ANTITHROMBIN PROTEIN FROM *Hirudinaria manillensis* : PRELIMINARY STUDIES ON ISOLATION, SEQUENCING OF OF THE ANTITHROMBIN PROTEIN AND CLONING OF THE DESIRED GENE

Looi Yat Seong, Fazilah Abd. Latif, Normah Norbib, and Nazlan Mohd. Said
Dept. of Bioprocess Engineering, Universiti Teknologi Malaysia
80990 Johor Bahru, Malaysia.

Abstract

Salivary secretions from leeches have been known to possess anticoagulant activity. Studies have been conducted to determine the amino acid sequences and isolation method of this protein. As most studies involved the European leech, *Hirudo medicinalis*, little studies have been conducted on *Hirudinaria manillensis*. With the trend of coronary heart disease and other related pathological diseases began to rise, antithrombin protein has potential in the pharmaceutical field. Thus, it is important for mast production of this protein. Molecular biology techniques have made possible cloning and expression of the genes responsible. However, isolation using chromatographic techniques together with the low yield and low activity of the recombinant antithrombin protein have prompt us to look into other alternative of purification and expression system. The N-terminal amino acid sequences of the antithrombin protein from Malaysian local leech were also studied.

Introduction

Medicinal leeches (*Hirudo medicinalis*) are known to prevent blood clots. Haycraft (1894) has shown that medicinal leechs contain anitcoagulant substances. Further studies have managed to isolate active anticoagulants from leech heads and thus the name hirudin was suggested (Franz, 1903). The action of hirudin is specific towards thrombin (Markwardt, 1994). The disadvantages of the current anticoagulants are impaired hemostasis whereas hirudin has been known to be pharmacologically inert with less side effects (Markwardt, 1994). Animal studies have shown that hirudin is more potent than heparin (Klocking *et al.*, 1988). This suggest that antithrombin protein may also have a great potential in the field of medical science.

Large scale isolation of hirudin from the whole leech was conducted by Badgy *et al.* (1973) using chromatography. Studies on the purification of hirudin were also conducted by ion-exchange chromatography (Walsmann and Markwardt, 1985) and affinity

chromatography (Markwardt, 1970). Steiner *et al.* (1990) also used HPLC to purify crude hirudin from *Hirudinaria manillensis*. However, the chromatographic process is tedious.

The complete amino acid sequences of hirudin from *Hirudo medicinalis* was published by Dodt *et al.* (1984) whereas Bergmann *et al.* (1986) managed to determine the nucleic acid sequences. Krstenansky *et al.* (1990) published the amino acid sequences of Hirullin P 18 isolated from *Hirudinaria manillensis*. The same study also found several conserved regions when compared with the hirudin sequence from European leech. The same finding was also observed in other related works (Electricwala *et al.*, 1991; Steiner *et al.*, 1992 and Scacheri *et al.*, 1993).

Thus far production of hirudin and antithrombin from *Escherichia coli* has given low yield (Dodt *et.al.*, 1986) and low activity (Scacheri *et al.*, 1993). Similar results were also seen in expressions with *Saccharomyces cerevisiae* (Loison *et al.*, 1988). This is due to the unsulfated froms of recombinant hirudin produced by *Saccharomyces* with ten times less affinity for thrombin (Stone and Hofsteenge, 1986) and having C-terminal proteolytic degradation (Heim *et al.*, 1994).

The objectives of this study were to determine the amino acid sequences of antithrombin protein isolated from Malaysian leeches (*Hirudinaria manillensis*) in comparison with the published sequences, looking into an alternative technique of purification and cloning of the antithrombin gene. The *Pichia pastoris* expression system which is easy and fast would be used. *Pichia pastoris* has also been known to express heterologous proteins either in intracellular or secreted and have managed to produce recombinant protein at levels of grammes per liter (Clare *et al.*, 1991). In comparison with *Saccharomyces cerevisiae*, recombinant proteins from *Pichia pastoris* are not hyperglycosilated and thus less antigenic (Cregg *et al.*, 1993). The activity of the obtained antithrombin protein in this study was determined by using a chromogenic assay (Chang, 1983) and the units in antithrombin unit (ATU).

Materials and Methods

Isolation and Purification

Leech samples (*Hirudinaria manillensis*) was pulverized in liquid nitrogen before mixing with Phosphate-Buffered-Saline (ratio of 1gm:10 ml of buffer). The mixture was then spun in an ultracentrifuge at 66226 X g and then proceed to a membrane dialysis process with the cut off at 3kD. The obtained sample was further ultracentrifuged and dialysed. The sample obtained from the third process of ultracentrifuge was then isolated by IEF (Isoelectric Focusing) techniqu with Rotofor (Bio-Rad) before further purification with Biologic medium pressure chromatography (Bio-Rad) and HPLC Biologic system (Bio-

Rad). The obtained protein was then subjected to ELISA for activity determination and finally to be lyophillized.

Amino Acid Sequencing

Amino acid sequencing at the N-terminal sequence was carried out on the lyophillised samples using the automated Applied Biosystem Procise Model 492. The equipment was linked on-line to PTH amino acid analyser which follows the Edman degradation method. The samples were first run on a 15% SDS-PAGE (1.5 hours, 200 volts) and then blotted on polyvynil diflouride (PVDF) membrane (1.2 hours, 100 hours) using the Pro-Spin cartridge (Bio-Rad) for sequencing.

DNA Extraction for Cloning

Extraction of genomic DNA from *Hirudinaria manillensis* was conducted with Easy DNA™ kit (Invitrogen). Leech samples (100 mg) was first pulverised with liquid nitrogen. The DNA was observed from 0.8% Agarose gel electrophoresis. The concentration and purity of the samples were determined by spectrophotometer. PCR with specific primers was conducted on the samples according to the PCR parameters by Scacheri *et al.* (1993). Further cloning of the PCR product would then be carried out.

Results and Discussions

Isolation and Purification

Table 1 : Isolation and purification results.

Process	Total Protein (mg)	Recovery (%)	ATU
1 st Centrifuge	2856	100	-
1 st Dialysis	2451	85.82	-
2 nd Centrifuge	2117	74.12	-
2 nd Dialysis	1572	55.04	-
3 rd Centrifuge	1339	46.88	60
ROTOFOR	53.56	1.87	210
Bioscale Q	2.68	0.09	1631
Biologic MA7Q	0.29	0.01	5075

As observed from table 1, the total protein content continue to decrease whereas the ATU value continue to increase. Therefore, the method applied is capable of purification with high quality with 17,500 ATU/mg protein at the final step of purification. As observed, the more steps involved in purification and isolation, the percentage of recovery continue to decrease.

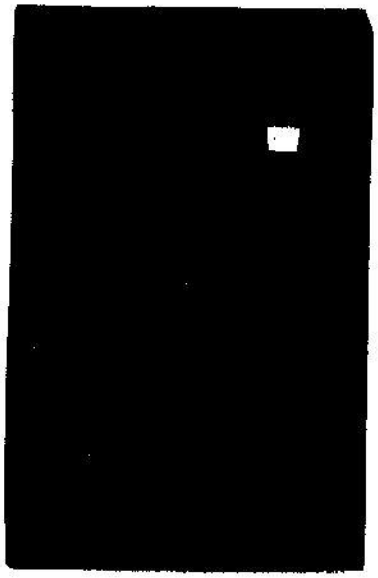
Amino Acid Sequencing

N-terminal analysis showed that the antithrombin protein lack the first cysteine residue at positioin 6 when compared to the published sequence of hirudin from *Hirudo medicinalis*. The obtained probable first 20 amino acid sequences from N-terminal were VDEKAEVTDGLCGDKTCSGA (Val-Asp-Lys-Ala-Val-Thr-Asp-Gly-Leu-Cys-Gly-Asp-Lys-Thr-Cys-Ser-Gly-Ala). However, it was reported that the C-terminal sequences are important for antithrombotic activity (Mao *et al.*, 1988) whereas the sequences at the N-terminal might be important for the structure of the protein.

DNA extraction

DNA extraction was successfully carried out with the DNA concentration at 348.5 µg/ml (n=10) whereas the purity of the DNA samples (n=10) were 1.72 as obtained form the ratio reading of OD_{260}/OD_{280}. The theoritical purity value is 1.8-2.2. The size of the genomic DNA from *Hirudinaria manillensis* is around 15,000 bp (Figure 1). This result coincides with the results obtained by Scacheri *et al.* (1993).

Figure 1 : Agarose gel electrophoresis of genomic DNA.
(Lane 7 on the right is the λ-DNA *Hind III* digest as the DNA marker. The first band of the marker from the well is 23,130 bp whereas the second band from the well is 9,416 bp. Lane 2 - 6 showing the genomic DNA of *Hirudinaria manillensis* in between the two bands of the marker)



Conclusion

The chromatographic method with IEF (Rotofor), medium pressure Biologic and Bioscale Q (BioRad) has managed to purify high quality of antihtrombin protein which are suitable for sequencing purposes. The obtained 20 amino acid sequences from the N-terminal of the antithrombin protein show a difference at position 6 when compared with the known amino acid sequences for hirudin. The kit is useful to obtain genomic DNA of high purity and quantity, the criteria needed for DNA samples for further studies (PCR and cloning for high level production of antithrombin protein).

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