body weight have no significant difference among the three groups. However, high dietary cholesterol and fat diets in both test and control group increased the average liver weight significantly as compared with the control group.

There are several ways that hepatic cholesterol can be increased. Hepatic cholesterol can be increased by an increase in receptors for low-density lipoprotein, LDL, on the surface of the hepatocytes, by increasing the high-density lipoprotein cholesterol, HDL-C, in blood as it can mediates reverse transport of cholesterol, the process by which cholesterol is removed from sites of deposition in the extra-hepatic tissues and is delivered back to the liver (Renn, 1997). Recent studies also showed that absorption of dietary cholesterol increase linearly with an increased intake of dietary cholesterol. Bile acid excreted into intestine can be reabsorbed and carried back to liver by albumin for cholesterol synthesis (Sehayek *et al*, 1998). Finally, de novo hepatic synthesis of fresh cholesterol can be initiated via increased activity of HMG-CoA Reductase, the key enzyme regulating the biosynthesis of cholesterol. However, there is only one pathway by which hepatic cholesterol can be removed away from liver. Hepatic cholesterol can be oxidized into bile acid by cholesterol-7α-hydroxylase (Souidi *et al*, 1998) and excreted as bile into intestine, then out of the body.

Both test and control groups showed a significant increase in hepatic cholesterol as compared to the normal group. This was due to the high cholesterol and fat diets in both groups as absorption of dietary cholesterol increased linearly with an increased intake of dietary cholesterol. However, the test group showed about 30% decrease in liver cholesterol as compared to the control group. Moreover, the plasma total cholesterol and high-density lipoprotein cholesterol level of the test group showed a significant decrease of more than 10% when compared with the control group. However, there was no significant difference in plasma triglyceride level between the test group and control group.

The present results showed that dietary APP supplementation generally increased the fecal excretion of neutral sterols. However, the difference is small. The different neutral sterols identified in feces were possibly the products of the bacterial modification of cholesterol in gut (Kellogg, 1974). These fecal neutral sterols are capable to diminish the intestinal cholesterol absorption, by which they lower the capacity of micelles and displace cholesterol from micellar binding during absorption (Ikeda & Sugano, 1983; Heinemann *et al*, 1991).

Three bile acids make up the majority of the total bile acids present in bile: cholic acid 40%, chenodeoxycholic acid 40% and deoxycholic acid 20% (Hofmann, 1976). Small amounts of ursodeoxycholic acid and lithocholic acid are also present (Einarsson & Angelin, 1986). The GLC profile showed that there is large amount of lithocholic acid. Lithocholic acid arises via bacterial dehydroxylation of

chenodeoxycholic acid, predominantly, in the colon; and is absorbed by passive mechanisms to enter the portal circulation for transport back to liver. Therefore, it is predicted that the bacterial action is huge at colon. The results showed that dietary APP supplementation increased the fecal excretion of acidic sterols (Gallaher & Schneeman, 1986). However, the mechanism was not clear.

Auricularia polytricha polysaccharide showed a marked hypocholesterolemic effect in plasma and liver in spit of its high cholesterol level as compared to the normal group. Therefore, APP can more or less protect the body from hypercholesterolemia.

4.4 Results (Exp. 2)

4.4.1 Body Weight and Food Intake

For the hamsters at week 8 and 12, there is no significant difference between the control and normal groups in food intake and body weight. For test group, the food intake was 5% more than control group with body weight 11% heavier than control group as shown in Table 4.2.

This study also showed that high dietary cholesterol increased the average liver weight in hamsters both in control and test (with 2% APP supplementation) group, as compared to the normal groups as shown in Table 4.1. Both test and control groups have an average liver weight of more than 15% and 20% higher than normal group at week 8 and 12 respectively. However, there was no significant difference in average liver weight between the test group and control group. Therefore, the increment of average liver weight was also due to the high cholesterol and high fat diets.

4.4.2 Effect of APP Supplementation on Hepatic Cholesterol

In the therapeutic study, hamsters were fed with high cholesterol diet for four weeks in order to boost it into hyperlipidemic status. After that, test group was fed with

high cholesterol diet with 2% APP supplementation for another eight weeks while control group was still fed with high cholesterol diet. This experiment was aimed to see whether APP supplementation could decrease cholesterol level in hamster consequently.

From the results, liver cholesterol was decreased by 14.3% ($P<0.05$) and 21% ($P<0.01$) in the test group at week 8 and 12 respectively, as compared with control group. However, both test and control groups have much higher liver cholesterol level than the normal group at both week 8 and 12.

4.4.3 Effect of APP Supplementation on Plasma TC and TG

Fasting plasma TC was significantly decreased by 19.3% ($P<0.05$) and 26.3% ($P<0.02$) in the test group at week 8 and 12 respectively, as compared with control group. However, TC in test and control were significantly higher than normal group at both week 8 and 12. The plasma TG level of the test group was higher than the control group at week 8 and lower than control group at week 12 but it was not statistically significant (Fig. 4.6).

4.4.4 Effect of APP Supplementation on Plasma HDL-C and LDL-C

Fasting plasma HDL-C was significantly decreased by 17.1% ($P<0.02$) and 20% ($P<0.05$) in the test group at week 8 and 12 respectively, as compared with control group. However, HDL-C in test and control were significantly higher than normal group at both week 8 and 12. Fasting plasma LDL-C was significantly decreased by 21.41% ($P<0.02$) and 47.1% ($P<0.01$) in test group at week 8 and 12 respectively, as compared with control group. However, HDL-C in test and control were significantly much higher than normal group at both week 8 and 12 (Fig. 4.7).

Table 4.3 shows the LDL/HDL ratio; the test group has a LDL/HDL ratio of 5.18% and 33.93% lower than the control group at week 8 and 12 respectively. However, the test group has a ratio of 8.75% and 18.16% higher than normal group at week 8 and 12 respectively.

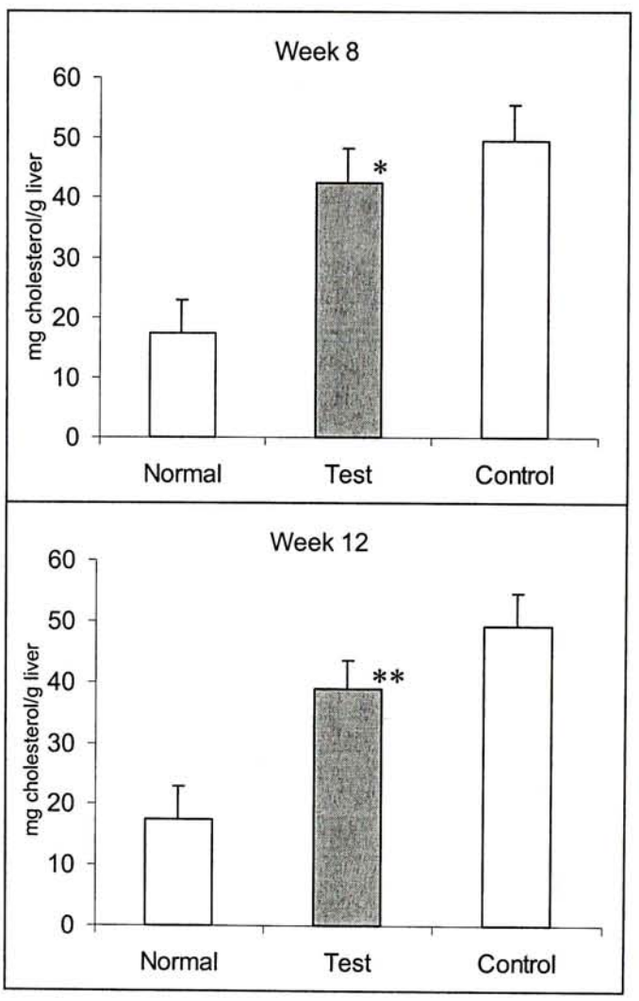


Fig. 4.7. Effect of APP on the liver cholesterol level. All male hamsters were fed with control diet (high cholesterol diet) for four weeks in order to push the hamsters into hyperlipidemic status. Then, hamsters were divided into three groups fed with normal diet, test diet (with 2% APP supplementation) and control diet respectively. After eight and twelve weeks, the liver cholesterol content was determined by GLC. (* $P<0.05$) (** $P<0.01$)

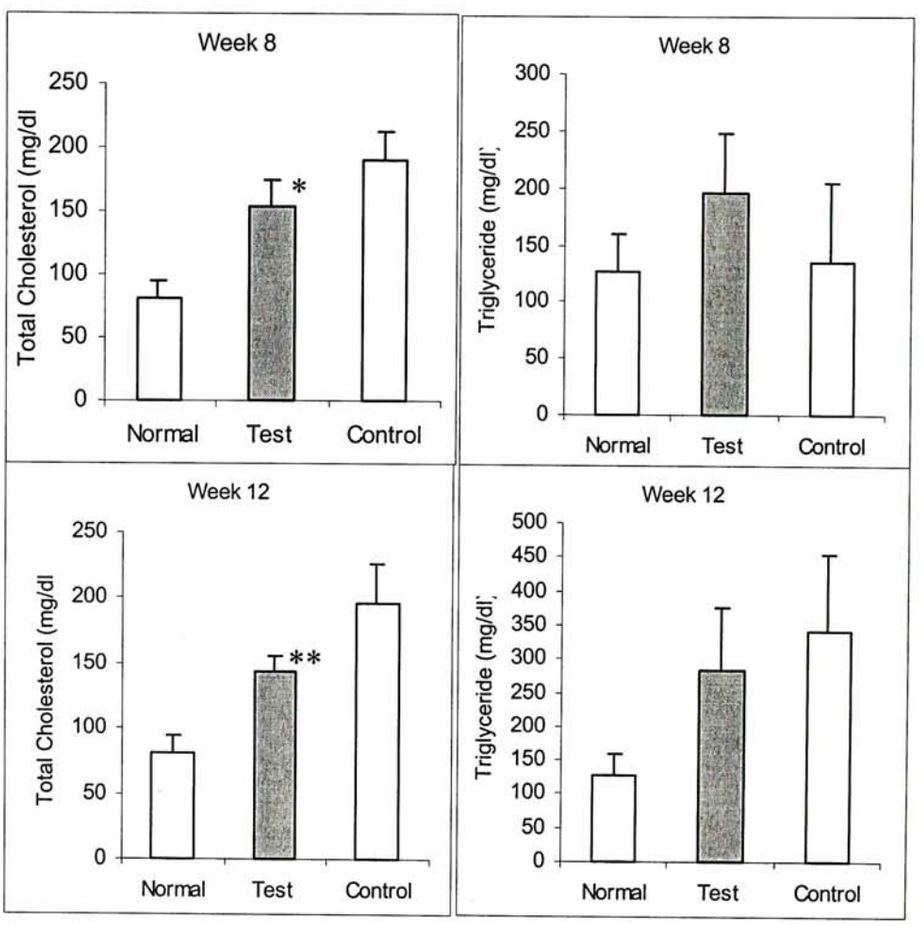


Fig. 4.8. Effect of APP on plasma total cholesterol and triglyceride level. All male hamsters were fed with control diet (high cholesterol diet) for four weeks in order to boost the hamsters into hyperlipidemic status. Then, hamsters were divided into three groups fed with normal diet, test diet (with 2% APP supplementation) and control diet respectively. After eight and twelve weeks, the plasma cholesterol content was determined by enzymatic diagnostic kit. (* $P<0.02$) (** $P<0.05$)

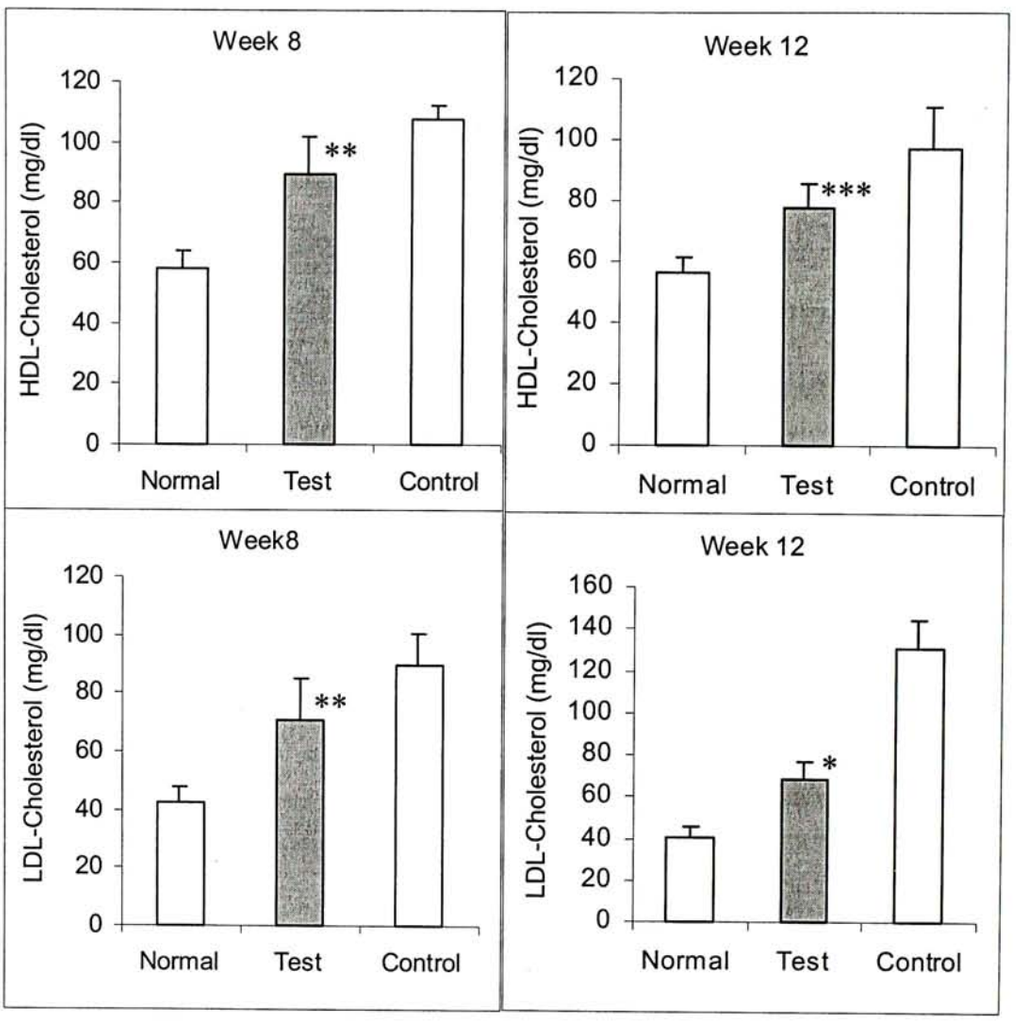


Fig. 4.9. Effect of APP on plasma HDL-cholesterol and LDL-cholesterol level. All male hamsters were fed with control diet (high cholesterol diet) for four weeks in order to push the hamsters into hyperlipidemic status. Then, hamsters were divided into three groups fed with normal diet, test diet (with 2% APP supplementation) and control diet respectively. After eight and twelve weeks, the plasma HDL- and LDL-cholesterol content was determined by enzymatic diagnostic kit. (* $P<0.01$) (** $P<0.02$) (***$P<0.05$)

Table 4.3. Effect of APP on the LDL/HDL Cholesterol Ratio. For experiment 2, all male hamsters were fed with control diet (high cholesterol diet) for four weeks in order to push the hamsters into hyperlipidemic status. Then, hamsters were divided into three groups fed with normal diet, test diet (with 2% APP supplementation) and control diet respectively. For experiment 3, male hamsters were fed with normal diet, control diet (high cholesterol diet), test diet (with 1% and 4% APP supplementation).

	LDL/HDL-Cholesterol Ratio		
Experiment 2	Normal	Test (2%)	Control
Week 8	0.7254	0.7889	0.8320
Week 12	0.7532	0.8900	1.347

	LDL/HDL-Cholesterol Ratio			
Experiment 3	Normal	Control	Test (1%)	Test (4%)
Week 4	1.1328	1.8771	1.2177	1.1000

4.5 Discussion (Exp. 2)

In experiment 1, the protective effect of APP was studied. The result showed that APP could prevent hypercholesterolemia in spit of high cholesterol level as compared with normal hamsters. In this experiment, the therapeutic effect of APP was studied. The aim was to see whether APP could lower cholesterol level in a pre-existing hypercholesterolemic state. The therapeutic effect of APP was tested at week 8 and week 12 in order to see whether it has progressive improvement on hypercholesterolemia (Hunninghake *et al*, 1994; Jensen *et al*, 1997).

The interrelations among diet-related risk factors and coronary heart diseases (CHD), particularly that between diet and plasma lipoproteins, were important for our studies. Cholesterol is transported in plasma by lipoprotein. The majority of plasma cholesterol is in low-density lipoprotein (LDL); hence elevations in total cholesterol reflect elevations in LDL. LDL appears to be atherogenic, because a high level of this lipoprotein is a significant risk factor for coronary heart disease (CHD) (Wilson *et al*, 1980). The remaining cholesterol in plasma in the fasting state is present in HDL. There is a clear inverse relation between HDL concentration and risk for CHD, such that HDL appears to be antiatherogenic (Abbott *et al*, 1988). Thus, high levels of total, and particularly LDL, cholesterol increase CHD risk; low levels of HDL may do so further. Therefore, reduction of LDL level is associated with reduced CHD risk (, 1984), slowing of atherosclerotic progression (Blankenhorn *et al*, 1987; Cashin-Hemphill *et al*, 1990;

Brown *et al*, 1990; Kane *et al*, 1990), and even, in some recent coronary angiographic studies, regression of established atherosclerotic lesions (Brown *et al*, 1990; Kane *et al*, 1990). Increases in HDL level may also be associated with reduced CHD risk (Gordon *et al*, 1989). Therefore, the ratio of LDL to HDL is a better reflection of CHD. The lower ratio gives a lower risk of CHD.

From the results, there are progressive decreases in hepatic cholesterol, total cholesterol, LDL-C and HDL-C. Moreover, LDL/HDL ratio in test group was significantly decreased by 5.18% and 33.93% at week 8 and 12 respectively as compared with control group. Therefore, APP has a positive effect on therapeutic response to hypercholesterolemia. Also, APP has an antiatherosclerotic effect on hypercholesterolemic body as it can lower LDL/HDL ratio significantly. The antiatherosclerotic effect of APP can be confirmed by regression of established atherosclerotic lesions. However, with the constraint of the size of aorta of hamster, the lesions were difficult for visualization. This methodology was performed in later experiment using rabbit as animal model.

4.6 Results (Exp. 3)

4.6.1 Body Weight and Food Intake

There is only a small difference in food intake and body weight among test group (both 1% and 4% APP supplementation), control and normal group. For test group, the food intake was slightly more than control group with a larger difference for test group with 4% APP supplementation. Moreover, increased food intake resulted in increased body weight as shown in Table 4.2.

In this experiment, the average liver weight of the test group and control was significantly increased as compared with the normal group. The test group had a lower average liver weight than control group. The results showed that increased percentage of APP has a lower average liver weight (Table 4.1).

4.6.2 Dose Response of APP Supplementation on Hepatic Cholesterol

In dose-dependent response study, hamsters were fed with high cholesterol diets with supplementation of 1% APP (low) and 4% APP (high dose) for four weeks.

From the results, liver cholesterol of the test and control groups were significantly higher than normal group (> 3 folds). However, the test group had lower

hepatic cholesterol than the control group. The liver cholesterol content was decreased by 16.44% and 41.83% significantly ($P<0.01$) in test group with 1% and 4% APP supplementation respectively (Fig. 4.10).

4.6.3 Dose Response of APP Supplementation on Plasma TG

From Fig. 4.11, fasting plasma TG of the test group and control group were significantly higher than normal group. However, the test group had lower TG than the control group. The plasma TG content was decreased by 15.75% and 10.41% significantly ($P<0.01$) in test group with 1% and 4% APP supplementation respectively.

4.6.4 Dose Response of APP Supplementation on Plasma HDL-C and LDL-C

From Fig. 4.12, fasting plasma HDL- and LDL-cholesterol of the control was significantly higher than the test group and normal group ($P<0.05$). However, the level of this two parameter has no significant difference between test group and normal group in this experiment. The plasma LDL-C content was decreased by 18.51% and 23.73% significantly ($P<0.05$) in test group with 1% and 4% APP supplementation respectively.

Moreover, the plasma HDL-C content was decreased by 27.7% and 30.15% significantly ($P<0.05$) in test group with 1% and 4% APP supplementation respectively.

Table 4.3 shows the LDL/HDL ratio, test group has a LDL/HDL ratio of 35.13% and 41.4% lower than control group with 1% and 4% APP supplementation respectively. Test group with 1% APP supplementation had a LDL/HDL ratio of 7.49% higher than normal group. However, there was no significant difference between test group with 4% APP supplementation and control group.

4.6.5 Dose Response of APP Supplementation on ALT and AST Activity

From Fig. 4.13, the results showed that plasma ALT activity has no significant difference among normal, control and test group. For AST activity, the level of normal group was lower than test and control for a less extent. However, there was no significant difference between test and control groups.

4.6.6 Dose Response of APP Supplementation on Fecal Output of Neutral and Acidic Sterols

From Fig. 4.14, the results showed that the fecal neutral sterol output at week 1 is much higher than at week 2, 3 and 4. The difference of fecal neutral sterol excretion is

small between control and test group (both 1% and 4% APP supplementation). In general, the neutral sterol in test group is higher than control group. However, we cannot draw a conclusion whether increase the percentage of APP can increase the amount of neutral sterol excretion.

From Fig. 4.15, the results showed that the fecal acidic sterol output at week 1 is also much higher than at week 2, 3 and 4. There is difference in fecal acidic sterol excretion among control and test groups (both test group) (Favier *et al*, 1997). Supplementation with APP always has higher fecal acidic sterol excretion. However, we have no conclusive pattern whether increase the percentage of APP can increase the amount of neutral sterol excretion. At week 2 and 4, test group with 4% APP supplementation have higher acidic sterol excretion than 1% of APP. However, at week 3, 1% APP supplementation has higher acidic sterol excretion than 4% APP.

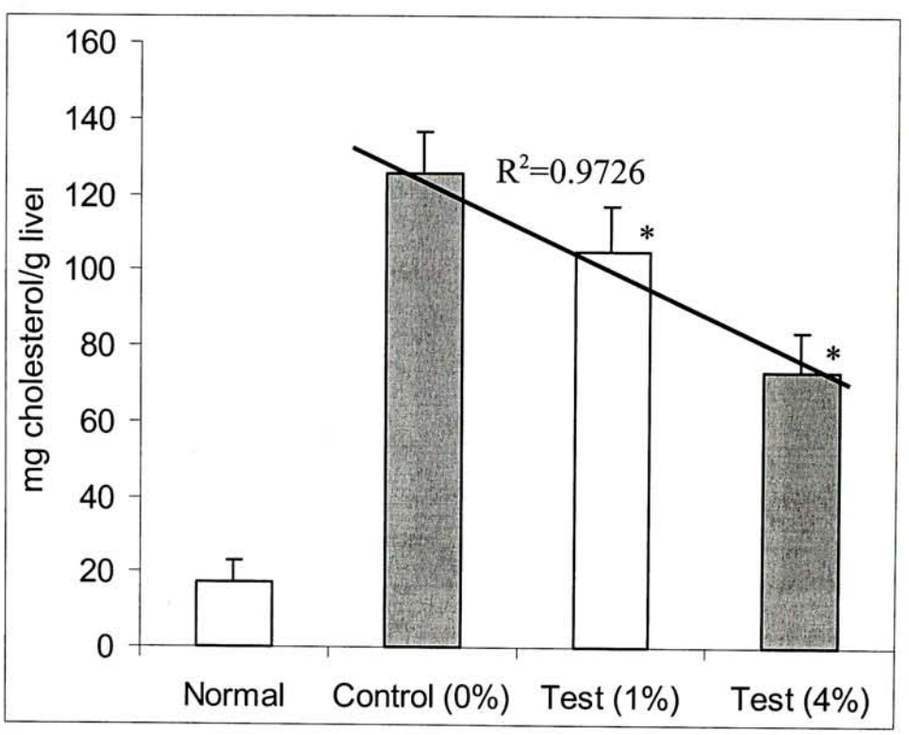


Fig. 4.10. Dose-dependent response of APP on hepatic cholesterol level. All male hamsters were fed with normal diet, control diet and test diet (with 1% and 4% APP supplementation). After four weeks, the liver cholesterol content was analyzed by GLC. (*P<0.01)

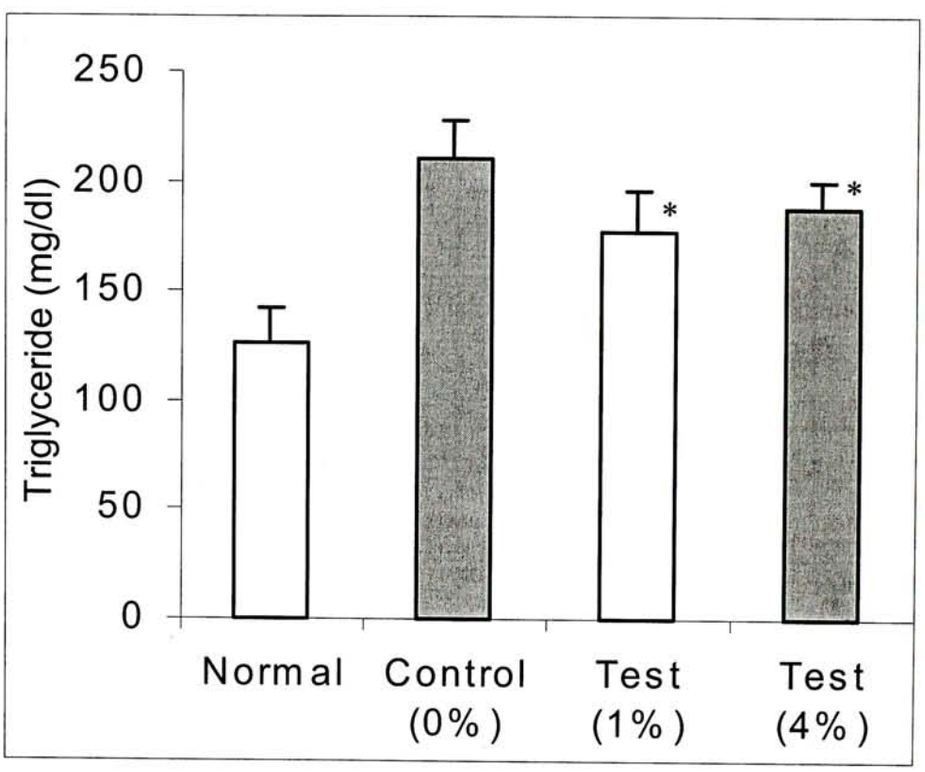


Fig. 4.11. Dose-dependent response of APP on plasma triglyceride. All male hamsters were fed with normal diet, control diet and test diet (with 1% and 4% APP supplementation). After four weeks, the plasma total cholesterol and triglyceride was determined by enzymatic diagnostic kit. (*P<0.01)

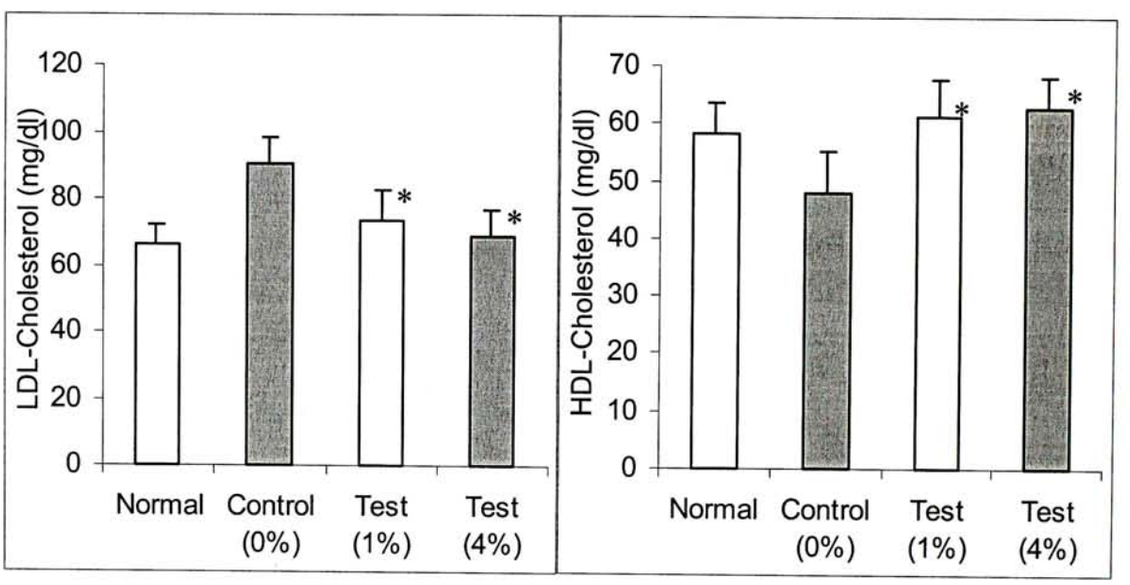


Fig. 4.12. Dose-dependent response of APP on plasma LDL-C and HDL-C. All male hamsters were fed with normal diet, control diet and test diet (with 1% and 4% APP supplementation). After four weeks, the plasma LDL-C and HDL-C was determined by enzymatic diagnostic kit. (*P<0.05)

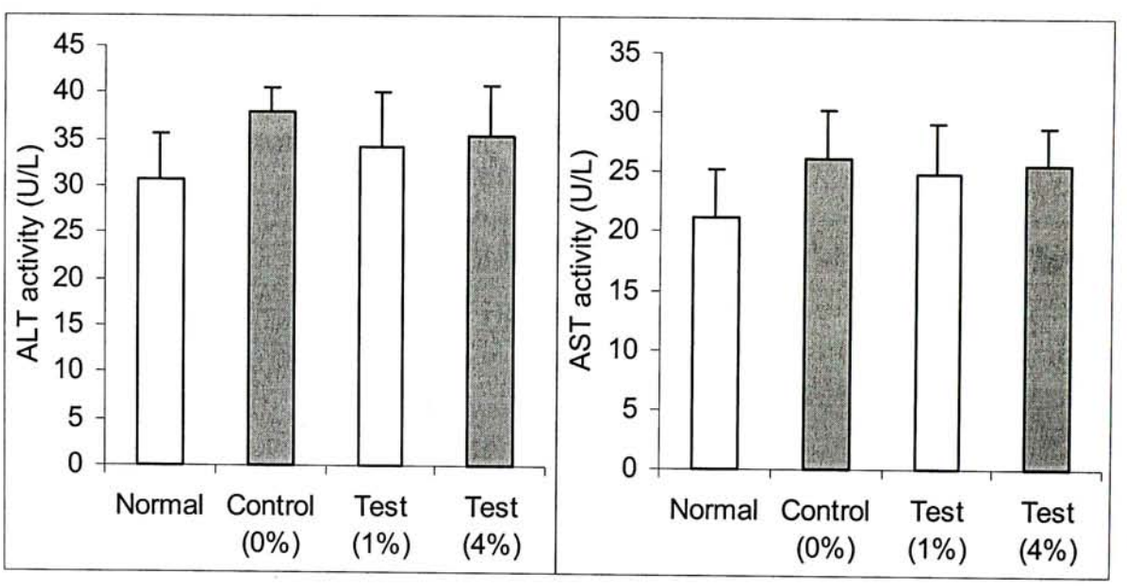


Fig. 4.13. Dose-dependent response of APP on plasma ALT and AST. All male hamsters were fed with normal diet, control diet and test diet (with 1% and 4% APP supplementation). After four weeks, the plasma ALT and AST was measured by enzymatic diagnostic kit.

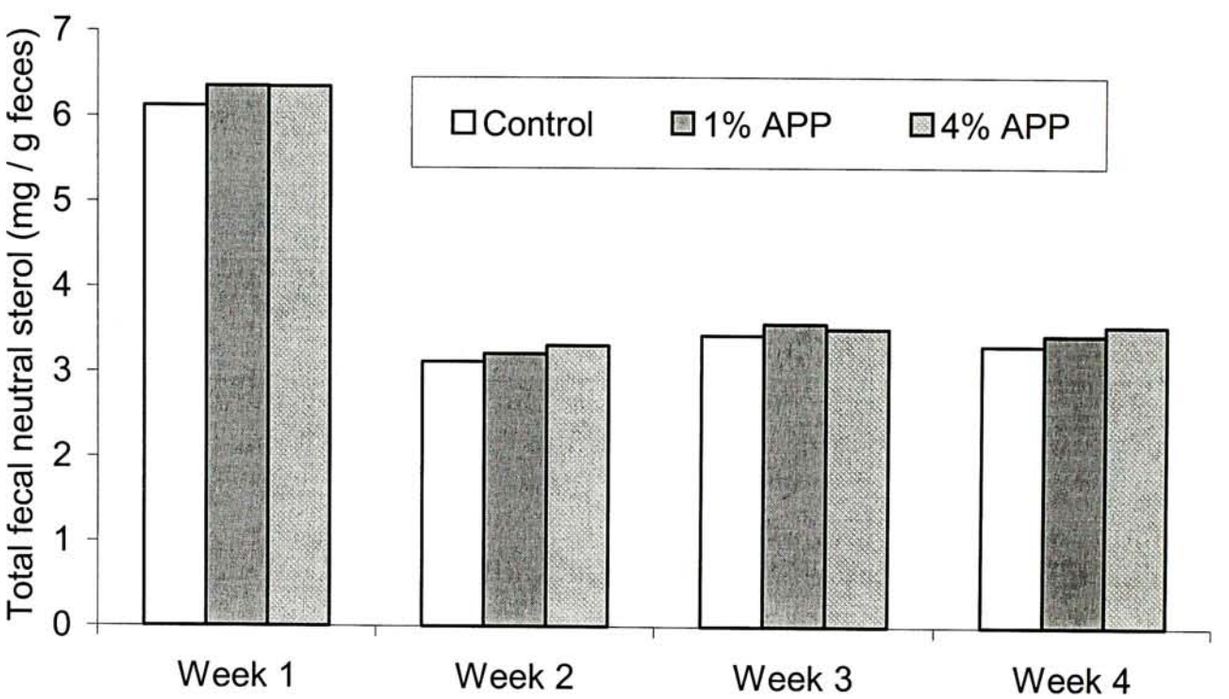


Fig. 4.14. Total fecal neutral sterol in the control and test group with APP supplementation. Test group diet was supplemented with 1% or 2% APP.

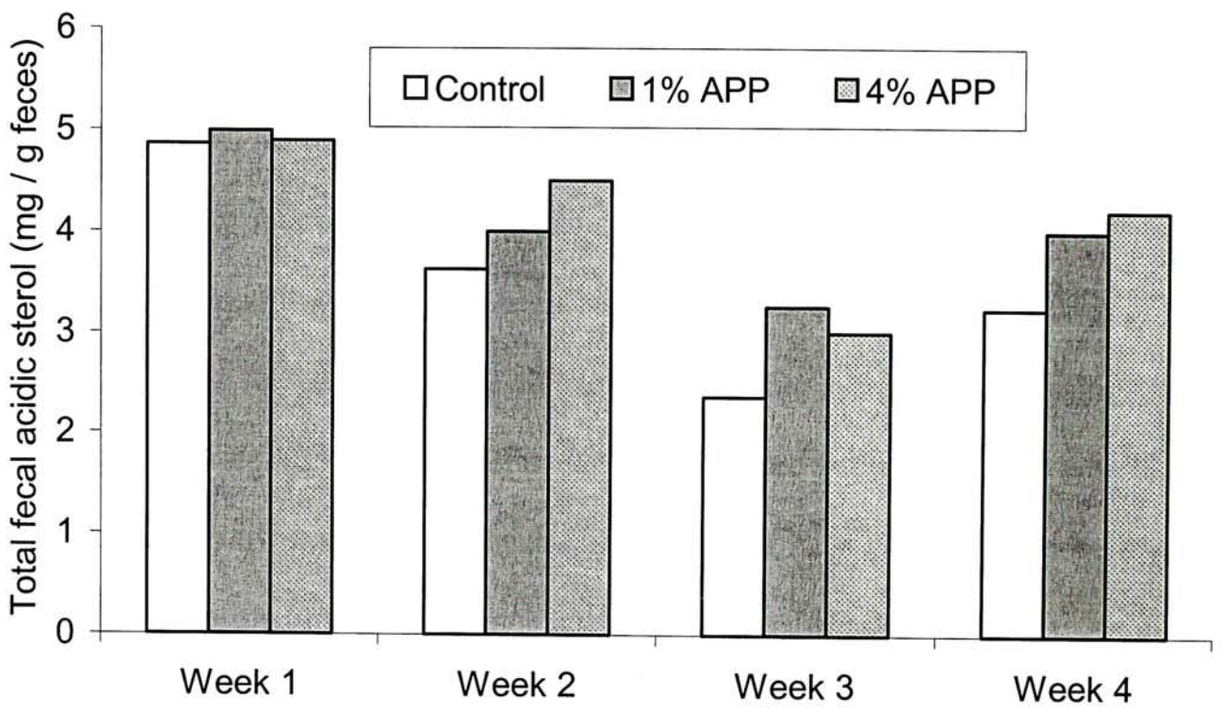


Fig. 4.15. Total fecal acidic sterol in the control and test group with APP supplementation. Test group diet was supplemented with 1% or 2% APP.

4.7 Discussion

In this experiment, the dose-dependent response of APP was under investigated (Bobek *et al*, 1998). One percent and four percent of APP was used to test whether there is a dose-dependent response relationship between amount of APP and its hypolipidemic effect. Moreover, the toxicity of APP was also measured by using AST and ALT assay.

Aspartate Aminotransferase (AST, EC 2.6.1.1) is widely distributed in tissues with highest concentration in liver, heart and skeletal muscle. Acute destruction of these tissues results in release of sizeable amounts of AST into systemic circulation. Alanine Aminotransferase (ALT, EC 2.6.1.2) is widely distributed in certain tissues with highest concentration in liver, kidney and heart. Above normal ALT values are also associated with toxic hepatitis, infectious mononucleosis and intrahepatic cholestasis. As a rule, ALT levels rise whenever liver cells are damaged. Therefore, the plasma AST and ALT were tested in order to find out whether APP exhibited toxic effect to liver cells.

From the results, we found that the hepatic cholesterol content decreased with increased percentage of APP. The triglyceride level of test group was significantly lower than in control group but this result was conflicted with the previous study. In previous studies, the plasma triglyceride level always has no significant difference between test and control groups. For LDL-C level, test group was significantly lower than control group and with similar level with normal group. However, it does not show any dose-dependent response relationship between percentage of APP supplementation and extent

of decrease in LDL-C. For HDL-C, test group was significantly higher than control group with a level even higher than normal group. Also, it does not show dose-dependent response effect. In spite of increased HDL-C, we are only concentrated on the atherosclerotic index, LDL/HDL ratio. This ratio is a better reflector of the degree of atherosclerosis. The test group showed much lower ratio than control group and the level was approximately equal to normal group for 4% APP supplementation. Therefore, this implies that APP supplementation can improve the state of atherosclerosis but this does not mean increase the amount of APP can have greater improvement. For ALT assay, it does not show significant difference among normal, control and test group. The ALT level of control group was only slightly higher than test group. From the result of AST assay, the same phenomenon appears. Therefore, the results showed that APP does not show any toxic effect up to recent investigations.

From the results of fecal sterol output, similar conclusion was drawn. Supplementation of APP generally increases neutral sterol excretion with small extent while greatly increase acidic sterol excretion. The purpose of this experiment is to test whether increase amount of APP can increase the sterol output. As a result, there is no direct relationship between percentage APP supplemented and both acidic and neutral sterol excretion.

Chapter Five:

Hypolipidemic Effect of Short Chain Fatty Acids

5.1 Introduction (Exp. 4,5)

An important physiological action of dietary fiber is the lowering of plasma cholesterol levels (Anderson, 1985). Inhibition of the absorption of bile acids and cholesterol, or suppression of cholesterol synthesis, may be involved in the effects of dietary fiber on lowering blood cholesterol. Previous investigations (Carr *et al*, 1996; Gallaher *et al*, 1993) showed that the viscosity of dietary fiber is related to its cholesterol lowering effect in hamsters fed with cholesterol-supplemented diets. However, other research (Younes *et al*, 1995) demonstrated that, in rats, a fermentable component, resistant starch, lowers plasma cholesterol levels more effectively than Cholestyramine®.

Ingestion of highly fermentable dietary fiber, sugar-beet fiber (SBF), decreases plasma cholesterol concentrations in rats (Aritsuka *et al*, 1989; Johnson *et al*, 1990), and the effect requires the bacterial actions of the large intestine (Nishimura *et al*, 1993). Recent studies demonstrated that a short-chain fatty acid mixture simulating the cecal fermentation products of SBF lowered plasma cholesterol levels to a degree similar to

that obtained by SBF feeding in rats (Hara *et al*, 1998a). This finding suggests that the suppressive effect of SBF on plasma cholesterol levels is at least partly caused by the cecal fermentation products, short-chain fatty acid (SCFA). One of these fermentation products, propionate, is viewed as a potential effective agent for lowering plasma cholesterol levels (Chen *et al*, 1984; Nishina & Freedland, 1990). However, the results of studies examining the effects of propionate on cholesterol metabolism are controversial (Berggren *et al*, 1996; Kishimoto *et al*, 1995). Also, studies showed that acetate was an effective component of the SCFA mixture for lowering plasma cholesterol levels, whereas propionate was not (Hara *et al*, 1998a).

The purposes of these studies (Experiment 4 and 5) were to determine whether the artificial SCFA decrease plasma cholesterol concentration and liver cholesterol (Moundras *et al*, 1994). Moreover, the effect of SCFA on de novo cholesterol synthesis was also examined by measuring the activity of HMG-CoA Reductase which is the key enzyme regulating the rate determining step in de novo cholesterol synthesis.

In this investigation, two separated experiments were conducted (Experiment 4 and 5) because fasting is needed for measuring plasma TG, HDL-C and LDL-C (Experiment 4). However, in measuring HMG-CoA Reductase activity, fasting is not needed because diets may have direct and acute response on this enzyme. Thus, the hamsters were not fasted in experiment 5. In experiment 5, one unit of activity is defined as one pmol/min/mg protein. The experimental conditions and diets were the same in these two experiments.

5.2 Results (Exp. 4,5)

5.2.1 Body Weight and Food Intake

For Pr, test and control group, the food intake was slightly higher than the other groups. However, there is no significant difference in body weight among all groups as shown in Table 5.1.

From Table 4.1, Pr and normal group has the lowest average liver weight (~4 g) while the test group has the highest liver weight (~6 g) and the other groups have a medium average liver weight (~4.5 g).

5.2.2 Effect of SCFA Supplementation on Hepatic Cholesterol

From the results, liver cholesterol was decreased significantly by 29.2% (Ac), 22.9% (Pr), 31.7% (Bu), 20.7% (Ac,Pr) and 38.4% (Test) as compared with the control group as shown in Fig. 5.1.

Table 5.1. Effect of APP and SCFA on body weight and food intake of hamsters and rabbits. For experiment 4 and 5, male hamsters were fed with normal diet, short chain fatty acids diet (with supplementation of 1% of sodium acetate (Ac), sodium propionate (Pr), sodium butyrate (Bu), 0.5% of each sodium salt of acetate and propionate (Ac,Pr) and control diet for four weeks. For experiment 6, male New Zealand rabbits were fed with normal diet, test diet (with 2% APP supplementation) and control diet for twelve weeks.

	Experiment 4,5		
	Ac	Pr	Bu
Initial body weight (g)	104.24 ± 7.87	104.02 ± 11.63	112.32 ± 8.66
Final body weight (g)	143.72 ± 8.57	137.38 ± 8.54	144.1 ± 6.02
Food intake (g/day)	7.49 ± 0.80	8.21 ± 0.82	7.47 ± 1.36

	Ac,Pr	Test (2%)	Control
Initial body weight (g)	109.84 ± 9.85	110.30 ± 6.44	100.44 ± 3.08
Final body weight (g)	141.32 ± 8.05	148.96 ± 4.07	144.8 ± 7.17
Food intake (g/day)	7.38 ± 0.95	8.66 ± 1.29	8.15 ± 1.59

	Experiment 6		
	Normal	Test (2%)	Control
Initial body weight (Kg)	3.50 ± 0.00	3.38 ± 0.26	3.30 ± 0.27
Final body weight (Kg)	3.70 ± 0.16	3.53 ± 0.49	3.06 ± 0.17
Food intake (g/day)	101.24 ± 15.66	86.83 ± 16.60	86.77 ± 12.15

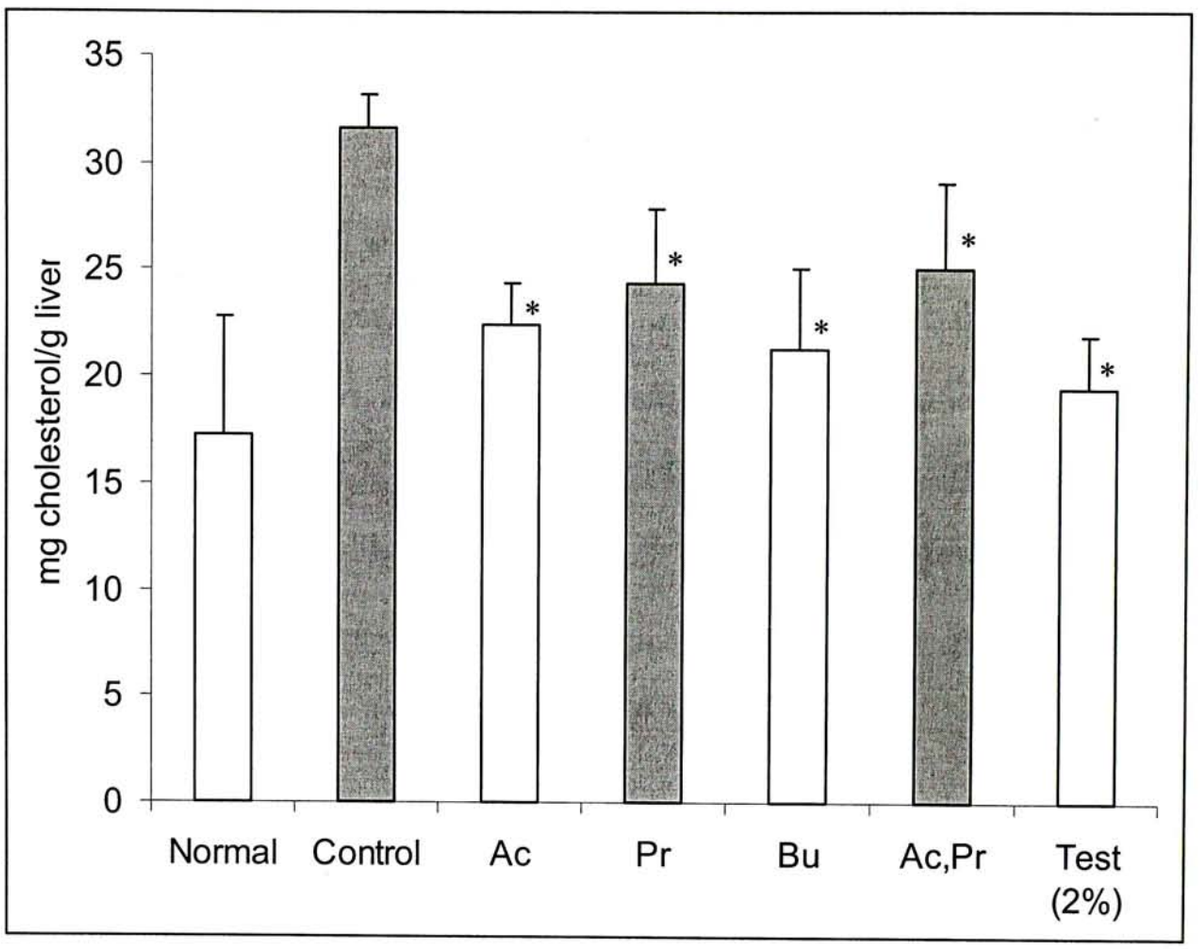


Fig. 5.1. Effect of APP and SCFA on liver cholesterol level. Male hamsters were fed with normal diet, control diet, short chain fatty acids diet (with supplementation of 1% of sodium acetate (Ac), sodium propionate (Pr), sodium butyrate (Bu), 0.5% of each sodium salt of acetate and propionate (Ac,Pr) and test diet (with supplementation of 2% APP) for four weeks. (*$P<0.01$)

5.2.3 Effect of SCFA Supplementation on Plasma TG, HDL-C and LDL-C

There is no significant difference among all the groups except for the normal group in plasma TG level as shown in Fig. 5.2. Fasting plasma LDL-C was decreased by 38.16% (Ac), 12.86% (Pr), 31.63% (Ac,Pr) and 40.4% (Test) significantly as compared with control group (Fig.5.3). However, there is no significant difference between group Bu and control. For group Ac and test group, the LDL-C has almost reached the normal level. For HDL-C, only group Ac and test group showed significant difference when compared with control group. HDL-C was increased by 10.9% and 18.5% in group Ac and test group respectively (Fig. 5.3). The LDL/HDL ratio was decreased by 43.94% (Ac), 9.09% (Pr), 7.58% (Bu), 36.36% (Ac,Pr) and 50.00% (test) compared with control group.

5.2.4 Effect of SCFA Supplementation on AST and ALT Activity

From Fig. 5.4, there is no significant difference for AST activity among all groups except group Bu as compared with control group, the level of AST activity was increased by 181.11%. For ALT activity, there is also no significant difference among all the groups except group Bu when compared with control group, the level of ALT activity was increased by 66.93%.

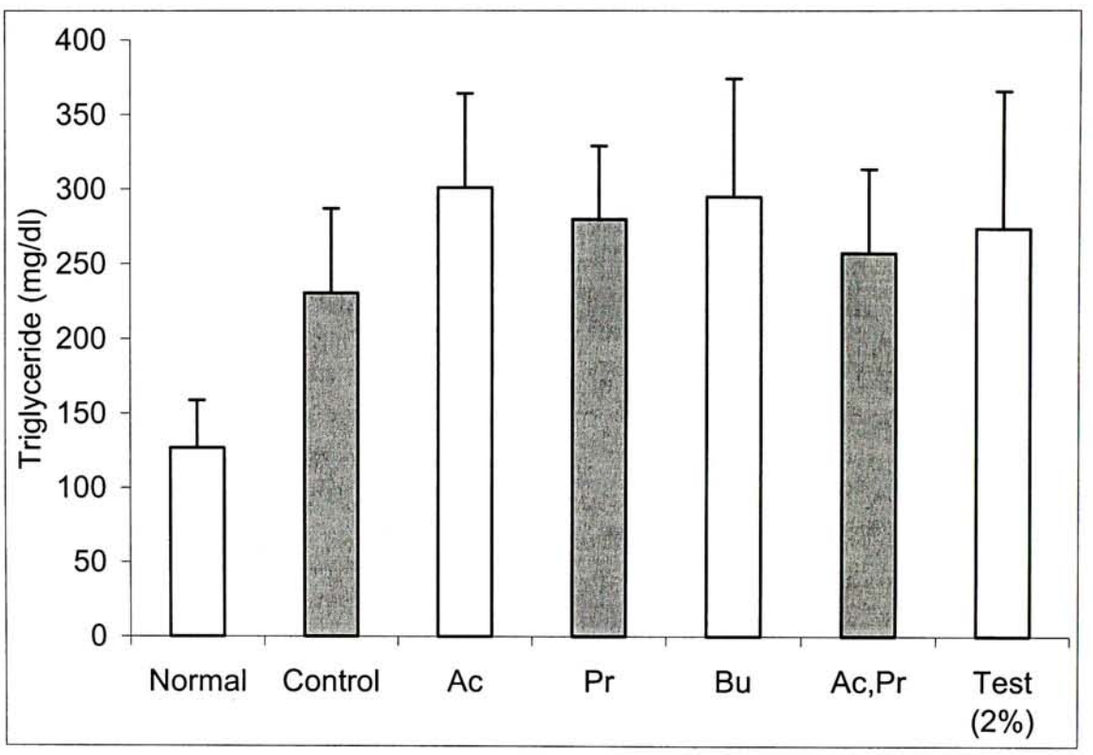


Fig. 5.2. Effect of APP and SCFA on plasma triglyceride level. Male hamsters were fed with normal diet, control diet, short chain fatty acids diet (with supplementation of 1% of sodium acetate (Ac), sodium propionate (Pr), sodium butyrate (Bu), 0.5% of each sodium salt of acetate and propionate (Ac,Pr) and test diet (with supplementation of 2% APP) for four weeks.

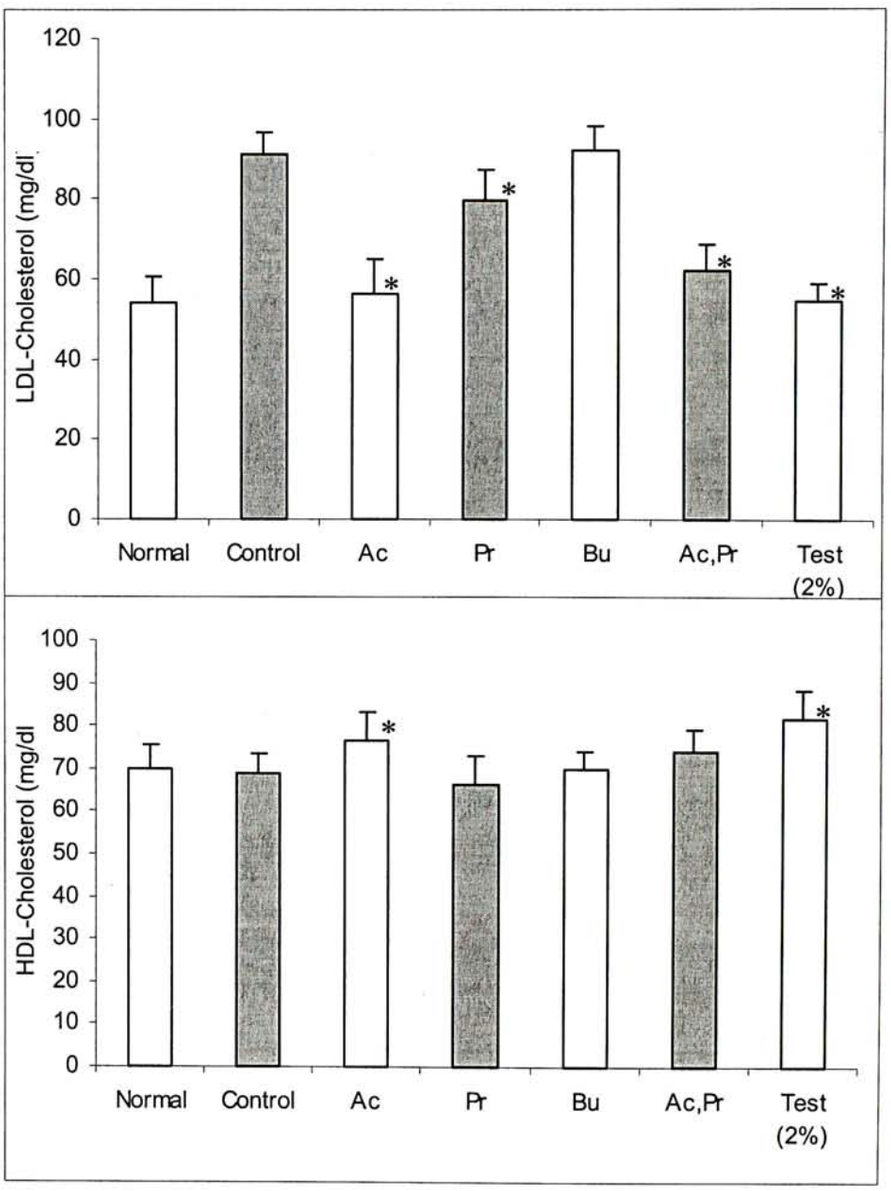


Fig. 5.3. Effect of APP and SCFA on plasma LDL- and HDL-Cholesterol level. Male hamsters were fed with normal diet, control diet, short chain fatty acids diet (with supplementation of 1% of sodium acetate (Ac), sodium propionate (Pr), sodium butyrate (Bu), 0.5% of each sodium salt of acetate and propionate (Ac,Pr) and test diet (with supplementation of 2% APP) for four weeks. (*$P<0.05$)

Table 5.2. Effect of APP and SCFA on LDL/HDL Cholesterol Ratio. For experiment 4, male hamsters were fed with normal diet, control diet (high cholesterol diet), test diet (with 2% APP supplementation) and SCFA diets (with supplementation of 1% of sodium acetate (Ac), sodium propionate (Pr), sodium butyrate (Bu), 0.5% of each sodium salt of acetate and propionate (Ac,Pr) for 4 weeks. For experiment 6, male New Zealand rabbits were fed with normal diet, control diet and test diet (with 2% APP supplementation) for three months.

	LDL/HDL-Cholesterol Ratio			
Experiment 4	Normal	Control	Ac	Pr
Week 4	0.77	1.32	0.74	1.20
	Bu	Ac,Pr	Test (2%)	
	1.22	0.84	0.66	

	LDL/HDL-Cholesterol Ratio		
Experiment 6	Normal	Test (2%)	Control
Month 0	0.68	0.74	0.66
Month 1	0.50	0.88	3.20
Month 2	0.56	0.86	3.35
Month 3	0.43	0.45	1.87

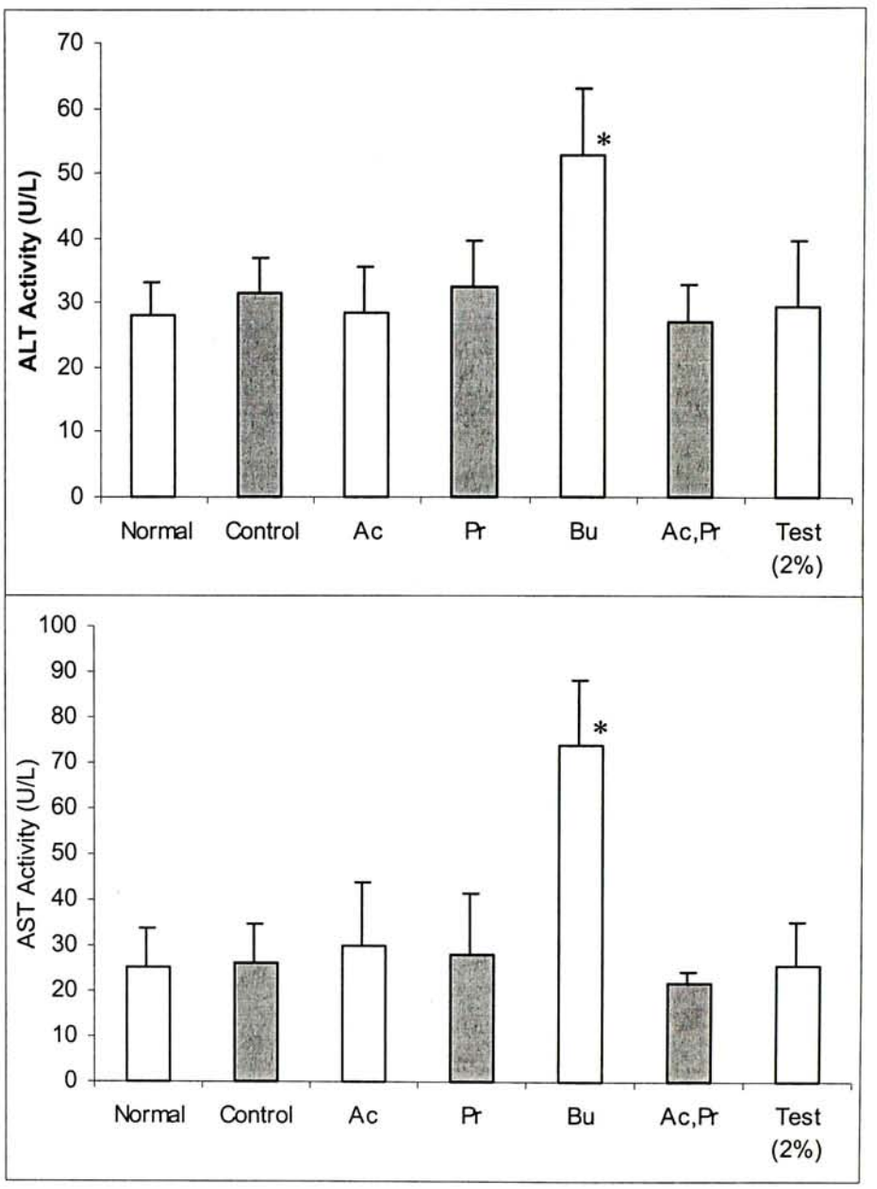


Fig. 5.4. The effect of different diets on plasma ALT and AST level. Male hamsters were fed with normal diet, control diet, short chain fatty acids diet (with supplementation of 1% of sodium acetate (Ac), sodium propionate (Pr), sodium butyrate (Bu), 0.5% of each sodium salt of acetate and propionate **(Ac,Pr)** and test diet (with supplementation of 2% APP) for four weeks. (* $P<0.05$)

5.2.5 Effect of SCFA supplementation on HMG-CoA Reductase Activity

All groups with supplementation of SCFA or APP showed significant difference in HMG-CoA Reductase activity compared with control group (Fig.5.5). The activity (pmol/min/mg protein) was decreased by 22.45% (Ac), 25.9% (Pr), 21.4% (Bu), 19.35% (Ac,Pr) and 30.95% (test).

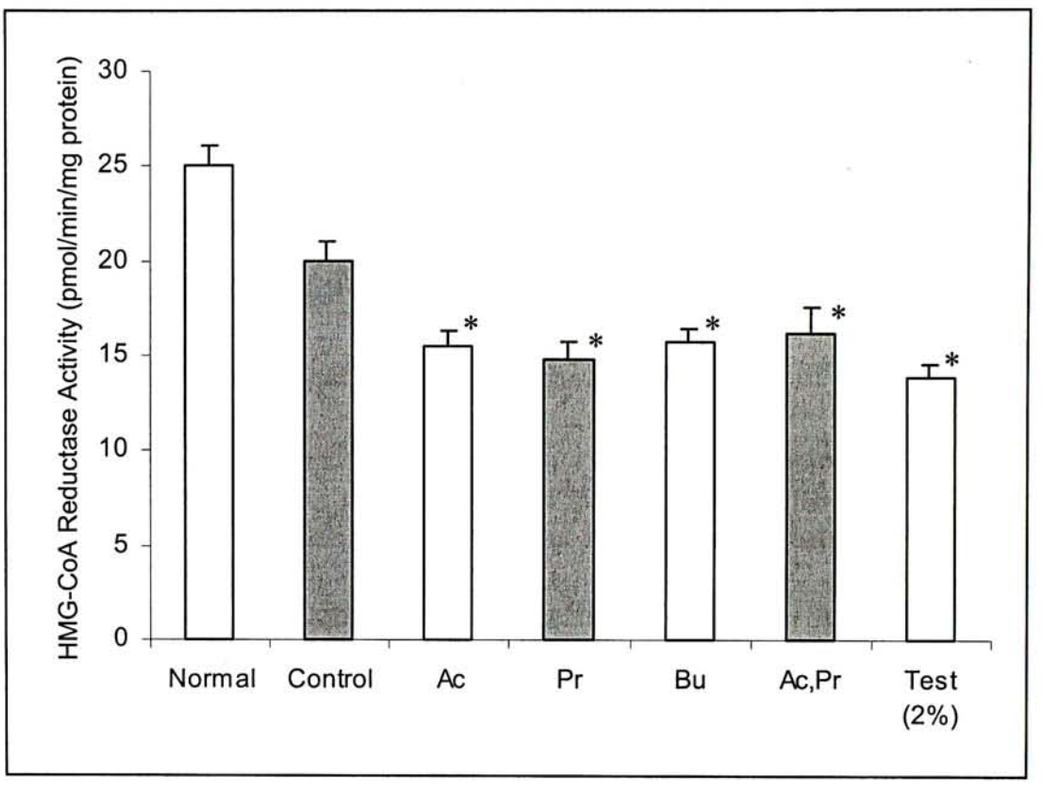


Fig. 5.5. Effect of APP and SCFA on HMG-CoA Reductase Activity in liver tissue. Male hamsters were fed with normal diet, control diet, short chain fatty acids diet (with supplementation of 1% of sodium acetate (Ac), sodium propionate (Pr), sodium butyrate (Bu), 0.5% of each sodium salt of acetate and propionate (Ac,Pr) and test diet (with supplementation of 2% APP) for four weeks. (*$P<0.05$)

5.3 Discussion (Exp. 4,5)

Feeding some dietary fibers lowers plasma cholesterol concentration. The mechanism is not fully understood. It is proposed that cecal and colonic fermentation of dietary fiber is associated with cholesterol-lowering effect of fibers (Anderson, 1985; Moundras *et al*, 1995). Study reported that the lowering effect of the fiber disappeared when the cecum was resected (Nishimura *et al*, 1993). This finding shows that the large intestine is involved in the reduction of plasma cholesterol in rats fed SBF which is a highly fermentable dietary fiber. The fermentation product is SCFAs. Therefore, we have examined the effect of artificial SCFA on plasma and liver cholesterol level. The results showed that acetic acid, propionic acid, mixture of acetic and propionic acid (Hara *et al*, 1998b) and APP could lower LDL-C but not butyric acid compared to control group. However, acetic acid and APP can slightly increase HDL-C and no significant difference for other groups. Among the three SCFAs, acetic acid exhibited highest degree of cholesterol lowering effect. This results shows that the major SCFA, acetic and propionic acids are responsible for the plasma cholesterol reduction.

Two possible mechanisms are proposed for the ceco-colonic-dependent decrease in plasma cholesterol. In the first mechanism, fiber or its fermentation products stimulate the large intestine, and humoral factors secreted from the large intestine or enteral nervous system modify cholesterol metabolism. Study (Goodlad *et al*, 1989) showed that colonic fermentation of dietary fiber elevates plasma enteroglucagon, and it is known

that the large intestine is important for production of this hormone (Kennedy *et al*, 1982). In addition, the enteric nervous system in the large intestine is possibly stimulated by fiber or its fermentation products. Some neuropeptides are known to influence cholesterol and bile acid metabolism (Cho *et al*, 1997; Farouk *et al*, 1992). Therefore, we also examined effect of SCFA on the de novo cholesterol synthesis by measuring the activity of HMG-CoA Reductase and the liver cholesterol level (Hara *et al*, 1999). From the result, all of the SCFAs and APP can decrease the activity of HMG-CoA Reductase activity significantly with similar extent among three SCFAs. Moreover, all three SCFAs can decrease hepatic cholesterol significantly compared with control group. The results showed that SCFA could decrease de novo cholesterol synthesis and thus liver cholesterol level. The lowering activity of HMG-CoA Reductase in control group compared with normal group was due to the high fat and cholesterol content in the diets. The results also showed that mixture of SCFA (acetic and propionic acid) exhibits the same effect as individual SCFA.

Furthermore, we also examined the toxicity of SCFA to liver. The result showed that butyric acid could greatly increase more than one fold of AST and ALT activity compared to control group.

Chapter Six:

Hypolipidemic and Antiatherosclerotic Effect of APP

6.1 Introduction (Exp. 6)

Epidemiological studies have shown a consistent and strongly significant correlation between total plasma cholesterol level and incidence of CHD (Stamler *et al*, 1986). Both CHD incidence and extent of atherosclerosis autopsy can be explained to a large extent by variations in mean total cholesterol levels between populations. Among individual within diverse populations, prospective studies of middle-aged men have shown a positive relation between CHD risk and total cholesterol level above 200 to 220 mg/dl (*J Chronic.Dis.*, **31**, 201-306, 1978). This association may be weak or absent in some populations with low mean total cholesterol levels or CHD risk (*Circulation*, **85**, 1083-1096, 1992).

Total cholesterol conc entration generally reflects LDL cholesterol level. Epidemiological studies between and within population also show a strong relation between CHD risk and LDL cholesterol levels (Cerda *et al*, 1994). A direct relation between LDL concentration and extent of coronary atherosclerosis has also been shown in several angiographics studies. Furthermore, clinical trials and angiographic studies provide good evidence that reductions in LDL cholesterol are associated with reduced

incidence of CHD events and slowed progression of atherosclerotic lesions (Kroon & Stalenhoef, 1997). Moreover, HDL cholesterol has inverse relationship with the degree of atherosclerosis.

Dietary cholesterol has been shown to increase atherosclerosis in susceptible animal species. The atherogenic effect of dietary cholesterol was first demonstrated in rabbits, but has also been observed in several other animals, including pigs, guinea pigs. We have employed New Zealand White Rabbits as animal model for testing whether supplementation of dietary fiber can stop the progression of atherosclerosis by using a high cholesterol diet as control.

The animals in which diets rich in dietary cholesterol and saturated fat produced atherosclerosis also have an increase in plasma cholesterol level in response to these diets. There is a strong positive correlation between changes in serum cholesterol level, especially LDL cholesterol level, and the development of atherosclerosis in these experimental models (McGill, Jr. *et al*, 1981a; McGill, Jr. *et al*, 1981b). An inverse relation between HDL level and atherosclerosis has been demonstrated in animals (Lusis *et al*, 1983) as well as in humans. In some species, such as rabbits, the diet-induced increase in plasma cholesterol level is due mainly to an increase in renmanent lipoprotein. However, this increase is seen, but to a lesser extent, in humans consuming similar diets. Therefore, we decided to make a relationship between LDL/HDL-C ratio to the degree of atherosclerosis. Increased ratio of LDL/HDL may increase the development of atherosclerosis as expected (Chait *et al*, 1993).

6.2 Results (Exp. 6)

6.2.1 Body Weight and Food Intake

From Table 5.1, the food intake of test and control group has no significant difference. However, the food intake of normal group was 14.23% and 14.29% significantly higher than test group and control group respectively. The average food intake was low in both test and control group as the food intake was decreased with time. The weight gain was 0.2 Kg and 0.15 Kg in normal and test group respectively. However, control group has weight loss of 0.24 Kg.

From Table 4.1, there was a large difference of the average liver weight between normal group (75.47 g) and test group (112.35 g) or control group (127.23 g). The average liver weight of test group was 11.7% lower than control group.

6.2.2 Effect of APP Supplementation on Hepatic Cholesterol

From the results, liver cholesterol level was decreased significantly by 14.08% in test group compared with control group as shown in Fig. 6.1. However, the liver cholesterol level of test group and control group was more than 10 folds higher than normal group.

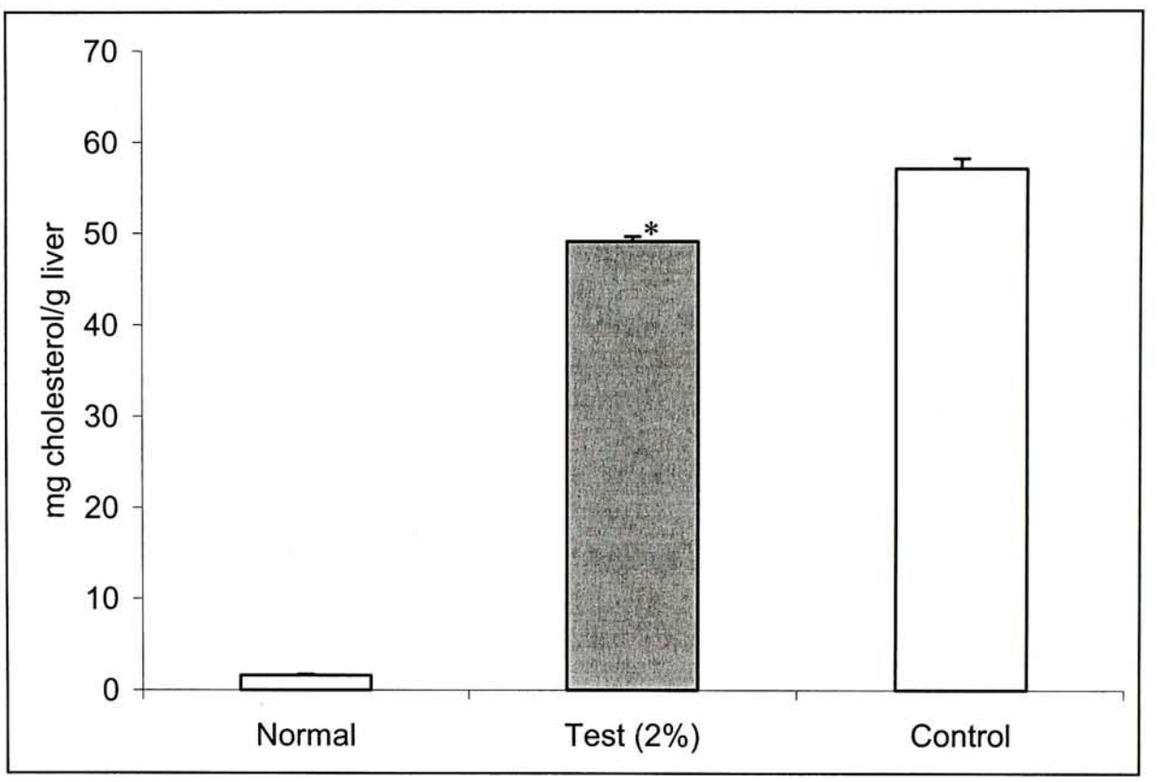


Fig. 6.1. Effect of APP on liver cholesterol level in rabbit. Male New Zealand rabbits were fed with normal diet, control diet and test diet (with supplementation of 2% APP) for three months. (*P<0.01)

6.2.3 Effect of APP Supplementation on Plasma TG, HDL- and LDL-C

Among the three months, the plasma TG level of both test and control group was significantly much higher than normal group. At the month 0 and 1, there is no significant difference between test and control group. The TG level in test group was 21.3% and 26.7% significantly lower than control group at month 2 and 3 respectively as shown in Fig. 6.2. For both HDL-C and LDL-C, normal group has a much lower level than control and test group. Except at month 0, LDL-C of test group showed a significantly lower level than control group at month 1, 2 and 3. Except at month 0, HDL-C of test group showed a significantly higher level than control group at month 1, 2 and 3. HDL-C was increased by 105.66%, 89.97% and 67.42% in test group at month 1, 2 and 3 respectively compared with control group. LDL-C was decreased by 43.67%, 50.96% and 59.25% in test group at month 1, 2 and 3 respectively as shown in Fig. 6.3. The LDL/HDL ratio of this experiment was shown in Table 5.2. The average ratio among three months was 0.54 ± 0.11, 0.73 ± 0.20 and 2.27 ± 1.26 in normal, test and control group respectively. However, this average value cannot represent the cardiovascular status of the rabbit. The change of ratio with time was more important. For normal group, this ratio does not fluctuate greatly. At month 0, there was no significant difference among three groups. For test group, the ratio was increased by 18.92% and 16.22% at month 1 and 2 respectively compared with month 0. However, the ratio was dropped to a level even lower than the ratio at month 0. For control group,

the ratio was increased by 384.85% and 407.58% at month 1 and 2 respectively compared with month 0. However, the ratio was dropped by 44.18% compared with month 2. At month 3, there was no significant difference of LDL/HDL ratio between normal and test group. However, control group has a ratio of 334.88% higher than normal group.

6.2.3 Effect of APP Supplementation on AST and ALT Activity

From Fig. 6.4, there is no significant difference for ALT activity between test and normal group. The ALT activity of control group was always higher than both test and normal group. However, there was a drop in activity in control group to the level of test group at month 2. At month 1, ALT activity of the test group was decreased by 37.77% significantly as compared with control group. For AST activity, the level of control group was always higher than both test and normal group. With similar phenomenon to ALT activity, there was no significant difference among three groups at month 2. Test group showed a significant decrease in AST activity by 42.84% and 47.31% at month 1 and 3 respectively.

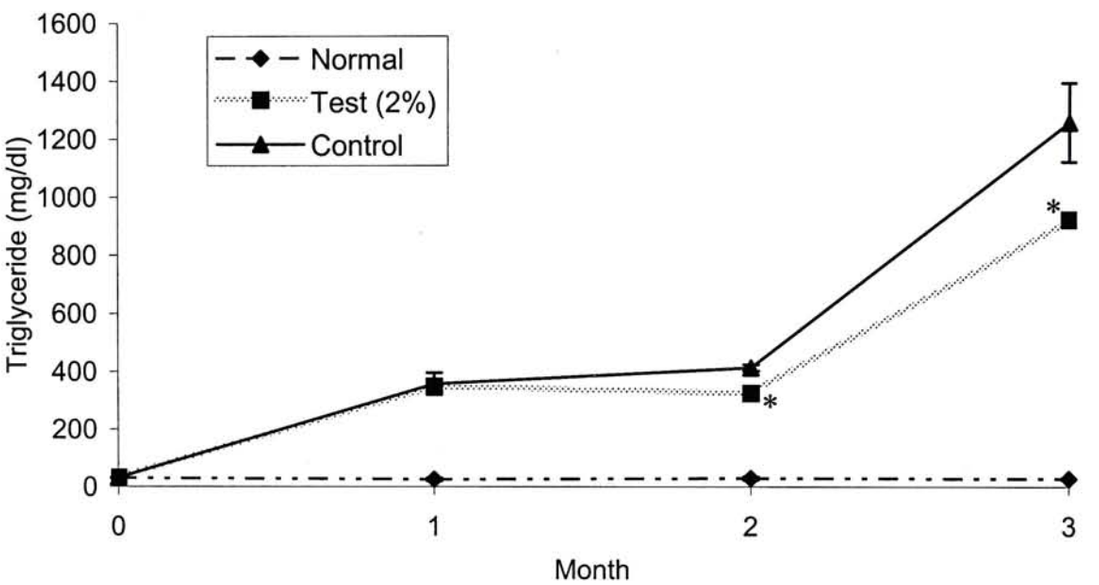


Fig. 6.2. Effect of APP supplementation on plasma triglyceride level. Male New Zealand rabbits were fed with normal diet, control diet and test diet (with supplementation of 2% APP) for three months. (* $P<0.05$)

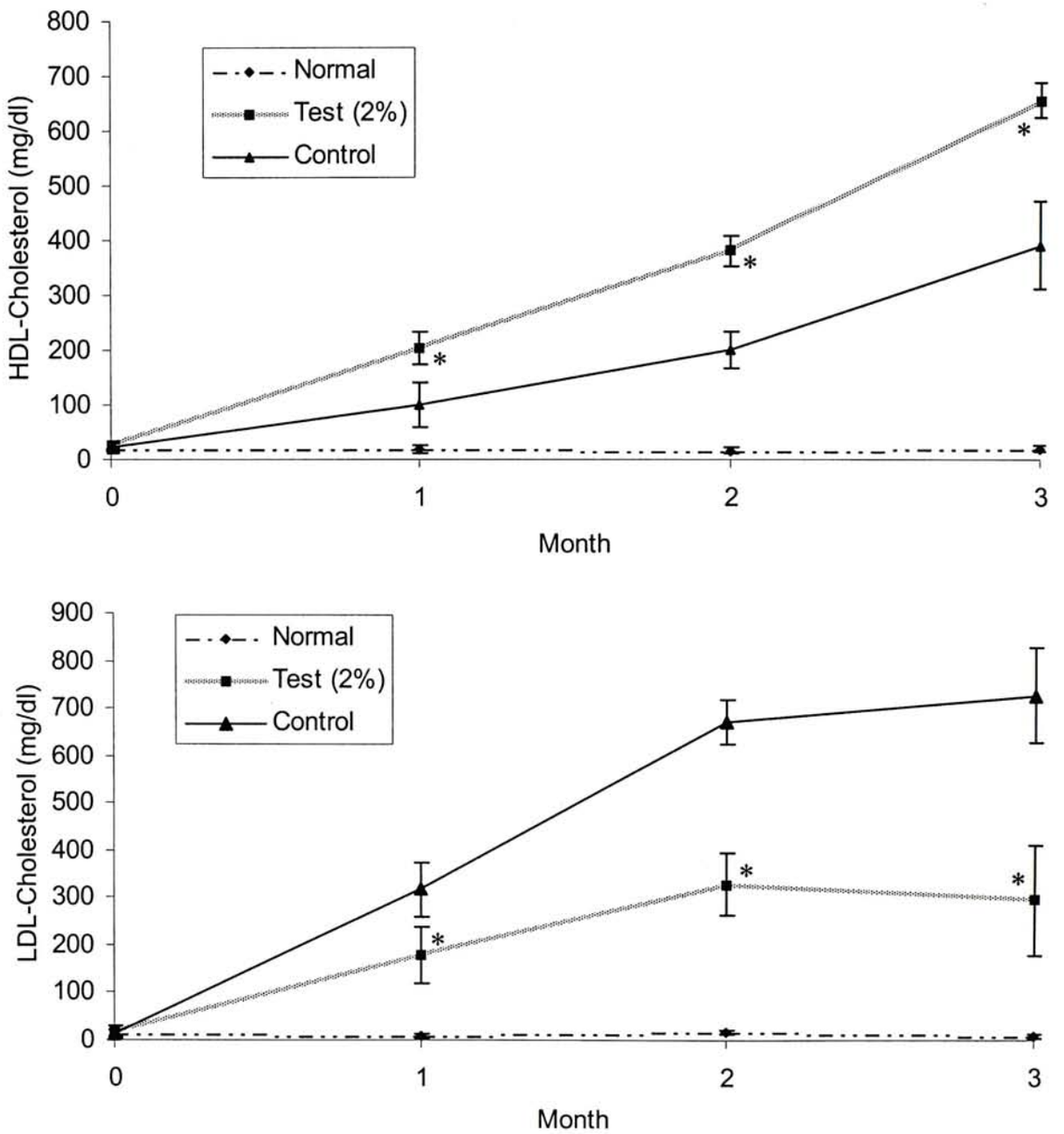


Fig. 6.3. Effect of APP supplementation on plasma HDL- and LDL-cholesterol level. Male New Zealand rabbits were fed with normal diet, control diet and test diet (with supplementation of 2% APP) for three months. (* $P<0.01$)

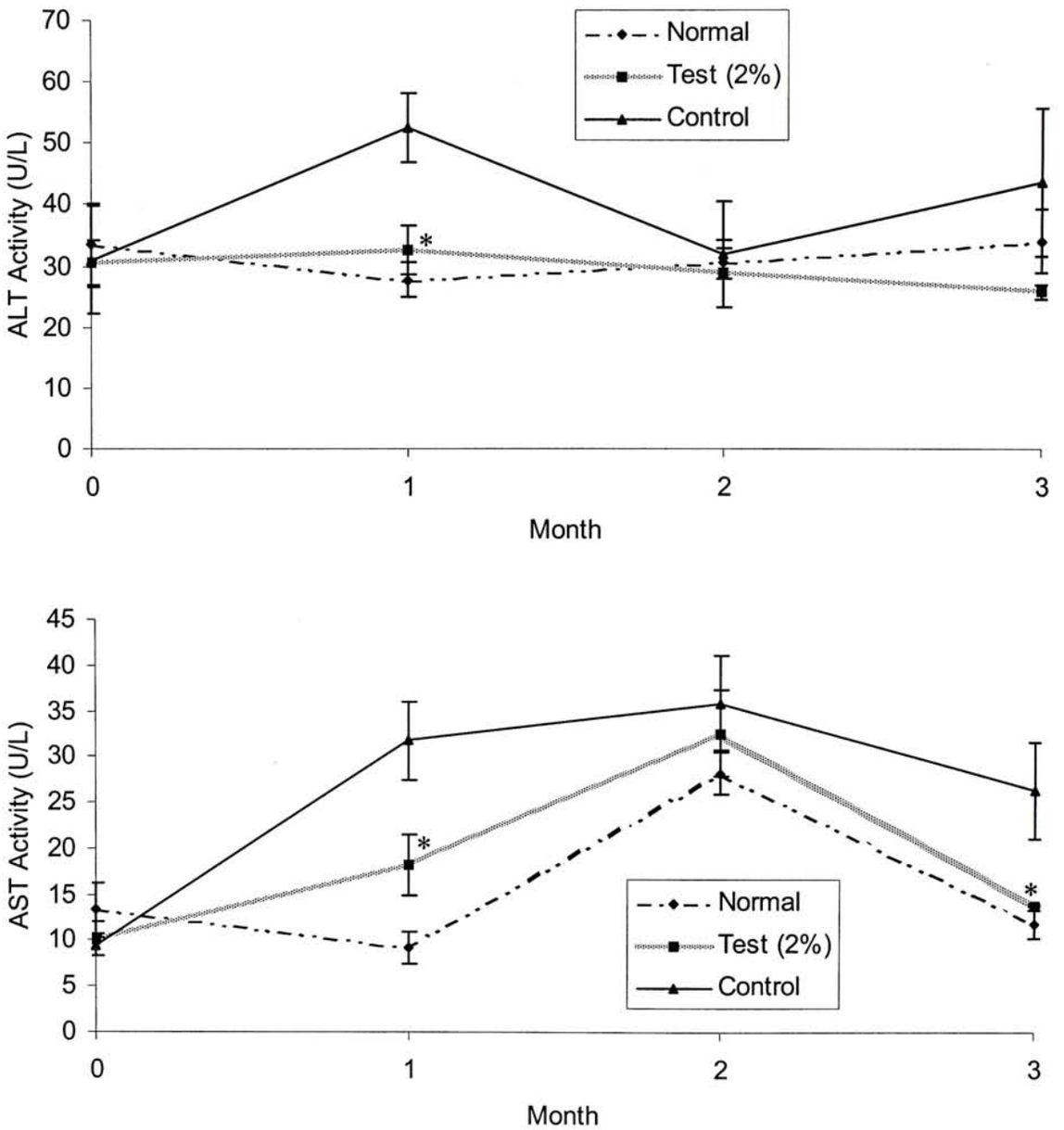


Fig. 6.4. Effect of APP supplementationThe effect of different diets on plasma ALT and AST level. Male New Zealand rabbits were fed with normal diet, control diet and test diet (with supplementation of 2% APP) for three months (* $P<0.05$).

6.2.5 Effect of APP supplementation on HMG-CoA Reductase Activity

Fig. 6.5 showed the HMG-CoA Reductase activity (pmol/min/mg protein) of normal (50.23), test (40.33) and control (43.43) group. The activity in normal was higher than both the test and control groups; while test group with 7.14% lower activity than control group ($P<0.05$).

6.2.6 Effect of APP supplementation on the Formation of Atheroma

The photographs of representative aortas in normal, test and control group were shown in Fig. 6.6. The regions stained red are represented to atheroma. By general observation, the area of atherosclerotic lesion is the highest in control which receive high cholesterol diet. In test group, there is still a small degree of lesion but it showed a regression of atherosclerosis compared with control group. By analysis of the area covered by atherosclerosis, the average value of percentage area covered was 8.80%, 24.85% and 58.51% in normal, test and control group respectively. All of the rabbit were fed for three months with different diet.

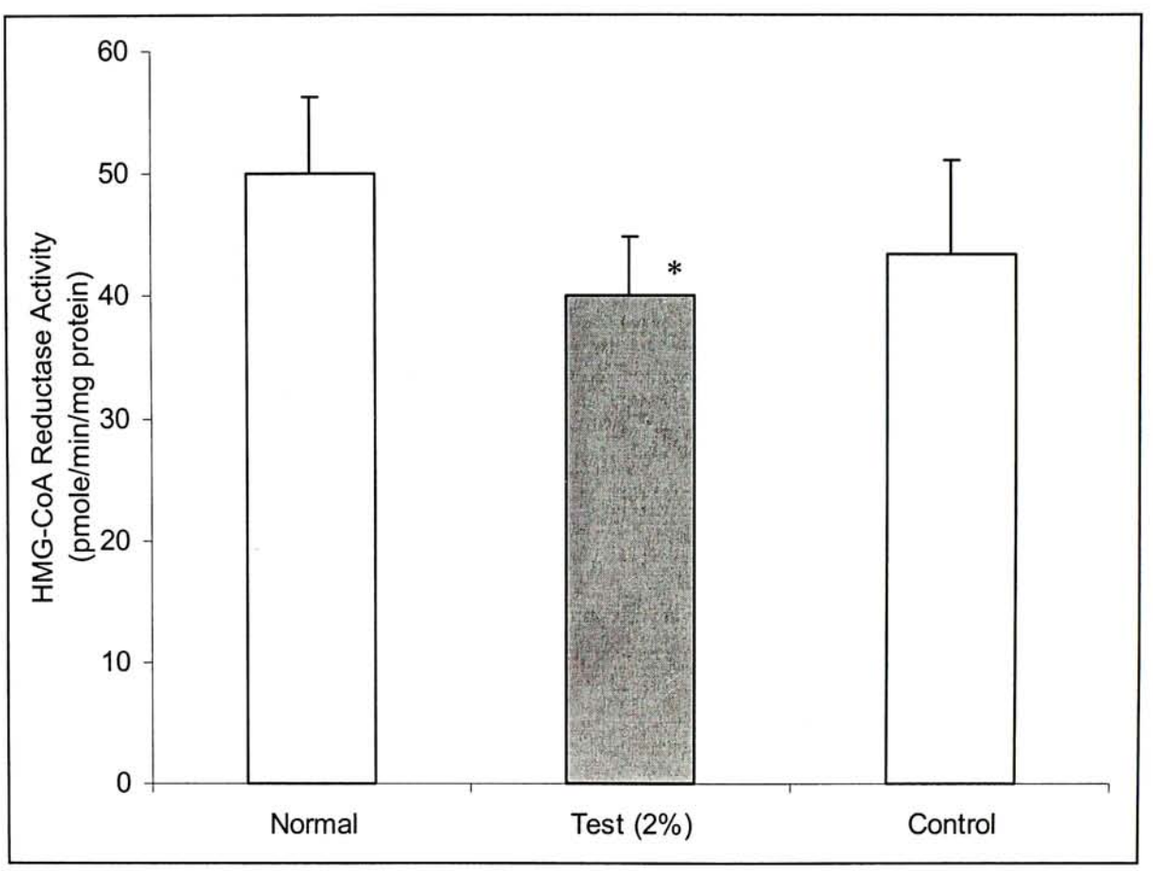


Fig. 6.5. Effect of APP supplementation on liver HMG-CoA Reductase level in rabbit. Male New Zealand rabbits were fed with normal diet, control diet and test diet (with supplementation of 2% APP) for three months. (* $P<0.05$)

Fig. 6.6. Photograph of representative aortas of rabbit receiving an atherogenic diet showing decreased aortic atherosclerosis receiving supplementation of APP. Rabbits were fed with normal diet without supplementation of cholesterol, test diet with supplementation of 1% cholesterol and 2% APP and control diet with only supplementation of 1% cholesterol.

Treatment	Aorta % Area Covered by Atherosclerosis
Normal	8.80 ± 5.95
Test (with 2% APP)	24.85 ± 7.70
Control (without APP)	58.51 ± 17.19

Table 6.1. Percentage of area covered by atherosclerosis in aorta in normal, test and control group. Normal diet contains no cholesterol. Test diet was a high cholesterol (1%) diet with supplementation of 2% APP. Control diet was only a high cholesterol (1%) diet.

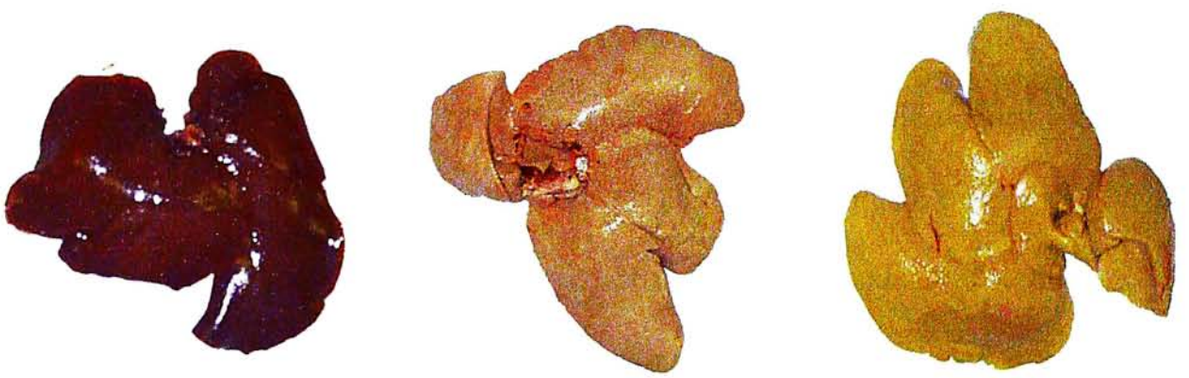


Fig. 6.7. Photographs of rabbit liver. Rabbits were fed with normal diet without supplementation of cholesterol, test diet with supplementation of 1% cholesterol and 2% APP and control diet with only supplementation of 1% cholesterol. The liver was harvested after three months treatment.

6.3 Discussion (Exp. 6)

Hypercholesterolemia is a major risk factor in the development of atherosclerosis (*JAMA*, **253**, 2080-2086, 1985; Ross, 1986) and cholesterol-lowering agents are used in selected patients to retard atherogenesis. As an alternative to the use of pharmaceutical agents, recent studies have shown that ingestion of water-soluble dietary fibers such as oat bran or pectin will lower cholesterol levels (Keys *et al*, 1961; Anderson *et al*, 1988). APP is also a water-soluble dietary fiber and the results showed that it has hypocholesterolemic effect. As a dietary supplement, APP reduces liver cholesterol levels (Keys *et al*, 1961; Kay & Truswell, 1977; Cerda *et al*, 1988) and lowers low-density lipoprotein (LDL) cholesterol. On the contrary, it can increase high-density lipoprotein (HDL) cholesterol and as a result, has a much lower LDL/HDL cholesterol ratio that is a good index of atherosclerosis. However, APP has little triglyceride lowering effect. Moreover, it does not show any toxic effect on liver cells as the AST and ALT activity was lower in test group compared with control group.

During the experimental period, we examined the external health status of the rabbits. For the control group without APP supplementation, the body weight of rabbits were dropped instead of weight gain and also the food intake was decreased. By observing the appearance of the liver, the control group with high cholesterol diet showed a pathogenic effect on liver. The rabbits of control group have some kinds of

jaundice as seen in Fig. 6.7. Therefore, we found that rabbit is an animal model that was very sensitive to dietary composition.

The pathogenic effect of liver was due to high concentration of cholesterol accumulation. High cholesterol diet lowered the liver cholesterol synthesis by down-regulating the HMG-CoA Reductase activity. APP can further lower the enzyme activity compared with the control group. High hepatic cholesterol in test and control group was mainly due to the intake of high cholesterol diet. The decreased hepatic cholesterol in test compared with control group may due to decrease in HMG-CoA Reductase activity. However, the underlying mechanism that APP can decrease the activity of HMG-CoA Reductase activity was unclear.

Very little is known about the effect of dietary fiber (APP) on atherosclerosis (Cerda *et al*, 1994). However, we can conclude that APP either regressed or prevented the development of atherosclerosis as it substantially reduced the aorta area occupied by atheromatous plaques.

Chapter Seven

General Discussion and Future Perspectives

Auricularia polytricha is an edible fungus which is a popular foodstuff in Oriental countries. This type of fungus is in the members of *Auriculariaceae* family. It has gelatinous fruiting bodies when it is fresh. It always sold in dry stuff in Chinese medicine store. This fungus was claimed to have hypolipidemic effect but without detailed experimental investigations.

The water-soluble fractions extracted from *Auricularia polytricha* was found to contain mainly polysaccharides which consist of mainly mannose, glucose and galactose as its monosaccharide. It was predicted to contain mainly glucomannan, galactomannan and glucogalactomannan. It showed a high solubility, viscosity and water-holding capacity. From the size-exclusion chromatography, *Auricularia polytricha* Polysaccharide (APP) was a heterogeneous mixture of water-soluble dietary fiber that has different molecular size. However, there is no information for its molecular structure. In order to have a clear picture of the structures, data from IR, NMR and even X-ray crystallographic analysis should be obtained.

From the results of the experiment, fasting plasma total cholesterol and low-density lipoprotein (LDL) cholesterol were lowered by supplementation of 2% of APP.

However, the high-density lipoprotein (HDL) cholesterol was increased or remains unchanged. Furthermore, the plasma triglyceride level fluctuated and there is no significant increased or decreased in TG level compared with control group.

For the protective study, a high cholesterol (0.5%) diet with supplementation of 2% APP was used to test whether APP can prevent the rise of plasma cholesterol level. The results showed that APP can lowered plasma cholesterol level and hepatic cholesterol level. Therefore, APP can protect body from hypercholesterolemia.

For the therapeutic study, hamsters were boosted into hypercholesterolemic status by feeding it with a high cholesterol diet. After 4 weeks, hamsters were fed with high cholesterol diet with supplementation of 2% of APP. After 4 weeks, the hypocholesterolemic effect of APP was not obvious but with some improvement. Eight weeks after supplementation of APP, the improvement was obvious and LDL-cholesterol was decreased by 47.1% compared to control group. Also, the liver cholesterol was decreased by 26.3% significantly. Therefore, APP can even lower cholesterol level in a pre-hypercholesterolemia body. For the dose-dependent response study, we try to use different amount of APP to see whether increased amount of APP can further improve hypercholesterolemia. The results showed that there is no dose-dependent response in plasma cholesterol level. However, there is a dose-dependent response in liver cholesterol level. The percentage of APP showed a linear relationship with liver cholesterol. Liver cholesterol decreased with increased percentage of APP supplementation. Moreover, by assay the activity of AST and ALT, APP does not show

any toxic effect toward liver. Similar activity was observed for increased percentage of APP supplementation.

APP was a water-soluble polysaccharide that can resist degradation from digestive enzyme. It can probably reach ceco-colonic region and degraded by anaerobic bacteria and produce short chain fatty acids (SCFA) (Treem *et al*, 1996). These fatty acids included mainly acetic acid, propionic acid and butyric acid that was claimed to have hypocholesterolemic effect. Therefore, the hypocholesterolemic effect of APP may be due to by-products of fermentation. In the experiment, we use artificial SCFA to test its hypocholesterolemic effect. As expected, the result showed that SCFAs have hypocholesterolemic effect with descending potency of acetic acid, propionic acid and then butyric acid. Experiment must be decided in future to prove whether APP can be fermented to produce SCFA (Daniel *et al*, 1997; Itoh & Mitsuoka, 1985) and compared the effect of fermented products with APP.

In experiment 5, the effect of APP and SCFA on HMG-CoA Reductase activity was tested. Both APP and SCFA can decrease the activity of HMG-CoA Reductase. Therefore, the de novo cholesterol synthesis was decreased with decreased in this enzyme activity. However, in the future, the activity of the enzyme (cholesterol 7-alpha hydroxylase) that convert hepatic cholesterol into bile acid must be assayed (Schmitz & McDonald, 1974; Jelinek *et al*, 1990). It is because bile acid is the only way that cholesterol can be excreted out of the body via feces. Therefore, increased activity of this enzyme can significantly decrease the pool of hepatic cholesterol.

Proposed mechanism included binding of steroids with water-soluble dietary fiber (Spiller, 1996). Therefore, we have determined the neutral and acidic sterol from feces output. In this mechanism, dietary fiber was acted as non-systemic agent (Story *et al*, 1997). However, in vitro experiment is difficult to conduct as the extracted polysaccharide was water-soluble (Ebihara & Schneeman, 1989). Excess bile acid was added for binding. However, there is a problem with the elimination of unbound bile acid. In experiment 1 and 3, APP can slightly increase the neutral steroid excretion and greatly increase the acidic steroid excretion. However, the dose of APP has no effect on both fecal neutral and acidic steroids excretion.

In experiment 6, we tested the antiatherosclerotic effect of APP. We can develop a relationship between plasma cholesterol level and degree of atherosclerosis. LDL-cholesterol was shown to have direct relationship with atherosclerosis while HDL-cholesterol has inverse relationship. Therefore, we used LDL/HDL ratio to construct a relationship with the degree of atherosclerosis. The degree of atherosclerosis was determined by the formation of atheroma that is stained by a lipophilic dye, Sudan III. The results showed that LDL/HDL-cholesterol ratio is much higher in control group than in test group. Moreover, the aortic area occupied by atheromatous plaques was much higher in control group. Therefore, we can conclude that APP can regress or prevent the development of atherosclerosis and there is a good relationship between LDL/HDL-cholesterol ratio and degree of atherosclerosis. Ratio was increased with the

higher probability of development of atherosclerosis. Therefore, APP may modify the lipoprotein profile in order to prevent formation of atherosclerosis.

Up to now, the hypocholesterolemic effect of dietary fiber was complicated and its detail mechanisms are still unclear. The hypocholesterolemic and antiatherosclerotic effects may be due to combined mechanisms and so more experiments must be decided to elucidate its underlying mechanisms.

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