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Structured Phospholipids from Commercial Soybean Lecithin Containing Omega-3 Fatty Acids Reduces Atherosclerosis Risk in Male *Sprague dawley* Rats which Fed with an Atherogenic Diet

Jaya Mahar Maligan, Teti Estiasih and Joni Kusnadi

Abstract - Structured phospholipids from commercial soybean lecithin with oil enriched omega-3 fatty acid form by product of tuna canning is alternative procedure to provides the stability of omega-3 fatty acid structure and increase these bioactive function in metabolism. Best treatment condition was obtain in 18 hours acidolysis reaction with 30% enzyme concentration, which EPA-DHA incorporation level was 127,47 mg/g and incorporation percentage of EPA-DHA was 51,04% at phospholipids structure. This structured phospolipids could reduce atherosclerosis risk in male Sprague dawley rat. Provision of structured phospholipids has significant effect ($\alpha = 0.05$) on changes in lipid profile, intima-media thickness of aorta rats (male Sprague dawley) fed atherogenic diet. Structured phospholipids intake can lower total cholesterol 78.36 mg/dL, total triglycerides 94,57 mg/dL, LDL levels 87.08 mg/dL and increased HDL level as much as 12.64 mg/dL in 10 weeks cares. Structured phospholipids intake also can prevent the thickening of the intima-media layer of the aorta.

Keywords— Structured phospholipids, commercial soybean lecithin, omega-3 fatty acid, atherosclerosis risk.

I. Introduction

One of the most important fatty acids for health is omega-3 fatty acid. Oil product of tuna fish (*Thunnus sp*) canning contains omega-3 fatty acid which is equivalent to omega 3 fatty acid in cod liver oil [1]. Omega-3 fatty acid product of tuna canning has limited stability of the low oxidation, therefore it need to look for the alternative of carrier agent of omega 3. The most stable omega-3 fatty acid is in form of phospholipids. Thus, the incorporation of omega-3 fatty acid to the structure of phospholipids is one of alternative to increase the stability. So, for health, there are double usages of structured phospholipids [2].

From the previous study, showed that the structured phospholipids which obtained from 18 hours acidolysis reaction with 30% enzyme concentration has the highest EPA-DHA fatty acid incorporation level and incorporation percentage [3]. The used of commercial soybean lecithin is more profitable as the carriers of omega-3 fatty acid, the price is cheaper and the quantity is higher also the process is not too difficult. The methods to make oil enrich omega-3 fatty acids product of tuna canning based on the crystallization of solvent in low temperature [4] which is more safety than urea crystallization which produced ethyl carbamate compound / urethane, which are dangerous and carcinogenic [5]. The method to corporate phospholipids of commercial soybean lecithin and omega-3 fatty acid was enzymatic acidolysis reaction, used lipase *R. miehei* [6].

Omega 3 fatty acids have hypocholesterolemia effect, such as omega 3 fatty acids of fish oil produce protective effect on aorta histopathology through hypolipidemia effect and decreased the thrombosis event [7]-[9]. Soybean lecithin will induct the reducing of plasma cholesterol in hypercholesterolemia rats and atherosclerosis lesion [10] [11].

Based on several studies mentioned above, it is important to study the potential of structured phospholipids in reducing the risk of atherosclerosis by in vivo testing in experimental rats. Tests were carried out by administering a structured phospholipid intake in the male white rat (*Rattus norvegicus*) *Sprague dawley* strain which fed with atherogenic diet for 10 weeks periods. The parameters observed include the examination of lipid profile (total cholesterol, total triglycerides, LDL and HDL level), histopathological examination and calculation of intima-media thickness (IMT) of the aorta.

II. Methods

A. Material and Tools

The materials used in this research were commercial soybean lecithin (PT. Panadia), fish oil (by product of tuna canning – PT. Aneka Tuna Indonesia), and Lipase *Rhizomucor meihei* (Sigma Co.). The chemicals used were mixed fatty acids standard, phospholipids standard BF3-methanol 14%, methylcloride, NaOH, benzene, KOH, HCl, acetone, SDS, phosphate buffer pH 7, hexane, sulfuric acid, TLC (silica gel G60 F254 as adsorbent), chloroform, methanol (p.a. Merck), ethanol, hexane, acetone, aquadest, dry ice, nitrogen, sucrose, CMC, Soybean oil, cholic acid, palm oil, yellow eggs, beef lard, formalin, NaCl, ethanol, xylol, hematoxylen-eosin (HE staining), paraffin, normal saline, propylene glycol, gelatin, cholesterol reagent kit (cholesterol, triglyceride, HDL & LDL precipitant, Diasys.co).

The tools used were water bath shaker, gas chromatography (Shimadzu), TLC Scanner (Shimadzu), TLC development tank, densitometer, oven, UV lamp, glassware, magnetic stirrer, rotavapour, pHmeter, microsyringe, refrigerator, filter flask, table balance, digital balance, freeze centrifuge, spectrophotometer, vortex, nitrogen gas sprayer, dissecting kit, feeding needle, spuit injection, microtube, incubator, microtome, microscope, haemocytometer, and microhematocrite.

B. Research Design

This research was conduct by provision of structured phospholipids to male rat (*Rattus norvegicus*) *Sprague dawley* strain. There were 5 treatments to the rats during the 10 weeks experiment periods. In every treatment includes 4 rats. The treatments were:

P0 : standard AIN-93M diet
P1 : atherogenic diet
P2 : atherogenic diet + commercial soybean lecithin
P3 : atherogenic diet + oil enriched omega 3 fatty acid
P4 : atherogenic diet + structured phospholipids

C. Analysis Methods

The analysis methods which are used are analysis of profile by thin layer chromatography phospholipids [12], profile and level of omega 3 fatty acids by gas chromatography (*in situ* transmethylation methods [13]). Analysis of lipid profile used Diasys reagent kit (total cholesterol, total triglyceride, HDL and LDL level), histopathology analysis of aorta and determination of intima-media thickness of aorta [14].

III. Results and Discussion

A. Lipid Profile Analysis

The lipid profile analysis was done to rat blood serum in three stage periods. It was done before adaptation period, after adaptation period (week 0) and every two weeks during 10 weeks periods of treatment. The average of lipid profile (cholesterol, triglyceride, HDL and LDL level) of rat during the maintenance can be seen on Figure 1,2,3 and 4. Then, the changing of lipid profile lipid average during the treatment can be seen on Table I.

Total Cholesterol

In this research, rat was fed with atherogenic diet (standard AIN-93M [15] modified by adding cholesterol, beef lard and cholic acid) which can increase the total cholesterol after period of adaptation during one week (week 0). It emphasized the total cholesterol in composition of atherogenic feed about 2000 mg/kg able to increase the total cholesterol. Adding 200 mg of cholesterol in 100 g feed can increase the cholesterol serum before the treatment up to hypercholesterolemia [16].

On Fig. 1, it could be seen on diet treatment of P1 group (positive control- atherogenic diet), the average of total cholesterol increase continuity from 106.15 mg/dL to 253.64 mg/dL in the end of treatment. In the diet treatment of P2 group, the average total cholesterol was decreased, the average total cholesterol about 233.57 mg/dL in the end. In P3 and P4 group treatment, the total cholesterol began decreased on the second week of treatment; it was about 134.21 mg/dL and 192.26 mg/dL in the end of the treatment period.

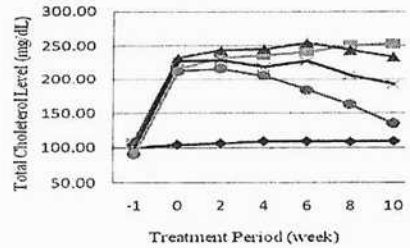

Fig. 1 Total cholesterol level of rat's treated by standard diet AIN-93M / P0 (◆), atherogenic diet/P1 (■), atherogenic diet + commercial soybean lecithin /P2 (▲), atherogenic diet + oil enriched with omega 3 fatty acid/P3 (X), and atherogenic diet + structured phospholipids/P4 (●)

Omega 3 fatty acid and soybean lecithin in structured phospholipids give synergic hypercholesterolemia effects. Phospholipids can decreased the plasma cholesterol by influenced absorption of cholesterol in intestine. Giving soybean phospholipids can improve the activity of LCAT enzyme [17]. Soybean phospholipids increasing the activity of lecithin-cholesterol acyltransferase (LCAT) in rat, it disturbed the absorption of cholesterol on ileum and increases the excretion of steroid through feces [18].

The mechanism of hypercholesterolemia omega 3 fatty acid was it could reduced the activity of HMGCoA-reductase [6], [19] and Acyl-coenzymA : cholesterol acyltransferase enzyme (ACAT) [20], also arranged the regulation of SREBP (Sterol Receptor Element Binding Protein) in reducing the regulation of gen PPARα (peroxisome proliferator activated receptor - alpha) [21] .

Omega 3 fatty acid contains of phospatidylcoline which can supressed the activity of fatty acid synthase significantly and increased the activity of carnitine palmytoil transferase and peroxisomal β-oxidation [22].

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TABLE I
Changes in Lipid Profile of Rats during the Period of Treatment

Parameter	Time Period	P0	P1	P2	P3	P4
Cholesterol Total	Week 0 (mg/dL)	104,42	215,64	231,92	226,99	212,56
	Week 10 (mg/dL)	109,42	253,64	233,57	192,26	134,21
	Average (mg/dL)	4,85^{c}	38,00^{c}	1,64^{bc}	-34,72^{b}	-78,36^{a}
Triglyceride Total	Week 0 (mg/dL)	64,36	114,65	109,57	117,97	104,9
	Week 10 (mg/dL)	68,55	162,28	111,90	94,44	84,92
	Average (mg/dL)	4,20^{b}	50,63^{c}	2,33^{b}	-23,52^{a}	-94,57^{a}
HDL Level	Week 0 (mg/dL)	57,12	52,56	56,67	55,26	55,96
	Week 10 (mg/dL)	56,31	43,82	54,46	57,64	68,60
	Average (mg/dL)	-0,87^{b}	-8,74^{c}	-2,21^{b}	2,39^{a}	12,64^{a}
LDL Level	Week 0 (mg/dL)	34,44	140,15	153,34	148,14	135,70
	Week 10 (mg/dL)	39,26	176,77	156,73	115,73	48,62
	Average (mg/dL)	4,82^{d}	36,32^{cd}	3,39^{c}	-32,41^{b}	-87,08^{a}

Note: Data are the average value of four replications
Values accompanied by different notations indicate significant differences in Tukey test ($\alpha = 0.05$)

Total Triglycerides

The provision of structured phospholipids contain of omega 3 fatty acid in which rat which fed with atherogenic diet could reduced the average of total triglyceride up to 95, 74 mg/ dL, besides giving only omega3 fatty acid reduced about 23,52 mg/dl (Fig. 2). It was different with giving phospholipids which not able to reduce the average of total triglyceride. It could increased the total triglyceride about 2,33% during the maintenance along 10 weeks.

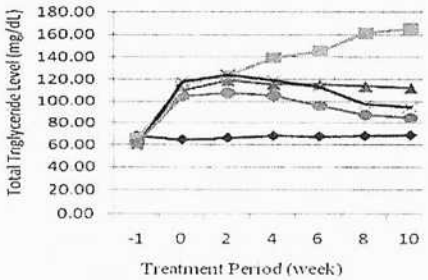

Fig. 2 Total triglyceride level of rat's treated by standard diet AIN-93M/P0 (◆), atherogenic diet/P1 (■), atherogenic diet + commercial soybean lecithin /P2 (▲), atherogenic diet + oil enriched with omega 3 fatty acid/P3 (X), and atherogenic diet + structured phospholipids/P4 (●)

The effect of reducing of the total lipids was possible because of the increasing of transport of lipid from blood to liver. Diet enriched phophatidylcholine from soybean lecithin will reduce the absorption cholesterol in ileum and decreased the cholesterol [23]. It controlled the biodynamic changing of lipid, by regulation of cholesterol homeostasis and fatty acid through the process of reducing the synthetic of cholesterol and fatty acids beside it increase the cholesterol oxidation became bile salt which increased the secretion of lipid.

Fish oil could reduce the level of triglyceride plasma significantly [24]. Omega 3 fatty acid increased the effect of hipotriglyseridemia by suppressed lipogenesis in liver by reduce the level SREBP-1c (*Sterol Receptor Element Binding Protein*–1c), it increased the regulation of oxidation fatty in liver and muscle through activation PPAR (*Peroxisome Proliferator-Activated Receptor*) and increased the rate of change of glucose become glycogenby pressing the regulation of HNF-4α (*Hepatocyte Nuclear Factor*-4α)[25]. Simultaneously, omega 3 fatty acid pressed the regulation of gen which coded protein which stimulated the fatty synthesis and increase the regulation of gen which protein coded that stimulated the fatty acid oxidation.

HDL Level

Phospholipids contains of omega3 fatty acid in rat's diet able to increase the average of HDL level about 12,64 mg/dL during ten weeks, besides omega 3 fatty acid only increased about 2,39 mg/dl. It compared to phospholipids input during the maintenance, reducing of the average HDL level about 2.21 mg/dl. The average of HDL level could be seen on Fig. 3.

The provision of phospholipids came from diet stimulated the production of HDL. CaCO2 cell in ileum will secreting ApoA-I which contains lipoprotein HDL [17]. The phospholipids which came from diet in ileum will incorporated directly in HDL then will increase the production of intestinal HDL.

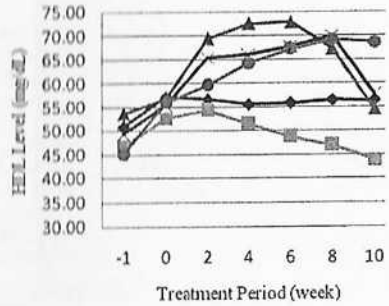

Fig. 3 HDL level of rat's treated by standard diet AIN-93M/P0 (◆), atherogenic diet/P1 (■), atherogenic diet + commercial soybean lecithin /P2 (▲), atherogenic diet + oil enriched with omega 3 fatty acid/P3 (X), and atherogenic diet + structured phospholipids/P4 (●)

DHA will increase the HDL level and HDL2 in heperlipidemia [26]. Omega-3 fatty acid, especially EPA and DHA was the inhibitor cholesteryl ester transfer protein (CETP). CETP is hydrophobic glycoprotein plasma which was synthesized by liver, its function transfer cholesterol ester [27]. CETP is the mediator to transfer cholesteryl ester from HDL become LDL or VLDL. The obstruction of CETP will increase the HDL level and support the transport back reaction of cholesterol.

LDL Level

The average of LDL rat which are given feed standard input tend to stable, between 35,77-39,29 mg/dL during maintenance. Atherogenic diet intake was able to increase the average of LDL level from 46, 88 mg/dl up to 176, 77 mg/dl. The provision of phospholipids was not able to reduce the average of LDL, it was increased from 153,34 - 256,73 mg/dL during ten weeks treatment periods. In other hand, the provision of omega 3 fatty acid and structured phospholipids which contains omega 3 fatty acids could reduce the average of LDL level during ten weeks periods. The reducing of LDL average was 148,14 - 115,73 mg/dl (P3) and 135,70 - 48,62 mg/dL (P4 group). The average of LDL content during 10 weeks treatment periods could be seen on Fig. 4.

Giving lecithin to the hypercholesterol rat could decrease the level of VLDL, IDL and LDL plasma also increase the level of HDL plasma. The changing of lipoprotein plasma maybe because of the role of lecithin which influenced the activity of lecithin:cholesteryl acyl transferase (LCAT), which increased the conformation of HDL plasma [28].

Omega 3 fatty acid in diet P3 and P4 reduced the average of LDL of mice. It caused by the role of omega 3 fatty acid in managed the lipogenesis which caused synthesis of VLDL decrease so the LDL level also decreases. Fatty acid disturbed the process of SREBP which stimulated the fatty acid oxidation. Peroxidation omega 3 fatty acid improving the degradation of apolipoproteinB meanwhile reduces the secretion of VLDL and LDL [26].

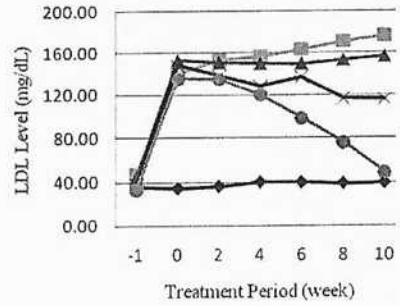

Fig. 4 LDL level of rat's treated by standard diet AIN-93M/P0 (◆), atherogenic diet/P1 (■), atherogenic diet + commercial soybean lecithin /P2 (▲), atherogenic diet + oil enriched with omega 3 fatty acid/P3 (X), and atherogenic diet + structured phospholipids/P4 (●)

B. Analysis of Rat's Aorta Histopathology and Intima-Media Thickness

Analysis of Rat's Aorta Histopatology

Results of hematoxylen-eosin staining in the aorta cross section and examination at 400x magnification light microscope could be seen on Fig. 5. It can conclude that the microscopic thickness of the aortic intima-media layer of P0 treatment group (standard diet AIN-93M) is the smallest compared with other treatment groups. The composition of the innermost layer of the aorta close to the lumen or direct contact with blood is the tunica intima is relatively flat not bumpy. It can be assumed that the arrangement and number of endothelial cells in aortic intima areas are still well preserved.

Boundary layer between the tunica intima with the media is not very clearly visible, and the tunica adventitia is also not clearly visible as well. The formation of foam cells in the tunica intima and smooth muscle cell migration into the tunica media tunica intima of the aorta have not seen.

Endothelial cells are very sensitive to the effects of oxidative stress and dyslipidemia conditions will lead to oxidative stress. This situation occurs due to disturbances of lipoprotein metabolism, are often referred to as the lipid triad, which includes increased levels of VLDL or triglycerides, decreased HDL levels and formation of a more atherogenic LDL [29]-[31].

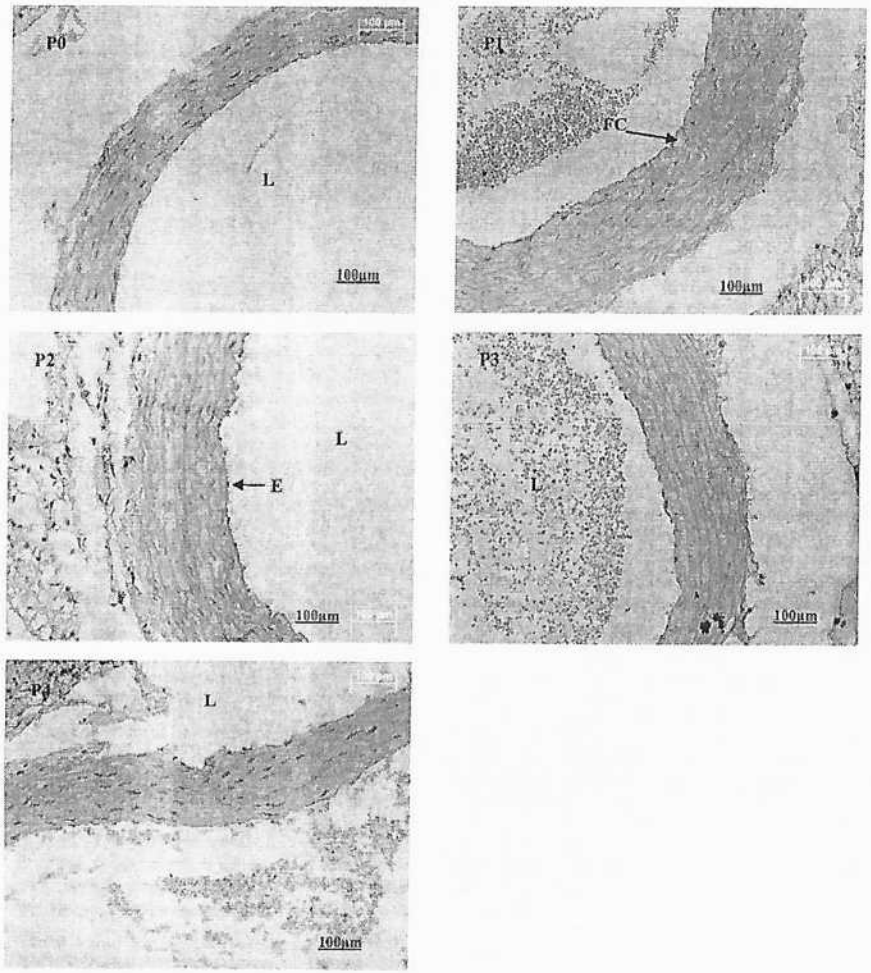

Fig. 5 Rat's aorta cross section (400x magnificent) treated by standard diet AIN-93M (P0), atherogenic diet(P1), atherogenic diet + commercial soybean lecithin (P2), atherogenic diet + oil enriched with omega 3 fatty acid (P3), and atherogenic diet + structured phospholipids (P4). Foam cell (FC), endotel cell (E) and lumen (L).

During the body's metabolism of protein molecules will be modified lipoproteins due process of oxidation, glycosylation and glycosydation with the end result will be an increase of oxidative stress and formation of radical oxygen species (ROS). Beside, sthe modified lipoprotein retention within the tunica intima would trigger atherogenesis.

The exposure of injury to the endothelium would trigger a variety of molecular and cellular mechanisms that induce atherosclerotic lesions. A high level of LDL is the main factor for endothelium and myocytes damage. The ability of oxidized LDL in causing foam cells will initiate atherosclerosis [32].

Analysis of Intima-Media Thickness (IMT) Rat's Aorta

The average of intima-media thickness of the aorta in rat which fed the standard AIN-93M diet during the treatment period was 103.81 µm, ranging from 102.96 to 104.86 µm. The thickness average of the aortic intima-media on atherogenic diet treatment groups (P1) was not much different from the group treated atherogenic diet + phospholipid (P2), it was 214.02 µm and 206.52 µm. While the average thickness of the intima layer of the aorta in the treatment group atherogenic diet + omega-3 (P3) was equal to 140.21 µm are not much different from the treatment of atherogenic diet + phospholipid-structured (P4), which is 131.82 µm. The intima-media thickness (IMT) average of aorta can be seen in Fig. 6.

Soy lecithin intake could increase the activity of paraoxonase (PON) and apolipoproteinA-1 (apoA-1 / apoA-I) in mice that the ApoE gene (ApoE null mice) has been disabled. Increased activity of both led to decreased lesion of atherosclerosis in ApoE-null mice. PON1 acts as an antioxidant that prevents oxidation of LDL during circulation, whereas apoA-1 is a major component of plasma HDL [10].

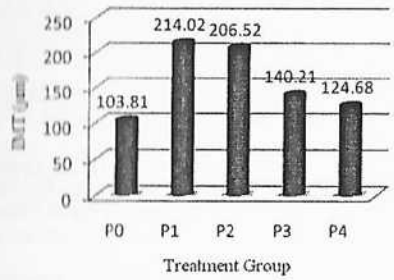

Fig. 6 Average of rat aorta's intima-media thickness treated by standard diet AIN-93M (P0), atherogenic diet (P1), atherogenic diet + commercial soybean lecithin (P2), atherogenic diet + oil enriched with omega 3 fatty acid (P3), and atherogenic diet + structured phospholipids (P4)

Fish oil intake in male wistar rats for 4 weeks resulted in a protective effect on histopathological aorta through hipolipidemia effect. Besides the intake of fish oil will decrease the activity of C-reactive protein (CRP) compared with the intake of saturated fatty acids and trans fatty acids. This CRP has effect on tissue inflammation [33].

Thickening of the aorta increases the risk of atherosclerosis, and exacerbated by the onset of inflammation and vascular pressure factor. Omega-3 fatty acids produce various eicosanoids and anti-inflammatory compound that can prevent the thickening of the aortic atherosclerosis and risk further into. Omega-3 fatty acids EPA-DHA in particular is able to prevent the occurrence of atherosclerosis by producing eicosanoids PGE1, PGI2, LXs, anti-inflammatory compounds such as resolvin and IL-4, IL-10 and TGF-β [27].

IV. Conclusion

Provision of structured phospholipids containing omega 3 fatty acids can reduce the risk of atherosclerosis on male *Sprague dawley* rats which fed with atherogenic diet. It may improve lipid profiles by lowering total cholesterol, total triglycerides and LDL levels and increase HDL levels. Besides, structured phospholipids can also reduce the occurrence of thickening of the aorta intima-media layer.

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Hypoglycemic Activity of Water Soluble Polysaccharides of Yam (*Dioscorea Hispida* Dents) Prepared by Aqueous, Papain, and Tempeh Inoculum Assisted Extractions

Teti Estiasih*, Harijono, Wenny Bekti Sunarharum, Atina Rahmawati

***Abstract*—** This research studied the hypoglycemic effect of water soluble polysaccharide (WSP) extracted from yam (Dioscorea hispida) tuber by three different methods: aqueous extraction, papain assisted extraction, and tempeh inoculums assisted extraction. The two later extraction methods were aimed to remove WSP binding protein to have more pure WSP. The hypoglycemic activities were evaluated by means in vivo test on alloxan induced hyperglycemic rats, glucose response test (GRT), in situ glucose absorption test using everted sac, and short chain fatty acids (SCFAs) analysis. All yam WSP extracts exhibited ability to decrease blood glucose level in hyperglycemia condition as well as inhibited glucose absorption and SCFA formation. The order of hypoglycemic activity was tempeh inoculums assisted->papain assisted- >aqueous WSP extracts. GRT and in situ glucose absorption test showed that order of inhibition was papain assisted- >tempeh inoculums assisted- >aqueous WSP extracts. Digesta of caecum of yam WSP extracts oral fed rats had more SCFA than control. Tempeh inoculums assisted WSP extract exhibited the most significant hypoglycemic activity.

Keywords: hypoglycemic activity, papain, tempeh inoculums, water soluble polysaccharides, yam (*Discorea hispida*)

I. INTRODUCTION

The genus *Dioscorea* or yam, which includes 600 to 700 species, is widely distributed throughout the world [1]. *Dioscorea hispida* or locally known as "gadung" is one of the *Dioscoreaceae* family, a family of creep plants that contains several bioactive compounds. *Dioscoreaceae* (*D. alata, D. batatas, D. bulbilfera, D. opposita*) has health beneficial compounds such as dioscorin [2, 3, 4], diosgenin [5, 6, 7], and water soluble polysaccharides (WSP) [8]. Dioscorin is the major storage protein in yam and functions against angiotensin, which converts enzyme to cause hypertension [9]. Diosgenin is used in making progesterone and other steroid drugs [10]. Recently, the biological activities of the polysaccharides of yam have attracted increasing attention in the biochemical and medical fields [8].

Water soluble polysaccharide of yam is a viscous mucilage containing glycoprotein [1, 11, 12]. Yam contains a large amount of water-soluble mucilage [1]. It has been reported that yam mucilage consists of glycoproteins and polysaccharides such as mannan and cellulose [13, 14]. Water soluble polysaccharides of *Dioscorea batata* consisted of acetylated mannan [8], viscous polysaccharides from wild yam consisted of mannose, arabinose, glucose, galactose, xylose and rhamnose [15] that attributed to soluble dietary fiber, meanwhile polysaccharides of *Dioscorea opposite* Thunb consisted mainly of mannose, and also contained uronic acid [16].

A high dietary fiber intake is emphasized in the recommendations of most diabetes and nutritional associations. It is accepted that viscous and gel-forming properties of soluble dietary fiber inhibit macronutrient absorption and reduce postprandial glucose response that associated with reduced diabetes risk [17]. Colonic fermentation of dietary fiber produces short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate [18]. Various short-chain fatty acids formed during fermentation depended on the specific carbohydrate as substrate, and had differences in physiological effects [19]. Some studies showed that WSP had hypoglycemic effect such as WSP. Nevertheless, there is no study to examine hypoglycemic activity of WSP from *Dioscorea hispida* tuber.

However, WSP of yam usually binds to protein and the existence of this protein is supposed to reduce the hypoglycemic activity. For this reason, we studied three extraction methods, namely aqueous, papain assisted, and tempeh inoculums assisted extraction to obtain crude WSP extract from *Dioscorea hispida*. The two later methods were aimed to partially remove protein from WSP. Papain is a proteolytic enzyme from papaya tree latex and widely used in food processing to hydrolyze protein. Meanwhile, tempeh inoculums consist of proteolytic microbes, *Aspergillus oryzae* and *Rhizopus oligosporus* that are supposed to hydrolyze WSP binding protein of *Dioscorea hispida*. Tempeh is an Indonesian traditional fermented soybean that involved proteolytic activity of these two molds during fermentation. Although proteolytic activities of papain and tempeh

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inoculums were not specific, applying both during WSP extraction was supposed to get more pure WSP and have more significant effect on hypoglycemic activities.

II.METHODS

A. Materials

Mature yam *Dioscorea hispida* tuber was obtained from local farmers, commercial crude papain and commercial tempeh inoculums were from local market. Other materials were SCFA standards, sugar standards (Sigma Co.), chemical reagent (analytical grade), alloxan monohydrate (Merck) glucose GOD FS, AIN 93M diet [20], white rats (*Rattus novergicus*) Wistar strain 2-3 month old and body weight of 131-239 g.

B. WSP Extraction

WSP extraction was conducted with three different methods, those were aqueous, papain assisted, and tempeh inoculums assisted extraction [21]. The preparation to obtain tuber slurry was done in similar way. Yam tuber was peeled and blanched by steaming, and then the tuber was crushed with water to get tuber slurry. The tuber slurry was sieved by sieving cloth and filtrate was added by papain for papain assisted extraction method, or tempeh inoculums for tempeh assisted extraction method. Meanwhile, aqueous extraction method was performed without any addition. The filtrate was incubated at 55°C for papain assisted extraction, meanwhile aqueous and tempeh inoculums assisted extractions were performed at ambient temperature. Starch separation was performed by centrifugation. Then, WSP was coagulated by solvent and dried in cabinet dryer. All WSP extracts were analyzed including free sugar composition by HPLC, protein by Kjedahl method, crude fiber, amylose according to AOAC methods [22], relative viscosity at concentration of 100 mg/100 mL at ambient temperature, and yield. Fresh tuber was analyzed for proximate, amylose, and HCN content.

Sugar analysis of polysaccharides was conducted as followed: About 10 g of WSP extracts was added by acetonitrile 80% and stirred until homogeneous. This solution was filtered by filter paper and evaporated to 1 mL of volume, and then filtered by millex 0.45 μm. Analysis was performed using sugar pack column at flow rate of 0.6 mL/min at 65°C, water as eluting solvent with refractive index detector.

C. Hypoglycemic activity

Hypoglycemic activity assay was referred to method of Ruzaidi et al. [23]. This experiment used 24 white rats (*Rattus novergicus*) strain Wistar. These rats were housed collectively at 20-25°C and were fed by AIN 93-M standard feeding *ad libitum*. Before experiment, the rats were adapted for a week. The treatment was conducted for 4 weeks. The rats were weighed every 3 days, and glucose blood level was analyzed every weeks. Rats were divided into 4 groups, 3 groups were treated by three types of WSP extracts and one group was control. Each group consisted of 6 rats.

The feed was referred to AIN 93 M [20] that consisted of 62.0692% maize starch, 14% casein, 10% sucrose, 4% soybean oil, 5% carboxy methyl cellulose (CMC), 3.5% AIN mineral mix, 0.18% L-cystine, 1.0% AIN vitamin mix, 0.25% choline bitartrate, and 0.0008% TBHQ. Yam WSB was fed orally for 28 weeks with dose of 400 mg/kg body weight. All rat groups were induced intraperitoneally to be hyperglycemia by alloxan 80 mg/g body weight 3 days before treatment. Only rats with blood glucose level of >126 mg/dL were used in this experiment. Every week, blood was taken retro orbital plexus after 16 hours fasting and analyzed for post-prandial blood glucose level. Blood sample was centrifuged at 4000 rpm and the supernatant was analyzed for blood glucose level that referred to glucose oxidase method [24]. This experiment procedure had been approved for ethical clearance from Veterinary Department – University of Brawijaya.

Glucose Response Test

Four group (control, treated by aqueous-, papain assisted-, and tempeh inoculums assisted-WSP extracts) of rats consisted of 3 normal rats were adapted to AIN 93-M feed for 3 days. Before blood withdrawn, rats were fasted for 16 hours and each rat was force fed by glucose at dose of 2 g/kg body weight and WSP extracts of 400 mg/kg body weight. This procedure followed method of Xie et al. [25] with some modification. Serum glucose concentration was analyzed at minutes 0, 30, 60, 90, and 120 after oral feeding of glucose and WSP extracts.

D. In Situ Glucose Absorption Test

At day 33th of experiment, rats were anesthetized and gut was withdrawn and reversed. Everted sac was immersed at WSP extract concentration of 10% and glucose concentration of 20%. Glucose absorption test was performed in a tube containing WSP extract, glucose, and everted sac. Physiological salt was poured into inner cavity of everted sac. The tube was placed at water bath shaker that have temperature of 36°C. Solution from inner everted sac was taken every 10 minutes at minutes 10, 20, 30, and 40. Glucose concentration of solution was analyzed by Nelson-Somogyi method.

E. SCFA Analysis

During surgical, caecum was taken and digesta was withdrawn for SCFA analysis. Digesta was centrifuged at 14000 rpm for 15 minutes. Supernatant of 1 μL was injected into gas chromatography (GC Shimadzu Seri GC 8), with column GP 1200 1% HPP30 on chromosorb waw 2 m in length, column temperature of 130°C, injector and detector temperature of 230°C. Carrier gas was N_2 with pressure of 1.25 kg/cm^2.

TABLE I
CHARACTERISTICS OF FRESH YAM (*Dioscorea hispida*) AND WATER SOLUBLE POLYSACCHARIDE (WSP) EXTRACTS OF YAM

Komponen	Fresh yam	Aqueous WSP Extract	Papain Assisted WSP Extract	Tempeh Inoculum Assisted WSP Extract
Yield (% db)	n.a.	9.15	12.02	11.05
Amylose (% db)	0.64	18	32	28
Protein (% db)	1.25	1.97	2.33	1.21
Crude Fiber (% db)	4.04	1.49	1.42	0.72
Free sugar				
* Glucose (ppm)	n.a.	101.10	840.29	1924.62
* Mannose (ppm)	n.a.	109.91	872.14	344.08
Water (% db)	80.23	n.a.	n.a.	n.a.
Ash (% db)	2.70	n.a.	n.a.	n.a.
Fat (% db)	0.57	n.a.	n.a.	n.a.
Carbohydrate (*by difference*, % db)	20.18	n.a.	n.a.	n.a.
Viscosity (cps)	n.a.	0.096	0.094	0.094
HCN (ppm)	19.81	16.06	2.29	6.88

n.a =not analyzed

F. Data Analysis

Data was analyzed using analysis of variance in nested experimental design, followed by Duncan Multiple Range Test.

III.RESULTS AND DISCUSSIONS

A. Characteristics of Yam WSP Extracts

Characteristics of fresh yam tuber and all WSP extracts were shown at Table 1. WSP extract yield was about 9-12% that higher than the yield of Gembili (*Dioscorea esculenta*) WSP (3-5%) [26]. Enzyme and microbial assisted extraction using papain and tempeh inoculum slightly increased the yield that it was supposed due to partially removing removing of WSP binding protein. Amylose content of WSP extracts was higher than fresh yam tuber. Amylose was an impurity in WSP extracts. *Dioscorea* starch has gelatinization temperature of 67-79°C [27] that during incubation at temperature of 55°C at papain asisited ESP extraction, some starch granules were supposed to gelatinize that increase their solubility and some concomitantly coagulated during WSP separation by coagulation. Protein content of WSP extracts was higher than that of fresh yam except tempeh inoculums assisted WSP extract. Protein in yam is a glycoprotein that binds to polysaccharides. During WSP extraction, protein was concomitantly extracted. It was expected that applying papain and tempeh inoculums during WSP extraction resulted more pure WSP extract including lower protein content. Papain is a proteolytic enzyme that expected to partially hydrolyze WSP binding protein, however higher protein content in papain assisted extracts compared to fresh yam was supposed to relate to papain contamination of WSP extract. Meanwhile, tempeh inoculums assisted WSP extract had slightly lower protein content than others due to proteolytic activity of tempeh molds although the decrease of protein content was not significant. Mold growth during fermentation produced biomass that contained protein [28]. The lowest crude fiber was found in tempeh inoculums assisted WSP extract. Tempeh inoculums beside has proteolytic acitivity also has amylolytic and cellulolytic acitivity [29, 20]. Previous study showed that *Rhizopus oligosporus*, one of tempeh inoculums mold, decreased crude fiber in rapeseed cake [31]. Free sugar of WSP extract consisted of glucose and mannose. Fu *et al.* [11] reported that mucilage of *Dioscorea* species of Keelung and Hualien No. 3 comprised of mannose (93,90-95,40%), meanwhile glucose, galactose, arabinose, and xylose were lower than 5%. Viscosity of all of WSP extracts was not significantly different. Slightly lower viscosity of papain assisted and tempeh inoculum assisted WSP extract was supposed due to partially liberation of protein. Cyanide (HCN) content decreased that due to solubility of free cyanide in the water during extraction. The lowest cyanide content was found at papain assisted WSP extract. Applying higher temperature of 55°C during incubation in extraction process might contribute to HCN vaporization.

B. Hypoglycemic Acitivity of Yam WSP Extracts

Yam WSP extracts had hypoglycemic activity that indicated by decreasing blood glucose level. Sharp decrease in blood glucose level was found in tempeh inoculums assisted WSP extract, followed by papain assisted WSP extract and aqueous extract (Fig. 1).

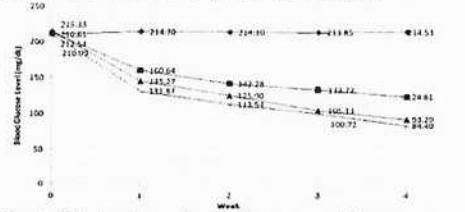
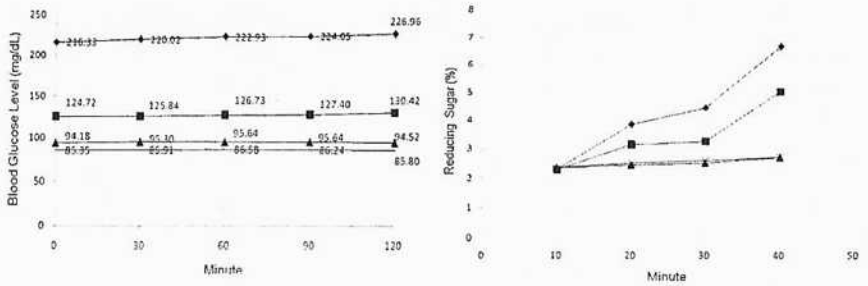


Fig. 1 Blood glucose level of rats treated by aqueous WSP extract (■), papain assisted WSP extract (▲), and tempeh inoculum assisted WSP extract (X) compared to untreated/control group (◆)



(a) (b)

Fig. 2 Glucose absorption inhibition in glucose response test (a) and *in situ* glucose absorption using everted sac (b). Aqueous WSP extract (■), papain assisted WSP extract (▲), tempeh inoculum assisted WSP extract (X), and untreated/control group (◆)

After three weeks treatment, hyperglycemic rats had normal blood glucose level for treatment by papain assisted WSP extract and tempeh inoculums assisted WSP extract, meanwhile aqueous WSP extract did not reach normal blood glucose level for 4 weeks treatment. Dietary fiber could decrease postprandial glucose because their viscous and gel forming properties that inhibits macronutrient absorption [17]. Gel structure was able to entrap glucose and other nutrient that slowed down absorption. However, there is no significant viscosity difference (Table 1) of all WSP extracts. Viscosity analysis was conducted at room temperature and the condition was different to that found in digestive tract. Other mechanism might be responsible to blood glucose level decline after yam WSP extracts feeding. The purity of extract might contribute to the ability of yam WSP extract in decreasing blood glucose level. We supposed that actually protein bound WSP in tempeh inoculums assisted WSP extract was the lowest, followed by papain assisted WSP extract. Both were more effective in decreasing blood glucose level than aqueous WSP extract.

It was interesting that tempeh inoculums assisted WSP extract had the highest ability in decreasing blood glucose level. In accordance, fermentation of Chinese yam (*Dioscorea batatas* Decne) flour by *Lactobacillus acidophilus, Streptococcus thermophilus*, and *Bifidobacterium bifidus* improved postprandial glucose [32]. Acidic hydrolysis of water soluble polysaccharides of *Tremella aurentia* decreased glucose plasma level due to formation of bioactive compounds [33]. Bioactive compounds formation such as aglycone isoflavones during tempeh fermentation depended on the substrates [34]. Bioactive compounds in *Dioscorea* were dioscorin [2, 3, 4], diosgenin [5, 6, 7], and WSP [8]. It was supposed that bioactive formation occurred during tempeh assisted WSP extraction that contributed to blood glucose level decline. Yam tuber contains diosgenin, a steroidal saponin that undergoes transformation into sapogenin [35]. Saponin in fenugreek (*Trigonella foenum*) could decrease blood glucose level [36]. Fermentation of *Dioscorea* by red mold *Monascus purpureus* NTU 568 resulted in more potent hypocholesterolaemic effect that supposed due to bioactive formation *monacolin* K [37]. Mc Cue et al. [38] reported that all of the soybean extracts possessed marked anti-amylase activity, with extracts of *R. oligosporus*-bioprocessed soybean having the strongest inhibitory activity, but only slight anti-glucosidase activity. There was a synergistic effect of tempeh inoculums and soybean as a substrate in hypoglycemic activity. Hence, it was possible that during tempeh inoculums assisted WSP extraction of yam *Dioscorea hispida* some bioactive compounds formed and contributed to hypoglycemic activity. This needed further research.

C. Glucose Response and In Situ Glucose Absorption Inhibition

Glucose response test showed that yam WSP extract was able to inhibit glucose absorption. This test revealed absorption after glucose ingestion. Glucose absorption inhibition was in order of papain assisted WSP extract>tempeh inoculums assisted WSP extract>aqueous WSP extract (Fig. 2a). Sharp rise in glucose blood level after glucose ingestion was found in untreated or control rats group. Similar results were found in *in situ* absorption test by using everted sac (Fig. 2b). Both methods indicated glucose absorption although both had differences. Glucose response test revealed glucose absorption inhibition in digestive tract and it did not indicate the effect of yam WSP extract in long time consumption such as SCFA formation. Meanwhile *in situ* glucose absorption test showed the ability to inhibit glucose absorption pass through intestines.

Papain assisted WSP extract showed the highest inhibition of glucose absorption either in GRT and in situ absorption test (Fig. 2ab). This was supposed due to the purity of WSP extract. More pure WSP extract, impurities that could interference polysaccharides action in glucose absorption inhibition was lower. Papain is a proteolytic enzyme that was supposed, to some extent,

partially hydrolyze WSP binding protein. Similar reason was applied to tempeh inoculums assisted WSP extract, although its glucose absorption inhibition was lower compared to papain assisted WSP extract.

It was supposed that glucose absorption inhibition was related to viscosity of WSP extracts, although all extract showed almost similar viscosity. Weak gel formation of yam WSP extract contributed to glucose absorption inhibition. Viscosity in digestive tract of each extract might be different. It was indicated by significant effect of types of extract on glucose absorption inhibition (α=0.05) meanwhile *in situ* glucose absorption inhibition was not affected by WSP extract types. Type of polysaccharides affected inhibition of glucose absorption. Polysaccharides of yam WSP mainly consisted of glucose and mannose or glucomannan. Previously, Chearskul et al. [39] showed that glucomannan taken before performing the oral glucose tolerance test can lower the rise of blood glucose. Similarly, Vuksan et al. [40] showed that glucomannan fiber added to conventional treatment may ameliorate glycemic control.

Dietary fiber decreases nutrition diffusion for absorption by intestinal mucosa thus decreases blood glucose level. Soluble dietary fiber was able to form gel in the existence of water in intestines. Gel formation slowed down the gastric emptying, fastened transit time, and controlled nutrition absorption. This affected glucose absorption and glycemic index of food products [18]. Previous research [41] showed that intake of diet containing cotton dietary fiber for diabetic patients resulted in lower rise of glucose compared to non cotton dietary fiber diet. Also, dietary fiber shortened transit time and bulk feces weight.

Furthermore, Madar et al. [41] revealed that physical properties of dietary fiber affected its ability in glucose absorption inhibition. Tempeh inoculum assisted WSP extract might be have different physical properties to papain assisted WSP extract. Tempeh inoculum contains *Aspergillus oryzae* and *Rhizopus oligosporus* that has proteolytic activity, as well as amylolytic and cellulolytic activities [29, 30]. Some WSP could be hydrolyzed by cellulolytic activity of tempeh inoculums. Therefore, physical properties of this soluble dietary fiber might be change that caused lower glucose absorption inhibition than papain assisted WSP extract.

D. Short Chain Fatty Acids

Not all the carbohydrate in the diet is digested and absorbed in the small intestine, and a significant portion arrives in the large bowel, where it is fermented by bacteria naturally present [18]. Dietary fiber could influence metabolism and SCFA production in colon [42]. Because fermentation in the hindgut may be elevated several hours after feeding, SCFA may be involved in the longer-term feeling of satiety [43]. Types of WSP extracts produced different composition of caecum SCFA (Fig. 3). In general, caecum of untreated rats had lower SFCA concentration then WSP extracts fed rats except aqueous WSP extract. Papain assisted WSP extract had the lowest SCFA concentration. This extract was supposed to be more pure than tempeh inoculums assisted WSP extract. Tempeh inoculums might be partially hydrolyze yam WSP due to cellulolytic acitivity. Meanwhile, in papain assisted WSP extraction, papain hydrolyze only protein. The purity of WSP extract perhaps affected ability to arrive in large bowel and fermentability in colon.

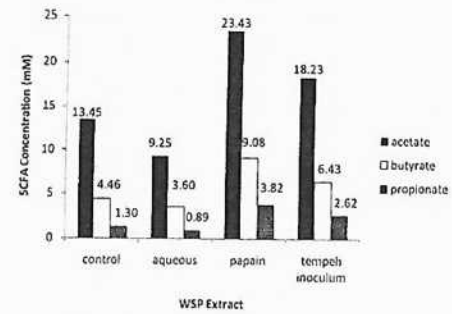


Fig. 3 Short chain fatty acids of caecum digesta of rats fed by WSP extracts

Certain indigestible oligosaccharides may benefit to gastrointestinal tract health via fermentation and proliferation of desirable bacterial species. Type of oligosaccharides influenced SCFA production [44]. Physicochemical properties of dietary fibers (DF) may influence their fermentation characteristics [45]. The physiological effects of these carbohydrates depend upon several factors including the extent of colonic fermentation and the fermentation products formed [46, 47]. The end products of colonic fermentation are SCFA and gases (CO_2, CH_4, and H_2) [45].

Mannose and glucose were the main sugar coumpounds of yam polysaccharides (Table 1). Glucomannan is one of fermentable polysaccharides [48]. Chen et al. [49] showed that fermentation of konjac glucomannan resulted in greater fecal acetate, propionate and butyrate concentrations and lower fecal pH. Predominant SCFA in caecum digesta was acetate, followed by butyrate and propionate. According to Lunn and Buttriss [18], all of these three SCFAs was predominant SCFA in colon Concentration of each SCFA was vary depending on fermented polysaccharides. In general, acetate was the highest abundance SCFA in digestive tract, and butyrate was the lowest. This research showed that *Dioscorea hispida* or yam WSP produced more butyrate rather than propionate. SCFA production is beneficial to health because of reducing hepatic glucose production [17]. Furthermore, increasing SCFA concentration in vena porta activates hepatic AMPK (*activated protein kinase*). APMK has a function to regulate energy production in cells and metabolic homeostatic regulation [50].

Nevertheles, not only fermentabililty of WSP contributed to blood glucose level reduction, but also some metabolic effects such as insulin sensitivity, hormone secretion modulation in digestive tracts, and other various related metabolism [17].

IV.CONCLUSIONS

WSP from *Dioscorea hispida* was hypoglycemic polysaccharides that able to reduce blood glucose level in hyperglycemia condition. Mechanisms of blood glucose level decline were glucose absorption inhibition and SCFA formation. Extraction methods in WSP extract preparation affected blood glucose level reduction. Tempeh inoculums assisted WSP extract had highest activity in reducing blood glucose level, meanwhile the highest glucose absorption inhibition was found in papain assisted WSP extract. It was supposed that there was a synergism between WSP extract and tempeh inoculums in reducing blood glucose level.

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