ISSN : 1410-5217
Acrredited : 83/Dikti/Kep/2009

Original Paper

THE INFLUENCES OF CHITOSAN FROM *Penaeus monodon* ON C-REACTIVE PROTEIN EXPRESSION IN AORTA AND CORONARY ARTERY OF SPRAGUE DAWLEY RATS BY HIGH FAT INDUCTION

Sri Isdadiyanto[1], Sukarti Moeljopawiro[2], Nyoman Puniawati[3], Hastari Wuryastuty[4]

[1] Department of Biology, Faculty of Science and Mathematic, Diponegoro University, Semarang, Indonesia
[2] Department of Biochemistry, Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia
[3] Department of Anatomy, Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia
[4] Department of Internal Medicine, Faculty of Veterinary Medicine,
Gadjah Mada University, Yogyakarta, Indonesia

Received : August, 1, 2012 ; Accepted : October, 5, 2012

ABSTRACT

The objective of this study was to analyze the influences of chitosan on C-Reactive Protein expression in aorta and coronary artery of Sprague Dawley rats by high fat induction. The animals for this study were 20 adult male rats divided into four groups, i.e. group I as the control was fed with basal diet containing normal fat for 3 months, group II was fed diet containing high fat for 3 months, group III was fed diet containing high fat and given chitosan 180 mg per kg body weight per day orally in 2 ml aquadest for 3 months, group IV was fed diet containing high fat for 3 months and after 1 month given chitosan 180 mg per kg body weight per day orally in 2 ml aquadest for 2 months. Each group consisted of five animals. After 90 days, the rats were necropsied and the hearts were collected to histopathological and immunohistochemical analysis by immunohistochemistry streptavidin-biotin method. C-Reactive Protein expression in aorta was negative. Chitosan was able to prevent atheroma plaque formation in coronary artery and CRP may involve in atherosclerosis.

Key words: Atherosclerosis, chitosan, CRP expression, endothelium, high fat diet

Corresponding: e-mail : isdadiyanto@yahoo.com

INTRODUCTION

Chitosan is the deacetylation's product of chitin which are found on the outer skin of *Penaeus monodon* (Hargono *et al.,* 2008). The last study was reported that natural biopolymer chitosan was synthesized using locally available shrimp type of *Penaeus monodon* (Sewvandi and Adikary, 2012).

Atherosclerosis, the most common cause of death in developing country is a complex process involving the interplay of genetic and environmental factors and the involvement of multiple cell types. Injury to the vascular endothelium is thought to iniate the atherosclerotic lesion (Maturana *et al.,* 2007).

Diet high lipid fed continuously is a major factor able to increase cholesterol and triglyceride level in plasma to cause hyperlipidemic. Hyperlipidemic for along term is able to cause atherosclerosis. Atherosclerosis is an artery disease that indicated with partial or whole thicking of blood vessels wall because of lipid accumulation with fibrous tissue formation, calcification is associated with alteration of tunica intima (Linder, 1992). The last study was explained that atherosclerosis was associated with increasing of LDL (Singh and Jialal, 2006). C-Reactive Protein, a major acute phase protein, has been associated with the presence of atherosclerosis, and has been found to predict acute cardiovascular events in prospective studies (Jialal *et al.,* 2004). Up to now, scientist still have been working to seek

ISSN : 1410-5217
Acrredited : 83/Dikti/Kep/2009

the way how to decrease or normalize cholesterol level in the blood. Alternative one was used to increase consumcion of dietary fiber that is chitosan. This fact, carapace of shrimp contains more chitosan compound that was effective to decrease cholesterol level in the blood (Hardjito, 2003).

Chitosan is derivation of chitin, an amino polysaccharide with acetylation process, to be found in exoskeleton and the shell of arthropoda like insect, crab, and shrimp. Chitosan and chitin is different on number of acetyl in their molecul. Chitin contains of 3-5% acetyl and can be made from chitin with acetylation in under 6 of pH (Vahouny *et al.,* 1983; Han, 2003; Fan *et al.,* 2006).

Chitosan is natural polymer, nontoxic, biocompatible and biodegradable (Hejazi and Amiji, 2003). Chitosan is able to bind fat in stomach before it is absorbed through the digestive system (Hardjito, 2003). Gallaher *et al*., (2002) state that the chitosan is a positively charged fiber that binds to negatively charged molecul like fat and bile salt to form an ionic binding. Fat binding of chitosan becomes a bulky masses so that they are not absorbed through the digestive system.

In accordance with Elleuch *et al.,* (2011), dietary fiber can be lower blood serum cholesterol levels and it was need for alteration process of cholesterol becomes bile salt. Wolever *et al.,* (1997) state that there are 4 mechanism decreases of cholesterol by dietary fiber i.e. 1. Bile acid binding in intestinum tenue that cause increase excresi of fecal bile acid, 2. Decrease absorbtion of fat and cholesterol, 3. Decrease absorbtion rate of carbohydrate that cause decrease levels of insulin serum then decrease of cholesterol and lipoprotein synthesis stimulus, and 4. Barrier cholesterol synthesis by short chain fatty acid that produced from soluble fiber fermentation in colon.

The objective of this experiment was to analyze the influences of chitosan on CRP expression in aorta and coronary artery of Sprague Dawley rats by high fat induction. Second objective of this experiment was to look for alternative natural material to overcome coronary heart disease by hyperlipidemic.

MATERIALS AND METHODS

Animal materials

Twenty male Sprague Dawley rats, 1,5 months of age were used as experimental animals. All rats were adapted in 20 single cages for 7 days and given basal diet containing normal fat and water *ad libitum*. After adaption, rats were divided into 4 groups of 5 each. Group I was fed with basal diet containing normal fat for 3 months, group II was fed diet containing high fat for 3 months, group III was fed diet containing high fat and given chitosan 180 mg per kg body weight per day orally in 2 ml aquadest for 3 months, group IV was fed diet containing high fat for 3 months and after 1 month given chitosan 180 mg per kg body weight per day orally in 2 ml aquadest for 2 months. After 90 days, each was fast at 12-14 hours. The rats were, then, necropsied and the hearts, including aorta, were collected to histopathological and immunohistochemical analysis.

Histopathological analysis (Farmilo and Stead, 2009)

Histopathological analysis of aorta and coronary artery used fixative 10% neutral buffered formalin, paraffin method, and Hematoxylin Ehrlich-Eosin staining method.

Immunohistochemical analysis of *CRP expression* (Key, 2009)

Immunohistochemical analysis with streptavidin-biotin method used primary antibody *Rabbit polyclonal Anti-C Reactive Protein antibody – Aminoterminal end-ab65842* and Invitrogen Histostain-SP kits.

Slide preparation were precoated with 0,1% poly-L- lysine in water, then air dried. All specimens were deparaffinized with xylene 3 x 2 minutes and rehydrated in a graded series of ethanol. Specimens washed with aquadest 2 x 2 minutes and then with phosphate buffer saline (PBS) 0,01 M pH 7,2 for 6 minutes. Specimens, then, were incubated in H_2O_2 3% (in absolute methanol 1: 10) for about 10 minutes. Specimens were incubated again in PBS for 6 minutes and then applied 2 drops (100 µL) or enough to cover completely tissue of serum

ISSN : 1410-5217
Acrredited : 83/Dikti/Kep/2009

blocking solution for 10 minutes. Specimens were applied 2 drops (100 µL) or enough to cover completely tissue of primary antibody (*Rabbit polyclonal Anti-C Reactive Protein antibody – Aminoterminal end-ab65842*) and incubated in moist chamber for 60 minutes and washed again in PBS for 5 minutes. Specimens were, then, applied 2 drops (100 µL) or enough to cover completely tissue of biotinylated second antibody for 10 minutes and washed again in PBS for 5 minutes. Specimens were, then, applied 2 drops (100 µL) or enough to cover completely tissue of enzyme conjugate (HRP-Streptavidin) for 10 minutes and washed again in PBS for 5 minutes. Specimens were added DAB (3'3 diaminobensidin) chromogen for 5 minutes. Specimens were washed with aquadest and incubated in hematoxylin counterstain for 3 minutes and washed with aquadest. Specimens were dehydrated with graded series of ethanol and added 2 drops (100 µL) of histomount to the slide and mount with coverslip. Immunohistochemical analysis of CRP expression was negative when did not form dark brown colour and positive when formed dark brown colour.

RESULTS AND DISCUSSION

Histopathologic analysis of aortas using Hematoxylin-Eosin staining method from rats that given normal diet for 3 months showed that there was no induce atheroma plaque. Aorta from rats given high fat diet induced atheroma plaque. Aorta from rats that given high fat and given chitosan 180 mg per kg body weight per day orally did not induce atheroma plaque. Aorta from rats that given high fat and after 1 month given chitosan 180 mg per kg body weight per day orally 40% did not induce atheroma plaque and 60% induced atheroma plaque.

Histopatologic analysis of coronary artery from rats that given normal diet for about 3 months showed that there was no atherosclerotic lesions. Coronary artery from rats that given high fat diet for about 3 months showed that induced atheroma plaques. Coronary artery from rats that given high fat diet + chitosan 180 mg per kg body weight per day orally showed that there was no atherosclerotic lesions. Coronary artery from rats that given high fat diet and then after 1 month given chitosan 180 mg per kg weight per day orally showed that there was several no atherosclerotic lesions and several induced atheroma plaques.

This experiment also be supported by experiment result from Wuryastuty *et al.,*(1995) and Adji (2006) stated that high fat diet that given in rats induced atheroma plaques in aorta. Libby and Theroux (2005) mentioned that high LDL cholesterol level as to cause accumulation of cholesterol in cell and induced atherosclerotic lesions in blood vessels wall.

Immunohistochemical analysis with streptavidin-biotin method using *Rabbit polyclonal Anti-C Reactive Protein antibody* of aortas showed that expression of CRP was negative for all treatments and was presented in **Table 1.** CRP expression of coronary artery for rats given normal diet was negative. CRP expression of coronary artery for rats given high fat diet was positive. CRP expression of coronary artery for rats given high fat diet and given chitosan 180 mg per kg body weight per day orally was negative. CRP expression of coronary artery for rats given high fat diet and after 1 month given chitosan 180 mg per kg body weight per day orally was positive. Immunohistochemical analysis positive when formed dark brown colour, this fact showed that rats given high fat induced inflammation in coronary artery and produced of CRP.

Immunohistochemical analysis with streptavidin-biotin method on CRP expression position is knowable when antigen (CRP) binding with anti CRP antibody in tissues, so that created presipitation and staining then is showed dark brown colour suitable with DAB (3'3 diaminobensidin) chromogen that used in this experiment. C-reactive protein is protein that may contribute to get inflammation in atheroma and also may be actively in early atherogenesis. Immunohistochemical analysis with streptavidin-biotin method on CRP expression of coronary artery rats are presented in **Table 2.** and **Fig. 1 - 4.**

ISSN : 1410-5217
Acrredited : 83/Dikti/Kep/2009

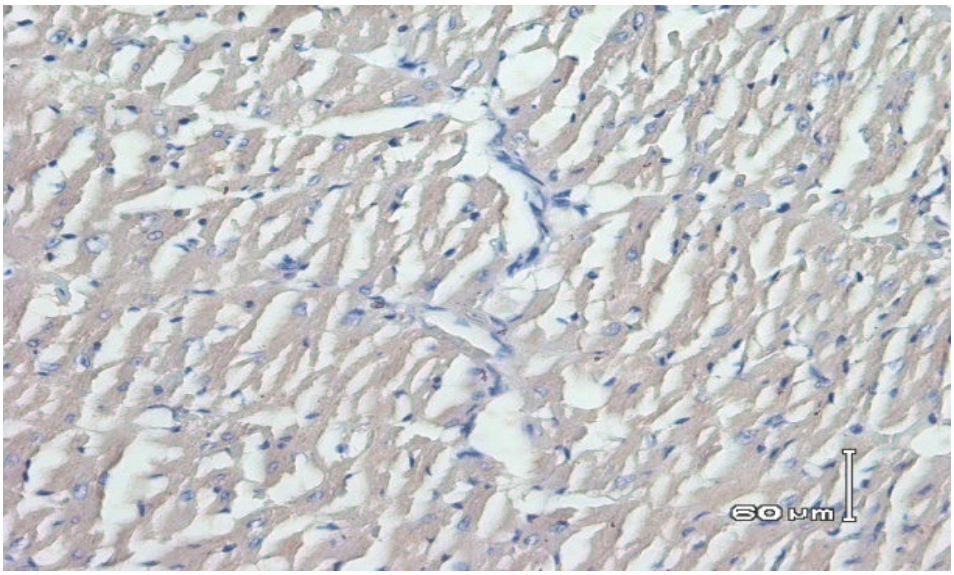

Fig. 1. Coronary artery of Sprague Dawley rats of Group 1, shows CRP expression negative. A. Lumen, B. Coronary artery wall (SB, 400x).

Table 1. Immunohistochemical analysis of CRP expression in aorta of Sprague Dawley rats

No.	Control	Group 1	Group 2	Group 3
1.	Negative	Negative	Negative	Negative
2.	Negative	Negative	Negative	Negative
3.	Negative	Negative	Negative	Negative
4.	Negative	Negative	Negative	Negative
5.	Negative	Negative	Negative	Negative

Table 2. Immunohistochemical analysis of CRP expression in coronary artery of Sprague Dawley rats

No.	Control	Group 1	Group 2	Group 3
1.	Negative	Positive	Negative	Positive
2.	Negative	Positive	Negative	Positive
3.	Negative	Positive	Negative	Positive
4.	Negative	Positive	Negative	Positive
5.	Negative	Positive	Negative	Positive

C-*reactive protein,* a major acute phase protein, is exclusively made in the liver and increased amounts of an acute inflammatory in the body. Be presumed that CRP also made in atherosclerotic lesions by smooth muscle and macrophage (Jialal *et al.*, 2004). Immunohistochemical analysis result with streptavidin-biotin method of aortas showed that expression of CRP was negative for all treatments, this fact means that CRP was not made in atheroma plaque in aorta. This result accords with experiment result of Adji (2007) which stated that although all aortas have atheroma plaque, the CRP may not be detected in the plaque. Atherosclerosis is a chronic inflammatory disorder. It is thought to begin with damage to the innermost layer of the artery called endothelium. According to Ross

ISSN : 1410-5217
Acrredited : 83/Dikti/Kep/2009

(1999), Endothelial dysfunctions includes increased endothelial permeability to lipoproteins and other plasma constituents, excression of growth factors that led to increased adherence of monocytes, macrophage and lymphocytes. These cells may migrate through the endothelium and situate themselves within subendothelial layer. In the vascular wall, macrophages accumulate lipids and become large foam cells. Foam cells, release growth factors and cytokines that promote migration of smooth muscle cells and promote neointimal proliferation, continue to accumulate lipid and support endothelial cells dysfunction. Foam cells, T-cells and smooth muscle cells eventually form the fatty streak. CRP in this experiment may not be detected in aorta tissue because possible CRP in circulation was not reached yet to atherosclerotic tissue and atherosclerotic tissue was not produced yet of CRP.

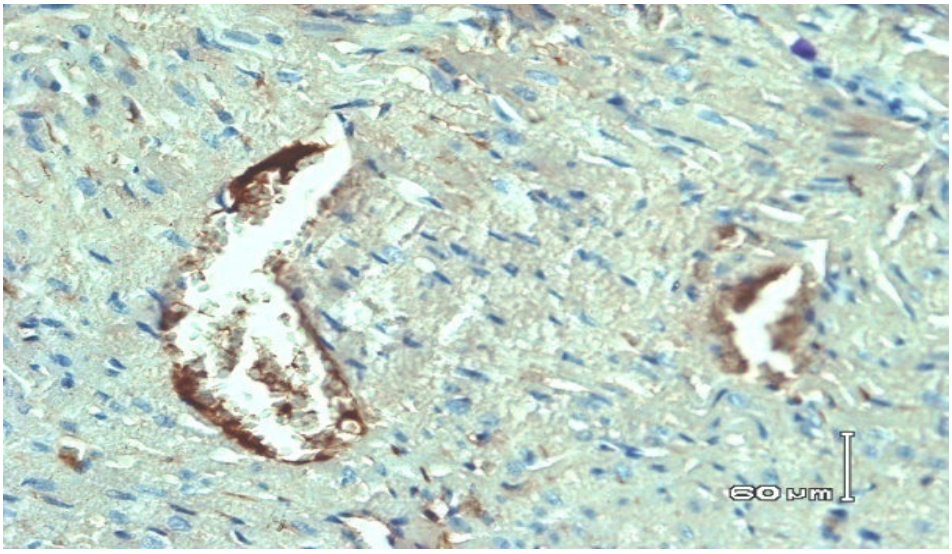

Fig. 2. Coronary artery of Sprague Dawley rats of Group 2, shows CRP expression positive. A. Lumen B. Coronary artery wall C. CRP showed dark brown colour (SB, 400x).

According to Key (2009) which stated that the principle of Immunohistochemical analysis with streptavidin-biotin method if there were many bounds of specific antigen and antibody so that created presipitation and staining then is appeared colour. Immunohistochemical analysis result with streptavidin-biotin method of coronary artery was positive for group II that rats given high fat diet **(Fig. 2.)** and group IV that CRP expression of coronary artery for rats given high fat diet and after 1 month given chitosan 180 mg per kg body weight per day orally (**Fig. 4.**). Positive result of CRP expression is meaning that treatment high fat lipid was induced inflammation in atheroma and also can be as a marker in early atherogenesis. This result accords with conclution of Pepys and Hirschfield (2003) which stated that increasing production of CRP was as an inflammation marker, may be to predict atherotrombocis events and atherogenesis process. It is also supported by experiment result from Jialal *et al.,* (2004) which stated that CRP was produced in atherosclerotic lesion (by smooth muscle and macrophage) and was produced because induction of lipid peroxidation and infection. Proinflammation effects and proatherogenic proved be present in endothelial cells consequence for decrease of nitric oxide, prostasiklin, increase of endotelin-1, cell adhesion molecul, and MCP-1.

CRP expression of coronary artery for rats given high fat diet and given chitosan 180 mg per kg body weight per day orally simultaneous was negative (**Fig. 3.**), same as group of control (**Fig. 1.**). This fact means that chitosan and high fat diet given simultaneous was able to prevent inflammation in coronary artery so that not be formed dark brown colour.

ISSN : 1410-5217
Acrredited : 83/Dikti/Kep/2009

Further, Jialal *et al.* (2004) so stated that CRP in smooth muscle cells will be induced increasing receptor AT-1 (Angiotensin type-1), increasing oxygen radical (ROS), increasing proliferation of vessel smooth muscle cell, increasing NFkb, increasing MAP cynase, and increasing iNOS. In endothelium cell, CRP will be induced decreasing eNOS, prostasiklin, increasing PAI-1, ET-1, MCP-1, ICAM, VCAM, E-selectin and IL-6. Whereas in monocyt (macrophage), CRP will be induced increasing ROS, sitokin (IL-1, TNFα, IL-6), chemotactic factor, tissue factor, uptake LDL oxidasied, adhesion in endothelium cell and MMP-1.

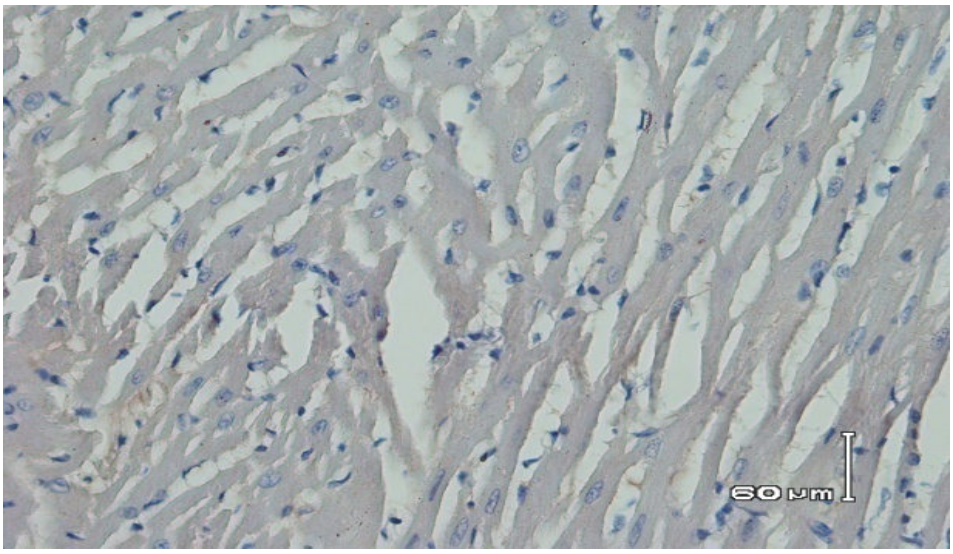

Fig. 3. Coronary artery of Sprague Dawley rats of Group 3, shows CRP expression negative. A. Lumen B. Coronary artery wall (SB, 400x).

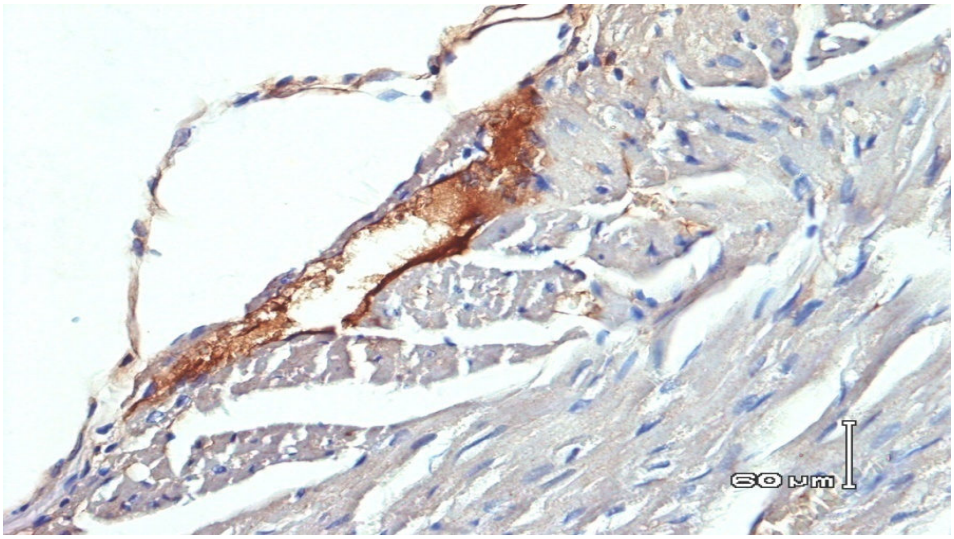

Fig. 4. Coronary artery of Sprague Dawley rats of Group 4, shows CRP expression positive. A. Lumen B. Endothelium cel C. CRP showed dark brown colour (SB, 400x).

ISSN : 1410-5217
Acrredited : 83/Dikti/Kep/2009

CONCLUSIONS

Based on the result of this study, it was concluded that chitosan was able to prevent atheroma plaque formation and it was as alternative natural material to overcome coronary heart disease by hyperlipidemic.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge to Prof. drh. H.R. Wasito, M.Sc., Ph.D., who has given advice, supporting and understanding of Immunohistochemical analysis.

REFERENCES

Adji, D. 2006. The relationships amongs high fat diet, surgery and LPS of Escherichia coli 0111:B4 in atherogenesis, The study to see the role of C-reactive protein in atherosclerosis. Disertation. Gadjah Mada University. Yogyakarta. Indonesia.

Adji, D. 2007. Immunohistochemistry method to detect C-reactive protein in atheroma plaques of Sprague dawley rats fed high lipid ration. *J. Sain Vet*. 25(I): 23-29.

Elleuch, M., D. Bedigian, O. Roissex, S. Besbes, C. Blecker, and H. Attia. 2011. Dietary fibre and fibre-rich by product of food processing: Characterizations, technological functionality and commercial application: A review. *Food Chem.* 124: 411-421.

Fan. D. X. Zhu. M. Xu. and J. Yan. 2006. Adsorption properties of Chromium by chitosan coated montmorillonite. *J. Biol. Sci.* 6:941-945.

Farmilo, A.J., and R.H. Stead. 2009. Immunohistochemical staining methods: Fixation and processing. Fifth Edition. Dako. North America. Carpinteria. California. Pp. 29-34.

Gallaher, D.D., C.M. Gallaher, G.J. Mahrt, T.P. Carr, C.H. Hollingshead, R. Heslink Jr., and J. Wise. 2002. A glucomannan and chitosan fiber suplement decreases plasma cholesterol excretion in overweight normocholesterolemic humans. *J. Am. Coll. Nutr.* 21: 428-433.

Han, S.M. 2003. Chitin and Chitosan for Versatile Application. The School of Polimer and High Performance Materials of Southern Mississipi.

Hardjito, L. 2003. Chitosan more full of life and safe. Examination Report. Faculty of Fisheries and Marine Sciences. Bogor Agricultural University. Bogor.

Hargono, Abdullah, and I. Sumantri. 2008. Production of chitosan is made of the *Penaeus monodon* shell waste and application to serum cholesterol reduction. *Reaktor*. 12:53-57.

Hejazi, R. and M. Amiji. 2003. Chitosan-based gastrointestinal delivery systems. *Journal of Controlled Release.* 89: 151-165.

Jialal, I., S. Devaraj, and S.K. Venugopal. 2004. C-Reactive Protein: Risk marker or mediator in atherothrombosis. *Hypertension*. 44: 6-11.

Key, M. 2009. Immunohistochemical Staining Methods: Immunohistochemistry staining methods. Fifth Edition. Dako. North America. Carpinteria. California. Pp. 57-59.

Libby, P. and P. Theroux. 2005. Pathophysiology of coronary artery disease. *Circulation*. 111:3481-3488.

Linder, M.C. 1992. Nutritional Biochemistry and Metabolism. Elseiver. London.

ISSN : 1410-5217
Acrredited : 83/Dikti/Kep/2009

Maturana, M.A., M.C. Irigoyen and P.M. Spritzer. 2007. Menopause, estrogens, and endotelial dysfunction: Current concepts. *Clinic Version Impresa*. 62: 1807-5932. Sao Paulo.

Pepys, M.B. and G.M. Hirschfield. 2003. C-reactive protein: A critical update. *J. Clin. Invest*. 111: 1805-1812.

Ross, R. 1999. Atherosclerosis An Inflammatory Disease. *N.E.J.M.* 340: 115-126.

Sewvandi, G.A. and S.U. Adikary. 2012. Synthesizing and characterization of natural biopolymer chitosan derived from shrimp type, *Penaeus monodon. Tropical Agricultural Research*. 23:272-276.

Singh, U. and I. Jialal. 2006. Oxidative stress and atherosclerosis, *Pathophysiology*. 13:129-142.

Wolever, T.M.S. R.A. Hegele. P.W. Connelly. T.P.P. Ranson. J.A. Story. E.J. Furumoto, and D.J.A. Jenkisn. 1997.Long-term effect of soluble-fiber foods on postprandial fat metabolism in dyslipidemic with E3 and apo E4 genotypes. *Am. J. Nutr.* 66:584-590.

Wuryastuty, H., R.Wasito and S. Raharjo. 1995. Peroxidation index: Methods and diagnostic value. A Reseach Report University Research for Graduate Education. Directorate General of Higher Education, Jakarta, Indonesia.

Vahouny, G.V. W.E. Connors, S. Subramanian, D.S. Lin, and L.L. Gallo. 1983. Comparative lymphatic absorbtion of sitosterol, stigmasterol, fucosterol and differential inhibition of cholesterol absorbtion. *Ann. J.Clin. Nutr.* 33: 576-589.