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Starvation Time and Successive Collection Effects on Leeches Saliva Collection Quantity and Proteins Quality and Quantity in Wet Season

(Kesan Masa Kebuluran dan Kejayaan Pengumpulan Kuantiti Air Liur Lintah serta Kualiti dan Kuantiti Protein dalam Musim Hujan)

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ABSTRACT

The salivary gland secretion of the haematophagous animals, leeches, has attracted the attention of therapists since the extreme old ages due to its wide range of medical properties. Thus, many researches have been done to develop and optimize new methods to collect leech saliva with high quality and quantity. In the present study, we aimed to evaluate the effects of starvation period and repeated collection on the quality and quantity of leech saliva extract LSE and its contents of proteins during the rainy season. Protein recovery in the LSE was also studied after first collection. It was found that leeches are able to produce protein-containing saliva whenever fed during the whole study period of 18 weeks with varied protein concentrations. The results showed that the highest protein concentrations (105-91 µg/mL) were produced after 12-15 weeks of starvation. The results of successive collection showed that leeches are able to produce proteins and peptides whenever they suck the solution after first collection with some varies in the concentrations. The concentrations varied between 0 and 72% of the initial concentration. Gel electrophoresis results showed absence for some bands when the concentrations are too low. Also the results showed that leeches are able to recover about 42% of their initial proteins concentration within four weeks of starvation after first feeding. The gel electrophoresis results showed the closeness between the first and second collections. To conclude, all test factors (starvation period, successive collection and recovery test) were shown to have an important impact on protein concentration of leech saliva and therefore its medicinal affectivity. The mentioned results are reported for the first time and they open the gate for further studies.

Keywords: Leeches; saliva; starvation; time; quality; quantity; wet season

ABSTRAK

Rembesan kelenjar air liur haiwan hematofaj, lintah, telah menarik perhatian ahli terapi sejak dulu kerana pelbagai sifat perubatan. Oleh itu, pelbagai kajian telah dijalankan untuk membangun dan mengoptimumkan kaedah baru untuk mengumpul air liur lintah dengan kualiti dan kuantiti yang tinggi. Dalam kajian ini, kami ingin menilai kesan tempoh kebuluran dan kutipan ulangan pada kualiti dan kuantiti air liur lintah ekstrak LSE dan kandungan proteinnya semasa musim hujan. Pemulihan protein di LSE itu juga dikaji selepas kutipan pertama. Didapati bahawa lintah boleh menghasilkan protein yang mengandungi air liur pada bila-bila masa setelah diberi makan sepanjang tempoh keseluruhan kajian selama 18 minggu dengan kepekatan protein yang berbeza-beza. Hasil kajian menunjukkan bahawa kepekatan protein tertinggi (105-91 µg/mL) telah dihasilkan selepas 12-15 minggu kebuluran. Hasil kejayaan kutipan menunjukkan bahawa lintah boleh menghasilkan protein dan peptida pada bila-bila masa mereka menghisap larutan selepas kutipan pertama dengan kepekatan berbeza. Kepekatan awal berbeza-beza antara 0 dan 72%. Hasil gel elektroforesis menunjukkan ketiadaan jalur apabila kepekatan terlalu rendah. Keputusan juga menunjukkan bahawa lintah mampu untuk memulihkan kira-kira 42% daripada kepekatan protein awal dalam tempoh empat minggu kebuluran selepas makan yang pertama. Keputusan gel elektroforesis menunjukkan kehampiran antara kutipan pertama dan kedua. Kesimpulannya, semua ujian faktor (tempoh kebuluran, kejayaan kutipan dan ujian pemulihan) telah terbukti mempunyai kesan penting ke atas kepekatan protein air liur lintah dan kesan perubatannya. Keputusan ini dilaporkan buat pertama kali dan membuka peluang untuk kajian lanjutan.

Kata kunci: Air liur; kebuluran; kualiti; kuantiti; lintah; masa; musim hujan

INTRODUCTION

Leech saliva extract (LSE) contains biologically active compounds which are mostly peptides and proteins (Abdualkader et al. 2013a, 2011; Sawyer, 1986b). Several studies have been conducted for the isolation and

characterization of the LSE (Abdualkader et al. 2013b; Baskova et al. 2004; Ghawi et al. 2013; Merzouk et al. 2012). The most challenging problem faced in the research and scientific studies about leeches saliva was to obtain sufficient amount of LSE for analysis and isolation of the

active compounds. In earlier studies, LSE was extracted by either killing the leeches and using the whole body (Bagdy et al. 1973) or only leeches' heads (Chopin et al. 2000; Salzet et al. 2000). These methods were laborious, time consuming, uneconomic and sacrificing required (Götz Nowak 2007). The studies about LSE have been expanded and developed using a new method to collect their saliva without sacrificing the leeches (Rigbi et al. 1987). This method depends on feeding the leeches on a phagostimulatory solution and then squeezing them from the posterior sucker upward to the mouth. This method may cause injury to leeches. A modified method was used in this study to collect leech saliva without sacrificing or causing any harm to the leech, and the leech can be used repeatedly (Alaama et al. 2011). Few studies have reported the influence of time factor in the secretion of considerable saliva quantity with high protein concentration (Rigbi et al. 1987).

The present work aimed to elucidate the effect of the starvation time of leeches on the quantity of LSE and also to evaluate the protein concentration in LSE during successive collections. Optimization of the time that should be given to leeches before submitting to another collection (recovery period) was also considered.

MATERIALS AND METHODS

LEECHES

Leeches were collected from Cheneh Lake, Terengganu, Malaysia during wet season in Malaysia i.e from September 2011 to March 2012. The leeches were identified as *Hirudinaria manillensis* (Lesson 1942) by Fred Hechtel (Biopharm, UK). They were kept in plastic containers with perforated cover to allow air circulation. The water inside the container was changed every three days.

CHEMICALS AND REAGENTS

The following chemicals and reagents were used in this study:- L-Arginine Hydrochloride; Tris base; Sodium Dodecyl Sulphate (SDS) electrophoresis grade; Acrylamide electrophoresis grade and ultra-low molecular weight peptide marker 1.02 – 26 kD were purchased from Sigma Aldrich (USA). Sodium Chloride; Bisacrylamid electrophoresis grade; Coomassie Blue electrophoresis grade; Acetone analytical grade and Tricin electrophoresis grade were supplied by Merck (Germany). Glacial Acetic Acid; Glycerol; Bromophenol Blue electrophoresis grade and Glycin electrophoresis grade were supplied from Fisher (UK). Tetramethylethylenediamine (TEMED) electrophoresis grade; Ammonium Per Sulfate (APS) electrophoresis grade and 2 - Mercaptoethanol electrophoresis grade were obtained from Bio Rad (USA). Glycerol electrophoresis grade was purchased from Ajax Finechem (Australia). Bradford reagent was supplied by Amrisco (USA).

METHODS

THE EFFECT OF STARVATION PERIOD ON THE CONCENTRATION OF PROTEINS IN THE LEECH SALIVA EXTRACT

The effect of starvation time on the quantity of leeches' saliva proteins was assessed. Briefly, five groups of leeches with 20 leeches for each group were starved for different periods of time. Saliva was collected according to what was described by Alaama et al. (2011). The period of starvation in weeks was recorded, the quantities of proteins in the LSE and the concentrations of total proteins were estimated for every batch by using the method described by Bradford 1976 using SECOMAM UV-Vis Spectrophotometer (Jena Analytic Germany).

THE EFFECT OF SUCCESSIVE COLLECTION OF LEECHES SALIVA ON THE QUALITY AND QUANTITY OF THE PROTEIN

Quantity A number of 20 leeches were maintained in clean plastic containers containing non-chlorinated water which was changed every three days. They were kept in the laboratory at room temperature with 12 h dark- light cycle. Prior to the first collection, all the leeches were starved for three weeks and then their saliva was collected weekly for six weeks. The amount of saliva collected and the concentration of proteins in the saliva were reported.

Quality The quality of proteins in the LSE was compared week by week for the total period of experiment using SDS- Tricine PAGE according to (Schägger & von Jagow 1987) using Mini-PROTEAN Tetra Cell: Bio Rad (USA), equipped with Power Pac Basic power supplier: Bio Rad (USA).

THE EFFECT OF STARVATION TIME ON THE RECOVERY OF PROTEIN IN THE LSE

Quantity The experiment was carried-out with thirty leeches that were divided into three groups of ten. All the three groups were subjected to feeding process at time zero. All three groups were then subjected to re-feeding process according to this schedule: First group after two weeks, second group after three weeks and finally, the third group after four weeks.

The amount of LSE and their protein concentrations for each group were quantified using Bradford method (Bradford 1976).

Quality The quality of the LSE and the possible changes in its composition was carried-out on all the LSE using SDS- Tricine PAGE technique (Schägger & von Jagow 1987).

RESULTS AND DISCUSSION

THE EFFECT OF STARVATION PERIOD ON THE CONCENTRATION OF PROTEINS IN THE LEECHES' SALIVA COLLECTION

Quantity The results showed an increase in the protein concentration in LSE until 12th week of starvation, after that it started to decrease. From the results, it was obvious that the concentrations of proteins are still reasonably high after weeks 15 and 18 (Table 1). The concentration of proteins and peptides in the LSE increased linearly until the twelfth week of the starvation period. The protein concentration was 39.97 µg/mL at the sixth week and increased to 105.35 µg/mL at twelfth week as shown in Table 1. When the period of starvation was maintained for more than 12 weeks, the concentration of proteins began to decrease, for instance at week 18th it was found to be 43.661 µg/mL. The decrease in the concentration of protein in the LSE after the twelfth week is believed to be related to the exhaustion of the leeches.

It is worth mentioning that there are no documented reports found on the effect of starvation period on the quantity of the proteins in the LSE. We found that the starvation period of leeches plays a significant role on increasing the quality of proteins and peptides in the LSE.

TABLE 1. Saliva protein concentration qualified using Bradford method

Group no	Period of starvation	Concentration mg/mL
1	6 weeks	39.97
2	9 weeks	59.2
3	12 weeks	105.35
4	15 weeks	91.41
5	18 weeks	43.661

THE EFFECT OF SUCCESSIVE COLLECTION OF LEECHES SALIVA ON THE QUALITY AND QUANTITY OF THE PROTEIN

Quantity The results in (Table 2) indicates that whenever leeches feed on the phagostimulatory solution, their saliva is spontaneously released with its content of protein. The

volume of saliva released or its protein content did not follow regular pattern. The volume of LSE varies from 15 to 50% of the initial volume. The only exception to this chaotic pattern is the concentration of protein in the saliva which is always less than the initial value. The protein concentration varies from 1 up to 72%. This phenomenon requires more investigation to be well understood.

Quality The quality of protein in LSE using SDS-Tricine PAGE is shown in Figure 1. It is obvious that whenever the concentration of protein in LSE dropped because of successive collection, it was accompanied with the absence of some bands. In week 4, the gel electrophoresis showed the existence of some bands even though the concentration was very low and undetectable by using Bradford method as shown in Table 2. In weeks 2 and 5 of collection, the composition was so close to week 0 because of the high concentration of protein in these weeks. The results showed that the variation in the number of bands in the LSE followed the same pattern of protein concentration. The variation in biological activities (antithrombin and antiplatelet aggregation) of LSE after successive feeding has been reported by Rigbi et al. 1987. Our study showed that this variation in biological activities of the saliva extract was linked to the variation in protein concentration accordingly.

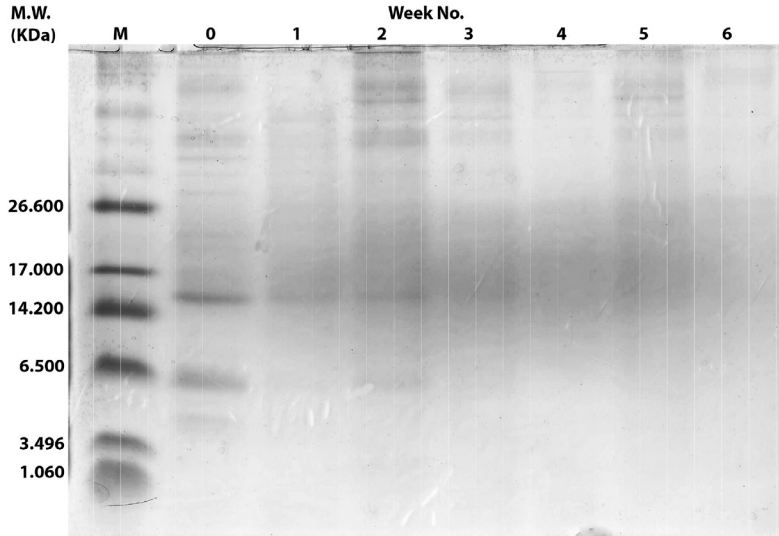
THE EFFECT OF STARVATION TIME ON THE RECOVERY OF PROTEIN IN THE LSE

Quantity After the first collection of leeches saliva, it was obvious that that leeches which were starved again for another 2 weeks, were able to produce about 26% of their initial quantity of saliva protein, while leeches which were starved for three weeks were able to produce 36% from the initial protein concentration. The highest recovery was obtained after four weeks of starvation after first feeding and leeches were able to produce 42% of their initial protein concentration. There are disagreement in the literature about the time interval effects on the recovery of protein in the LSE. It is worthy to mention that Rigbi and his team mentioned one week interval period to collect leeches' saliva after the first collection is suitable for leeches to produce good protein quantity (Rigbi et al.

TABLE 2. Protein recovery after the first feeding

period weeks	V µl	C µg/mL	Recovery of protein concentration%
0	20	25	100
1	10	3	12
2	7.5	18	72
3	5	8	32
4	4	0*	0*
5	7	12.02	48.08
6	3	6.06	24.24

V: the volume of extracted saliva, C: the concentration of protein in the saliva, recovery: calculated as follow $(C_n/C_0) \times 100$ and C_n the concentration of protein in n week, C_0 the concentration in week 0, *: no protein concentration was detected using Bradford method but gel electrophoresis showed that the protein is present in this sample



Note: Lane M: peptide marker, Lane 0-6 represent the weeks 0-6, respectively, when the saliva was extracted

FIGURE 1. Protein composition of the saliva extract after successive extraction

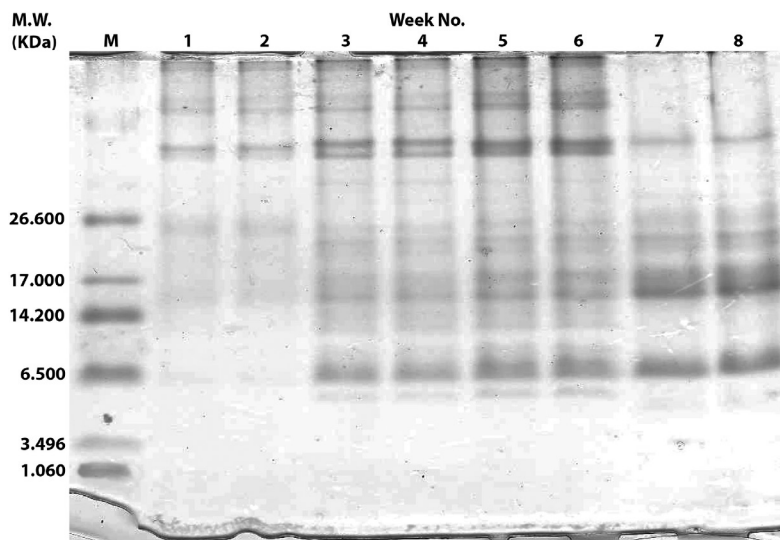
1987). While Baskova (1984) reported 2 weeks intervals for leeches to be starved to give usable quantity of proteins without scientific justification. Our study indicated that four weeks period was enough for leeches to recover almost half of their initial saliva concentration. While one or two weeks periods helped leeches to recover small amounts of their initial saliva concentration with some difficulties in leeches feeding.

Quality Figure 2 shows the quality of proteins using SDS-Tricine PAGE. The composition of LSE showed closeness between week 0 and other 4 weeks, with minor variation

in the concentration, the same bands were there but not with the same concentrations. This supports what was reported above regarding protein concentration variation. It is worthy to mention that no documented reports were found in the literature on the effects of time on protein quality in LSE. This result is reported here for the first time.

CONCLUSION

The leeches starvation period is playing a vital role on the quality and quantity of the proteins in the LSE. It is found that 12 weeks is the maximum starvation period to produce



Lane M: peptide marker, lanes 1-8 represent the week number at which the saliva was extracted in duplicate, lanes 1-2 week 2, lanes 3-4 week 3, lanes 5-6 week 4, lanes 7-8 week 0

FIGURE 2. Protein composition of the saliva extract

sufficient concentration and usable amount of proteins in the LSE. Hence it is mentioned that leeches should not be starved more than 12 weeks because the concentration of proteins will decrease. Also it is reported that leeches can be used for successive saliva collection. However there is variation in protein quality and quantity. It is very important to mention that leeches can recover 42% of their initial protein concentration if they are starved for 4 weeks after first collection. Based on the results of this study, researchers in this field are now able to decide which time is more suitable to collect saliva from leeches after the first extraction. Many results were reported here for the first time, hence more studies are needed to elucidate many undiscovered facts about LSE.

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