ANTIOXIDANT ACTIVITY OF HYDROLYZED MELINJO (*GNETUM GNEMON*) SEEDS PROTEIN AGAINST NEUTROPHIL SUPEROXIDE RADICAL IN VITRO

[1]A.A. ISTRI PUSPITA SARI DEWI, [2]I DEWA AYU SUSILAWATI, [3]TRI AGUS SISWOYO

[1,2]Department of Biomedical Faculty of Dentistry, Jember University, Indonesia
[3]Facultyof Agriculture, Jember University, Indonesia
E-mail: [1]121610101087@students.unej.ac.id, [2]dewasusi@unej.ac.id, [3]triagus.faperta@unej.ac.id

Abstract— Superoxide radical (O_2^-) is one type of reactive oxygen species (ROS) that play important role in pathogenesis of various inflammatory diseases. The main source of ROS is inflammatory cell neutrophil, that will produce a large amount of ROS to response against antigen. This study purposed to analyze antioxidant activity of the hydrolyzed melinjo (*Gnetum gnemon*) seeds protein (Gg-PH) against superoxide radical produced by neutrophil. This in vitro study used neutrophil isolated from human peripheral blood vein using Histopaque Gradient Centrifugation method. Briefly, neutrophil were incubated with antigen and antioxidant for 1 hour and 18 hours. Antioxidant activity were demonstrated by reduction of superoxide production. Experiments were divided into three groups, (1) neutrophil + antigen + Gg-PH, (2) neutrophil + antigen + glutathion (GSH), (3) neutrophil + antigen. The antigen is formyl-methionyl-leucyl-phenylalanine (fMLP). Superoxide production was analyzed by means of Nitro Blue Tetrazolium (NBT) assay. Intracellular superoxide were demonstrated microscopically, whereas extracellular superoxide spectrophotometrically. Result showed that incubation of Gg-PH for 1 hour reduced intracellular superoxide production significantly ($p<0,05$), but not for extracellular superoxide ($p>0.05$). Incubation for 18 hours could not prevent superoxide production, both intracellular and extracellular. Inconclusion Gg-PH had antioxidant activity against intracellular superoxide radical for 1 hour incubation but not in extracellular. Effect of dosis and duration of incubation need to be analyzed further.

Keywords— Antioxidants, *Gnetum gnemon* Protein, Neutrophil, Superoxide Radical, Nitro Blue Tetrazolium (NBT)

I. INTRODUCTION

Free radicals are atoms or molecules that have unpaired electrons in its outer layer [1]. The most important free radicals in the body is reactive oxygen species (ROS)[2].One of them is superoxide radical (O_2^-) [3].

Neutrophil is the first line cell that play a role in body defense mechanism [4]. In doing so, neutrophil develope microbisidal mechanism,mainly oxidative mechanism (respiratory burst) which is performed by ROS[5]. Respiratory burst accompany by production of a large amounts of superoxide radical that occured when neutrophilstimulated by antigen through the reduction of oxygen (O_2) by Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase[6].

Free radical is important to destroy antigen, but if it is produced more excessivethan cellular antioxidant, it will damage the cell itself[7]. Reactivity of free radical and tendency to form a new radical if it meets other molecules causes chain reaction. Cell damage and chain reaction are dangerous because it can cause a variety of inflammatory diseases[8], [9].

Free radicals can scavenge using antioxidants. Antioxidants are compounds that in low concentrations can stabilize free radicals by donating electrons and inhibiting the chain reaction without change its function[10].One of natural source of antioxidants that have been studied is melinjo (*Gnetum gnemon*) seeds[11].

Previous research showed that hydrolyzed melinjo (*G.*gnemon) seeds protein (Gg-PH) has a potential active as an antioxidant[12], however no studies have been developed its activity against radicals produced by human cell. This study purposed to analyze antioxidant activity of Gg-PH against human neutrophil superoxide radicalin vitro.

II. DETAILS EXPERIMENTAL

2.1. Materials and Procedures

Human neutrophils were isolated from venous blood obtained from healthy adult donor after compliting informed consent and in accordance with ethical approval fromMedical Faculty, University of Jember, Indonesia. *G. gnemon* seeds were collected from the collection of Center for Development of Advanced Sciences and Technology (CDAST), University of Jember, East Java, Indonesia. Glutathione (GSH) was purchased from Sigma, Pyrogallol was purchased from Sigma,2,2-Azino-bis(3-ethylbenzothiazoline-6sulfonic Acid)(ABTS) was purchased from Wako, Histopaque 1119 was purchased from Sigma, Lymphocyte Separation Medium was purchased from MP, N-Formyl-Met-Leu-Phe (fMLP) was purchased from Sigma, Nitro Blue Tetrazolium (NBT)was purchased from ScyTek, Penicillin-Streptomycin was purchased from Sigma, Fungizone-Amphotericin B was purchased from Gibco, and Hanks'Balanced Salt Solution (HBSS) was purchased from Gibco.

This study consisted of two stages. The first stage aimedto obtain Gg-PH protein that have antioxidant activity against 2,2-Azino-bis(3-ethylbenzothiazoline-6 sulfonic Acid) (ABTS) radical (general antioxidant activity)and furthermore we testagainst superoxide radical using Pyrogallol

method. The concentration of Gg-PH protein which had the highest antioxidant activity against superoxide radical Pyrogallol then applied as an antioxidant protein in the next stage. The second stage aimed to examine the activity of Gg-PH protein against neutrophil superoxide radical using Nitro Blue Tetrazolium (NBT) assay.

Melinjo Seeds Extraction. Extraction of *G. Gnemon* seeds was done by method described by Siswoyo[11]. After washing and peeling, 123 g of sample seed then was grounded in a blender, and the resulting meal was extracted with distilled water (1:3) for 30 minutes. The homogenate result filtered with filter paper to remove larger particulate and then centrifued (10000 rpm for 10 min at 15 ^{0}C). Supernatant obtained called *Gnetum gnemon* crude protein (Gg-CP).

Melinjo Seeds Protein Isolation. The pH of Gg-CP was set to 8, then centrifued 10000 rpm for 10 min at 15 ^{0}C. pH of resulting supernatant was then regulated to obtain pH 4 and incubatedfor 30 minutes. After that, the solution was centrifued (10000 rpm for 10 min at 15 ^{0}C). Protein precipitate was then dissolved in distilled water and the pH was set to 8 [11]. Result of isolation called *Gnetum gnemon* protein isolates (Gg-PI).

Melinjo Seed Protein Hidrolysis. Gg-PI was hydrolyzed using alcalase enzyme for 5 hours at 50 ^{0}C. Termination of enzymes actually was done by heating at 95 ^{0}C for 10 min and then centrifuged (5000 rpm for 1 min at 27 ^{0}C) [13]. Supernatant was analyzed as *Gnetum gnemon* protein hydrolysates or hydrolyzed melinjo seed protein (Gg-PH).

Determination of Soluble Protein. Total protein soluble was measured by the method of Bradford [14], used 5 µl protein sample, 45 µl distilled water, and 950 µl Bradford. The absorbance was measured with a spectrophotometer λ 595 nm. The results obtained compared with standard Bovine Serum Albumin (BSA).

Degree of Hydrolysis. Measurement degree of hydrolysis by the method of trinitro-benzene-sulfonic-acid (TNBS) [13], 10µl Gg-PH was added to 415 µl Na phosphate buffer 0.2 M pH 8, and 200 µl 0.1% TNBS. The mixture then was dark incubated for 30 min at 50^{0}C, after that added 400 µl sodium sulfite (Na_2SO_3) 0.1 M, and incubated for 15 minutes. Degree of absorbance was measured using spectrophotometer λ 420 nm. A solution of 1.5 L-Leucine was used as a standard. The percentage of the degree of hydrolysis (DH) was determined by equation DH= h/h_{tot} x 100%, h is the number of hydrolyzed peptide bonds and h_{tot} is the number of peptide bonds per equivalent protein.

ABTS Radical Scavenging Activity. ABTS radical scavenging activitiy of Gg-PH as described by You [15] with slight modification. ABTS solution was prepared by mixed of 7 mM ABTS and 2.45 mM potassium persulfate. The mixture then dark incubated for 16 h at room temperaturebefore use. Working stockmade by diluted ABTS solution with 0.2 M sodium phosphate buffered saline (pH 7.4) until absorbance of 0.70 ± 0.02(λ734 nm). Absorbance of solution measured at λ734 nm. ABTS scavenging activity calculated as $[(A_c - A_s)/A_c]$ x 100%, where $A_c$is absorbance of control and A_s is absorbance of sample.

Superoxide Radical Scavenging Activity. Superoxide radical scavenging activityused method of autooxidation of pyrogallol described by Tang [16] with slight modification. Gg-PH 0.1 mL was mixed with 1.8 mL of Tris-HCI buffer 50 mM (pH 8.2). Mixture incubated for 10 min, then 0.1 mL of pyrogallol 10 mM (dissoluble in 10 mM HCI) added. Absorbance of solution measured for 4 min (λ 320 nm). Oxidation rate was calculated as the slope of the absorbance line (A_s). Autooxidation rate pyrogallol for control measured with 1.0 mL of double distilled water (A_0). The superoxide radical scavenging activity calculated as $[(A_0 - A_s)/A_0]$ x 100%.

Neutrophil Superoxide Radical Scavenging Activity. Neutrophil superoxide radical scavenging activities of Gg-PH were determined using NitroBlue Tetrazolium (NBT) method [17].This assay is conducted by observe neutrophil containing blue NBT formasan deposit. Neutrophilswere prepared fromheparinized whole blood. Neutrophil isolation was done by method of double Ficoll Hypaque centrifugation using Ficoll Hypaque (d=1.119 g/mL) and Lymphocyte Separation Medium (LSM). Speed of centrifugation was 1900 rpm for 30 min at temperature 21 ^{0}C. The neutrophil pellet obtained was then added by HBSS to final volume 1200 µl and it was further divided into 12 chambers (@100 µl). Antimicrobial agents were 114 µl Peniciline-Streptomycine and 36 µl Fungizone Amphotericin that diluted in 7200 µl HBSS.

This experiment was divided into 3 groups, there were Gg-PH, GSH, and FMLP groups. Each group was further divided into two subgroups, 1 hour group and 18 hours group. Each group consisted of two chambers. Gg-PH group was exposed to 30 µg Gg-PH, 10^{-7} M fMLP & 250 µl NBT, GSH group was exposed to 10 µg GSH, 10^{-7} fMLP & 250 µl NBT, whereas the FMLP group was induced by 10^{-7} fMLP & 250 µl NBT. Control group was used HBSS. The mixture was incubatedin an incubator shaker at 37 ^{0}C. Intracellular superoxide production was observed microscopically (light microscope) used safranin as counter strain. Visualization used Opti Lab magnetization 10^3. Percentage of neutrophil cell that containing formazan blue particle was determined by evaluating 100 cells randomly under a microscope. Extracellular superoxide production was observed spectrophotometrically using microplate readerλ 630 nm. HBSS was used as a standard solution.

Data Analysis. The intracellular and extracellular production of superoxide radical were analyzed by Two Way Annova and Post Hoc LSD.

III. RESULTS AND DISCUSSION

Isolationof melinjo seed protein was done by method of isoelectric precipitation. This isolation was produced Gg-PI at concentration 5.919 µg/µl. Isoelectric precipitation is a method of deposition protein based on isoelectric point. The solubility of protein becomes minimal when it approached isoelectric point (pH 4) as a result of an increase in electrostatic interaction between protein molecules so that electrostatic interaction with water being minimal. This isolation aimed to separate antioxidant protein from other substance contained in melinjo seeds that have antioxidant activity, so that the antioxidant activity in this study pure from protein melinjo [18].

Hydrolisis of melinjo seed protein produced Gg-PH at concentration 3.187 µg/µl. This hydrolysis process was produced a mixture of small peptides and amino acids (Gg-PH) by solving of complex peptide bond. Hydrolisis aims to disrupt the primary structure or protein denaturation, which increase the accessibility of soluble oxidative amino acid [19], [20].Increase exposure to soluble amino acid peptide causes reaction of peptide and free radical increase, so that the antioxidant activity of Gg-PH also increaseand higher than Gg-PI dan Gg-CP[21], [22].Degree of hydrolysis was 38% means that decomposition of complex peptide bonds into simple peptide bonds and various amino acids was about 38% (from 100% hydrolisis total value)[13].

ABTS assay showed Gg-PH had scavenging activity80%, it washigher than Gg-PI dan Gg-CP at the same concentration (6 µg) that used. Gg-PH peptide size that aresmaller and simpler enhance bonding between peptide and free radical thus increasing itsantioxidant activity[23].Gg-PH scavenging activity against ABTS radical shown the ability of Gg-PH donates electron or hydrogen atom so that free radical compounds being more stable[24]. This ABTS assay was conducted as general screening of antioxidant potential Gg-PHand determine stage of purification protein with highest scavenging activity, that in this study was Gg-PH so it was used as an antioxidant protein in superoxide neutrophil assay [25], [26].

Pyrogallol assay showed Gg-PH at concentration 30 µg had the highest antioxidant activity (30,38%). This indicated Gg-PH 30 µg waseffective to scavenge production of superoxide radicals Pyrogallol and this concentration was used in superoxide neutrophil assay. Concentration above 30 µg showed stasioiner condition, it may occured because Gg-PH binding ability of superoxide radical in this Pyrogallol assay maximum at concentration 30 µg. Gg-PH scavenging activity against superoxide radical Pyrogallol demonstrate the ability of Gg-PH donates electrons or hydrogen atoms[27].Gg-PH that had been tested the antioxidant activity against ABTS radical and superoxide radical Pyrogallol applied as an antioxidant protein in superoxide neutrophil assay.

Gg-PH has antioxidant activity because it is contains of cysteine amino acid withthiol/sulfhydryl group (-SH) that can react with free radicals or electron deficient compound, and stabilize free radical by donating electron along H atom. Thiol (-SH) that oxidized will form a stable covalent disulfide bond/cystine (R-S-S-R) with reaction as follow $2RSH \rightleftharpoons RS\text{-}SR + 2H^+ + 2e^-$[11], [28], [29].

The average numbers of neutrophil that produce intracellular superoxide radical is presented in Fig. 1, whereas the average of extracellular superoxide radical production is presented in Fig. 2. Microscopic pictures of neutrophil intracellular superoxide radical assay are presented in Fig. 3.

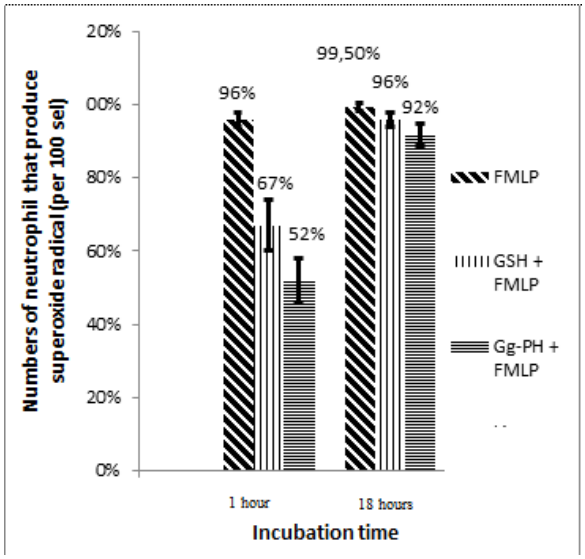


Fig 1. The average number of neutrophil that produce superoxide radical intracellularly in percent (per 100 cells) (NBT assay). Gg-PH can scavenge superoxide radical production in 1 hour incubation.

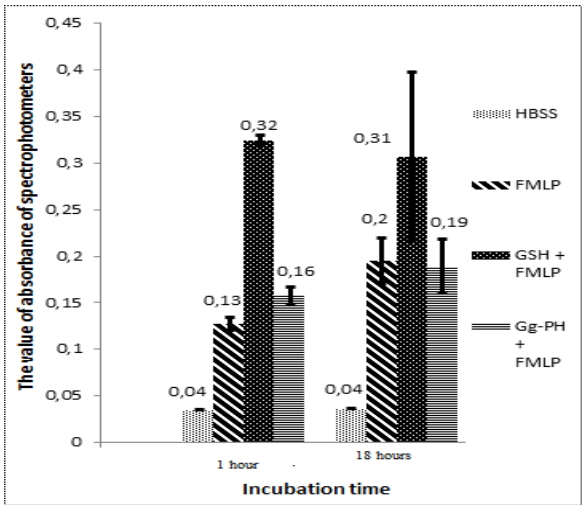


Fig 2. Extracellular superoxide radical production (NBT assay). The average of spectrophotometric absorbance values using a microplate reader at λ 630 nm. Gg-PH has absorbance value higher than FMLP in 1 hour incubation.

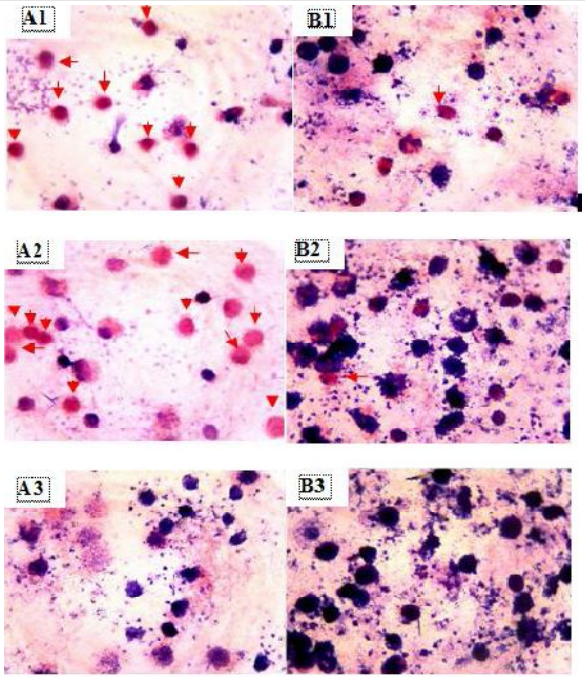


Fig 3. Intracellular superoxide radical production of neutrophil after 1 hour and 18 hours incubation (NBT assay). Magnetization (10^3). Arrow pointed neutrophil cells that do not produce superoxide radicals. Fig. A is 1 hour incubation; Fig. B is 18 hours incubation. Fig. A1 and B1 are Gg-PH group (neutrophil exposed by Gg-PH & fMLP); Fig. A2 and B2 are GSH group (neutrophil exposed by GSH & fMLP); Fig. A3 and B3 are FMLP group (neutrophil induced by fMLP).

Production of intracellular superoxide in Gg-PH group was significantly lower than ($p<0.05$) FMLP and GSH groups at 1 hour incubation. This demonstrates the ability of antioxidant protein Gg-PH donate electron to free radical so it becomes more stable and the chain reaction can be stopped [9]. Oxidative stress cell occurs in 18 hours incubation because concentration of Gg-PH and cellular antioxidant such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH)are no longer able to scavenge superoxide radical that produced continously. This excessive activation of neutrophil causes oxidative stress that triggers degranulation and lysis of neutrophil as shown in this study (Fig. 3). When neutrophil lysis, ROS [superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-)], myeloperoxidase enzyme (MPO), hydrolytic and proteolyticenzymes of neutrophil granula will spill into tissue then cause damage to variety of organic molecules around this [5], [7].

There was no differed significantyl ($p>0.05$) of extracellular superoxide production. Production of extracellular superoxide radical as in Fig. 2, the absorbance values of Gg-PH and GSH arehigher than FMLP at 1 hour incubation, whereas in 18 hours incubation absorbance of Gg-PH is almost same as FMLP. Absorbance value of Gg-PH should be lower than FMLP. This phenomenon may occured because many of superoxide radicals neutrophil released intracellularly, so that superoxide radical that released in extracellular slightly. Other factors that may caused are the use of different type of solvent, neutrophil cell that probably detached from cover glass become readable thereby disrupting the spectrophotometric analysis, and limitationof this study that did not count pH of each group.

Superoxide radical produced by neutrophil in its oxidative antimicrobial activity. This activity through a system of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase [6]. NADPH oxidase is activated when antigen FMLP activate G protein-coupled receptorsuch as FPR (Formyl Peptide Receptor) and FPR-L1 (Formylpeptide Receptor-Like 1) at the plasma membrane of neutrophil [30], [31].This activation causes translocation of cytosolic proteins (p47phox, p67 phox, Rac1/Rac2, p40phox) to the membrane bound cytochrome b_{558} (gp91phox, p22phox) that catalyzed by the flavocytochrome enzyme [3], [9]. This complex function as an electron transport system, which transport electrons from cytosolic NADPH to molecular oxygen (O_2) to reduce it to superoxide radical (O_2^-) together with the subsequent reactive products such as hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) which are bactericidal and cytotoxic [32], [33]. Superoxide radicalthat formed intracellular and extracellular will reduce Y-NBT to form water insoluble blue formasan particles (NBT) that was measured microscopically and spectrophotometrically in this study [17]. Production of superoxide radical in neutrophil is higher than monocytes and macrophages because amount of oxidase componentsof neutrophil such as p22phox, p67phox, and p47phox more than monocytes and macrophages. Content of these functional proteins have positive interrelationship with their function to produce superoxide radical [34].

CONCLUSIONS

In conclusion hydrolyzed melinjo (*G.* Gnemon) seed protein (Gg-PH) can scavengeproduction of neutrophil intracellular superoxide radical for 1 hour incubation but not in extracellular. Cell oxidative stress occuredin 18 hours incubation because concentration of Gg-PH that used was not able to scavenge superoxide radical that continued to be produce for 18 hours. Further studies are needed to analyze effect of dosis and duration of incubation.

ACKNOWLEDGMENTS

This researchpartly supported by High Education Ministry (BPOPTN Project) of Indonesia.

REFERENCES

[1] Clarkson PM, Thompson HS,"Antioxidants: what role dothey play in physical activirty and health," Am J Clin Nutr., vol. 72, pp. 637S-46S, 2000.

[2] Halliwell B, Whiteman M, "Measuring reactive species and oxidative damage in vivo and in cell culture: how

should you do it and what do the results mean," Br J Pharmacol., vol. 142, pp. 231-55, 2004.
[3] Forman HJ, Torres M,"Reactive Oxygen Species and cell Signaling," Am J Respir Crit Care Med., vol. 166, pp. S4-S8, 2002
[4] Kirkwood KL, Nisengard RJ, Haake SK, and Miyasaki KT,"Immunity and inflammation: basic concepts in Carranza's Clinical Periodontology 10th ed," Philadelphia: W.B. Saunders, 2006.
[5] Susilawati IDA,"Induction of*Porphyromonas gingivalis* against neutrophils kolagenolisis activity on type IV collagen (In vitro studies kolagenolisis mechanism of atherosclerotic plaques,"Unpublished,Dissertation, Malang: Brawijaya University's postgraduate program, 2008.
[6] Barry-Lane PA, Patterson C, van der Merwe M, Hu Z, Holland SM, Yeh ET, and Runge MS,"P47phox is required for atherosclerotic lesion progression in ApoE (-/-) mice," J Clin Invest., vol. 108, pp. 1513-1522, 2001.
[7] Sanmugapriya E, Venkataraman S,"Studies on hepatoprotective and antioxidant actions of *Strychnos potatorium* Linn. Seeds on CCI_4 induced acute hepatic injury in experiments rats," J. Ethnopharmacol., vol. 105(1-2), pp. 154-160, 2006.
[8] Droge W,"Free Radical in The Physiological Control of Cell Function," PhysiolRev., vol. 82, pp. 47-95, 2002.
[9] Chapple ILC, Matthews JB,"The role of reactive oxygen and antioxidant species in periodontal tissue destruction," J. Compilation Periodontology, vol. 43, pp. 160-232, 2007.
[10] Valko M, "Free radical, metal, and antioxidant in oxidative stress inducced cancer," J. Chem. Biol. Interact., vol. 160, pp. 1-40, 2006.
[11] Siswoyo TA, Mardiana E, Lee KO, and Hoshokawa K, "Isolation and Characterization of Antioxidant Protein Fractions from Melinjo (Gnetum gnemon Linn) Seeds," J. Agric. Food Chem., 2011; 59: 5648-5656.
[12] Siswoyo TA, Hoshokawa K,"Development production of a new generation antihypertensive *Gnetum gnemon* protein as a material neutraceutical commercial," Prosiding InSINas, vol. 0652, pp. 217-222, 2012.
[13] Adler-Nissen J, "Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid," J. Agric. Food Chem., vol. 27, pp. 1256-1262, 1979.
[14] Bradford MM, "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding," Analytical Biochem., vol. 72, pp. 248-254.
[15] You L, Zhao M, Regenstein JM, and Ren J,"Change in the antioxidant activity of loach (*Misgurnus anguilicaudatus*) protein hydrolysates during a stimulated gastrointestinal digestion," Food Chem., vol. 120, pp. 810-816, 2010.
[16] Tang X, He Z, Dai Y, Xiong YL, Xie M, and Chen J,"Peptide fractionation and free radical scavenging activity of zein hydrolysate," J. Agric. Food Chem., vol. 58, pp. 587-593, 2010.
[17] Choi HY, Kim JW, Cha YN, and Kim C, "A Quantitative Nitroblue Tetrazolium Assay for Determining Intracellular Superoxide Anion Production in Phagocytic Cells," J. Immunoassay & Immunochemistry, vol. 27, pp. 31-44, 2006.
[18] Ortiz Sara EM, Wagner JR,"Hydrolysates of native and modified soy protein isolates: structural characteristics, solubility, and foaming properties," J. Food Research International, vol. 35, pp. 511-518, 2001.
[19] Diaz M, Decker EA,"Antioxidant mechanisms of caseinophosphopeptides and casein hydrolysates and their application in ground beef," J. Agric. Food Chem., vol. 52, pp. 8208-8213, 2005.
[20] Hernandez LB, Davalos A, Bartolome B, and Amigo L, "Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS," J. Agric. Food Chem., vol. 53,pp. 588-593, 2005.
[21] Park EY, Mukarami H, Mori T, and Matsumura Y,"Effect of protein and peptide addition on lipid oxidation in powder model system," J. Agric. Food Chem., vol. 53, pp. 137-144, 2005.
[22] Sakanaka S, Tachibana Y, "Active oxygen scavenging activity of egg-yolk protein hydrolysates and their effects on lipid oxidation in beef and tuna homogenates," Food Chem., vol. 95, pp. 243-249, 2006.
[23] Elias RJ, Kellerby SS, and Decker EA,"Antioxidant Activity of Proteins and Peptides," Critical Reviews in Food Science and Nutrition, vol. 48, pp. 430-441, 2008.
[24] Shimada K, Fujikawa K, Yahara K, and Nakamura T, "Antioxidative Properties of Xanthone on the Auto Oxidation of Soybean in Clycodextrin Emultion," J. Agric. Food Chem., vol. 40, pp. 945-948, 1992.
[25] Arnao MB,"Some Methodological Problems in the Determination of Antioxidant Activity using Chromogen Radical: a Practical Case," Trens Food Sci. Technol., vol. 11, pp. 419-421, 2000.
[26] Wang, Wen B, Yun HK, Haeng SL, Ki YK, Xi PD, and Sang K,"Analysis of Antioxidant Enzyme Activity during Germination of Alfafa under Salt and Drought Stress," Plant Physiology and Biochem, vol. 47, pp. 550-557, 2009.
[27] Nelson DL, Cox MM,"Lehninger Principles of Biochemistry Fourth Edition," New York: W.H. Freeman and Company, 2005.
[28] Marks D, Marks A, and Smith C, "Basic Medical Biochemistry: A Clinical Approach,"Jakarta: EGC, 2000.
[29] Witt D,"Recent Development in Disulfide Bond Formation," Department of Organic Chemistry, Chemical Faculty, Gdansk University of Technology, Narutowicza, Gdansk, Poland, Synthesis, New York: Thieme Stuttgart, vol. 16, pp. 2491-2509, 2008.
[30] Dalpiaz A, Spisani S, Biondi C, Fabbri E, Nalli M, and Ferretti ME,"Studies on Human Neutrophil Biological Functions by Means of Formyl-peptide Receptor Agonist and Antagonist," Current Drug Targets-Immune, Endocrine & Metabolic Disorders, vol. 3, pp. 33-42, 2003.
[31] Paclet MH, Clare D, Peter K, Jasminka GZ, Anthony ES, and Lodewijck VD,"N-Formyl peptide receptor subtypes in human neutrophils activate L-plastin phosphorylation through different signal transduction intermediates," J. Biochem., vol. 377, pp. 469-477, 2004.
[32] Clark RA,"Activation of the Neutrophil Respiratory Burst Oxidase," J. Infectious diseases, 1999.
[33] Dahlgren C, Karlsson, "A. Respiratory burst in human neutrophils," J. Immuno. Methods., 1999.
[34] Yagisawa M,"Superoxide Release and NADPH Oxidase Components in Mature Human Phagocytes: Correlation between Functional Capacity and Amount of Functional Proteins," Biochemical and Biophysical Research Communication,vol. 288, pp. 510-516, 1996.

★★★