

Isolation of Antithrombin Protein from Local Leeches (*Hirudinaria manillensis*)

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Abstract

Hirudinaria manillensis is a buffalo leech widespread in tropical parts of Asia. It is the same family as the European medicinal leech (*Hirudo medicinalis*) which has been used extensively in studying antithrombin protein called Hirudin. The purpose of this study is to isolate and purify antithrombin protein or "hirudin-like" protein from local leeches (*Hirudinaria manillensis*). From this study we found that the Rotofor cell (Isoelectric focussing cell) brought about a significant purification of antithrombin protein from local leeches in a minimum number of steps.

INTRODUCTION

The medicinal leech has been used therapeutically for thousands of years. Its traditional role was in systemic removal of blood, newer applications include removal of local accumulations of blood in threatened skin necrosis (1). Other apparent benefits of leeches is the release of their salivary anticoagulant, Hirudin. Hirudin was first discovered in 1884 (2), however biological hirudin only had been isolated in late 1950s (3,4) this is due to the extinction of leech species and the development of modern biotechnology involving cloning and expression of cDNA coding for Hirudin. There are quite a number of important substances, were isolated and studied from different leech species (table 1)(5). From these survey, they showed that apart from *Hirudo medicinalis*, antithrombin protein is not reported in other leech species.

Hirudin is a potent anticoagulant occurs naturally in leeches and it is a small non-glycosylated protein composed of 65 to 66 amino acids. Its tertiary structure is maintained by three disulfide bond and reported to be resistant to heat and variable pH(6,7,8). Previous studies showed that Hirudin is a specific inhibitor of thrombin and does not act on thrombocytes, also it does not require other coagulation factors or plasma constituents, unlike some other anticoagulant such as Heparin. It is also reported to be a non-immunogenic reactions. Recombinant Hirudin has a wide utilities as an anticoagulant. For example it is used for prevention of post operative thrombosis, treatment of disseminated intravascular coagulation, for antithrombin ointments and anticoagulant in blood collection.

The focus of this study is to isolate and purify the antithrombin protein from buffalo leech as in Malaysia. The ATU (Anti-Trombin Unit) activity of the purified molecule will be determined and the amino acids sequence of the protein will be monitored. If the ATU activity of the purified antithrombin protein is better or the same as the reported Hirudin, we would like to clone the antithrombin protein gene in order to get large scale production of natural anticoagulants for medical application purposes.

MATERIALS AND METHODS

Samples

Leeches were collected at a few places in N.Sembilan, Pahang and Kelantan with an average of about 2gm/leech and let them starved for at least 2 weeks.

Total Protein Extractions

Two methods were employed, first was the modified method of Harvey (1986) by using liquid nitrogen, then homogenization with phosphate buffer. Homogenizer mini container G-04248-10 (Waring blender) was used for the second method.

Proteins Tests

Protein concentration were determined by Bradford method using BIO-Rad Reagent and measuring absorbance at 595 nm.

SDS-PAGE

Analytical analysis at every steps of purification was carried out by using 15%T, 2.7% C SDS-PAGE

Purification Stages

Purification of antithrombin protein was carried out by using twice sample runs in an isoelectric focussing cell (ROTOFOR), then through Fast Protein Liquid Chromatography (FPLC) and finally analytical conformation through HPLC.

ATU Test

Determination of inhibition of purified antithrombin protein were measured by their ability to inhibit thrombin from releasing colored p-nitroaniline from chromogenic substrates Tos--Gly-Pro-Arg-pNA. The increase of absorbancy was monitored at 405nm with 5 min. periods. The ATU units at every steps of purification are determined.

RESULTS AND DISCUSSION

Overall methods for extraction and purifications of antithrombin protein from local leeches are shown as in figure 1. Using homogenizer mini container blender, total protein of 112.8 mg/g leech (fig.2) were obtained compared to only 82.7 mg/g leech(fig.3) using the modified method of Harvey. From our calculations we obtained the smallest molecular weight protein molecule to be approximately 7 kD and 8 kD respectively. From these results show that method of using homogenizer mini container is better in order to get the highest amount of total protein. However, the final method will be selected depending on the ATU activity.

Isoelectric focusing in the Rotofor cell was operated for 4 hours at 12 W constant power at 4°C. Twenty fractions were collected, their pH value and absorbance at 280 nm were measured (fig 4) and aliquots were analyzed on Coomassie blue stained gels. We pooled the fractions at hirudin's pI value around 3.5-

4.5 . The fractions were assayed on 15% SDS-PAGE (fig 5). We found that only two molecular weight protein are pooled at the same pI value.

To finally confirm the purity of the antithrombin protein, further purifications through Rotofor, FPLC and HPLC are required. The most important aspects have to be done is the determinations of the ATU activity at every stages of purifications. At this points we already tested the ATU activity of a few stages but the results could not be produced at the moment until reproducibility data gathered.

CONCLUSIONS

The results show the purification of antithrombin protein from local leeches can be carried out significantly by using Isoelectric focussing cell (Rotofor). Further and thorough work of purification has to be carried out in order to get the purified antithrombin protein with a desirable ATU activity.

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Table 1. List of various components isolated from different leech species

Species	Components isolated
<i>Hirudo medicinalis</i>	Hirudin, eglin, hyaluronidase collagenase, apyrase, bdellin
<i>Haementeria ghilianii</i>	Hementin, antistasin, ghilantenn Inhibitors to trypsin, plasmin, chymotrypsin and elastase
<i>Haementeria officinalis</i>	
<i>Macrobdella decora</i>	Decorsin
<i>Haementeria luzi</i>	Plasminogen activator
<i>Nephelopsis obscura</i>	Inhibitors to trypsin, elastase and chymotrypsin
<i>Erpobdella punctata</i>	
<i>Hemopsis marmorata</i>	

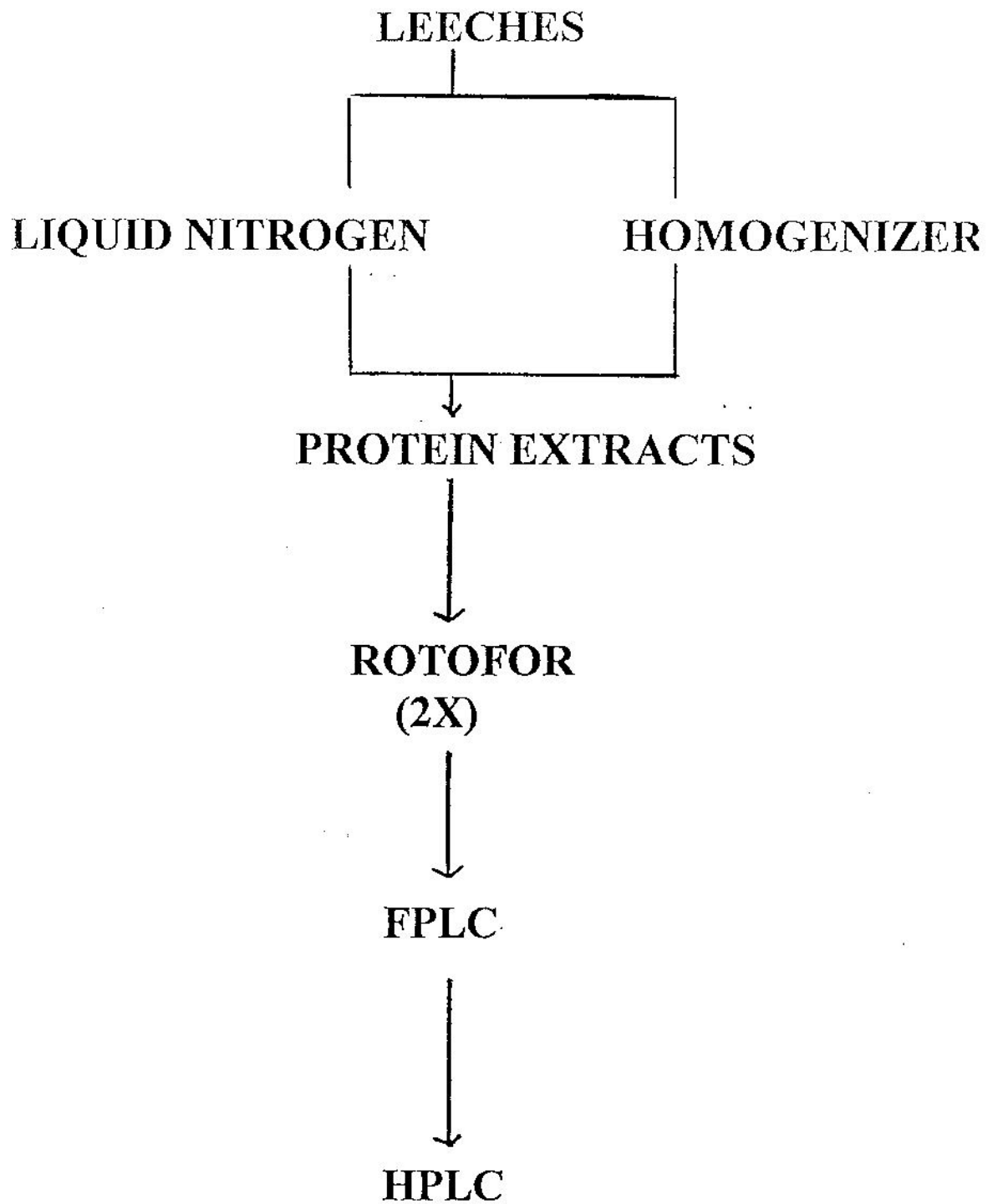


Figure 1

RESULTS

TOTAL PROTEIN BY HOMOGENIZER MINI CONTAINER METHODS

112.80 mg/g

15% SDS-PAGE

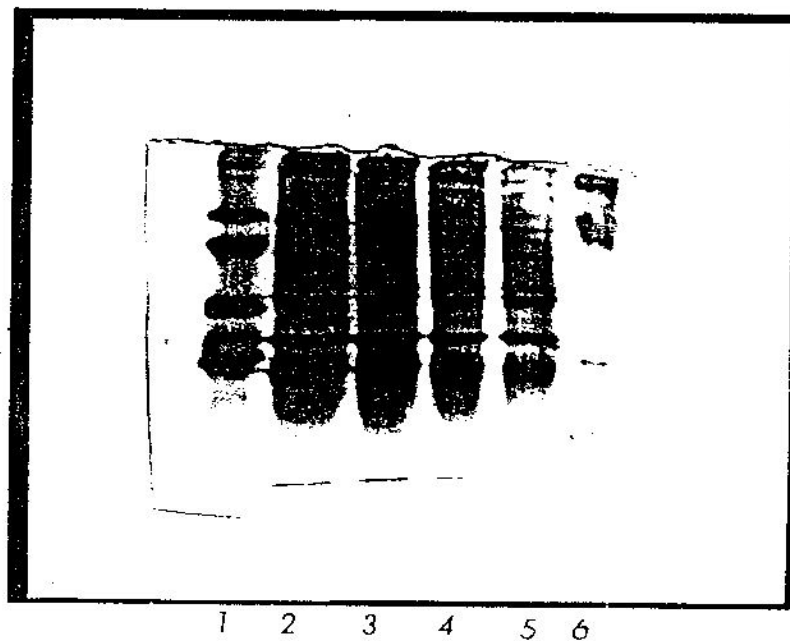


Figure 2

RESULTS

TOTAL PROTEIN BY MODIFIED METHODS OF HARVEY (1986)

82.70 mg/g

15% SDS-PAGE

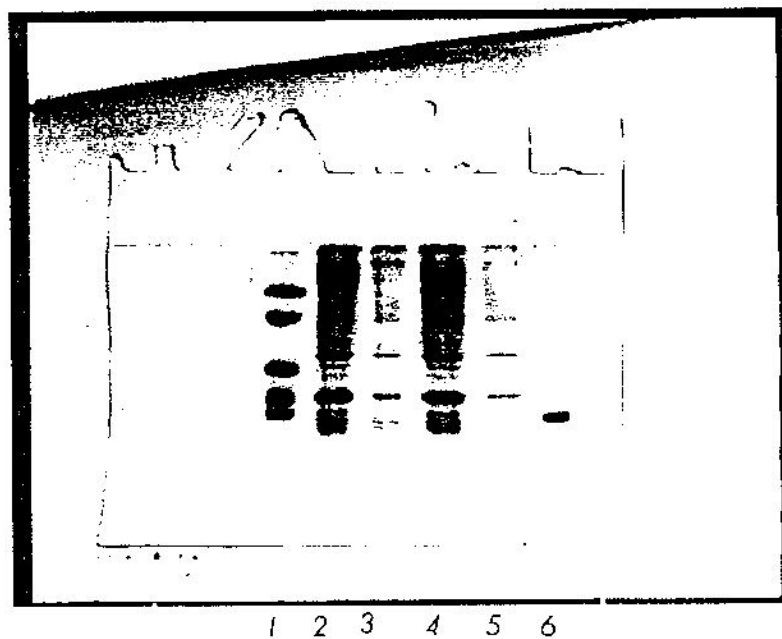
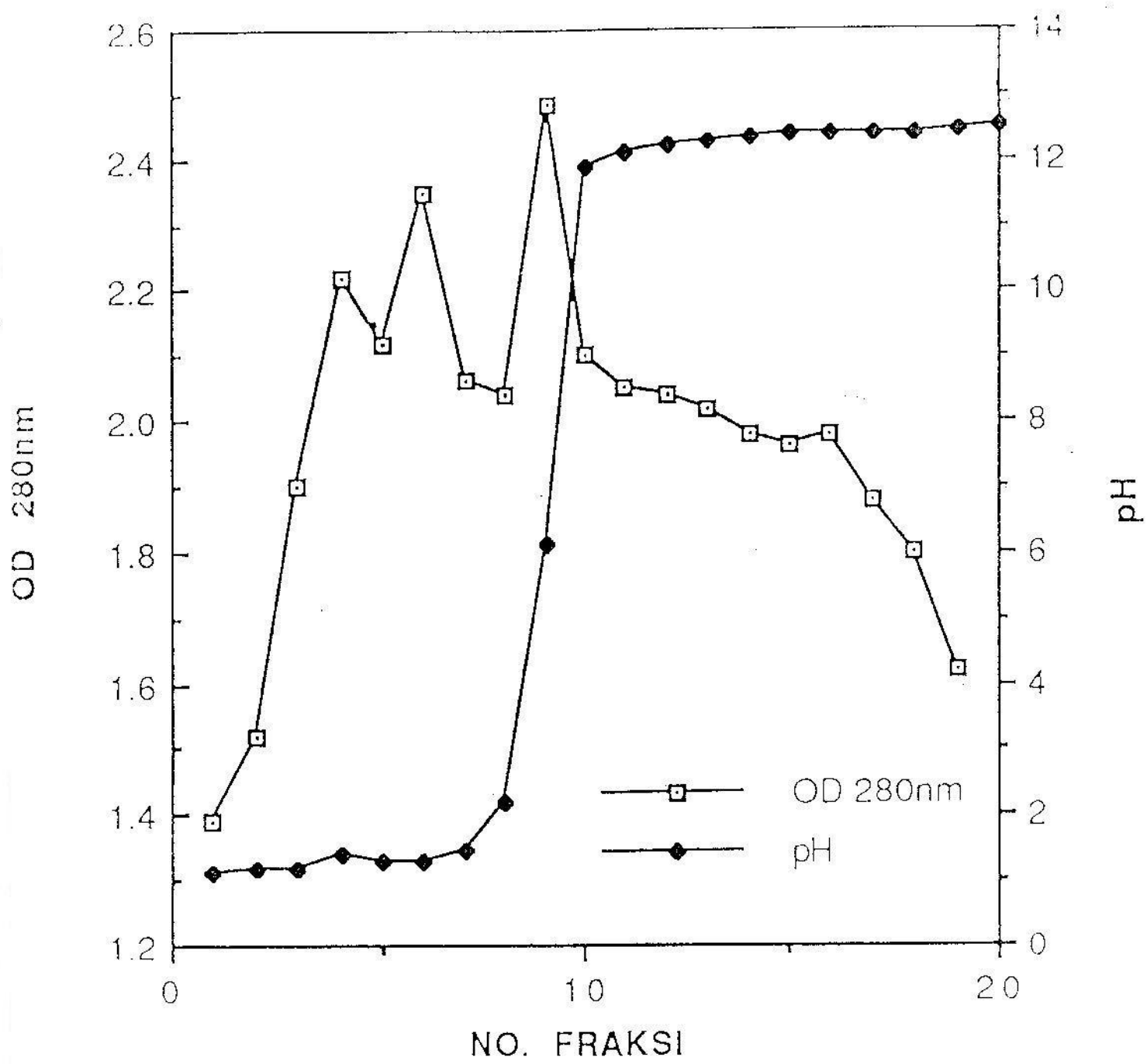


Figure 3



GRAF : ANALISA HASIL DARI ROTOFOR

Figure 4

1 2 3 4 5 6 7 8 9 10 11 12



RAJAH 3 : SDS-PAGE 15% T, 2.7% C YANG MENUNJUKKAN
PENYINGKIRAN PROTEIN BERMOLEKUL TINGGI OLEH
ROTORFOR

DIMANA : LANE 1-4 ADALAH PROTEIN MARKER
LANE 5 ADALAH 'CRUDE EXTRACT' LINTAH
LANE 6 ADALAH 'CRUDE EXTRACT' YANG TELAH
DINYAH GARAM
LANE 7-12 ADALAH FRAKSI YANG MENUNJUKKAN
BACAAN OD TINGGI SELEPAS ROTORFOR

Figure 5