Characterization and optimization of lyophilization and storage conditions of *Leech saliva* extract from the tropical leech *Hirudinaria manillensis*

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Abstract: The medicinal Malaysian leeches have been used in traditional medicine to treat many different ailments. In this study, leech saliva extract (LSE) was collected from the medicinal Malaysian leech *Hirudinaria manillensis*. Gel electrophoresis of LSE was carried out to estimate the peptide and protein molecular weights of its content. Results showed that LSE contains more than 60 peptides and proteins with molecular masses ranging from 1.9-250kDa. Thrombin time assay *in vitro* was employed to assess the collected LSE antithrombin activity. First, to study its stability, LSE was lyophilized under the following different conditions: pre-freezing temperature, type of container and lyophilization cycle. Pre-freezed LSE sample at -20°C and lyophilized for 24 hours retained about 100-95% of its original biological activities. Second, the LSE antithrombin activity was monitored for a period of six months. Storage temperature, type of the container and photosensitivity effects on antithrombin activity of the lyophilized (solid state) and non-lyophilized (liquid state) were investigated. Results showed that storage temperature drastically affected the biological activity of LSE with -20°C as the optimum temperature. Samples stored at ambient temperature and +4°C were light photosensitive and adversely affected when stored in polypropylene tubes. Lyophilized samples were more stable than non-lyophilized ones over the period of study. To sum up, in order to have a biologically active stock of LSE, it has to be lyophilized for no more than 24 hours following freezing at -20°C and has to be stored at -20°C in glass tubes protected from light.

Keywords: Antithrombin, leech, lyophilization, protein, storage conditions.

INTRODUCTION

The salivary gland secretion of the medicinal leeches was known to contain a variety of peptides and proteins with a wide range of therapeutical benefits and chemical properties (Koh and Kini, 2009, Corral-Rodríguez *et al*., 2010, Baskova *et al*., 2004). For example, most important biologically active compounds identified from leech extract includes: peptides such as *hirudin* from the European leech *Hirudo medicinalis* (Markwardt, 1970), and *hirudin-like* peptide from the medicinal Malaysian leech *Hirudinaria manillensis* (Seong *et al*., 1997) with a direct antithrombin activity.

On the other hand, the complex nature of protein-based compounds (the primary, secondary, tertiary, and quaternary structures) makes them physically and chemically unstable, which in turn are serious challenges to formulation scientists. Lyophilization is a conventional method of transforming instable protein solutions into a more stable solid form; however, proteins may undergo many degradation reactions during this process, during either freezing and/or the drying steps, leading to inactive protein preparations (Frokjaer and Hovgaard, 2000, Wang, 2000).

The aim of the current study is to characterize the peptides and proteins of the salivary gland secretion of the medicinal Malaysian leech, *Hirudinaria manillensis*, by estimation of their molecular weights. In addition, we tried to optimize the freeze-drying conditions of LSE involving pre-freezing temperature, lyophilization vessel type, and lyophilization cycle period. Additionally, the effect on biological activities for a period of six months of LSE storage temperature, storage container type, and photosensitivity were also investigated. The antithrombin activity of the extract was taken as the indicator of LSE stability during all experimental procedures.

MATERIALS AND METHODS

Leech sampling and maintenance

The medicinal Malaysian leeches *Hirudinaria manillensis* (Lesson, 1842) were collected from the natural lake Cheneh, located in Trengganu, Malaysia. They were maintained in well-aerated plastic containers filled with un-chlorinated tap water. Water was regularly changed every two days. The collected leeches were kept under 12h: 12h light and dark cycle at the room temperature (25±1°C).

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Chemicals and reagents

Thromboclotin® (thrombin reagent) and Control N® (control plasma) were purchased from Siemens Healthcare Diagnostic (Germany). Sodium chloride, glacial acetic acid (analytical grade, 99.7%), glutaraldehyde (50% in H_2O), tricine (ULTROL grade, ≥100.9%), *bis* acrylamide (electrophoresis grade), hydrochloric acid (ACS reagent, 37%), and bromophenol blue (ACS reagent) were purchased from Merck (Germany). Arginine hydrochloride, acrylamide (electrophoresis grade, ≥99%), trizma base (analytical grade, 99.9%) and sodium dodecyl sulfate (SDS, electrophoresis grade, ≥98.5%) were procured from Sigma Aldrich (Germany). Ammonium persulfate (APS, electrophoresis purity grade), β-mercaptoethanol (β-ME, electrophoresis purity grade), and N,N,N□,N□-tetramethylethylenediamine (TEMED, electrophoresis purity grade) were from Bio-Rad Laboratories (USA). Coomassie blue G250, glycine (analytical grade, ≥100.1%) methanol (analytical grade, 99.99%) and glycerol (analytical grade, 100%) were all purchased from Fisher Scientific (USA). Prestained protein molecular weight markers (range from 20- 118kDa) were obtained from Fermentas Life Science (Canada). Ultra-low range molecular weight peptide markers (1.060- 26.600kDa) were purchased from Sigma Aldrich (Germany).

Thrombin time assay was carried out using Sysmex CA-50 coagulometer (Japan). Centrifugation was executed using Universal 32R Centrifuge produced by Hettich ZenTrifugen (Germany). Lyophilization was performed using Christ freeze-drier model Alpha 1-4LD (Germany). Mini-Protean Tetra Cell Gel Electrophoresis Apparatus which is used to cast and run the gels was purchased from Bio-Rad Laboratories (China).

Collection of leech saliva extract

Leech saliva extract was collected from starved leeches through leech feeding device which has been previously described (Abdualkader *et al*., 2011). Briefly, leeches were fed on the phagostimulatory solution (PHS) of 0.001M arginine in normal saline (Rigbi *et al*., 1987) filled in parafilm membrane wrapped a glass funnel (Abdualkader *et al*., 2011, Dickinson and Lent, 1984). The fed leeches were forced to regurgitate whatever they sucked through immersion in ice containers (Abdualkader *et al*., 2011). Only those colorless salivary fluids vomited by leeches were pooled and centrifugated at 2500rpm and +4°C for 10min. The supernatant was filtered through 0.45µm Sartorius® filter paper and named leech saliva extract (LSE), which will be used during all the following experimental procedures.

Molecular weights determination

Molecular weights of the peptides and proteins of LSE were estimated by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis methods (SDS-PAGE). The analysis was carried out using tris-glycine-SDS-PAGE method described by Laemmli (Laemmli, 1970) in two conditions containing 15% and 16% resolving gel respectively. An alternative method with slightly higher percentage resolving gels, 16.5%, 18% and 20%, was used according to tricine-SDS-PAGE method (Schagger, 2006). Protein bands were visualized by Coomassie blue dye method (Ahmed, 2005). Molecular weights were estimated by reference to the prestained protein molecular weight markers and the ultra-low range molecular weight peptide markers.

Thrombin time assay in vitro

The antithrombin activity of LSE was ascertained using thrombin time (TT) assay *in vitro*. All procedures were carried-out according to the standard protocols provided with Thromboclotin® reagent and the coagulometer manual. A mixture of fresh citrated plasma and LSE samples was incubated for 3min in the coagulation analyzer wells. Then, thrombin reagent was added and the time until coagulation starts was recorded (Abdualkader *et al*., 2011, Schmied *et al*., 1995). The percentage increase in thrombin time (%TT) was calculated from the following equation:

$$\%TT = \left(\frac{\text{TT of the sample} - \text{TT of the citrated plasma}}{\text{TT of the citrated plasma}}\right) \times 100$$

The effect of lyophilization conditions and lyophilization-vessel type on the antithrombin activity of LSE

The collected LSE was aliquoted in separated tubes each of which contains 1ml. Two types of tubes were used: glass and polypropylene tubes. Additionally, these samples were frozen at -20°C or at -40°C. Thereafter, the frozen samples were lyophilized for 12, 24, 48 or 72 hours. Finally, the %TT of each lyophilized sample was determined and compared to fresh LSE.

The effect of storage conditions and type of storage-container on the antithrombin activity of LSE

Tubes (glass or polypropylene) with 1 ml of lyophilized or non-lyophilized LSE were stored at variable storage temperature conditions (room temperature, +4°C and -20°C). Some tubes at room temperature were protected from light by wrapping with aluminum foil. The antithrombin activity (%TT) of each sample was monitored for a period of six months and compared to fresh LSE.

STATISTICAL ANALYSIS

All measurements were repeated in triplicates, the results were expressed as the mean ± the standard error of the mean (SEM) and analyzed by One Way ANOVA and General Linear model (GLM) repeated measure ANOVA, followed by Tukey's HSD post hoc test for multi-comparison using the Statistical Package for the Social

Sciences SPSS 18.0 software, and ρ□0.05 was considered statistically significant.

RESULTS

Molecular weights determination

Results revealed that LSE contains a wide range of high molecular weight (HMW) and low molecular weight (LMW) peptides and proteins. The calculated molecular weights separated by tris-glycine-SDS-PAGE ranged from 7.2kDa to 120.9kDa with thirty well separated bands (figs. 1a and b). On the other hand, tricine-SDS-PAGE resulted in 37 different bands with molecular weights ranging from 1.9kDa to 252.4kDa (figs. 2a, b and c).

The antithrombin activity of LSE

It was found that LSE prolonged TT in a dose-dependent manner (data not shown). A citrated plasma containing 40µl of LSE showed a thrombin time value of 36.97±1.07sec, corresponding to 62.85±4.73% increase compared to thrombin time of the pure citrated plasma (table 1).

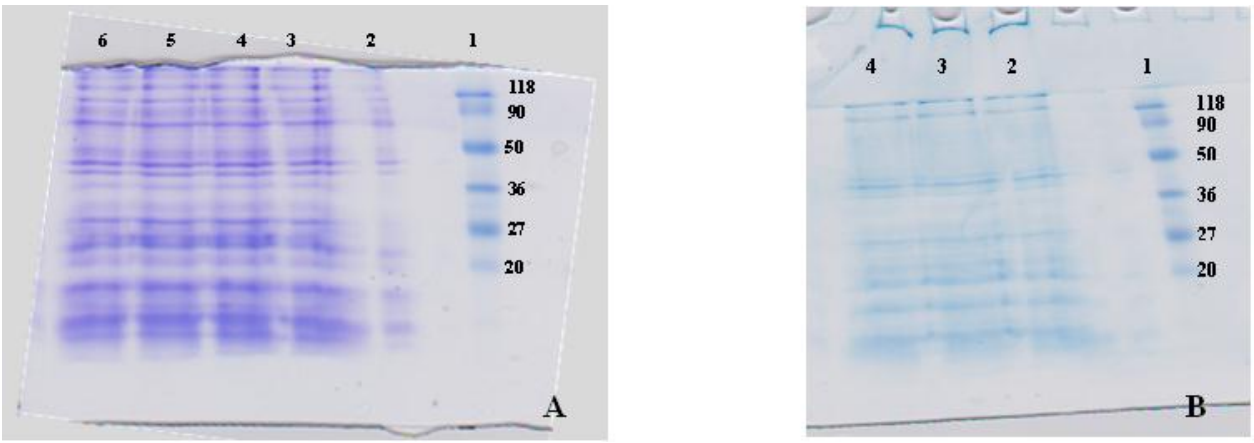

Fig. 1: Separation of LSE peptides and proteins by Tris-glycine-SDS-PGE method with 15% resolving gel (A) or 16% resolving gel (B). Gels were stained by Coomassie blue brilliant dye. Lane 1: Prestained protein markers and their molecular weights (kDa); Lane 2, 3, 4, 5 and 6: leech saliva extract.

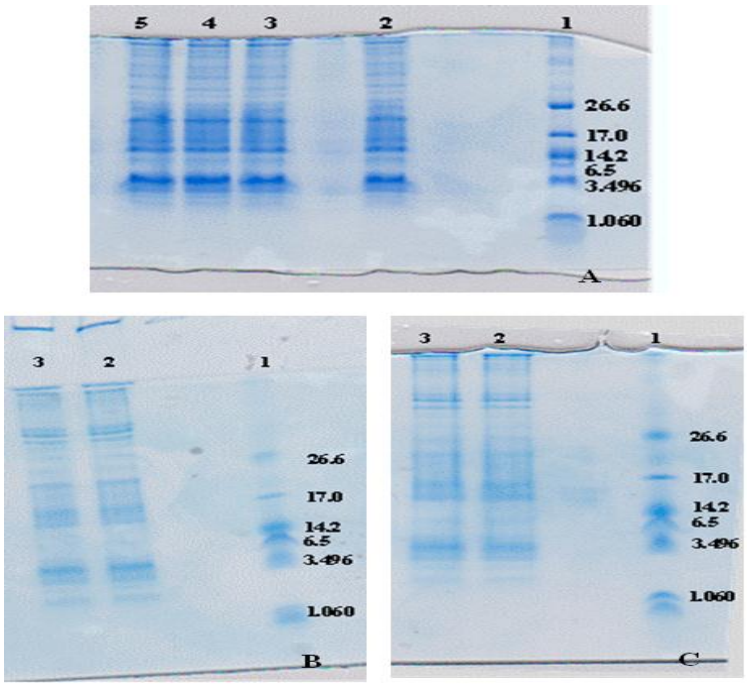

Fig. 2: Separation of LSE peptides and proteins by Tricine-SDS-PGE method with 16.5% resolving gel (A), 18% resolving gel (B) and 20% resolving gel (C). Gels were stained by Coomassie blue brilliant dye. Lane 1: peptides markers with their molecular weights (kDa); Lane 2, 3, 4 and 5: leech saliva extract.

The effects of lyophilization conditions and lyophilization -vessel type on the antithrombin activity of LSE

The results of the study (table 1) revealed that pre-freezing (freezing before lyophilization) at -40°C significantly (ρ□0.05) decreased the antithrombotic activity of LSE by 31-34% compared to the activity of the fresh LSE. Whereas, pre-freezing at -20°C yielded lyophilized saliva with antithrombin activity (%TT= 60-65%) almost similar to that of the fresh sample (%TT= 62%), regardless the vessel type.

However, it was found that type of the container (glass or polypropylene) had no significant effect on LSE bioactivity during the lyophilization process.

Furthermore, results revealed that lyophilization of LSE for more than 24hours led to a dramatic decrease (ρ□0.001) of the antithrombin activity by 67-80%. On the other hand, the extract that was lyophilized for 12-24 hours retained about 95% of its original activity.

The effects of storage conditions and type of storage container on the antithrombin activity of LSE

Antithrombin activity of LSE stored at room temperature

After one day of storage, all samples stored at room temperature significantly (ρ□0.001) and partially lost their antithrombin activity in comparison to the activity of the fresh LSE, which was taken as reference control.

The non-lyophilized LSE (extract in liquid state) kept in glass tubes and protected from light lost 62.2% of its initial activity after one day of storage, and exhibited more than 90% decline after three days. The non-lyophilized samples stored in glass tubes and exposed to light lost more than 90% of their activity after one day. Similarly, the non-lyophilized samples kept in polypropylene tubes either in light or in dark place showed more than 90% downfall in their antithrombotic activity after a storage period of one day.

The lyophilized LSE (extract in solid state) maintained in glass tubes and protected from light for one, three and seven days lost about 26.5, 75 and 95% of their biological activity, respectively. While, the same samples when exposed to light for one day lost about 48% of their initial biological activity, and a total loss of 90% after 3-7 days of storage. The lyophilized LSE kept in polypropylene tubes in dark place for one day lost 57% of its antithrombin activity, and more than 90% at the third day of storage. Likewise, the lyophilized LSE kept in polypropylene tubes and exposed to light experienced 80-99% loss of its antithrombin activity during the period of the study.

At room temperature, it was found that light significantly affected LSE antithrombin activity. The lyophilized LSE stored in glass tubes and protected from light lost 26.5% of its activity during the first day in comparison to 48% (ρ<0.05) loss for those lyophilized samples exposed to light. In the same manner, the non-lyophilized samples maintained in polypropylene tubes and protected from light showed 62.2% decrease in their antithrombin activity after one day of storage, meanwhile the same samples stored in polypropylene tubes in light lost about 92% (ρ<0.001) of their activity.

Moreover, type of the container was found to significantly affect the antithrombin activity of LSE stored at room

Table 1: The effect of lyophilization conditions and lyophilization-vessel type on the antithrombin activity of LSE

Sample and lyophilization condition		TT (sec)		%TT [3]
		Citrated plasma	Plasma Mixed with LSE	
Fresh LSE (glass/Polypropylene tubes)		22.70	36.97±1.07	62.85±4.73
Pre-freezing at -20°C [1]	Polypropylene container	15.90	26.37±0.64	65.83±4.00
	Glass container	15.90	25.37±0.29	59.54±1.83
Pre-freezing at -40°C [1]	Polypropylene container[α]	15.90	22.40±0.75	40.88±4.72
	Glass container [α]	15.90	22.73±0.33	42.98±2.06
The period of lyophili zation cycle[2]	Lyophilized 12h	15.9	25.40±0.46	59.75±2.88
	Lyophilized 24h	15.9	25.30±0.83	59.12±5.24
	Lyophilized 48h [β]	17.20	20.70±0.40	20.35±2.33
	Lyophilized 72h [β]	17.90	20.03±1.01	11.92±3.25

[1]Samples were aliquoted in 1-ml tubes and lyophilized for 24 hours.
[2]Samples were aliquoted in 1-ml glass tubes and lyophilized for different periods.
[3]%TT was estimated from the equation mentioned in materials and methods section. Thrombin time of each sample was monitored during a period of seven days at regular intervals using Sysmex CA-50 coagulometer.
[α]ρ□0.05 when compared with fresh LSE. [β] ρ□0.001 when compared with fresh LSE.
All results are expressed as the mean of three measurements± SEM and analyzed by One Way ANOVA, followed by Tukey's HSD post hoc test using the Statistical Package for the Social Sciences SPSS 18.0 software, and ρ□0.05 was considered statistically significant.

temperature. The lyophilized samples stored in glass tubes exhibited 26.5-47.8% decline in activity compared to 57.1-84.5% decrease for lyophilized samples stored in polypropylene tubes ($\rho<0.05$). Similarly, the non-lyophilized samples stored in glass tubes and protected from light showed 62% decrease in comparison to 92% ($\rho<0.001$) loss of activity for those samples stored for one day in polypropylene tubes and protected from light.

Additionally, findings showed that the non-lyophilized samples (extract in liquid state) undergo a significant and a faster loss of activity compared to the lyophilized counterparts (extract in solid state) stored in the same conditions. For example, the non-lyophilized LSE stored in glass tubes and protected from light lost 62.2% of their initial activity compared to their lyophilized counterpart, which lost 26.5% of their bioactivity after one day of the study ($\rho<0.001$). After 3-7 days of storage, significant differences between samples were not observed because samples lost a great part of their biological activity (75-95%). All results are presented in fig. 3.

Antithrombin activity of LSE stored at +4°C

Fig. 4 displayed that no significant loss of activity was observed during the first seven days of the study of all samples, regardless storage conditions. Whereas, all samples exhibited a significant decrease ($\rho \square 0.001$) in their biological activities after 15 days of storage when compared to the biological activity of the fresh saliva (control reference). The non-lyophilized samples kept in glass tubes retained about 100-97% of their biological activities during the first seven days of storage. Thereafter, a sharp decline in their activities occurred after 15 days. The non-lyophilized samples which were stored in glass tubes lost about 45% of their initial activity after 15 days of the study and more than 90% after longer storage. For polypropylene tubes, the non-lyophilized saliva samples held about 100-95% of their antithrombotic activity during the first seven days of the study. Thereafter, they lost about 47% of their activity after 15 days and experienced more than 90% decline after a storage period of 30 days or more.

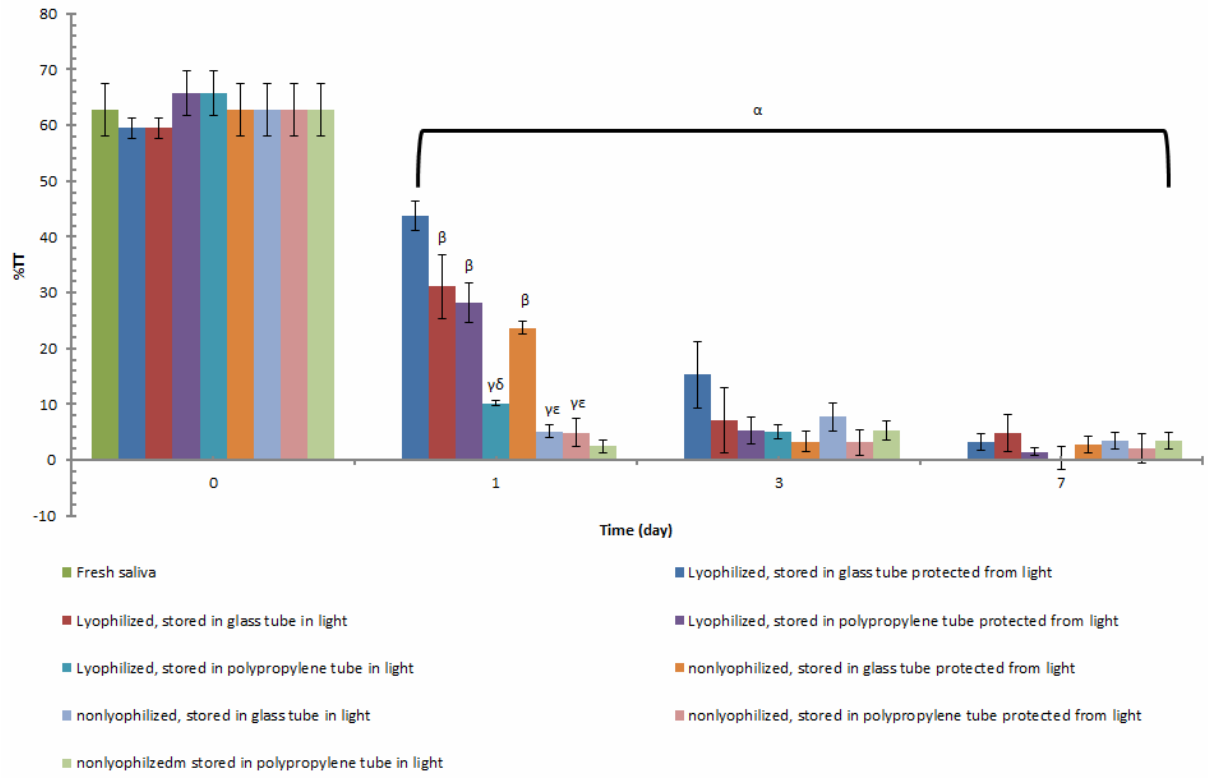

Fig. 3: The antithrombin activity of LSE stored at room temperature.
Thrombin time of each sample was monitored during a period of seven days at regular intervals using Sysmex CA-50 coagulometer.
$^{\alpha}$ significant when compared with fresh LSE (reference control).
$^{\beta}$ significant when compared with lyophilized LSE stored in glass tubes and protected from light.
$^{\gamma}$ significant when compared with lyophilized LSE stored in polypropylene tubes and protected from light.
$^{\varepsilon}$ significant when compared with non-lyophilized LSE stored in glass tubes and protected from light.
$^{\delta}$ significant when compared with lyophilized LSE in glass tubes in light.
Results are expressed as the mean ± SEM and analysed by General Linear model (GLM) repeated measure ANOVA, followed by Tukey's HSD post hoc test for multi-comparison using the Statistical Package for the Social Sciences SPSS 18.0 software, and $\rho \square 0.05$ was considered statistically significant.

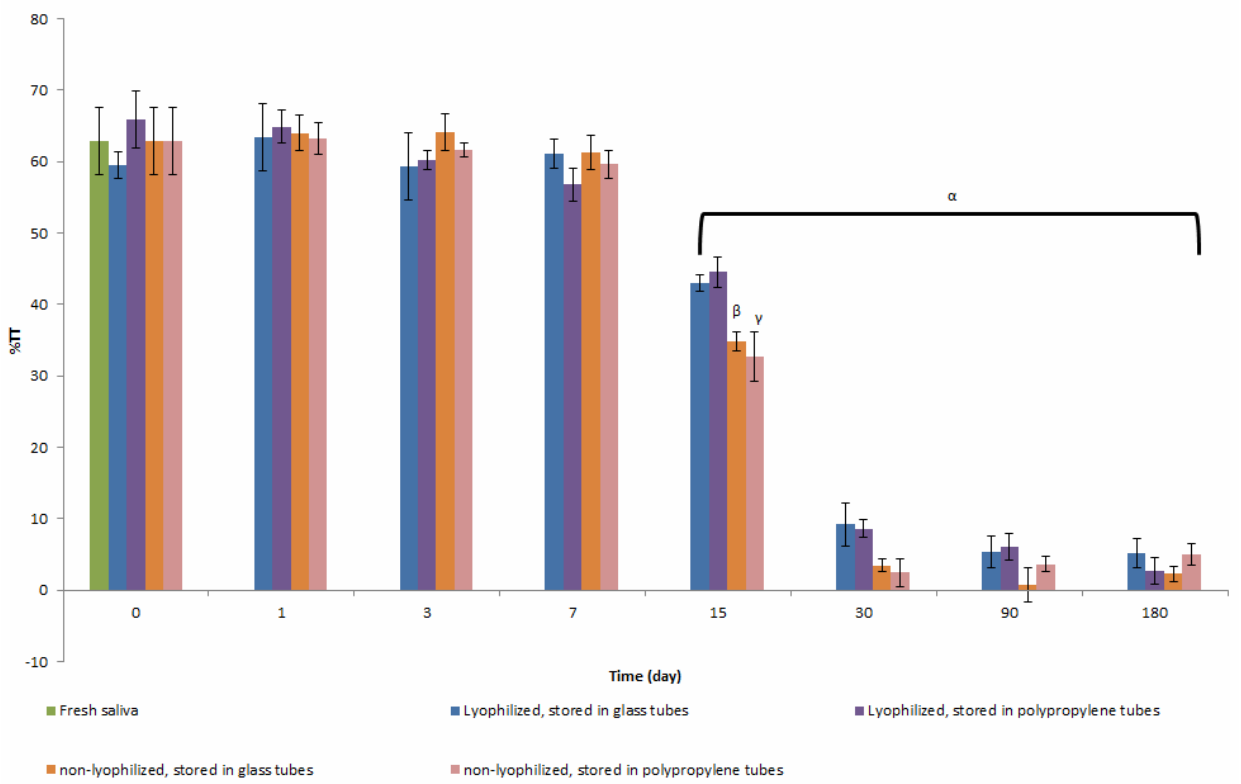

Fig. 4: The antithrombin activity of LSE stored at +4°C.
Thrombin time of each sample was monitored during a period of 180 days at regular intervals using Sysmex CA-50 coagulometer.
$^{\alpha}$ significant when compared with fresh LSE (reference control).
$^{\beta}$ significant when compared with lyophilized LSE stored in glass tubes.
$^{\gamma}$ significant when compared with lyophilized LSE stored in polypropylene tubes.
Results are expressed as the mean ± SEM and analysed by General Linear model (GLM) repeated measure ANOVA, followed by Tukey's HSD post hoc test for multi-comparison using the Statistical Package for the Social Sciences SPSS 18.0 software, and $\rho \square 0.05$ was considered statistically significant.

The lyophilized LSE samples stored in glass tubes retained about 100% of their initial anticoagulant activity after a storage period of seven. After fifteen days, they lost 27% of their activity. After 30 days, 80-90% loss in their bioactivity was recorded. On the other hand, after three days of storage in polypropylene tubes, the lyophilized saliva samples experienced about 0-9% decrease of their biological activity. Then, a gradual decrease of 13-32% after 7-15 days was noticed. After 30 days, a sharp downfall (85-95%) occurred.

Noticeably, the effect of the type of container on the antithrombin activity of stored samples was minor. On the other hand, state of the extract (lyophilized versus non-lyophilized saliva) was shown to have a significant effect on the biological activity after 15 days of storage. Whereas, the non-lyophilized samples (liquid) were more prone to activity loss than the lyophilized ones (solid). The non-lyophilized LSE samples kept in glass tubes lost 45% of their activity, while their lyophilized counterparts showed only 27% decrease after 15 days of storage ($\rho<0.05$-0.001). Likewise, the non-lyophilized samples maintained in polypropylene tubes revealed 47% decline compared to 32% downfall for those lyophilized samples ($\rho<0.05$) for the same period of 15 days. After 30-180 days, all stored samples lost 81-98% of their activity.

Antithrombin activity of LSE stored at -20°C

The findings presented in Table 2 revealed that the non-lyophilized LSE samples stored in glass tubes experienced 0-6% decrease in their activity (statistically not significant) during the first 15 days of storage. After 30 days, they lost about 10% of their activity. Further storage (90-180 days) resulted in a significant 12-15% loss ($\rho \square 0.05$) of their antithrombin activity.

Similarly, the non-lyophilized LSE samples kept in polypropylene tubes lost approximately 0-5% (statistically not significant) of their bioactivities during the first 15 days of the study, and about 13-16% loss ($\rho \square 0.05$) after a longer storage period (30-180 days). Noticeably, the results revealed that the lyophilized LSE stored in glass tubes experienced only 0-5% decrease in the biological activity during the 180-day study period.

The statistical analysis showed the decrease was not significant when compared to fresh LSE samples.

Moreover, the lyophilized samples stored in polypropylene tubes displayed a non-significant loss of about 3-6% during the first fifteen days of the study and a statistically significant ($\rho \square 0.05$) decline of about 13-20% during a longer storage period (30-180 days). At this temperature of storage (-20°C), the impact of type of container and state of the extract were not of statistically significant.

DISCUSSION

The molecular weights of LSE peptides and proteins

A comparison between our findings and the literature data revealed a striking coincidence to what has been already known in terms of leech extract peptides and proteins. We previously evidenced that LSE contained more than 25 peptides and proteins with a molecular weight ranging from 3.7-80kDa, and many of them matched well-known biologically active proteins isolated from the same leech species (Alaama *et al*., 2011), whereas the current results were more extensive and presented a wider range of LMW and HMW peptides and proteins (1.9-252kDa) with more than 60 well-separated bands.

Interestingly, amongst the calculated molecular weights, some were in a good agreement with four previously isolated proteins from the leech *H. manillensis*. First of all, the band of 7.2kDa was very close to the molecular weight of the antithrombin protein, *hirudin-like* (#7kDa), isolated from the extract of the Malaysian leech *H. manillensis* (Seong *et al*., 1997). This band could be attributed to antithrombin protein, *bufrudin* (#7kDa), which was purified from *H. manillensis* (Electricwala *et al*., 1991). The estimated molecular weights ranged from 52.5-58.2kDa were in a good agreement with the protein named *manillase*. The latter was isolated from the tropical leech head extract *H. manillensis* collected from Bangladesh. *Manillase* was found in a glycosylated form (58±2kDa) and non-glycosylated form (54±2kDa) and it was found to possess a hyaluronidase activity (Kordowicz *et al*., 2000).

Furthermore, a comprehensive search in the literature showed that our findings match many antithrombin agents from other leech species, particularly the band of 7.2kDa which is in accord with *hirudin* identified from the salivary gland secretion of the European leech *Hirudo medicinalis* (Haycraft, 1884, Markwardt, 1970). The band of 5.9kDa is in a close similarity with *granulin-like* (#6kDa) from *Hirudo nipponia* (Jin Hong and Won Kang, 1999). Additionally, *theromin* (14.5kDa) from *Theromyzon tessulatum* head extract (Salzet *et al*., 2000) corresponds to the estimated molecular weights 14.7-14.9kDa. On the other hand, the bands of 4.6kDa and 64.9kDa were in a good agreement with previously known platelet aggregation inhibitors. Namely, *decorsin* (4.3kDa) which was isolated from *Macrobdella decora* (Seymour *et al*., 1990), and *calin* isolated from *Hirudo medicinalis* saliva (Deckmyn *et al*.,1995). Some calculated weights were very similar to other leech-derived anticoagulants; for example, the band of 7.2kDa could be assigned to *tridegin* (7.3kDa) which was purified from the anterior and posterior parts of *Haementeria ghilianii* with a Factor XIIIa inhibitory activity (Finney *et al*., 1997). Moreover, the estimated mass 8.9kDa is identical to the factor Xa inhibitor called *therostatin* (8.9kDa) isolated from the anterior parts of the leech species *Theromyzon tessulatum* (Chopin *et al*., 2000). Two fibrinolytic enzymes named *hementerin* (80kDa) and *hementin* (82kDa) identified from *Haementeria depressa* and *Haementeria ghilianii*, respectively (Chudzinski-Tavassi *et al*., 1998, Swadesh *et al*., 1990) were parallel to two bands, 80kDa and 82.2kDa, which we estimated from LSE of the medicinal Malaysian leech.

The antithrombin activity of LSE

Our findings showed that salivary gland secretion obtained from the medicinal Malaysian leech possessed a remarkable thrombin inhibitory activity, since it arrested thrombin ability to form the insoluble fibrin clot from the soluble fibrinogen that resulted in a prolonged thrombin time from 22.70 sec to about 36.97 sec. We have already demonstrated a linear dose-response relationship of the antithrombin activity. The protein concentration that can increase thrombin time (TT) by two folds (IC_{100}) was 62.682±1.705µg/ml (Abdualkader *et al*., 2011). On the other hand, some researchers characterized a direct thrombin inhibitor named *thrombin-like* (#7kDa) from the medicinal Malaysian leech extract (Seong *et al*., 1997).

The effects of lyophilization conditions and lyophilization-vessel type on the antithrombin activity of LSE

This study demonstrated that LSE would lose about 30% of its antithrombin activity in the pre-freezing step at -40°C. Whereas, pre-freezing at -20°C allow LSE to maintain its biological activities at a level similar to fresh LSE. Similarly, LSE remained 95% biologically active when lyophilized for 24 hours or less. In contrast, it lost about 67-80% of its initial biological activities when lyophilized for 48 hours or more. However, the type of the container (polypropylene or glass) in which LSE was kept during lyophilization seems to have no effect on LSE biological activities. To the best of our knowledge, this is the first time a detailed study has been carried-out to evaluate the effect of lyophilization parameters on the stability of the biological activities of leech saliva extract. Some researchers mentioned in their findings that the antithrombotic activity of *hirudin* extracted from *H. medicinalis* decreased sharply by 40-60% after lyophilization, but without giving a rational explanation

for that fact (Rigbi *et al*., 1987), and our finding could be the explanation of the observed fact.

As a general rule, lyophilization is a two-step process involving an initial step of freezing of a protein solution followed by a vacuum drying step. Both steps have been considered carefully during the freeze-drying process because of their potential effects on protein stability. For instance, it is known in general that one or more factors like: temperature, concentration, pH changes, and dehydration could affect biological activities of any protein preparation (Wang, 2000). Similarly, LSE in our study can easily be affected by one or more of these factors at various degrees starting from a slight decrease in activity to a total inactivation.

Specifically, at a low temperature, some proteins, called "cold labile proteins", lose their native structure by denaturation and subsequently their bioactivity (Ó'Fágáin, 2010). This low temperature-induced protein denaturation was proposed to be the result of protein instability due to the decreased solvation forces interaction and increased hydration (solubility) of the non-polar group of the protein chain (Graziano *et al*., 1997, Jaenicke *et al*., 1990, Dill, 1990).

Another mechanism to explain the alteration in stability at different temperatures (-20° and -40°C in our study), was suggested based on freeze-induced inactivation due to the formation of ice-water interfaces at which proteins can adsorb. Larger ice-water interfaces will be generated by fast freezing. Conversely, smaller interfaces will result from slow freezing (Strambini and Gabellieri, 1996).

The time-dependent stability of LSE during freeze-drying can be explained by the formation of a dry product layer resulting from the continuous drying step. The formation of such a layer will slow-down the diffusion of water vapor leading to an increase in product temperature (Overcashier *et al*., 1999, Adams and Ramsay, 1996). The resultant rise in temperature may cause a product collapse, because the temperature should be below the glass transition temperature (T_g') or the eutectic melting temperature (T_{mf}) (Wang, 2000, Overcashier *et al*., 1999).

It was known that in order to perform a successful lyophilization process, the containers should contain a minimum amount of the protein solution at a depth not more than 2 cm in order to obtain free sublimation of water from the surface of the ice crystal (Felix, 1998). Besides, a suitable depth of the liquid will ensure an easier and better circulation of the resulted vapor by vacuum (Ó'Fágáin, 2010). Generally, the smaller the filling volume, the more efficient is the lyophilization cycle and the lower loss of biological activities (Wang, 2000). That is why samples were aliquoted in 1-ml tubes in the current study.

The effects of storage conditions and storage container on the antithrombin activity of LSE

To the best of our knowledge, it is the first time, at least in Malaysia, a study to evaluate the effect of storage temperature, light and type of container on the antithrombin activity of LSE in both solid and liquid states was carried-out. The current results demonstrated that storage temperature is the most important factor affecting the anticoagulant activity of LSE. For better storage conditions, -20°C is the most preferable temperature.

Additionally, this study stated that non-lyophilized LSE (liquid form) showed more rapid loss in its biological activity than the lyophilized form (solid state). For instance, storage of non-lyophilized LSE in glass tubes in dark place at -20°C for six months resulted in roughly 12% decline in the biological activity compared to only 4% loss for lyophilized saliva extract stored at similar conditions for the same period.

In liquid state, protein solution can be affected dramatically by storage temperature. In fact, the biological activity of a protein depends principally on protein folding in an individually unique globular conformation. This conformational folding is a result of stabilizing forces (hydrophobic interaction, hydrogen bonds, van der Waals 'forces, etc.) and destabilizing forces (local and nonlocal protein's conformational entropy). After all, high temperatures will promote physical degradation and aggregation rate due to the destruction of the native conformational state of the protein. Besides that, high temperature will increase intra-molecular and inter-molecular reactions due to the increase in kinetic energy needed to overcome the activation energy (Chi *et al*., 2003). In the solid state of protein preparation, storage temperature is a critical factor that may affect protein stability. The influence of temperature on solid protein activity upon storage cannot be described by one simple pattern due to the complexity and variations of protein structure. Generally, it can be said that the higher the temperature, the lower is the stability (Ford and Dawson, 1994, Wang, 2000). It was proposed that high temperature can stimulate both physical aggregation and chemical degradation of solid proteins. In addition, storage temperature above (T_g) can destabilize a solid protein due to crystallization of the amorphous components of the freeze-dried protein formulation (Wang, 2000, Hancock and Zografi, 1997, Aldous *et al*., 1995).

Furthermore, our findings suggested light would accelerate LSE degradation which was clearly apparent when the extract was stored at an undesirable temperature (ambient temperature). When the tubes were kept in the fridge (+4°C/-20°C), the effect of light was not important since there is no light inside.

Table 2: The antithrombin activity of LSE stored at -20°C

Time (day)	Fresh LSE [1]	%TT [2]			
		Lyophilized LSE (solid state) [3]		Non-lyophilized LSE (liquid state)	
		Glass tubes	Polypropylene tubes	Glass tubes	Polypropylene tubes
0	62.85±4.73	59.54±1.83	65.83±4.00	62.85±4.73	62.85±4.73
1	-	61.55±1.60	62.58±1.23	60.94±1.78	62.99±1.64
3	-	64.78±0.96	63.31±0.42	59.75±2.02	63.73±1.83
7	-	62.38±0.74	60.15±2.20	62.94±0.37	61.45±2.81
15 [α]	-	62.80±1.14	61.51±1.55	62.37±1.14	59.78±1.55
30 [α]	-	56.25±1.95	56.65±1.02	56.65±1.24	52.76±2.55
90 [α]	-	55.86±1.47	55.31±3.21	53.30±0.84	54.40±1.45
180 [α]	-	57.23±0.36	53.25±0.42	55.14±1.79	53.88±1.17

[1] LSE: leech saliva extract was considered as zero point with which all samples were compared.
[2] Samples were aliquoted in 1-ml tubes, freezed at -20°C, then lyophilized for 24 hours. During storage, light effect was irrelevant since the fridge was not equipped light.
[3] %TT was estimated from the equation mentioned in materials and methods section. During storage, light effect was irrelevant since the fridge was not equipped light. Thrombin time of each sample was monitored during a period of 180 days at regular intervals using Sysmex CA-50 coagulometer.
[α] $\rho \square 0.05$ when compared with fresh LSE (reference control) except for lyophilized samples stored in glass tubes.

In fact, many pharmaceutical proteins were reported as photosensitive. Photosensitivity is a very complex process resulting in oxidative modifications in protein structure and loss of activity. The compounds that can initiate photooxidation were termed as photosensitizers which have some mutual structural characteristics such as aromatic rings and/or high conjugation enabling the absorption of light photons and being excited (Frokjaer and Hovgaard, 2000). When a protein molecule absorbs photons, photoionization takes place leading to degradation by-products (Carstensen and Rhodes, 2000). The most sensitive amino acids to light exposure-induced oxidation are Histidine (His), Methionine (Met), Tryptophan(Trp), Cysteine (Cys) (Frokjaer and Hovgaard, 2000) and Tyrosine (Tyr) (Carstensen and Rhodes, 2000). Additionally, the type of the container was found to affect LSE biological activity over a storage period of six months. At ambient temperature and +4°C, samples which were kept in polypropylene tubes displayed lower activity than those in glass tubes. On the contrary, the role of the container in LSE stability was not considerable when samples stored at -20°C. Container-induced loss of protein activity can be referred to two major mechanisms, surface adsorption and surface-induced denaturation (Burke *et al*., 1992). Proteins unfold when adsorb at interfaces. Adsorption occurs via the interaction between the hydrophobic amino acids and hydrophobic surfaces or between the hydrophilic amino acids (polar amino acids) and charged surfaces (Frokjaer and Hovgaard, 2000). As a rule of thumb, at lower temperature, interactions take place at slower rates due to decline in activation energy (Sinko, 2006) which clarifies the reason of LSE stability at -20°C regardless type of container. In addition, surface adsorption is related to protein concentration (Frokjaer and Hovgaard, 2000) which is beyond the scope of this study since all samples belong to the same extract and consequently contain identical total protein concentration.

CONCLUSION

The salivary gland secretion collected from the medicinal Malaysian leech *H. manillensis* contained a wide range of HMW and LMW peptides and proteins. We found that LSE possessed an antithrombin activity. Optimization of lyophilization conditions suggested that the maximum bioactivity was achieved when the extract was pre-freezed at -20°C and lyophilized for no more than 24 hours. Type of the container was found to have no impact on the antithrombin activity of the extract during the lyophilization process. Optimization of storage conditions showed that the optimum storage temperature was -20°C regardless of storage conditions. Whereas, type of container, light and extract state affected LSE bioactivity at a higher temperature. Taken together, we recommend that LSE should be stored in the lyophilized state, in glass tubes, protected from light and at -20°C.

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