Mukh Syaifudin, Devita Tetriana, Darlina,Siti Nurhayati / International Journal of Engineering Research and Applications (IJERA) ISSN: 2248-9622 www.ijera.com
Vol. 3, Issue 1, January-February 2013, pp.314-319

Studies On The Protein Profiles Of Gamma Ray Induced Blood Stage Of *Plasmodium berghei* For Developing Candidate Of Malaria Vaccine

Mukh Syaifudin[1], Devita Tetriana[1], Darlina[1] and Siti Nurhayati[1]
1. Center for Technology of Radiation Safety and Metrology, National Nuclear Energy Agency, Jl. Lebak bulus Raya No. 49 Jakarta Selatan INDONESIA

ABSTRACT

Ionizing radiation is a known physical agent that had been widely used to attenuate the invasive blood stage of malaria parasite, merozoite, in vaccine materials development. In this study, we determined the protein profiles of blood stage *Plasmodium berghei* of ANKA strain post gamma irradiation. Parasitized bloods obtained from various percentage of parasite density, days post infection, doses (125, 150 and 175 Gy) and dose rates (380.2 and 640.4 Gy/hour) of gamma ray for irradiation and species of rodent parasites were separated on 10% SDS-PAGE gels under reducing conditions after boiled for 5 minutes. We found that protein profile of infected blood was different from uninfected blood with higher number of bands in infected samples indicating some exported proteins to the host cells for pathology in parasitized erythrocyte. Higher dose and dose rate of irradiation resulted in more protein bands on acrylamide gel. Based on further analysis 150 Gy and 380 Gy/hour was the most appropriate dose and dose rate to develop vaccine candidate. There was no difference in protein profiles of non irradiated parasited blood in days post infection, however the profile was depended on the percentage of parasitemia. Protein concentration that was analyzed by Lowry method was decreased with increasing of irradiation dose. Protein profile of *P. berghei* was different with those of other species of rodent plasmodia. It is concluded that profile of protein was dependent on the percentage of parasitemia i.e. depended on the composition of parasite's life cycle stages, and dose and dose rate of irradiation. Protein profile was also affected by some technical factors that will be discussed furthermore in the paper.

Keywords : gamma rays, malaria vaccine, P. berghei, protein profile, SDS-PAGE

INTRODUCTION

Malaria remains the most prevalent devastating parasitic disease worldwide. With the widespread development of drug resistance in the parasite, vaccination is considered to be an approach that will complement other strategies for prevention and control of the disease in future [1]. It has become well established that protective immune responses to the parasite are directed at stage-specific antigens. Proteins expressed at different stages of the life cycle of *Plasmodium sp.* are likely to be involved in the generation of natural immunity to malaria and a number of these proteins have been highlighted as candidates for a subunit vaccine [2]. Vaccines that mimic the antigenicity of infectious organisms may ultimately prove to be the most effective strategy for achieving protective immune responses [3,4]. Injection with radiation-attenuated parasites induces sterile immunity and protects >90% of human recipients for more than 10 months [5]. Studies in animal models also demonstrate that intravenous injection of mice with attenuated *Plasmodium berghei* induces sterile immunity to challenge with viable parasites [6,7].

Since clinical symptoms of malaria manifest only during the blood stage, a vaccine against this stage of the parasite life cycle would prevent or reduce severity and complications of the disease, and perhaps eliminate malaria if sterile immunity could be achieved. Much attention has therefore been given to parasite molecules that interact with the host cells during red blood cell (RBC) invasion as potential targets of host immune responses. A number of proteins have been identified on the merozoite surface or in the apical organelles that play a role in RBC invasion and are thought to be targets of immunity [8]. Merozoites, which are the extracellular developmental form of the parasite that invade erythrocytes, expose on their surface a protein complex, which is the processed product of a 190-200 kDa precursor known as merozoite surface protein-1 (MSP-1) [9]. One of cell components affecting parasite virulence factors is proteins. These proteins are also can be used as candidate of malaria vaccine [10,11].

Irradiation produces some chemical changes, which, although lethal to living organisms like malaria parasites, lead to the production of small amounts of radiolytic products [12]. They are formed from biopolymers like the proteins and are the result of reactions with hydroxyl radicals and hydrated electrons generated from water molecules by gamma rays. Free radicals may cause breaks in the protein chains or changes in their secondary or tertiary structure, resulting in modifications of their physicochemical properties such as fragmentation, crosslinking and aggregation [13]. However, the

knowledge on the alteration of protein profile of parasites after irradiation is still limited and it is needed to study the vaccine efficacy. These changes can be confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Parasites and mouse. *Plasmodium berghei* (strain Antwerpen-Kasapa**,** ANKA) and *P. yoelii* infected mouse bloods were received from Eijkman Institute for Molecular Biology, Indonesian Ministry of Research and Technology and National Institute of Health Research and Development, Indonesian Ministry of Health. Male Swiss-Webster mice (6–8 weeks old) were purchased from National Institute of Health Research and Development, Indonesian Ministry of Health and were housed at the Biomedical Laboratory of The Center for Technology of Radiation Safety and Metrology, National Nuclear Energy Agency of Indonesia (BATAN) animal facility and handled according to institutional guidelines. All procedures were reviewed and approved by the Animal Care and Use Committee National Institute of Health Research and Development, the Indonesian Ministry of Health.

Infection of mouse. Mice were intraperitoneally (IP) injected with about 1 x 10^6/ml *P. berghei* parasitized blood and control mice were sham injected with uninfected blood. Each batch consisted of equal numbers of infected and control mice. Parasitemia was monitored by Giemsa-stained blood smears using light microscopy. Parasitemia and survival of mice were evaluated. Mice were sacrificed at days post-infection to harvest blood, liver and spleen for further analysis.

Parasitemia monitoring. Parasitemia in mice from each infected group were monitored daily by conventional Giemsa staining starting on day 3 after infection. Thin blood films were prepared by tail bleeding, air dried, and methanol fixed before staining. Staining was done with a 10% Giemsa solution for 10 minutes at room temperature. Slides were evaluated at a 1000× magnification (oil-immersion) using a Nikon E200 Eclipse microscope by reading 20 fields per slide.

Irradiation of blood stage parasites. Infected bloods of mouse with 20-25% parasitemia were irradiated *in vivo* within a gamma irradiator (Cobalt-60 of IRPASENA) of the Center for Application of Isotope and Radiation, BATAN to a dose of 0, 125, 150 and 175 Gy at a low dose rate (380.0 Gy/hour) and high dose rate (640.0 Gy/hour). Radiation dose was calculated from the machine specific estimate of 1505 Rads per minute.

SDS-PAGE electrophoresis. SDS-PAGE was performed according to the method of Laemmli, 1970 [14] and Theisen [15] to the following group of samples : doses and dose rates of gamma ray for irradiation of *P. berghei*, and other species of rodent parasites (*P. yoelii*), and days post infection and percentage of parasite density for non irradiated parasites. It was carried out on Bio-Rad gels composed of stacking gel (5% w/v) using 1.0 M Tris-glycine buffer containing 0.4% SDS at pH 6.8 and resolving gel (12%, w/v) using 1.5 M Tris-glycine buffer containing 0.4% SDS at pH 8.8. Protein sample was dissolved in phosphate buffer (5 mg/ml) and mixed with a solubilization buffer Tris-HCl 6.22 mμ (pH 6.8) which contains 2% (w/v) SDS, 50% glycerol, a pinch of bromophenol blue and reduced with 0.9 mμ 2-mercaptoethanol in boiling water for 5 min. Protein sample was loaded onto each well and electrophoresis (Bio-Rad Laboratories, Hercules, CA) was conducted at constant current of 100 volts for 90 minutes by a Bio-Rad electrophoresis constant power supply unit. A standard broad range of protein marker was also run into every gel. After electrophoresis, gels were stained with Commassie-250 blue solution for one night after being treated with fixing solution (methanol-acetic acid-H_2O-p-formaldehyde) for 1 hour and photographed with Gel-doc imaging system (Bio-rad).

Determination of protein concentration with Lowry methods. After irradiated with gamma rays at 150-200 Gy, the content of protein was determined according to Lowry method [16]. The blood sample was digested by dissolving it in acetone (1:1) and sonicated for 15 minutes. Five milliliter of Lowry solution I was added into 1 ml of sample and let stand for 10 minutes and followed by the addition of Lowry solution II and incubated in room temperature for 30 minutes. After that it read with spectrophotometer at wave length of 700 nm.

RESULTS AND DISCUSSION

Results of experiment showed that on SDS-PAGE gels, there were a difference in the protein profile between control blood (uninfected blood) and infected blood. Based on analysis with Geldoc-Image *software* it was known that infected blood contained more bands of protein compared to uninfected blood that showing parasites protein inside the blood or indicating some exported proteins to the host cells for pathology in parasited erythrocyte (Figure 1). Irradiation caused the alteration of protein profile in infected blood but there was no significant difference among irradiation doses of 125, 150 and 175 Gy where several new bands appeared after irradiation. Irradiation with 150 Gy altered protein profiles where the 15 kDa protein was not appeared but there

was no different protein profiles found at higher dose (175 and 200 Gy).

There was a difference in protein profiles between species of rodent Plasmodium examined. Profile of protein in blood was depended on the percentage of parasite in red blood cells (parasitemia) and depends on irradiation dose rate. This protein can be used as vaccine candidate by determining molecular weight and extract them with blotting method. Profile of protein is influenced by several factors such the stability of electrical voltage used during electrophoresis and condition of the samples itself and SDS gels.

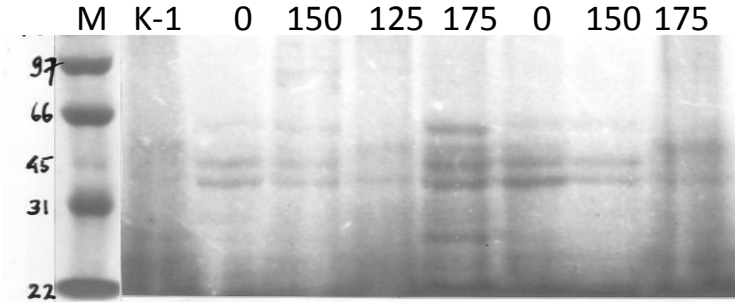

Figure 1. Profile of protein in blood of mouse infected with *P. berghei* post irradiation. M : *broad range* standard marker with numbers showing molecular weight of 22, 31, 45, 66 and 97 kDa. K-1 : non infected blood.

Higher dose and dose rate of irradiation resulted in more protein bands on acrylamide gel. Based on further analysis 150 Gy and 380 Gy/hour was the most appropriate dose and dose rate to develop vaccine candidate (Figure 2). Results of experiment on the protein profile post irradiation of low and high dose rates showed that new bands with the size of 77 and 105 kiloDalton (kDa) were observed after irradiation with higher dose rate for doses of 150 dan 175 Gy. It was predicted that *merozoite surface protein*-1 (MSP-1) with size of 26 kDa (p26) was detected for all samples.

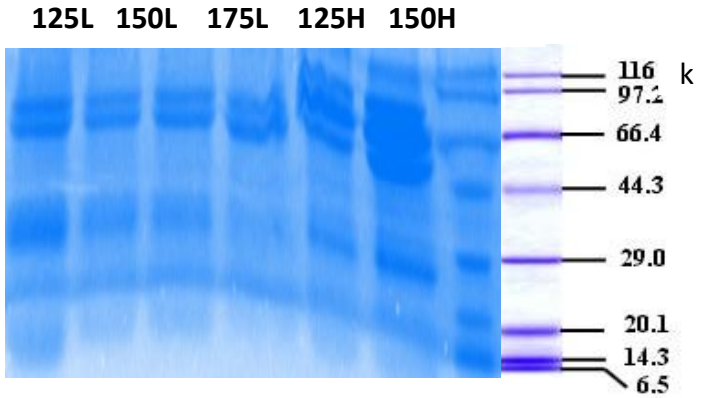

Figure 2. Protein prifile of mouse blood infected with *P. berghei* post gamma irradiation of doses 125, 150 dan 175 Gy at low (125L, 150L dan 175L) and high (125H, 150H, 175H) dose rates. Std : standard marker.

There was no difference in protein profiles in days post infection (Figure 3), even though there were some stages of parasites during its development (rings, tropozoites and schizonts) based on microscopy observation. It will be more clearly if the samples were further analyzed such as two-dimensional differential in-gel electrophoresis (2D-DIGE) protein quantification.

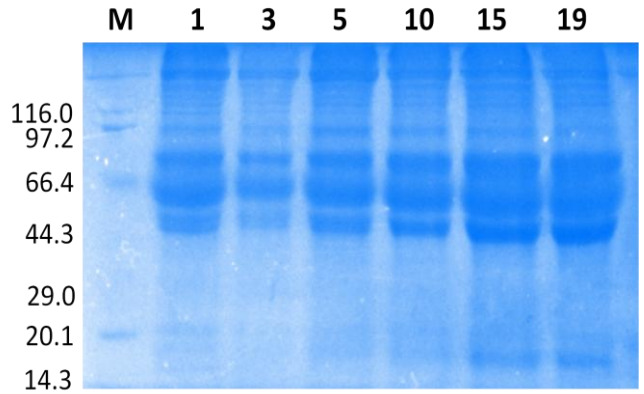

Figure 3. Protein profiles of non irradiated *P. berghei* in days post infection into mouse.

Study on the protein profile of several percentages of parasitemia in infected mouse blood showed that profile of protein was dependent on the percentage of parasitemia i.e. depended on the composition of parasite's life cycle in blood stages. The profiles of proteins of *P. berghei* infected mouse blood are quite different among 0%, 1% and 10% samples analyzed (Figure 5). The higher number of bands is seen in 1% sample that probably due to higher number of stage of life cycle of parasites whether rings, trophozoites or schizonts in that sample when it was taken.

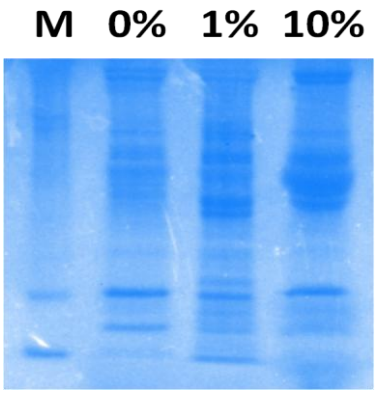

Figure 5. Profile of proteins in *P. berghei* infected mouse blood at different percentages of parasitemia. M: standard protein marker of broad range.

Protein profile of *P. berghei* was different with those of other species of rodent plasmodia. There were some protein bands seen in SDS page of *P. yoelii* (35, 36 and 60 kDa) (Figure 6) that was not found in *P. berghei*.

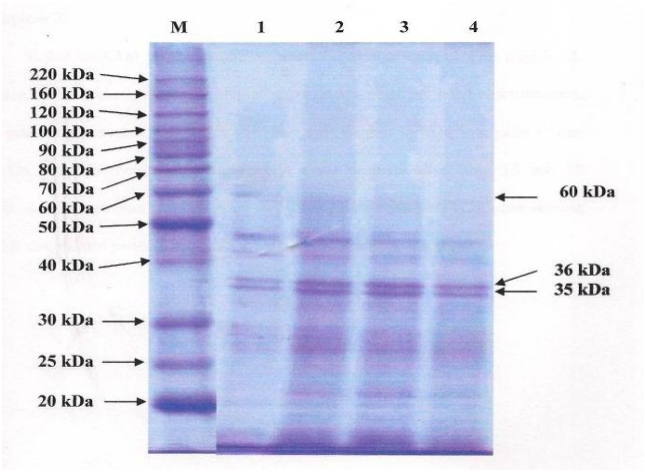

Figure 6. Profile of protein of *P. yoelii* (another rodent parasite) post gamma irradiation. M : standard marker; Row 1 : 0 Gy, 2 : 125 Gy, 3 : 150 Gy and 4 : 175 Gy. Notes : kDa is unit of molecular weight of protein. Protein of 35, 36 dan 60 kDa were predicted as *merozoite surface protein* (MSP) of *P. yoelii*.

Protein concentration was analyzed according to procedures of Lowry method. Irradiation with different doses on *P. berghei* of erythrocytic stadium showed the alteration of total protein concentration (Figure 6). Concentration of protein was decreased according to the increase of irradiation dose. Concentration of total protein for dose of 150 Gy was 435 mg/ml and for dose of 200 Gy was 315 mg/ml. It was predicted that irradiation caused the splitting of protein (peptide) bonds. Statistical analysis showed that there was an influence on the protein concentration.

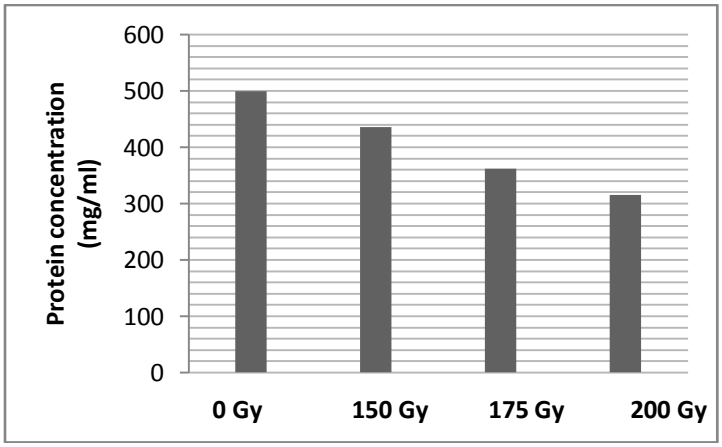

Figure 6. Concentrations of protein of *P. berghei* of erythrocytic stadium post gamma irradiation.

In developing countries malaria is currently believed to be the third most common cause of death among children, after deaths from respiratory infections and diarrheal diseases. It means that malaria is the one of the most important parasite diseases in the world. The research and development of cost-effective deployable vaccine will be needed to facilitate eradication of malaria [17]. However, the number of vaccine against protozoan diseases, malaria, is still limited. Despite this there is the belief that a vaccine against malaria is both practical and possible [18]. In developing a vaccine that could be used against malaria, we have attempted to produce explore the protein profile of irradiated blood of mouse that subjected to SDS-PAGE under reducing conditions.

The alteration of intensity (concentration) of protein may be caused by the damage that induced by radiation such as gamma rays, either its structure or its peptide bonds. The structural alteration can be caused by denaturation or degradation of protein or DNA alteration. This DNA alteration may cause the increase in synthesis of certain protein or the production of new protein. Denatured protein would have two possibility impacts, the development of peptide bonds and the breakage of protein into smaller pieces without molecular development. These two denaturations are depends on molecular condition. First, it occurred in polypeptide bonds, while the second is occurred in molecular parts that bind to secondary bond. The degradation of protein may cause its function as protein and structural degradation may resulted from the lost of side group [19].

A critical focus of vaccine development process has been to determine the minimum dose of radiation that adequately attenuates all parasites, and thus ensures that the vaccine will not cause malaria [20]. Thus the dose of radiation used to attenuate plasmodium is the most crucial factor. To determine the dose of gamma-irradiation which will produce attenuated parasites without affecting the pre-erythocytic and erythrocyte stabilities is the ultimate goal of many studies on irradiation vaccine. When dosed adequately, the parasite survives and remains infectious to the hepatocyte. However, liver stage development terminates during early hepatocyte infection [21]. The safety and efficacy of irradiated parasites is dependent on a precise irradiation dose; too little irradiation allows the parasite to complete liver stage development and cause blood-stage infection, too much irradiation completely inactivates the parasites, and inactivated parasites do not induce significant protection.

SDS-PAGE is a method used to analyze and isolate small amounts of protein. In this technique, the sample to be fractionated is denatured and coated with detergent by heating in the presence of SDS and a reducing agent. The SDS coating gives the protein a high net negative charge that is proportional to the length of the polypeptide chain.

The sample is loaded on a polyacrylamide gel, and high voltage is applied, causing the proteins to migrate toward the positive electrode (anode). Since the proteins have a net negative charge that is proportional to their size, proteins are separated solely on the basis of their molecular mass, a result of the sieving effect of the gel matrix. The molecular mass of a protein can be estimated by comparing the gel mobility of a band with those of protein standards. Sharp protein bands are achieved by using a discontinuous gel system, having stacking and separating gel layers that differ in either salt concentration or pH or both [22].

Several factors can affect the migration of proteins on SDS-PAGE and cause them to migrate at a slightly different rate than predicted based solely on its molecular weight [23]. The first factor is incomplete reduction of the sample that often characterized by the presence of multiple bands at and around the predicted size of the protein. Second factor is differences in SDS binding where the amino acid composition of each protein is unique, and the different side chains present on the individual amino acids cause each protein to bind SDS with varying affinity. This difference in binding can cause significant differences in the actual mobility of the protein compared to the predicted. The third factor is degradation of the protein sample where often bands at less than the predicted size are the result of degradation of the protein sample prior to electrophoresis. It can be overcome by using fresh lysates containing protease inhibitors that have been properly prepared and stored. The fourth factor is inappropriate polyacrylamide concentration and samples of specific molecular weights migrate best in certain percentage SDS-PAGE gels.

CONCLUSION

Results showed that profile of protein was different among infected and non infected samples, and irradiated and non irradiated samples of blood stage protein, and depended on the rate of irradiation used to attenuate parasites as well as the percentage of parasitemia. No different in protein profile in days post infection. Protein concentration was depended on the irradiation dose of gamma rays used to attenuate the parasites. Several factors affecting the results of protein profile analysis with SDS-PAGE.

REFERENCES

1. Keto GB, West SE., Steiger D, Beck HP, Weiss N, Hynes NE. A DNA delivery system targeting dendritic cells for use in immunization against malaria: a rodent model. Tanzania Health Research Bulletin 2005; 7: 142-148.
2. Soares IS, Levitus G, Souza JM, Del Portillo HA, Rodrigues MM. Acquired immune responses to the N- and C-terminal regions of *Plasmodium vivax* merozoite surface protein 1 in individuals exposed to malaria. Infection and Immunity 1997; 65: 1606-1614.
3. Abbas D, Lichmant A, Pober J. Cellular and molecular immunology, 2nd ed. Philadelphia, WB Saunders Co., 1994.
4. Hodes RJ. Aging and the immune system. Immunology Review 1997; 160: 5-8.
5. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, Sacci, J, de la Vega P, Dowler M, Paul C. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. J. Infect. Dis. 2002; 185: 1155-1164.
6. Kramer LD, Vanderberg JP. Intramuscular immunization of mice with irradiated *Plasmodium berghei* sporozoites. Enhancement of protection with albumin. Am. J. Trop. Med. Hyg. 1975; 24: 913-916.
7. Vanderberg JP, Nussenzweig RS, Most H. Protective immunity produced by the bite of X-irradiated mosquitoes infected with *Plasmodium berghei*. J. Parasitol. 1970; 56: 350-351.
8. Wipasa J, Elliott S, Xu HJ, Good MF. Immunity to asexual blood stage malaria and vaccine approaches. Immunology and Cell Biology 2002; 80: 401-414.
9. Chappel JA, Holder AA, Monoclonal antibodies that inhibit *Plasmodium falciparum* invasion *in vitro* recognize the first growth factor-like domain of merozoite surface protein-1. Molecular and Biochemical Parasitology 1993; 60: 303-312.
10. Wizemann TM, Adamou JE, Langermann S. Adhesins as targets for vaccine development. Emerg. Infect. Dis. 1999; 5: 395-403.
11. Tetriana D, Darlina, Armanu, Syaifudin M. Pengaruh radiasi gamma terhadap profil protein *Plasmodium berghei* stadium eritrositik, Prosiding Seminar Nasional Keselamatan, Kesehatan dan Lingkungan IV dan International Seminar on Occupational Health and Safety I, Depok, 27 Agustus 2008, 282-291.

12. Alexander P, Hamilton LDG, Irradiation of Proteins in the Solid State: II. Chemical Changes Produced in Bovine Serum Albumin. Radiation Research 1960; 13(2): 214-233.
13. Robins D. The preservation of food by irradiation. IBC Tech Serv Ltd. UK 1991; 112.
14. Laemmli EK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685.
15. Theisen M, Frederiksen B, Lebech AM, Vuust J, Hansen K. Polymorphism in *ospC* gene of *Borrelia burgdorferi* and immunoreactivity of OspC protein: implications for taxonomy and for use of OspC protein as a diagnostic antigen. J. Clin. Microbiol. 1993; 31: 2570-2576.
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951; 193: 265-275.
17. Greenwood BM, Control to elimination: implications for malaria research. Trends in Parasitology. 2008; 24: 449-454.
18. Wang R, Smith JD, Kappe SHI. Advances and challenges in malaria vaccine development, Expert. Rev. Mol. Med. 11: e39. doi:10.1017/S1462399409001318.
19. Syaifudin M. Biochemical indicator in cells to ionizing radiation. ALARA Bulletin; 2005; 4: 125-131.
20. Syaifudin M, Tetriana D, Darlina, Nurhayati S The feasibility of gamma irradiation for developing malaria vaccine. Atom Indonesia Journal 2011; 37(3): 91-101.
21. Silvie O, Semblat JP, Franetich JF, Hannoun L, Eling W, Mazier D. Effects of irradiation on *Plasmodium falciparum* sporozoite hepatic development: implications for the design of pre-erythrocytic malaria vaccines. Parasite Immunology 2002; 24: 221-223.
22. Hanes BD. One-dimensional polyacrylamide gel electrophoresis. In: Gel Electrophoresis of Proteins *: A practical approach*, 2nd edition, Hanes, B.D. and Rickwood, D., ed., IRL Press, Oxford, UK, 1990, 1-148.
23. ANONIM, Factors affecting migration of proteins in SDS-PAGE gels, Novus Biologicals. http://www.novusbio.com/support/antibody-guide/factors-affecting-migration-proteins-sds-page-gels