The Differences of Electrophoretic Profile and Snake Venom Phospholipase A2 (svPLA$_2$) Activity from the Venom of Javan Spitting Cobra, *Naja sputatrix*, based on Body Scales Color and Storage Condition

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Abstract: *Naja sputatrix* (Javan spitting cobra) is one of medically important snake species in Indonesia which have various dorsal scales color. This research purposes to examine the differences of venom general profile and its phospholipase A$_2$ (svPLA$_2$) activity from some *N. sputatrix* with different dorsal scales colors, and to examine the activity of *N. sputatrix* svPLA$_2$ in different storage conditions. A total of 6 *N. sputatrix* from East Java with various dorsal scales color were milked. Venom storage was performed at -80, 4 and 37°C in a maximum period of 14 days. Venom profile and phospholipase A$_2$ activity were examined through 15% SDS-PAGE and acidimetric method using egg yolk substrate respectively. Statistical analyses were performed to evaluate svPLA$_2$ activity in every dorsal color and storage condition. Few protein bands range in 16 – 22 kDa are only found in the venom of the certain dorsal color snake. Protein bands at 37°C were found to have the lightest intensity among other groups. The svPLA$_2$ activity of brown dorsal *N. sputatrix* is found as the highest activity. An interaction between the storage temperature factor and period factor has effects on post-storage svPLA$_2$ activity. Storage in 37°C effects on svPLA$_2$ activity declining compared to the control group and other experimental groups.

1 INTRODUCTION

Elapidae, Viperidae, and Colubridae snakes can produce venom as their secreted product which is useful in foraging activity and survival mechanism (Vitt & Caldwell, 2009; Warrel, 2010). Venomous snakes are present around the world. Thus, the conflict between human and venomous snake becomes a global health problem. The total of the conflicts around the world, in 2008, reaches the number of 421.000 – 1.841.000 envenomation cases per year (Kasturiratne et al., 2008). Snakebite problem does not get enough attention and is included in Neglected Tropical Disease (NTD) since 2009 (Gutierrez et al., 2013; Williams et al., 2019). Tropical and subtropical area, including Indonesia, are susceptible to snakebite problem (Hijaz et al., 2018; Megawati, 2014; Safitrih et al., 2016; Pratama & Oktafany, 2017).

Naja sputatrix is only one of various venomous snakes in Indonesia that is considered medically important. *N. sputatrix* is classified to Catagory I venomous snake because of its high venom and its habitat preference that is near to human (Warrel, 2010). *N. sputatrix* in Indonesia can naturally be found in Java, one of the most populated islands in Indonesia, also in Lombok, Sumbawa, Padar, Rinca, Komodo, Flores, Adonara, Lomblen and Alor Islands. This snake is a terrestrial organism that often found in rice fields and swamps near residential areas (Iskandar, et al., 2012). *N. sputatrix* has a total body length of 1,5 meters with a wide head and an elongated hood. The dorsal scales color of this species is varied. *N. sputatrix* in West Java have blackish gray dorsal scales color, while those from East Java and Islands of Southeast Nusa (Nusa Tenggara) have silver to brown color (Das, 2010). The scales color of this snake is possibly a result of an adaptation process. The snakes with darker scales live in rain forest with high relative humidity, while the snakes with lighter scales color live in dry soil habitat (Kurniawan, et al., 2017).

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The venom of *N. sputatrix* is a dangerous mixture solution for humans containing many protein and non-protein components. It generally contains major and minor components: three-finger toxins, cytotoxin, short-chain-α-neurotoxin, long-chain-α-neurotoxin, muscarinic toxin-like protein, snake venom metalloproteinase, snake venom serine protease, phospholipase A_2, Kunitz-type serine protease inhibitor, cobra venom factor, phosphodiesterase, nucleotidase, L-amino acid oxidase, nerve growth factor, acetylcholinesterase, and many more (Tan et al., 2017). Phospholipase A_2 (PLA_2), a major component of the venom, is an enzyme that hydrolyzes glycerophospholipid (Sunagar et al., 2015). This enzyme has been extensively explored because of the stable structure (Vija et al., 2009; Kang et al., 2011). Many physiological and pathological effects are caused by $svPLA_2$: presynaptic neurotoxin, edema, necrosis also hemolysis (Sunagar et al., 2015; Doley & Kini, 2009; Asad et al., 2014). Some PLA_2 antidote potentials are also estimated useful to handle or alleviate snakebite effects (Xiao et al., 2017).

The abundance of each component in venom is varied in every single snake, even in the same species with differences in the locality. The composition of the venom can be affected by geographic condition, habitat, season variation, diet preference, the age of the snake, also sexual dimorphism (Tan et al., 2015; Sarhan et al., 2017). Other than the factors described in these studies, the profile of venom from different dorsal scale colors was not researched well yet. This makes the factor of dorsal scales color is needed to be considered. It would be very important in research to use fresh-milked snake venom in wildlife to get a holistic description of snake venom. Considering the importance, the storage of the venom before it arrives in the research center is very important to note. The previous study put forward the results that $svPLA_2$ and the venom of *Crotalus molossus molossus* were generally stable in various temperature storage for 7 days. The study however told that the results might be generalized for other snakes, but further researches are still required to conduct (Munekiyo & Mackessy, 1998). This research purposes to examine whether the differences of venom profile and enzymatic activity, which is represented by $svPLA_2$ as a major component of the venom, of some *N. sputatrix* with different dorsal scales colors are present or not. Besides, this research also purposes to evaluate the activity of *N. sputatrix* $svPLA_2$ in different storage conditions. The activity of $svPLA_2$ was evaluated after the crude venom was stored at various temperatures for 14 days.

2 MATERIALS AND METHODS

2.1 Sample Preparation

The examination of *Naja sputatrix* venom profile and $svPLA_2$ activity were carried out to examine the difference or similarity among different dorsal scales color snakes, also different storage condition. The samples used in this research comprises in total of 6 East Javan *N. sputatrix* individuals. Black dorsal scales color snakes were collected from Malang, the brown dorsal scales snakes from Jombang, and yellow dorsal scales snakes from Bangil. The snakes used in this research have a total length of 1,2 – 1,5 meters. Venom milking was conducted after the snakes 3 days fasting. Venom milking was done in a beaker glass which covered with parafilm. N. sputatrix venom from each dorsal color scales was pooled separately. These samples were used to examine whether dorsal scales color would affect the venom profile and $svPLA_2$ activity or not. Meanwhile, snake venom solution from black dorsal scales snakes was pooled together and aliquoted into some different storage condition groups to evaluate the effect of storage temperature in 7, 9 and 14 days. All fresh-milked venom were centrifuged at 4000 rpm for 5 minutes at 4°C. The supernatant was stored for further analysis. Storage temperature used to evaluate the effect of dorsal scales color was -80°C, while 37, 4 and -80 °C were used to store snake venom sample that would be evaluated under different storage condition. Three times replication was used at any data collection.

2.2 Protein Concentration

The whole crude protein and the $svPLA_2$ examinations were done in Molecular Biology Laboratory of Life Science Central Laboratory and *Institut Biosains*, University of Brawijaya, Indonesia. The protein concentration of the venom solution was measured by spectrophotometry principle, based on the absorbance value of the sample in wavelength 280 nm by using the NanoDrop instrument. The protein concentration data were used to equalize sample for further assays, both visualization by SDS-PAGE or $svPLA_2$ activity assay.

2.3 Snake Venom Electrophoresis (SDS-PAGE)

Naja sputatrix crude venom solutions were subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The preparation of the venom solution comprises the addition of reducing buffer followed by incubation at 100°C for 15 minutes. 10-20 µl venom solution containing 23 µg protein was loaded to each gel well. The standard marker used was from Jena Bioscience BlueEye Prestained Marker 10-245 kDa. Electrophoresis was conducted at separating gel 15% and stacking gel 3% with a constant volt of 120 V. CBB R-250 staining process was used to visualize the protein separation results of the venom solution.

2.4 Phospholipase A_2 Assay

Phospholipase A_2 assay was conducted based on the acidimetric method. Phospholipid substrate suspension was prepared from chicken egg yolk, CaCl2 18 mM and sodium deoxycholate 8,1 mM 1:1:1 (v/v/v). The material used was mixed well and adjusted to pH 8,0 by using NaOH 1 M. One hundred microlitres venom solution containing 50 µg protein was mixed into 15 mL substrate suspension. The decrease of 1 pH unit between 5 – 65 s was considered equal to the release of 133 µmol fatty acids (Tan & Tan, 1998).

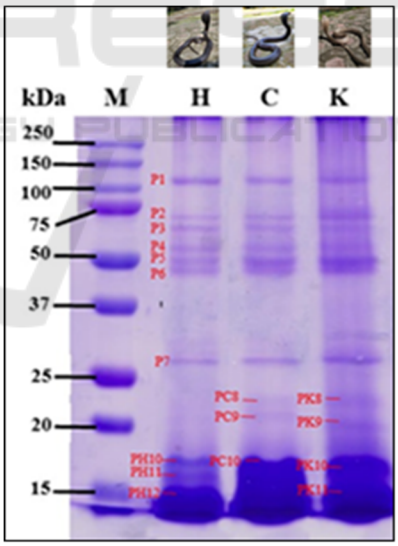
2.5 Statistical Analysis

The results of *the Naja sputatrix* $svPLA_2$ activity were analyzed statistically. We performed a Kolmogorov-Smirnov test, Lavene statistic, One Way ANOVA, and Games-Howell test to evaluate the $svPLA_2$ activity in each dorsal scales color snake. To evaluate whether interactions among storage factors were happening or not, we performed univariate analysis and the test of between factors interactions. Further investigation was done by Tukey test to compare the results between the control group and other experimental groups.

3 RESULT AND DISCUSSION

3.1 N. *sputatrix* Venom Electrophoretic Profile

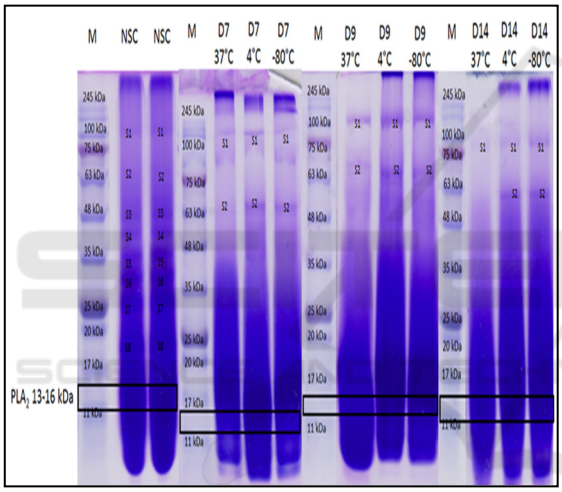
The evaluation of N. sputatrix venom electrophoretic profile based on the dorsal scales color shows that the venom proteins range 16 – 134 kDa. There are seven protein bands, labeled as P1 until P7. The P1 bands have similar molecular weights and characteristics among the three kinds of venom. The same conditions are also found on P2 until P7. Those bands have molecular weight on 26, 54, 60, 65, 80, 92, 134 kDa (Figure 1, P1 – P7). However, the venom from yellow dorsal scales N. sputatrix has a higher intensity in the 2nd, 5th, 6th, and 7th bands. Other than that, the 8th until 11th bands from all samples used show various molecular weights and intensity. Venom from black dorsal scales N. sputatrix does not show both 8th and 9th protein bands as the other samples show. Venom from brown dorsal scales snakes observed to have 20 and 22 kDa proteins (Figure 1, PC8 and PC9). Yellow dorsal scales snakes observed to have 19 and 21 kDa proteins (Figure 1, PK8 and PK9). Differences are also observed in the ≤ 20 kDa protein bands. Three thick protein bands (16 – 17 kDa) are observed in the venom of black dorsal scales snakes (Figure 1, PH10, PH11, and PH12); a thick protein band (16 – 18 kDa) in the venom of brown dorsal scales snakes (Figure 1, PC10); two protein bands (16 – 17 kDa) in the venom of yellow dorsal scales snakes (Figure 1, PK10, PK11).



M: marker
H: black dorsal scales
C: brown dorsal scales
K: yellow dorsal scales, P1 – P8: protein bands appearing in three samples
PH: protein band only appearing in black dorsal snakes
PC: protein band only appearing in brown dorsal snakes
PK: protein band only appearing in yellow dorsal snakes

Figure 1: Molecular weight profile of N. sputatrix protein venom on SDS-PAGE 15%.

Meanwhile, *N. sputatrix* venom samples in which the profile are evaluated under different storage conditions are mostly composed of proteins that have a molecular weight of ≤ 35 kDa, which can be seen as the thick band showed in the crude venom separation. Separation by SDS-PAGE of *N. sputatrix* crude venom does not show a well-separated band (Figure 2). However, the analysis of control group results in a better separation of the venom protein. Few protein bands (19, 27, 30, 34, 39, 44, 64 and 125 kDa) are visualized well in the control group. Those bands, in contrast, are not visualized well in the other experimental group, except for the S1 and S2 bands. Electrophoretic visualization of the stored *N. sputatrix* venom solutions shows a lower intensity, with the lowest intensity is showed in the experimental groups under 37°C storage temperature.



NSC: control group
D7: 7 days storage
D9: 9 days storage
D14: 14 days storage.

Figure 2: N. sputatrix venom visualization on 15% SDS-PAGE in variation of storage condition.

Black outline squares show protein with molecular weight range on 13 – 16 kDa. N. sputatrix svPLA2 is estimated to be in the range.

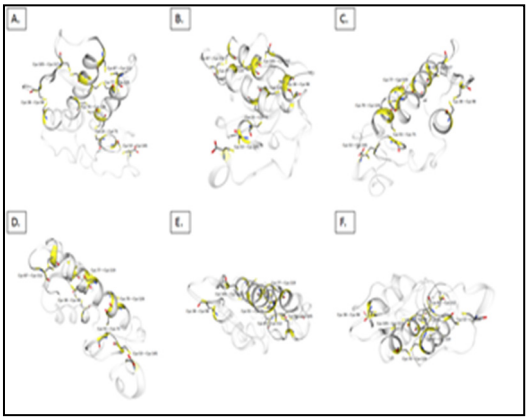


Figure 3: Dissulfide bonds in the three-dimentional structure of N. sputatrix svPLA2 (Q92084).

Dissulfide bonds are represented by yellow parts. A) Front side, B) Back side, C) Right side, D) Left side, E) Upper side, F) Under side.

3.2 The Activity of N *sputatrix* svPLA$_2$

N. *sputatrix* svPLA2 from the black dorsal scale snake performs the lowest activity (144,08 µmoles/minutes/mg) compared to the brown and yellow dorsal scale color snakes (Figure 4). Statistic analysis with a confidence level of 95% shows that a significant difference is present between the svPLA2 activity of the black dorsal scales snakes and the brown dorsal scales snakes. On the other hand, the difference of svPLA2 activity in yellow dorsal scales snakes with both black and brown dorsal color snakes are not significant (Figure 4). This condition indicates that the ability to hydrolyze biological cell phospholipid membrane in brown dorsal snakes venom is higher than the other venom, and is found significantly different from the venom of black dorsal snakes, and not significantly different with the venom of yellow dorsal snakes. Along with it, the venom from brown dorsal color might be riskier to raise various pathophysiological effects in following the envenomation compared to the two other snakes.

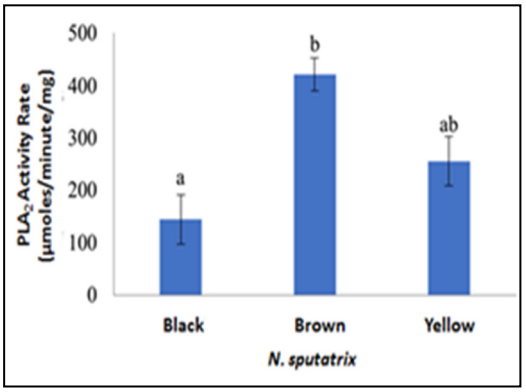


Figure 4: The snake venom phospholipase A2 activity rate of Javan spitting cobra (N. sputatrix) with different dorsal scales color. Letter a and b define the statistic notation among sample groups.

To confirm the stable electrophoretic results of svPLA2 under different storage conditions, we performed the svPLA2 activity assay. In this research, N. sputatrix svPLA2 activity is affected by the interaction between storage duration and temperature factors. However, svPLA2 in our research are estimated stable during the storage at both 4 and -80°C for two weeks long. There are no significant differences between svPLA2 activity from the control group and from the venom solutions which are kept at 4 and -80°C for 7 - 14.

On the other hand, the activity of svPLA2 that had been stored at 37°C does not remain stable since the first 7 days of storage. The N. sputatrix svPLA2 activities in 37°C storage temperature are found similar in 7, 9 and 14 days storage duration, where only reach about ¾ of svPLA2 activity in the control group sample (Figure 5). This may indicate the damage of svPLA2 native form, which affects its performance.

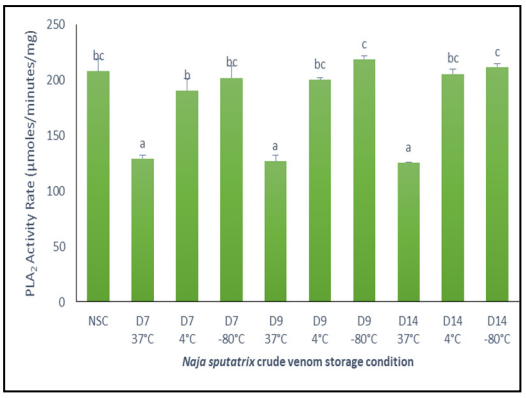


Figure 5: Effect of interaction between temperature and period of time storage on svPLA2 activity rate of N. sputatrix venom. Letter a, b, c define notation among sample groups based on statistical data.

All protein bands from the venom of each dorsal scales color snake (Figure 1) are estimated as four venom protein families, those are Phospholipase A2 (svPLA$_2$), Cysteine-Rich Secretory Protein (CRiSP), Snake Venom Metalloproteinase (SVMP), and Nerve Growth Factor (NGF) (Table 1). PLA$_2$ is estimated as the protein family from protein bands 16 – 19 kDa. Even though PLA$_2$ is known as a major component of venom, the toxic effects caused by this enzyme are varied. This enzyme also synergistically works with the other venom components to support the toxicity potential of cobra venom (Wong et al., 2017; Tan et al., 2017).

Protein bands 16, 18, 20, 21 and 26 kDa in the SDS-PAGE results are estimated as CRiSP. This protein family possesses the inhibition the smooth muscle contraction through the blockade of cyclic nucleotide-gated (CNG) and L-type calcium channels. Protein bands with molecular weight on 22 and 26 kDa can also be estimated as NGF family, a non-enzyme protein that effects on apoptosis induction and cytotoxic activity (Tan et al., 2017; Wong et al., 2017). Few higher molecular weight venom proteins (80, 65, 60 and 54 kDa) are estimated as SVMP, which effects on local and systemic bleeding induction, hemostatic disruption through the properties of procoagulant or anticoagulant, inflammation and tissue necrosis (Sanhajariya et al., 2018).

Table 1: Protein family estimation of the protein bands appearing in N. sputatrix venom solution 15% SDS-PAGE.

Protein family prediction	**Band**	**Molecular weight (kDa)**	**References**
SVMP	P3	80	Lauridsen et al., 2017; Shan et al., 2016; Xu et al., 2017
	P4	65	
	P5	60	
	P6	54	
NGF	P7	26	Xu et al., 2017
	PC8	22	
CRiSP	P7	26	Xu et al., 2017; Sanhajariya et al., 2018; Shan et al., 2016
	PK8	21	
	PK9	20	
	PH10, PC10, PK10, PH11, PK11, PH12,	16 - 18	
PLA$_2$	PK9, PH10, PC10, PK10, PH11, PK11, PH12	16-19	Shan et al. 2016; Xu et al., 2017

Venom protein visualization in this research is in accordance to the previous studies. Liu et al. (2018) conducted research which found that few protein families are identified on the whole venom of Naja atra, those are SVMP, Venom Complement C3, CRiSP, PLA_2, NGF, and 3FT. Xiao et al. (2017) found that the venom of *Naja naja*, *Naja melanoleuca*, *Naja nigricollis*, and *Micrurus fluvicus* consist of acetylcholinesterase, SVMP, a serine protease, CRiSP, PLA_2, and 3FT. The protein families found in previous studies are estimated appearing in the sample used. Even few differences are found in the separation profile, the differences are in the same protein families among the three kinds of venom used. This indicates that the color of Javan spitting cobra dorsal scales might have effects in the abundance or characteristics of venom protein bands. The unclear results lead to the need for further research with more supportive methods that a holistic analysis of cobra venom in the consideration of dorsal color could be done better.

Under different storage condition, the protein separation through SDS-PAGE of *Crotalus molussus molussus* venom showed few variations. The storage conditions under 4, -20, and -80ºC, in general, did not affect the visualized protein band. The similar results also found in previous researches. The visualization of venom protein through SDS-PAGE appears to be not affected by the storage temperature (-80, -20, 4 and so 20ºC) for 1-7 days long (Egen & Russell, 1984; Munekiyo & Mackessy, 1998). Other than cold storage, the proteins of snake venom are found stable in dried storage even until more than 50 years. Few degradations in the protein may happen but limited to the functionally unimportant peptides (Jesupret et al., 2014). In contrast, the venom solution storage under 37ºC caused some changes in the protein bands. The decrease in intensity, absence, and appearance of some bands in the visualization may indicate an autolytic degradation of some proteins in the venom solution (Munekiyo & Mackessy, 1998). This also possibly happens in the *N. sputatrix* venom solution stored at 37ºC, which can be observed from the decrease of intensity in the separation results.

The differences in $svPLA_2$ activity of *N. sputatrix* from Jombang, as the highest activity among other groups, are possibly an effect of their habitat and so prey availability. Variation in snake venom (Casewell et al., 2014) component is an adaptation to choose prey. These phenomena exist in both interspecific and intraspecific levels. The venom system is an important adaptation that evolved independently in every animal lineage. The toxins in snake venom are encoded by a few gene families, in which each gene family can produce related isoform that had been produced from gene duplication during the evolution process. Birth and death model of toxin-gene evolution is often used as a mechanism that brings out toxin gene paralogue, with the evidence that natural selection does facilitate the encoded protein subfunctionalization or neofunctionalization. This process produces a toxin complex that synergistically works to cause death in prey. Venom evolution in the advanced level enables the changes in prey capture from mechanic (constricting) to chemical (venom). It plays an important role in snakes diversification. The diversity in snake venom is caused by the new toxin gene recruitment, or the diversification of existing toxin genes, that happened before and during the evolution (Xu et al., 2017). The $svPLA_2$ enzyme is coded by the ancestor's physiological gene that experiences convergent and divergent evolution several times. PLA2 in snake venom is a single chain polypeptide consists of 115 – 125 amino acid residues with a molecular weight of 13 – 15 kDa and has a high homolog sequence in many cobra species. However, the pharmacology of $svPLA_2$ in envenomation cases are contributed in various way even the sequence was generally homolog. *Naja melanoleuca*, an African cobra, has a very high PLA_2 activity that reaches 2120,66 μmoles/minutes/mg. Meanwhile, $svPLA_2$ activity of Asian cobras range from 864,04 – 1157,56 μmoles/minutes/mg. Asian cobras used in the research are *Naja sputatrix*, *Naja naja*, *Naja kaouthia*, *Naja atra*, *Naja sumatrana*. Some African cobras, for example, *Naja katiensis*, *Naja nigricollis*, *Naja pallida*, *Naja mossambica* and *Naja nubiae* have $svPLA_2$ activities that do not show many differences with those in Asian cobras venom. Some other African cobras, *Naja senegalensis*, *Naja haje*, *Naja annulifera*, and *Naja nivea* have very low $svPLA_2$ activity (Tan et al., 2019).

A slight difference in venom molecular weight profile, also with the significant differences in the $svPLA_2$ activity which is found in our research could indicate that some different protein kinds and/or abundance might exist in different dorsal scales color snakes, and are related to the habitat of the snakes. However, the differences are not studied further because of data limitations. Further studies with more supportive methods were needed to confirm these results.

Under different storage conditions, we evidence that $svPLA_2$ activity of *N. sputatrix* venom is influenced by the interaction of temperature and

storage time. The activity of $svPLA_2$ is observed decreasing significantly under the temperature of 37ºC. These results are not in accordance to the previous study by Munekiyo and Mackessy (1998). Munekiyo and Mackessy did research that results in the stable activity of some enzymes, including $svPLA_2$ that had been stored in various temperatures: -80, -20, 4 and 37°C for 7 days long. The $svPLA_2$ enzyme activity, specifically, are maintained under the storage of 37°C . PLA_2 enzyme is considered as a stable enzyme in various temperature conditions even in the presence of proteolysis enzymes because of its small size and molecular structure (Vija et al., 2009; Kang et al., 2011). Our examination on a member of *N. sputatrix* $svPLA_2$ family shows that there are 7 dissulfide bonds in the structure of *N. sputatrix* svPLA2. Dissulfide bonds play an important role in maintaining this molecule stability through decreasing the protein entropy in the unfolding condition (Xiao et al., 2017; Fass, 2012).

With the exception in the research conducted by Munekiyo and Mackessy (1998), *Naja naja* venom PLA_2 shows an optimum temperature at 45 – 55°C after its incubation at 37°C for 60 minutes (Shashidharamurthy & Kemparaju, 2006). A similar condition also found in the venom of *Ecis ocellatus* and *Crotalis durissus terificus* (Sallau et al., 2008; Toyama et al., 2003). In addition, *Bothrops asper* $svPLA_2$ have an optimum temperature at 52°C after its incubation in the temperature range 6 – 92°C for 30 minutes (Avila et al., 2004). These indicate that the stable feature of PLA_2 at various temperature are phenomena performed by $svPLA_2$ after its incubation at a various temperature in a relatively short time, which are not intended to storage condition.

The crude venom solutions which we stored at 37°C show a visual difference compared to the other storage condition groups. The solutions are more turbid and contained some precipitation. The presence of precipitation is an indication of protein changes. Wrong storage could lead to the instability and formation of precipitation. The precipitation usually is the non-native form of protein which is irreversible. The aggregate formation that leads to protein precipitation could occur in some conditions: changes of pH, freeze-thawing cycles and high-temperature exposure (including the temperature of 37°C). The aggregate appearance could decrease the amount of native-form $svPLA_2$ protein molecule in the *N. sputatrix* venom solution, which can be observed from the decrease of *N. sputatrix* $svPLA_2$ rate activity which had been stored in 37°C. The $svPLA_2$ that have already aggregated with another protein would undergo conformation changes that it could not function on the substrate (Carpenter et al.. 2002; Calamai et al., 2005).

Protease activity might be a factor that leads to the svPLA2 degradation at the 37°C venom solution. Protease could degrade endogenous inhibitor and/or svPLA2, which impacts to the decrease of svPLA2 activity. Munekiyo & Mackessy in their study detected the degradation of endogenous protein inhibitor in the venom stored for 7 days at 37°C (Munekyo & Mackessy, 1998). Endogenous protein inhibitor has a crucial role in maintaining the whole venom quality. Venom protein in the whole venom solution form might undergo proteolysis caused by protease, that impacts on the venom impotent. Besides, the protease in the venom solution could also damage the cells that make up the venom gland. An endogenous inhibitor is found inside the venom solution and functions to inhibit the protease, including its activity to damage the svPLA2 (Francis et al., 1992; Francis & Ivan, 1993).

Another factor that might influence the svPLA2 activity inside the venom solutions which had been stored at 37ºC is a growth of microbes. Snpake venom has been known for its antibacterial potential, however, there is association between bacterial infection and venomous snakebite cases. Ten years of research (2001 – 2010) conducted in North Taiwan pointed that Morganella morganii and Enterococcus are the most abundant bacteria identified in the victim's wound culture (Chen et al., 2011). The snakebite victims in KwaZulu Natal, South Africa, have an infection of a few kinds of bacteria, for example, Morganella morganii, Enterococcus faecalis, Proteus sp., and Salmonella enterica. The bacteria were collected from necrosis tissue samples from the victim (Wagener et al., 2017). The microbes mentioned above can be found in the intestinal track of human and other warm-blooded animals (Lee et al., 2009; Dubin & Eric, 2014; Drzewiecka, 2016;); which is associated to the storage temperature we performed.

Microbial analysis at oral swab and venom samples in recent research pointed that microbial diversity of venomous snake oral cavity is dependent on the food type and water resource as result from faeces that might enter the oral cavity or venom gland. Bacteria from the oral cavity (including fangs) and venom solution are found to be appearing in two different clusters, indicates that the venom gland might be a different ecological niche. The bacteria community in both the oral cavity and venom gland was different. The bacteria in the

venom solution also show that those are viable even though the venom are air-dried or lyophilized. The research also pointed out that two new strains for Enterococcus faecalis appeared as a result of adaptation to the venom. (Esmaeilishirazifard et al., 2018).

Protein cold storage at -80°C until 4°C is an easy method to store protein solution, specifically for a stable protein in a short time of period range to 4 weeks. The storage at 4°C usually accompanied by the addition of stabilizer solutions like sucrose, glycine or glycerol to reduce the protein concentration. This is important to decrease the degradation of protein risk as an effect of the kinetic process inside the protein solution (Carpenter et al., 2002). Snake venom solution, however, does not need a stabilizer solution due to its stable profile in 4°C storage. Venom storage at -80°C (or lower) could also decrease the degradation risk to one year. A factor that needs to be considered is a freeze-thawing cycle that might denaturate protein on the ice surface during freezing or thawing (Cao et al., 2003), however, research studies also pointed its stability during freeze-thawing cycles (Egen & Russell, 1984; Munekiyo & Mackessy, 1998).

4 CONCLUSIONS

To conclude, we evidence differences in protein bands' abundance and characteristics. Different dorsal color N. sputatrix. The differences we found are estimated as a similar protein family. The svPLA2 activities of these snake venom solutions also show a significant difference between black and brown dorsal color. Yet the yellow dorsal color snakes do not show a significant difference of svPLA2 activity with both black or brown dorsal color N. sputatrix. Considering the variation in storage condition, svPLA2 visualization of Javan spitting cobra venom in this research were remain similar, accompanied by the decrease in band intensity in the 37°C condition. The svPLA2 activity of N. sputatrix based on the storage condition is influenced by the factor of storage temperature and time with a significant different results in the 37°C storage temperature condition. We suggest the storage of venom solution in a relatively short time until 14 days would be performed at 4 or -80°C.

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