Detection and Purification of Hirudin from Local Leeches Using Gel Filtration Chromatography

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Abstract

It has long been known that local leech contain a substance with anticoagulant properties and the isolation of this was first made in 1984 as was reported by Weinberg (1994). In this study the purification of the blood-thinning agent hirudin, protein profiling and quantification from different parts of leech body are reported. The result shows the sample from leech head gave the highest result in protein concentration with a value of 69.33 μg/ml compared with the sample from the whole body and headless leech which was 36.67 μg/ml and 26.67 μg/ml, respectively. Sample from leech head was subsequently used for purification by gel permeation chromatography on Sephadex G-75. Fractions which contain significant amounts of protein with absorbance value of more than 0.2 at 595 nm were tested on their anti-coagulant activity. The result shows that that Peak III (fraction number = 23, optical density = 0.256, protein concentration = 82.00 μg/ml) gave the longest time of blood to clot (123 ± 15 minutes) and did not contract but 380 ±17 minutes later when compared with Peaks II (fraction number = 21, optical density = 0.293, protein concentration = 94.33 μg/ml) and V (fraction number = 36, optical density = 0.214, protein concentration = 68.00 μg/ml) where the blood showed clotting relatively earlier within 13±3 and 23±3 minutes and which contract much sooner at 24±0.6 and 42±3 minutes later, respectively. Crude protein and eluted fractions which contain significant amounts of protein with the longest time of blood to clot were subjected to SDS-PAGE analysis. The result shows that the molecular weight of hirudin was about 11.36 kDa. From the purification determined by Sephadex G-75 Gel Filtration technique the profiles of leech crude extract from different parts of body indicated that the anti-coagulant agent or hirudin is only found in substantial quantities in the head (salivary glands) of leeches.

Key words: *Hirudinea* sp., hirudin, Gel Filtration technique, protein purification and profiling

Introduction

Hirudin is the generic name for a family of closely related homologous peptides that are all found in the cranial salivary glands of medicinal leech (Wallis, 1996). In 1984, the blood-thinning agent (anticoagulant) hirudin was isolated for the first time from leech salivary glands (Weinberg, 1994). Hirudin was extracted from the homogenized heads of medicinal leeches and enriched by precipitation procedures followed by ion-exchange chromatography and gel filtration (Badgy *et al.*, 1976; Markwart, 1957; Walsmann, 1991). Electrophoresis is a method normally used to separate and characterize a mixture of charged molecules, especially proteins and nucleic acids. SDS-PAGE is one of the common laboratory techniques in the biomedical and life sciences, and is usually used to resolve proteins in biological samples according to their size. The principle of the technique is simple, and it has been in use for many years (Laemmli, 1970).

Gel filtration (GF) separates proteins solely on the basis of differences in molecular size. Separation is achieved using a porous matrix to which the molecules, for steric reasons, have different degrees of access (smaller molecules have greater access and larger molecules less). The matrix is packed into a chromatography column, the sample mixture applied, and the separation accomplished by passing an aqueous buffer (the mobile phase) through the column. Protein molecules that are confined in the volume

outside the matrix beads will be swept through the column by the mobile phase and the sample is thus eluted in decreasing order of size. Generally, the protein zones eluted are detected by an in-line UV monitor and fractions are collected for subsequent specific analysis or further preparation steps (Hagel, 1998).

Material and Methods

Source and rearing of leeches

Local leech used in the study was provided by PT Dynamic Consultant Co., Kota Bharu, Kelantan. The non-chlorinated freshwater in the aquarium tank placed indoor was aerated, with 50% of the water changed once every 3 days. The temperature, pH and light intensity were maintained at 27.82 ± 6.22 °C, 6.6 ± 1.5 and 100-150 lx, respectively. The leeches were starved for 2 weeks before starting this study.

Preparation of leech crude extract

The procedure outlined by Claude (1935) was followed for the preparation of crude extract from the locally raised leeches. For this experiment, 10 g of live leeches was taken and the crops emptied of the blood by placing them in a container filled with crystals of sodium chloride for 15 minutes and then washed with distilled water. They are then cut into small pieces and grinded using a blender. The extraction was made with a volume of distilled water equal to six times the weight of the pulp. After centrifugation at 1500 rpm for 60 minutes at 4 °C, the supernatant fluid was filtered through coarse paper. Finally, the crude extract was stored at 4 °C for 24 hours. The preparation of crude extract was similarly done for leeches without head (headless) and with the leeches head comprising the first to six body segments or annuli only (Claude, 1935). The optical density (OD) value for the three parts was recorded and compared according to their protein concentrations.

Protein quantification

For the preparation of the protein reagent, Coomasie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95 % ethanol and followed with the addition of 100 ml phosphoric acid. The resulting solution was diluted with ddH_2O to the final volume of one liter. Final concentration in the reagent were 0.01% (w/v) Coomasie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5(w/v) phosphoric acid.

As for protein assay, varying concentrations of protein solutions were prepared from the stock containing 1mg/ml BSA. The concentrations prepared were 0 (the blank), 10, 20, 30, 40, 50 and 60μl of the protein stock solution and were added to individual tubes and adjusting the volume to 100 μl with ddH2O. 1000μl of the diluted dye reagent was added to all test tubes and the content was mixed by inversion. The absorbance was measured at A_{595} nm after 2 minutes. Standard curve was plotted for protein determination.

Preparation of gel-degassing

Powdered gel weighing 7 g was added gradually to an excess amount of buffer (0.01 M sodium phosphate buffer, pH 7.0) and allowed to swell to about 84-87 ml of gel suspension and the gel supplied as slurry was suspended in a two-to fourfold excess of buffer and 95% of the gel was allowed to settle out. The remaining gel and supernatant solution was aspirated by suction. The gel was equilibrated at room temperature for 3 hours. Immediately prior to pouring the column, the gel was degassed by placing the slurry in a thick-walled Ehrlenmeyer flask with a side-arm. The top side was sealed with a large rubber stopper which fits over the mouth of the flask. The vacuum tubing was attached to the sidearm and connected to a vacuum line. The flask was swirled for 15 minutes and when bubbles no longer appeared, the gel was totally degassed.

Column assembly and packing

The end of column was connected to a 50 ml syringe. The column was flushed with wash buffer (0.01 M sodium phosphate buffer, pH 7.0), leaving a few ml at the bottom. The column was mounted vertically on the fixture away from direct sunlight and high traffic movement. The media slurry was re-suspended and poured into the column in one continuous step. The bottom outlet of the column was opened until the gel

bed reached a constant height. The column was equilibrated overnight with wash buffer until pH of gel suspension inside the column is equivalent to pH 7 (pH of wash buffer).

Protein separation

As the starting material, 30 ml of crude protein extract from leech head was used with concentration of 69.33μg/ml. The protein determination prior to fractionation was made using a 1.5 x 30 cm BioRad Econo chromatoghraphy column filled with Sephadex G-75 which was equilibrated overnight with 0.01 M sodium phosphate buffer, pH 7.0. The protein was loaded onto the column and eluted twice the column volume (80ml) of the equilibration buffer. Fractions (3 ml per tube) were collected throughout the experiment and absorbance of proteins was measured at 595 nm by spectrophotometer (Thermo scientific). Protein concentrations were determined separately for each fraction with absorbance value of more than 0.2 at 595 nm measured by Coomasie protein assay. The procedure was repeated on samples obtained from headless leech and the whole body with the initial protein concentration of 26.67 and 36.67μg/ml respectively.

Protein activity and profiling

Fractions which contain significant amounts of protein with absorbance value of more than 0.2 at 595 nm were tested for their anti-coagulant activity by adding 0.5 ml of the extract to 2 ml of fresh rabbit blood and the time of clotting recorded by using the drop method (Prakasam *et al.*, 2005). Crude protein and eluted fractions which contain significant amounts of protein with the longest time of blood to clot were subjected to SDS-PAGE analysis using MP3 Bio-rad minigel apparatus with 12% resolving buffer. The crude extract was mixed with 1:1 of sample buffer [13.16 % (v/v) 0.5 M Tris-HCl, pH 6.8, 26.32% (v/v) glycerol, 21.05% (w/v) SDS, 2.11% (w/v) Bromophenol blue]. The mixture was then boiled for 5 minutes. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was prepared as described by Leammli (1970). Electrophoresis was carried out in running buffer [0.3% (w/v) Tris-base, 1.44% (w/v) glycine, 0.1% (w/v) SDS; pH 8.3] at constant current of 100 V for 75 minutes. The gels were then stained with Coomassie Brilliant Blue R-250 [0.2 % (w/v) in 28% (v/v) isopropanol and 11% (v/v) acetic acid] overnight with gentle agitation at room temperature and de-stained with solution containing 10% (v/v) acetic acid and 10% (v/v) isopropanol for 1 hour. Molecular weight was evaluated by making comparison with standard molecular weight markers (SDS-PAGE Molecular Weight Standard, Low Range, Bio-Rad).

Results

Protein quantification

Protein quantification was carried out by using the assay method adopted by Bradford with Bovine Serum Albumin (BSA) as a standard to determine unknown concentration of substance in particular volume. Table 1 shows the protein concentration of leech crude extract from different body parts of leeches. The result shows the sample from leech head gave the highest protein concentration of about 69.33 μg/ml compared with either the sample from the whole body or headless leech. Similarly for the total protein content, the sample extracted from leech head produced 2.08 mg protein far more in quantity than from either of the two sources.

Table 1: Protein concentration of extracts from different body parts

Sample	Total crude (ml)	Protein concentration (µg/ml)	Protein (mg)
Whole body	30	36.67	1.10
Headless leech	30	26.67	0.8
Leech head	30	69.33	2.08

Protein profiling

Sephadex G-75 Gel Filtration chromatoghraphy technique was used to separate the protein fractions for purification of the hirudin protein. The protein profile of the fractions from the crude extract obtained from leech head is shown in Figure 1. Four distinct peaks were obtained, namely Peaks II – IV. That

for the fraction at the first peak eluted during the wash was assumed as representing proteins that did not adhere to the column. The second, third, fourth and the fifth peaks represented proteins that adhered to the column very well. The result shows that Peak II gave the highest optical density (OD) reading with a value of 0.293 compared with Peaks III, IV and V with optical density values of 0.256, 0.18 and 0.214 respectively. Only peaks with OD readings greater than 0.2 were chosen for subsequent protein quantification. For protein quantification Bradford assays with Bovine Serum Albumin (BSA) was used as a standard to determine unknown concentration of substance in a particular volume. Table 2 shows the different protein concentration for the corresponding peaks. The result shows that the sample from Peak II and the fraction number 21 gave the highest protein concentration of 94.33 μg/ml compared with the samples from the Peaks III (fraction number 23) and V (fraction number 25) which was 82.00μg/ml and 68.00μg/ml, respectively.

Table 2: Protein concentration of crude extracts of leech head for corresponding peaks

Peaks	Number of fractions	Optical density (O.D)	Protein concentration (µg/ml)
I	-	-	-
II	21	0.293	94.33
III	23	0.256	82.00
IV	-	-	-
V	36	0.214	68.00

*Peak I: Assumed as represented proteins that did not adhere to the column
*Peak IV: The O.D value < 0.2 not considered for determination of protein concentration

The protein profile of the fractions from the crude extracts obtained from whole body produced five distinct peaks is shown in Figure 2. The result shows that Peak I produced the highest optical density with a value of 0.197 (fraction number 20) compared to the other four peaks and the lowest at Peak III with a value of 0.06 (fraction number 29). As the optical density values for all peaks were below 1.5 there was no necessity to determine the protein concentration of the sample.

Similarly, the protein profile of the fractions obtained from crude extract of headless leech also produced five distinct peaks but at different fraction numbers to that profile shown by extracts obtained from whole body (Figure 3). The first peak eluted during the wash was assumed to represent proteins that did not adhere to the column. As the optical density values for all peaks were below than the required value of 0.2 there was therefore no necessity to determine the protein concentration of this extract. For subsequent experiments only samples from crude extracts obtained from leech head was used as the source for protein since the optical density value obtained was greater than 0.2 compared with two other sources.

The crude protein bands of the crude extracts of leech head on 12 % SDS-PAGE is presented in Figure 4. The figure shows a broad range of protein ranging from more than 97.4 kDa to less than 14.4 kDa. From the electrophoretic bands shown on the 12% polyacrylamide gel, there were 9 profile bands of unknown proteins from the leech head sample before purification was made by Sephadex G-75 Gel Filtration.

The protein profile after purification was carried out is shown in Figure 5. Only one band appeared after purification and the molecular weights of the band was about 11.36 kDa. The molecular weights of the unknown protein were determined by using the curve generated by plotting the log of the molecular weight of the broad range standard vs. the relative mobility (Hames & Rickwood, 1990).

Determination of hirudin protein activity

As described earlier, the sample of the crude extracts of leech head was obtained from purification by using Sephadex G-75 Gel Filtration chromatography technique and Table 3 shows the time taken for blood to clot and contract at three different peaks. The activity of protein hirudin was determined based on the time of blood to clot. The result of present study showed that the Peak III gave the longest time for blood to

clot (123.33±15.28 minutes) and only contracted 380.00±17.32 minutes later when compared with Peaks II and V where the blood tested was shown to clot in a much shorter time of 13.33±2.89 and 23.33±2.89 minutes and contracted much sooner within 24.33±0.58 and 41.67±2.89 minutes, respectively. The control treatment with pure rabbit blood gave the lowest result when clotting occurred within 1±0.0 minute by which time contraction also took place.

Table 3: Time taken for blood to clot and contract from samples of crude extracts of leech head obtained at three different peaks

Peaks	Clotting time (min)	Time for blood clot to contract (min)
II	13.33±2.89	24.33±0.58
III	123.33±15.28	380.00±17.32
V	23.33±2.89	41.67±2.89
Control 1	1±0.0	1.17±0.29
Control 2	3.33±0.58	3.33±0.58

Data in the table were means and standard deviations (mean ± S.D.)
Peak II: Fraction number = 21, O.D = 0.293, protein concentration = 94.33 µg/ml
Peak III: Fraction number = 23, O.D = 0.256, protein concentration = 82.00 µg/ml
Peak V: Fraction number = 36, O.D = 0.214, protein concentration = 68.00 µg/ml
Control 1: Blood rabbit only
Control 2: Blood rabbit treated with distilled water

Discussion

In this study, new information on detection and purification of hirudin protein from local leeches are reported. Accurate determination of protein concentration is fundamental for all quantitative measurement of biochemical interactions. Before the purification process was carried out, initial studies on protein content was conducted to determine in which part of the leeches anatomy contained large concentration of protein. The protein concentrations between different body parts of local leeches were determined by Bradford assay method with Bovine Serum Albumin (BSA) which was used as a standard to determine unknown concentration of a substance in a particular volume and absorbance of proteins measured at 595 nm. This is different with the study conducted by Orevi (1992) who used the method adopted by *Lowry et al.* (1951) to determine the protein concentration from the saliva of *Hirudo medicinalis* and protein absorbance was measured at 280 nm. Generally, the Bradford method will only detect proteins with a molecular weight greater than 3-5 kDa and this particular protocol is recommended for 20-140 μg protein (200-1400 μg/ml) (Rosenberg, 1949). The results of present study showed that leech head gave the highest protein concentration of 69.333 μg/ml compared with the sample from either the whole body or headless leech (Table 4.5).

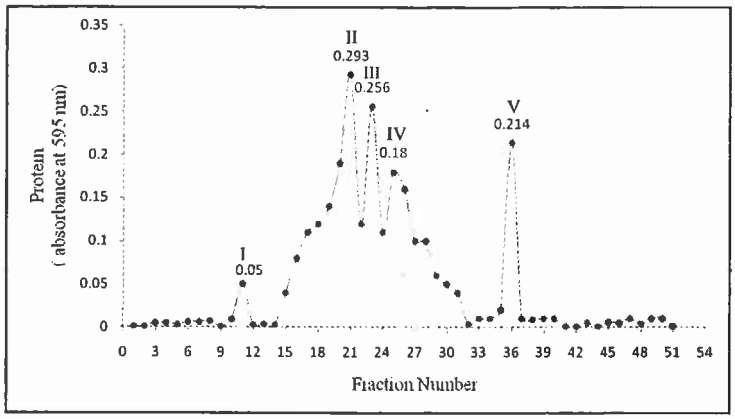


Figure 1: Purification of protein obtained from leech head

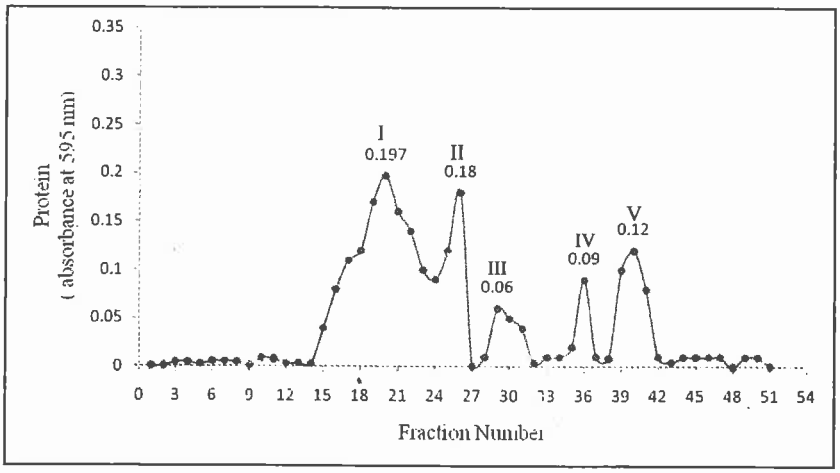


Figure 2: Purification of protein obtained from the whole body of leeches

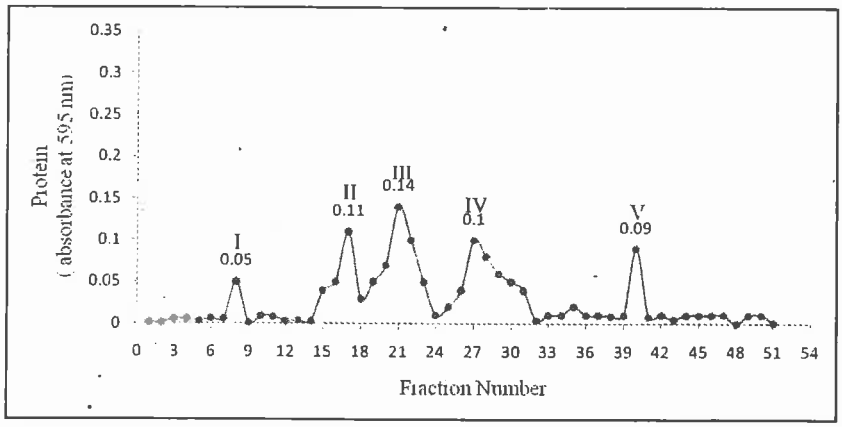


Figure 3: Purification of protein obtained from headless leeches.

Although Walsmann (1991) on the heels of what Markwardt (1957) had achieved finally succeeded in the preparation of a nearly 50% pure hirudin sample. However, the way to reach this goal was long and stony and, as a first step required the development of appropriate chemical extraction procedures to obtain sufficient amounts of starting material and this material was the head of the leech. In the original report on some properties of hirudin, Bodong (1905) noted that 10% of a leech head contained sufficient hirudin to keep rabbit blood uncoagulated *in vitro* for more than two days. This statement was supported by Claude (1937) who argued that extracts prepared from the leech head were usually purer than those prepared from the whole body. In his study, the effects of extracts from different parts of the leech on skin permeability resulting in the greatest spread was obtained from the isolated head and gonads with the area of spread from 0.5 ml of extract with 0.25 ml India ink (as an indicator) was 91.2 and 80.7 sq. cm., respectively, compared with the extract from leech body which was only 66.9 sq. cm. According to Nowak *et al.* (2007) about 600 mg of natural pure hirudin could now be prepared from about 15,000 leech heads.

The purification process of leech crude extract was continued with the use of Sephadex G-75 gel filtration. Gel filtration chromatography is also known as molecular sieving, gel permeation and size exclusion chromatography (SEC). The primary objective of gel filtration is to achieve rapid separation of molecules based on size (Rosenberg, 1949). For the Sephadex G-75 Gel Filtration, the useful working range (molecular weight) was around 3 kDa to 70 kDa. In this study, the size of the columns used was 1.5 x 30 cm. The height of the dextran filling was about 25cm. The main advantages of the gel chromatographic technique presented in this paper over the existing techniques are that it is rapid and simple to operate. The

columns can be used repeatedly over a considerable period of time. The bed volume can be maintained constant by adding freshly soaked Sephadex G-75 for any lost by leaching during the washing procedure. After the solution has penetrated the gel bed the latter was carefully covered with buffer and the elution started. The gel bed will be cracked when dry and it cannot be used again. In the present study, the first 44 ml was eluted with wash buffer and the assumptions made that the represented proteins did not adhere to the column very well. In the study conducted by Verena-Steiner *et al.*,(1990), initial purification of leech crude extract by gel permeation chromatography on Sephadex G-50 and anion-exchange chromatography on Q Sepharose.fast-flow removed most contaminants and yielded a highly active extract. The result of the present study showed that purification by using Sephadex G-75 gel filtration also gave clear indication that most of the contaminants were also removed according to the large result obtained in protein quantification and SDS-PAGE analysis. Generally, the high peak does not necessarily indicate that it contained a high amount of hirudin protein. For example, as can be seen in Table 3 although the Peak II gave the highest optical density (0.293) and highest in protein concentration (94.33 μg/ml), the time taken for blood to clot when treated with leech extract was only 13.33±2.89 minutes when compared with the Peaks III and V which was 123.33±15.28 and 23.33±2.89 minutes respectively. The result clearly indicates that this particular local species not only has the hirudin protein from its extract but it also contained many other unknown proteins. In this respect, more detailed studies should be made on this local species to ascertain the molecular structure and function of some of the unknown proteins.

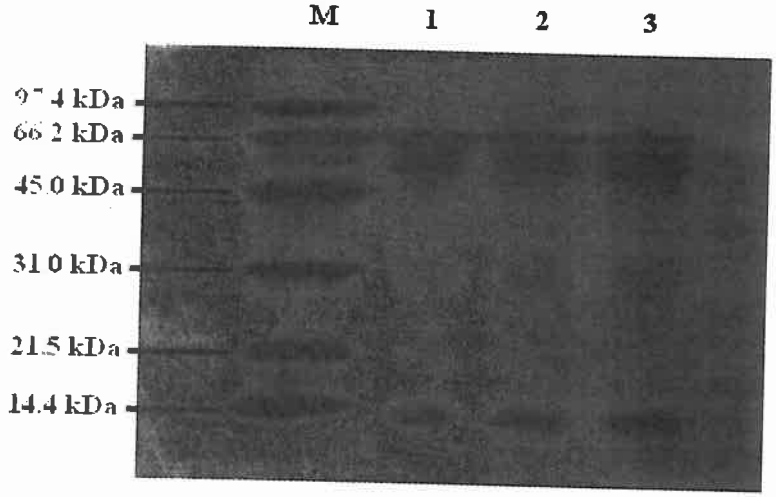


Figure 4: Electrophoretic patterns (12% polyacrylamide gel) on crude extract from leech head. Lane 1-3: crude extract from leech head loaded with 0.69 µg per well. M: SDS-PAGE Molecular Weight Standards, Low Range (BIO-RAD).

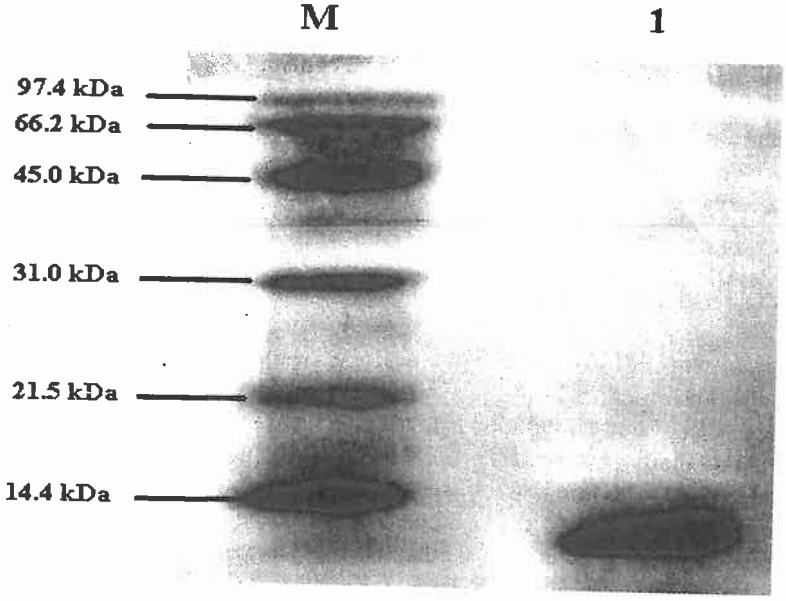
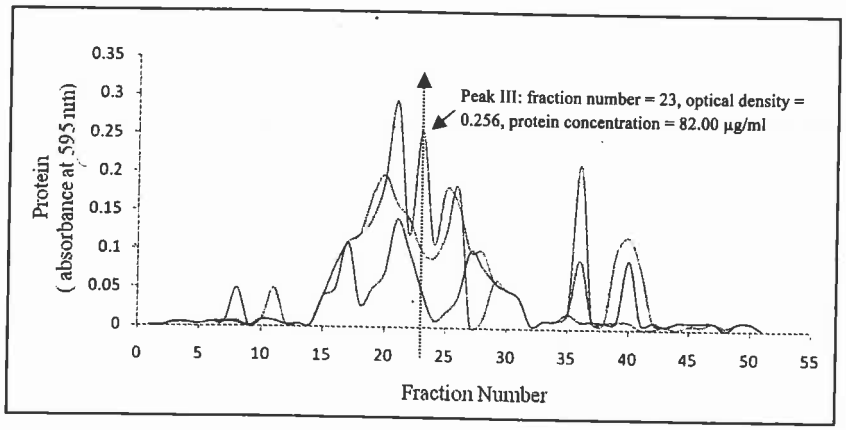


Figure 5: Electrophoretic patterns (12% polyacrylamide gel) on purification of crude extract sample of leech head by Sephadex G-75 Gel Filtration Chromatography. (Peak III: Fraction Number = 23, O.D = 0.256, protein concentration = 82.00 µg/ml). Lane 1: protein extract from leech head loaded with 0.82 µg per well. Lane M: SDS-PAGE Molecular Weight Standards, Low Range (BIO-RAD).

Generally, the activity of hirudin was determined based on its inhibition to thrombin-catalyzed reactions and expressed as antithrombin units (ATU) (Grießbach *et.al.*, 1985 and Spanagel *et. al.*, 1991). Markwardt (1970) stated that the fast and specific reaction of hirudin with thrombin was utilized for quantitative determination of the inhibitor where it was found that one mole of hirudin formed complexes with 1 mol of thrombin. This corresponds to the antithrombin activity of pure hirudin of which 1 μg inhibits about 5 μg of human thrombin. Therefore, the activity of hirudin is measured in antithrombin units (ATU), where 1 ATU is the amount of hirudin that neutralizes 1 IU of thrombin. He also stated that pure hirudin contains approximately 10,000-15,000 ATU/mg of protein, depending on the thrombin used for standardization. In this study, the protein was tested on their anticoagulant activity by adding a drop of the leech extract onto the pure rabbit blood and time to clot was recorded. This test was used since it is a simple and quick method whereby the longer the time taken for blood to clot the higher the anticoagulant activity of that sample. This test was previously used by Claud (1937) in his study to determine the spreading properties and anticoagulant activity of leech extract.

Electrophoretic methods have become important tools for analyzing and characterizing macromolecules. According to Weber & Osborn (1969), 0.01 mg of protein was usually applied per gel, but the amount of protein could be lowered if the gel was stained for longer period. In this study, the amount of protein loaded from leech head extract was 0.82 μg into the gel, but the gel was stained overnight. Polyacrylamide gel electrophoresis has been done to separate proteins according to their sizes or molecular weight and this method has been optimized to get clear banding pattern of protein profile. Optimization of the gel to 12% produced the clearest banding pattern from SDS-PAGE analysis where the separation was carried out at a constant current of about 400mA starting at 100V which lasted about 90 minutes until the band's marker had reached the bottom of the gel.

To date, there are very few reports of the molecular weight of hirudin. In the present study, the peak that gave the longest time of blood to clot (Peak III: fraction number = 23, optical density = 0.256, protein concentration = 82.00 μg/ml) was chosen for electrophoretic analysis and the result shows that there was only one band appearing on the 12 % gel electrophoresis with the molecular weight of 11.36 kDa compared with the protein profile from unpurified extract which produced 9 bands of unknown protein. SDS-PAGE analysis (Figures 4 and 5) showed that purification of leech crude extract was achieved by using Sephadex G-75 Gel Filtration as evidenced by the bands that appeared before purification compared with that after purification. Baskova (1976) determined the molecular weight of hirudin purified by electro-focusing to be 7.1 kDa which was close to the molecular weight determined from amino acid sequence. She also found that hirudin can dimerize in aqueous media and detected the molecular structure in a highly purified preparation with the two-fold molecular weight of about 14 kDa. In addition, Markwardt (1992) stated that the pure anticoagulant agent obtained is a carboxyhydrate-free single chain mini-protein containing 65 amino acids with a molecular weight of about 7 kDa.



(The blue line (——) represented purification of protein obtained from leech head, the red line (——) from the whole body and the black line (——) from the headless leech).

Figure 6 : Comparison of the protein profile of crude extracts from different body parts.

Tan *et al.* (2002) studied the effect of geographical factors on the hirudin gene's variation of the leech *H. manillensis*. By isolating genomic DNA and amplifying hirudin gene by the PCR method, cloning and measuring sequence as well as comparing *H. manillensis* from Guangdong Province with the hirudin gene, *H.manillensis*, from Manila, it was discovered that the geological factors have variation effect on the hirudin gene of *H.manillensis*.

In this study, the molecular weight of hirudin was found to be about 11.36 kDa and this fact is reinforced by all the other information gathered where the molecular weight of hirudin in this study was similar to those of other studies and what small differences observed might due to the different leech species used. This study is one of the few conducted to verify the presence of an anticoagulant agent, hirudin, whose molecular weight was found to be 11.36 kDa. This evidence alone may not be sufficient to provide a satisfactory explanation on the actual molecular weight of hirudin without first conducting proper molecular studies on the local leeches.

Hirudin is the generic name for a family of closely related homologous peptides and usually found in the cranial salivary glands of medicinal leech, a fact confirmed by several workers ((Wallis, 1996; Weinnberg, 1994). In the present study, it is evident that the anti-coagulant agent (hirudin) was also from the salivary glands located in the leech head.

Conclusion

The purification process of the crude extract of local leeches by chromatographic gel electrophoresis technique indicated the presence of the hirudin protein which is largely found in the head of the leech body. This protein when tested on pure rabbit blood demonstrated its anti-coagulant activity whose molecular weight was found to be 11.36 kDa.

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