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**SYNTHESIS AND CHARACTERIZATION OF A RECOMBINANT THROMBIN
INHIBITOR**

by

Joel Lwande

**A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Chemistry**

**Western Michigan University
Kalamazoo, Michigan
December 2004**

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2004

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Joel Lwande

SYNTHESIS AND CHARACTERIZATION OF A RECOMBINANT
THROMBIN INHIBITOR

Joel Lwande, M.S.

Western Michigan University, 2004

The final step of the blood coagulation cascade is catalyzed by thrombin, a serine protease. Like most enzyme activities, thrombin activity encounters inhibition from both direct and indirect inhibitors. A new thrombin inhibitor peptide named theromin was recently isolated from the leech *Theromyzon tessulatum*, and it is the most potent thrombin inhibitor isolated to date. Theromin is made up of 67 amino acid residues, out of which 16 are cysteines engaged in 8-disulfide bridges

We synthesized the theromin gene and expressed the protein in *Eschericia coli*. We purified the recombinant protein and carried out thrombin inhibition assays that enabled us determine the kinetic parameters of the protein. Our studies have resulted in a faster and easier way of obtaining large amounts of the pure recombinant theromin. Our results indicate that both the dimeric and monomeric forms of the recombinant theromin inhibit thrombin activity better than hirudin, a well known thrombin inhibitor. We also show that the monomeric form of theromin has a high tendency to oxidize into its dimeric form.

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CHAPTER I

INTRODUCTION

Blood Coagulation

Many organisms maintain a well balanced system that restricts blood clotting/coagulation, the body's major defense against blood loss to the site of injury. The circulatory system must be self-sealing; otherwise continued blood loss from even the smallest injury would be life threatening. Bleeding is brought to a rapid stop in most cases except in catastrophic occurrence. This defense mechanism is achieved through hemostasis, a process made up of many sequential processes also known as the blood coagulation cascade (Davie et al., 1991). A better understanding of the role of the blood coagulation cascade (Figure 1) and its inhibitors both in normal and pathologic responses is enhanced by the availability of more specific reagents that intervene in the coagulation process (Esmon, 1993). Several factors known as coagulation factors are involved in the cascade with each performing a specific role (Halkier, 1991).

Hemostasis is a complex physiologic mechanism that maintains blood in a fluid state within the circulation, yet provides important defense mechanisms against bleeding when injury occurs to a blood vessel. The intraluminal (inside) lining of the blood vessels, also called the vascular endothelium, provides a surface that will not

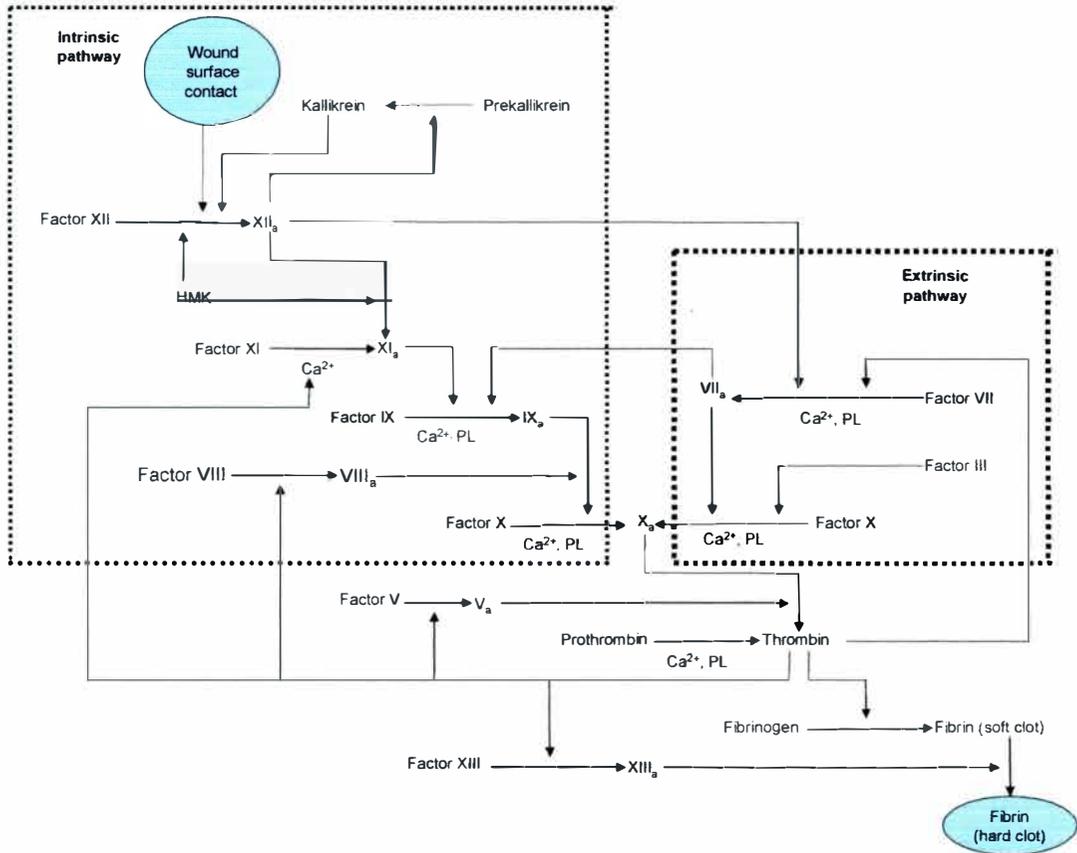


Fig. 1. Schematic Showing the Intrinsic and Extrinsic Pathways of the Coagulation Cascade Leading to Fibrin Formation (modified from Bowen, 2002). The initial stages of the cascade are divided into two pathways, the intrinsic and extrinsic pathways. The active form of a factor is designated by the subscript a and apart from fibrin, all other active clotting factors are serine proteases. PL = phospholipid membrane.

permit clot formation under normal circumstances. However, the coagulation system is initiated in response to injury or rupture of endothelium or injury to the blood vessel, which allows exposure of blood to the extravascular tissue (Bloom et al., 1994). The responses of the coagulation system are coordinated with the formation of the hemostatic plug that occludes the bleeding vessel (Kaplan et al., 1979; Zucker et al., 1979) leading to stoppage of minor bleeding.

Triggering the Blood Coagulation System

Due to the importance of the role played by hemostasis and clot formation in protecting the organism, multiple pathways can initiate clot formation to prevent bleeding following injury. The coagulation of blood is mediated by both cellular components (platelets, but also other inflammatory cells) and soluble plasma proteins (coagulation factors). In response to vascular injury, circulating platelets (unpigmented enucleated blood cells) adhere, aggregate, and provide cell-surface materials (phospholipids) for the assembly of blood clotting enzyme complexes (Midathada et al., 2004). As the platelets aggregate, they release several physiologically active substances, including serotonin (5-hydroxytryptamine) and thromboxane, that stimulate vaso-constriction (Golino et al., 1994), thereby reducing the blood flow at the injury site. Finally, the aggregating platelets and the damaged tissue initiate blood clotting/coagulation (Esmon, 1993). The extrinsic pathway of blood coagulation is initiated when blood is exposed to non-vascular-cell-bound tissue factor in the subendothelial space (Mann et al., 1998; Hoffman and Monroe III, 2001). Tissue factor binds to activated factor VII (factor VIIa) to form an enzyme complex. The formation of this complex is the key to the initiation of coagulation (Nemerson and Esnouf, 1973; Repke et al., 1990; Nemerson, 1992). The resulting enzyme complex activates subsequent factors in the cascade: factors IX and X, respectively. Factor IX activated by the tissue factor VIIa pathway in turn activates additional factor X, in a reaction that is greatly accelerated by a cofactor, factor VIII. Once activated, factor X converts prothrombin to thrombin (factor IIa) in a reaction

that is accelerated by factor V. In the final step of the coagulation pathway, thrombin cleaves fibrinogen to generate fibrin monomers, which then polymerize and link to one another to form a stable clot. Thrombin is a potent activator of platelets, thereby amplifying the coagulation mechanism.

Control and Inhibition of Clotting

The blood clotting cascade is organized to permit enormous amplification of its triggering signals, but remains tightly controlled to prevent any fatal consequences. To prevent widespread clotting following injury, hemostatic inactivation of coagulation occurs whenever clotting is initiated. The potentially explosive nature of the coagulation cascade is offset by natural anticoagulant mechanisms (Davie et al., 1991). The maintenance of adequate blood flow and the regulation of cell-surface activity limit the local accumulation of activated blood-clotting enzymes and complexes (Davie et al., 1991). Thrombin, when formed, binds to thrombomodulin that activates protein C and protein S pathways that inactivate factors Va and VIIIa (Esmon, 2000), thereby dampening further thrombin generation. Antithrombin III, a circulating protein, inactivates factors Xa, and IIa (prothrombin) in a reaction that is accelerated by the presence of heparin (Egeberg, 1965). Endothelium, when activated, release tissue-type plasminogen activator (t-PA) and other activators that convert plasminogen to plasmin, a serine protease that acts on fibrin to dissolve preformed clots. Plasmin digests non-crosslinked fibrin and fibrinogen thereby producing fragments known as degradation products (FDP) (Kowalski, 1968). FDPs inhibit both

platelet aggregation and the action of thrombin on fibrinogen. Another recently identified plasma protein known as tissue factor pathway inhibitor (TFPI) blocks the activity of the tissue factor-factor VIIa (FVIIa-TF) complex (Bajaj et al., 1987). TFPI forms a complex with factor Xa (FXa) then the TFPI-FXa complex binds to the FVIIa-TF complex inhibiting its activities.

Table 1. Human Blood Coagulation Factors (Modified from Halkier, 1991)

Factor Number	Common name
I	Fibrinogen
II	Prothrombin
III	Tissue factor/thromboplastin
IV	Ca ²⁺
VI	Proaccelerin
VII	Proconvertin
VIII	Antihemophilic factor
IX	Christmas factor
X	Stuart factor
XI	Plasma thromboplastin antecedent (PTA)
XII	Hageman factor
XIII	Fibrin-stabilizing factor (FSF)
	Prekallikrein
	High molecular weight kininogen (HMK)

Thrombin

Thrombin is one of the factors in the coagulation cascade and is also known as factor IIa. It belongs to the family of enzymes known as serine proteases which are characterized by the presence of a catalytic triad of residues in their active site. The catalytic triad (Figure 2) is made up of aspartate, histidine and serine residues (Rawlings and Barrett, 1994). Serine proteases catalyze the hydrolysis of covalent peptidic bonds based on nucleophilic attack of the targeted peptidic bond by a serine. Several enzymes fall under the same category with the common ones being chymotrypsin, trypsin and elastase. Thrombin plays an essential role in the formation of blood clots by catalyzing the final steps of the coagulation cascade involving the conversion of fibrinogen into fibrin (Ratnoff and Bennett, 1973).

Thrombin consists of two disulfide-linked polypeptide chains: in humans, a 36-residue A chain and a 259-residue B chain. The thrombin B chain is homologous to trypsin and has similar specificity but is far more selective: It cleaves only certain Arg-X and, less frequently, Lys-X bonds with a clear preference for a Pro preceding the Arg or Lys.

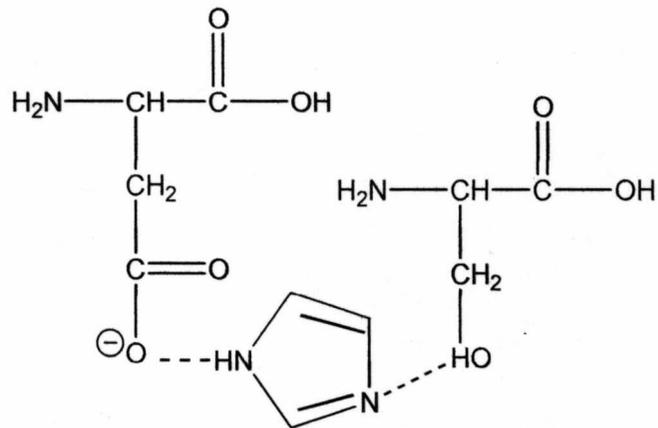


Fig. 2. The Catalytic Triad of Residues in Serine Protease Active Site. Hydrolysis of covalent peptidic bonds is catalyzed based on nucleophilic attack of the targeted peptidic bond by a serine.

Human thrombin is synthesized as a 579-residue zymogen, prothrombin (factor II), which is activated by two proteolytic cleavages (Figure 3) catalyzed by activated Stuart factor (factor Xa), the product of the preceding step of the clotting cascade in an enzyme complex called prothrombinase (Lundblad, 1976). The cleavage of prothrombin's Arg 271-Thr 272 and Arg 320-Ile 321 bonds releases its N-terminal propeptide and separates the A and B chains. Thrombin then autolytically cleaves its Arg 285-Thr 286 bond, thereby trimming away the N-terminal 13 residues of the A chain to yield α -thrombin. The A chain is covalently linked to the B chain through a single disulfide bond (Winzor et al., 1964; Braughman et al., 1967; Fenton et al., 1977).

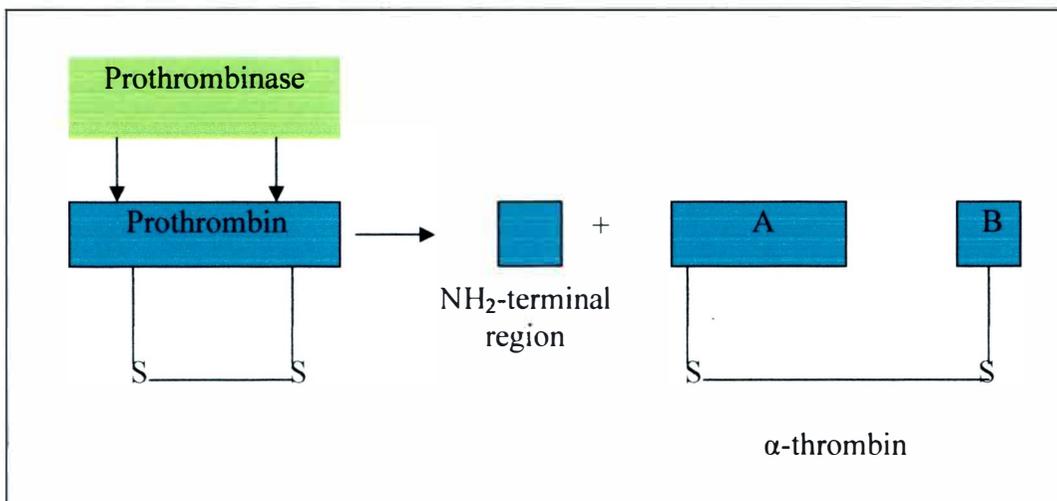


Fig. 3. Proteolytic Activation of Prothrombin. The zymogen prothrombin is activated to give α -thrombin, a serine protease. Proteolysis of two peptide bonds in prothrombin is catalyzed by prothrombinase resulting in an NH_2 -terminal region and the heterodimer, α -thrombin composed of two covalently linked chains (A and B) through a single disulfide bond.

α -thrombin cleaves four bonds in fibrinogen to generate fibrin monomers (Blombäck and Blombäck, 1972), which spontaneously assemble to form a clot (Figure 4) (Budzinski et al., 1983). Thrombin is a unique molecule that functions both as a procoagulant and anticoagulant (Narayanan, 1999). In its procoagulant role it activates platelets through its receptor on the platelets. It regulates its own generation by activating coagulation factors V and VIII resulting in a burst of thrombin formation (Pieters et al., 2001). It also activates factor XIII thereby effecting the cross-linking of fibrin monomers to produce a firm fibrin clot (Greenberg, 1987). Thrombin activates factor XI, thus preventing fibrin clots from undergoing fibrinolysis (Narayanan, 1999). The clotting promotional activities of thrombin can be inhibited by either complex formation with thrombomodulin, a receptor protein on the endothelial

membrane of the blood vessel, or by heparin cofactor II or the antithrombin III/heparin complex. Following complex formation with thrombomodulin, thrombin's ability to cleave fibrinogen and activate factors V and VII is reduced. The same complex will enhance activation of protein C, an anticoagulant. Given all these activities, thrombin stands out as an excellent target for development of new clotting inhibitors.

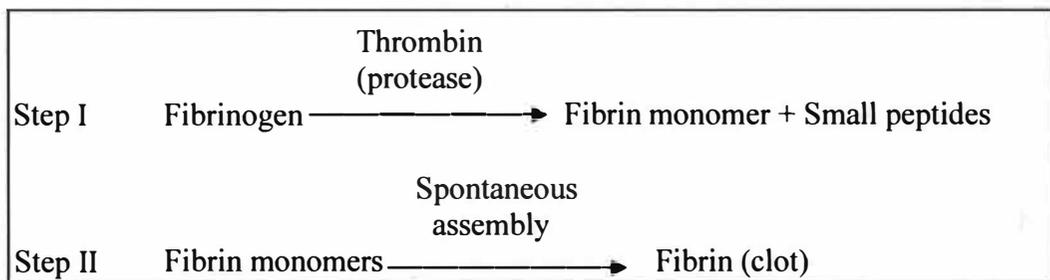


Fig. 4. Final Steps in the Blood Clotting Cascade. Thrombin cleaves four bonds in fibrinogen to generate fibrin, which spontaneously assembles to form a clot.

Thrombin Inhibitors

Like most enzyme activities, the blood coagulation cascade has inhibitors that target different sites along the cascade to prevent blood clot formation. Thrombin activities encounter both direct and indirect inhibition by several inhibitors which include various factors (protease inhibitors) produced by blood-sucking animals such as leeches (Sawyer, 1986 and 1991), and ticks (Jacobs et al., 1990; Waxman et al., 1990). Other animals in the same category include hookworms (Capello et al., 1995) and bats (Gardell et al., 1991). Such factors are produced by these animals to maintain the blood in a fluid state during intake and subsequent digestion (Sawyer, 1986).

Table 2. Some of the Inhibitors Extracted from Leeches

Products	Activity	References
Theromin	thrombin inhibitor	(Salzet et al., 2000)
Hirudin	thrombin inhibitor	(Markwardt, 1994)
Ghilanten	inhibitor of factor Xa	(Blankenship et al., 1990)
Gelin	inhibitor of elastase/chymotrypsin	(Electricwala et al., 1993)
Eglin	inhibitor of elastase/chymotrypsin	(Seemuller et al., 1977)
Hementin	fibrinogenolytic enzyme	(Budzynski et al., 1981)
Antistasin	inhibitor of factor Xa	(Blankenship et al., 1990)
Bdellins	acts on the extracellular matrix	(Fritz and Krejci, 1976)
Guamerins	acts on the extracellular matrix	(Jung et al., 1995; Kim et al., 1996)
Hirustasin	acts on the extracellular matrix	(Sollner et al., 1994)

A variety of coagulation inhibitors isolated from the leech have been studied including hirudin (Markwardt, 1994) and haemadin (Strube et al., 1993). A potent antithrombosis drug (Shionoya, 1927), hirudin was extracted from the salivary glands of the leech *Hirudo medicinalis* (Haycraft, 1884) and its structure has been determined (Markwardt et al., 1967). Other factors isolated from the leech include antistasin and ghilanten (Blankenship et al., 1990), both of which inhibit coagulation factor Xa. Eglins (Seemuller et al., 1977), bdellins (Fritz and Krejci, 1976), guamerins

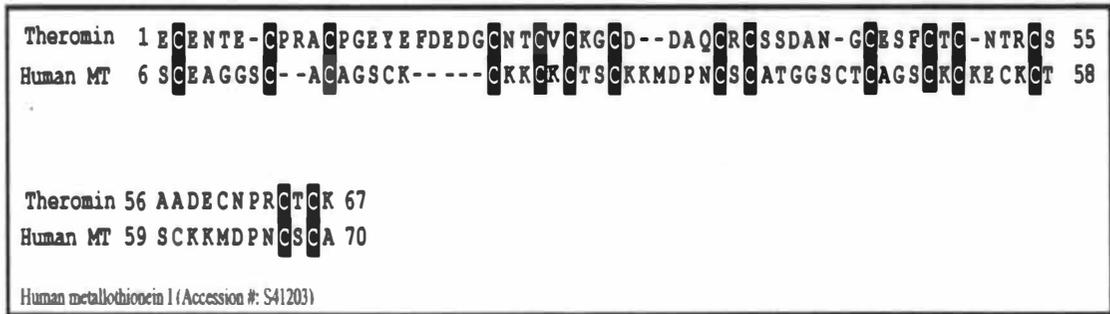
(Jung et al., 1995; Kim et al., 1996), and hirustasin (Sollner et al., 1994), act on the extracellular matrix.

Theromin

A newly isolated peptide named theromin, obtained from the leech *Theromyzon tessulatum*, inhibits the action of thrombin (Salzet et al., 2000). It is the most potent thrombin inhibitor isolated to date (Salzet et al., 2000). Theromin's specificity was determined (Salzet et al., 2000) and used to compare it with other serine proteases already isolated from leeches (Salzet et al., 1999). In their study, theromin, a homodimer, was found to have no inhibition toward trypsin, chymotrypsin, elastase, cathepsin G, plasmin, or factor Xa. It, however, demonstrated inhibition of thrombin with the S-2238 chromogenic substrate with a K_i value of 12 ± 5 fM (Salzet et al., 2000), a value that is lower than that obtained with hirudin (21 fM) (Markwardt, 1994) and haemadin (100 fM) (Strube et al., 1993) under the same conditions. In their study (Salzet et al., 2000), theromin was found to be a strict tight binding thrombin inhibitor. They also showed that theromin monomer (reduced and *S*- β -pyridylethylated) had little thrombin inhibition, leading them to suggest that dimerization is necessary to give the protein an active folded configuration for complete binding to thrombin. In the same study, they reported that the reduced molecule (monomer) does display anti-thrombin activity (they don't mention how much activity), a reason that led them to suggest that each monomer possesses an active site. They also suggest that this could explain the low value of 12 fM for

theromin (dimer) compared with 21 fM for hirudin (monomer). They concluded that each theromin monomer acts in synergy to block thrombin. Other results reported in their work demonstrated that theromin is anionic and rich in cysteine residues just like hirudin (Markwardt, 1994). Hirudin is a natural single-chain peptide of 65 amino acid residues with three intra-chain disulfide bridges and a sulfated tyrosine residue (Markwardt et al., 1967; Markwardt and Walsmann, 1981; Walsmann and Markwardt, 1985; Fortkamp et al., 1986). The hirudin N terminus is globular and very compact because of the presence of the three-disulfide bridges.

The amino acid sequence of theromin is quite different from that of previously isolated thrombin inhibitors (Salzet et al., 2000) and instead it bears resemblance to a known class of metal-binding peptides, namely the metallothioneins (Huffman, unpublished observations). Theromin is a homodimer of 67 amino acid residues, out of which 16 are cysteines engaged in 8-disulfide bridges (Salzet et al., 2000). Metallothioneins also contain a high proportion of cysteine residues in their primary amino acid sequence (Winge et al., 1985). For example, 15 of the 16 theromin cysteines align well with a domain of a zinc-containing MT-I homolog (Figure 5) isolated from human monocytes (Pauwels et al., 1994). Metallothionein proteins bind the physiological metal ions Cu (I) and Zn (II), and toxic metal ions like Cd (II) into clusters bridged by sulfurs of the amino acid cysteine (Riordan and Vallee, 1991 and Zangger et al., 1999).



Human metallothionein I (Accession No. S41203)

Fig. 5. Sequence Alignment of Theromin with a Domain of a Zinc-containing MT-I Homolog Isolated from Human Monocytes (Huffman, D.L., unpublished observations).

While it is known that cysteine residues of metallothioneins participate in metal ion binding, the role played by the same residues in theromin has not been established. There is a possibility that metal ions such as Zn (II) and Cu (I) affect the function of theromin. Therefore, a potential action for a metal-binding peptide may exist in the blood-clotting event. In a separate study, it was demonstrated that yeast metallothionein gained stability and resistance to proteolytic digestions on binding to Ag (I), Cd (II) and Zn (II) metal ions (Nielson and Winge, 1984). It showed resistance to the action of proteases when its metal-binding sites were saturated, but was extensively digested in the metal-free state (Nielson and Winge, 1984). This points to another important role played by metal ions in metallothioneins and suggests a similar protective role for metal ions in theromin.

Significance of This Study

The blood clotting process is tightly controlled in order to limit coagulation to the site of injury. Thrombin, a serine protease, catalyzes a final step of the blood coagulation cascade. Typically, inhibition of thrombin is accomplished by administration of the drug heparin, which stimulates the production of the endogenous inhibitor antithrombin. Theromin has been reported to be the most potent thrombin inhibitor isolated to date. After its extraction from the leech, some studies were carried out on theromin (Salzet et al., 2000), but there is need for more studies to be carried out given the potential of theromin as an anticoagulant. For example there is a good possibility that metal ions such as Zn (II) and Cu (I) are essential to the function of theromin. No study has been carried out to establish if theromin binds metal ions. Whether metal ions such as Zn (II) and Cu (I) play a role in the function and/or structural stability of theromin is unknown. To be able to carry out further studies on theromin, it is necessary to devise an easier and faster way of getting large amounts of the protein. There is also need for further characterization of theromin.

Objectives of the Study

There were three objectives in this study. The first objective was to synthesize the theromin gene and integrate it into an expression vector to produce the recombinant theromin gene. The second objective was to transform the recombinant theromin gene into *E. coli* for protein over-expression followed by extraction and purification of the protein. The third objective was to characterize the protein by using

it to carry out thrombin inhibition assays that would be compared to hirudin, a well studied thrombin inhibitor. The K_m and K_i of the recombinant thrombin were to be determined by the use of Lineweaver-Burk plots of the resulting thrombin inhibition assay readings.

CHAPTER II

MATERIALS AND METHODS

General

All the primers were purchased from Integrated DNA Technologies, Inc. All restriction endonucleases and enzymes plus the relevant buffers were purchased from New England Biolabs Inc. or Life Technologies. The deoxyribonucleotides (dNTPs) were purchased from Sigma Chemical Co., while the bacterial strains and plasmids were from Novagen. GST Pure agarose was purchased from Life Technologies. The QIAGEN plasmid DNA isolation kit was purchased from Qiagen Inc. GST•Bind™ Kits and Factor Xa kits were purchased from Novagen. Dimethylsulfoxide (DMSO) was purchased from J. T. Baker Chemical Co. To correct the mutation in the synthesized gene, a Quik Change Sigh Directed Mutagenesis kit was purchased from Stratagene. Isopropyl- β -D-thiogalactopyranoside (IPTG) was purchased from INALCO SPA (Milano, Italy) and used to induce protein expression. To filter extracted protein, 0.22 μ m PES (Polyether sulfone) filters were purchased from Corning Inc. Stirred Ultra-filtration Cells obtained from Amicon Bioseparations were used for protein concentration. α -thrombin was purchased from Haematologic Technologies Inc (HTI). We purchased the chromogenic substrate (Chromozyme TH) from Roche Inc.

Design and Synthesis of Theromin Gene

The published theromin amino acid sequence (Salzet et al., 2000) was converted to the corresponding DNA sequence with highly expressed *E. coli* codons based on the *E. coli* high-frequency codon database (Gouy and Gautier, 1982). The resulting 201 DNA base pair sequence was elongated by addition of an extra 30 DNA base pairs to accommodate the stop codon plus two restriction sites, NcoI and BamHI (Figure 6).

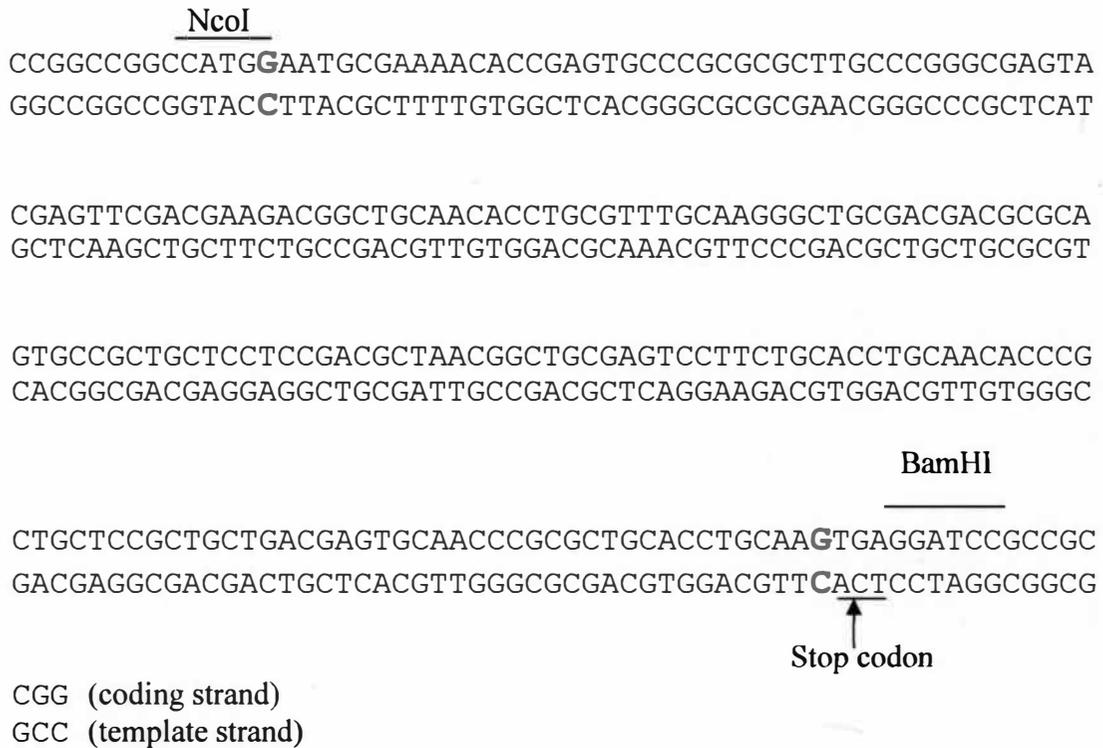


Fig. 6. Engineered Theromin DNA Sequence. The double strand sequence was engineered based on the *E. coli* high-frequency codon database (Gouy and Gautier, 1982). The DNA base pairs shown in red mark the first and the last base pairs of the 201 DNA base pair theromin gene. The NcoI and BamHI restriction sites are also marked. The stop codon was designed to appear immediately after the last theromin DNA base pair.

The double stranded 231 base pair DNA sequence was encoded by 10 over-lapping primers (Figure 7). The primers varied in length (Table 3) and overlapped by varying numbers of bases and each had a different melting temperature (T_m), and a different GC ratio (Table 4).

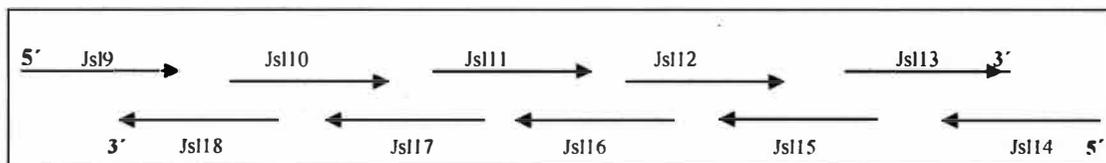


Fig. 7. The 10 Overlapping Primers Including 5 Forward (Jsl9, Jsl10, Jsl11, Jsl12 and Jsl13) and 5 Reverse (Jsl14, Jsl15, Jsl16, Jsl17 and Jsl18). Jsl9 has the Restriction Site NcoI While Jsl14 Has the Restriction Site BamHI.

Table 3. Sequences for the Designed 10 Over-Lapping Primers. The primers vary in size.

Primer	Sequence
Jsl9	GGCCGGCCATGGAATGCGAAAACACCGAGTGCCCGCGCGC
Jsl10	GGCGAGTACGAGTTCGACGAAGACGGCTGCAACACCTGCG
Jsl11	CAAGGGCTGCGACGACGCGCAGTGCCGCTGCTCCTCCGAC
Jsl12	CGGCTGCGAGTCCTTCTGCACCTGCAACACCCGCTGCTCC
Jsl13	GACGAGTGCAACCCGCGCTGCACCTGCAAGTGAGGATCC
Jsl14	GCGCCGGCGGCGGATCCTCACTTGCAGGTG
Jsl15	CGCGGGTTGCACTCGTCAGCAGCGGAGCAGCGGGTGTGTC
Jsl16	CAGAAGGATTCGCAGCCGTTAGCGTCGGAGGAGCAGCGGC
Jsl17	GCGTCGTCGCAGCCCTTGCAAACGCAGGTGTTGCAGCCG
Jsl18	GTCGAACTCGTACTCGCCCGGGCAAGCGCGCGGGCACTCG

Table 4. The Varying Sizes of Primers Each with a Different GC Ratio and Melting Temperature (T_m)

Jsl9	- 40 bp,	$T_m = 80.3^\circ\text{C}$,	GC = 70.0%
Jsl10	- 40 bp,	$T_m = 75.2^\circ\text{C}$	GC = 62.5%
Jsl11	- 40 bp,	$T_m = 80.3^\circ\text{C}$	GC = 72.5%
Jsl12	- 40 bp,	$T_m = 78.3^\circ\text{C}$	GC = 67.5%
Jsl13	- 40 bp	$T_m = 77.0^\circ\text{C}$	GC = 65.0%
Jsl14	- 30 bp	$T_m = 76.0^\circ\text{C}$	GC = 73.3%
Jsl15	- 40 bp	$T_m = 79.7^\circ\text{C}$	GC = 70.0%
Jsl16	- 40 bp	$T_m = 76.6^\circ\text{C}$	GC = 65.0%
Jsl17	- 39 bp	$T_m = 78.3^\circ\text{C}$	GC = 66.7%
Jsl18	- 40 bp	$T_m = 80.0^\circ\text{C}$	GC = 72.5%

Different Polymerase Chain Reaction (PCR) steps were used to construct the theromin gene (Altmann et al., 1995) with the first step involving the use of each of the overlapping pairs of primers. Only one pair of overlapping primers was added to each reaction tube. One microliter of each primer from an original stock of 500 μM was used. In each tube was added 10 μL of 2 mM dNTPs, 1 μL of Deep Vent[®] (exo-) DNA polymerase (2 U/ μL) (New England Biolabs) and 10 μL of the Thermopol buffer (10X) (New England Biolabs). Sterile de-ionized H_2O was added to bring the total reaction volume to 100 μL . Using the Mini Cycler PTC-150 PCR system (MJ Research), 30 cycles of amplification were carried out by using the following program (95°C, 20 s; 50°C, 20 s; 72°C, 30 s). This was followed by a 5 minute final extension at 72°C.

Five different pieces of double stranded DNA were obtained after the first PCR reaction. The reaction products in each tube were purified using QIAquick Spin PCR Purification Kit (Qiagen, Inc.) according to the kit instructions from the

manufacturer. One μL of $30 \text{ ng}/\mu\text{L}$ from each purified product was then used in subsequent steps of extension PCR. The second step involved the addition of the first two overlapping double strand DNA pieces from step one into a new PCR reaction tube. Two flanking (outer) primers were also added (Figure 8). The procedure in step one was used in amplification with each subsequent round of reaction involving purified product from the preceding extension reaction. In each case a molar ratio of 1:1 was maintained for each of the two double stranded DNA pieces being joined. This process was performed sequentially to complete the synthesis of theromin gene. The final theromin gene product was analyzed on 2% agarose gel electrophoresis. The gene was excised from the gel and purified using the QIAquick Gel extraction kit (Qiagen Inc.) following the kit instructions from the manufacturer.

Cloning of the Theromin Synthetic Gene

Both the synthesized gene (insert) and the plasmid (pET24d) were subjected to double digestion using the restriction endonucleases NcoI and BamHI (Figure 9). Two hundred ng of the insert and plasmid were each added to a different reaction tube. A total of $0.5 \mu\text{L}$ ($20 \text{ units}/\mu\text{L}$) of BamHI and $1 \mu\text{L}$ ($10 \text{ units}/\mu\text{L}$) of NcoI was added to each reaction tube. $3 \mu\text{L}$ of BamHI buffer ($10\times$) and $0.3 \mu\text{L}$ of BSA ($10\text{mg}/\text{mL}$) were added to each reaction mixture. Sterile deionized water was added to a final reaction volume of $50 \mu\text{L}$. Reaction tubes were incubated at 37°C for 12 hours.

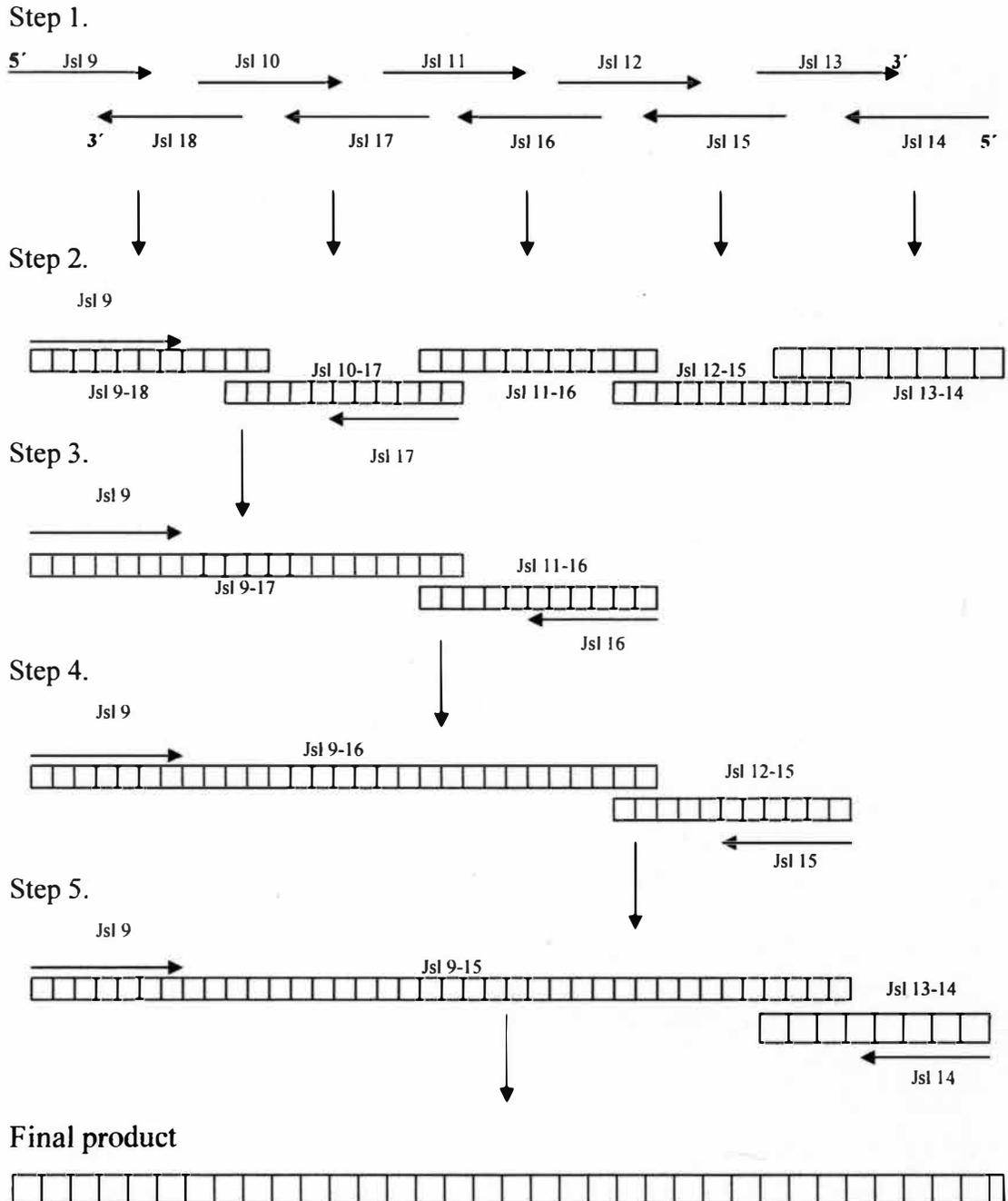


Fig. 8. A Schematic Showing the Addition-extension PCR. One piece from step one is added each time until the final product is achieved. The leading primers, Jsl9 and Jsl14 have the restriction sites Nco I and BamH I respectively. Horizontal arrows are DNA primers, segmented boxes are double-stranded DNA products.

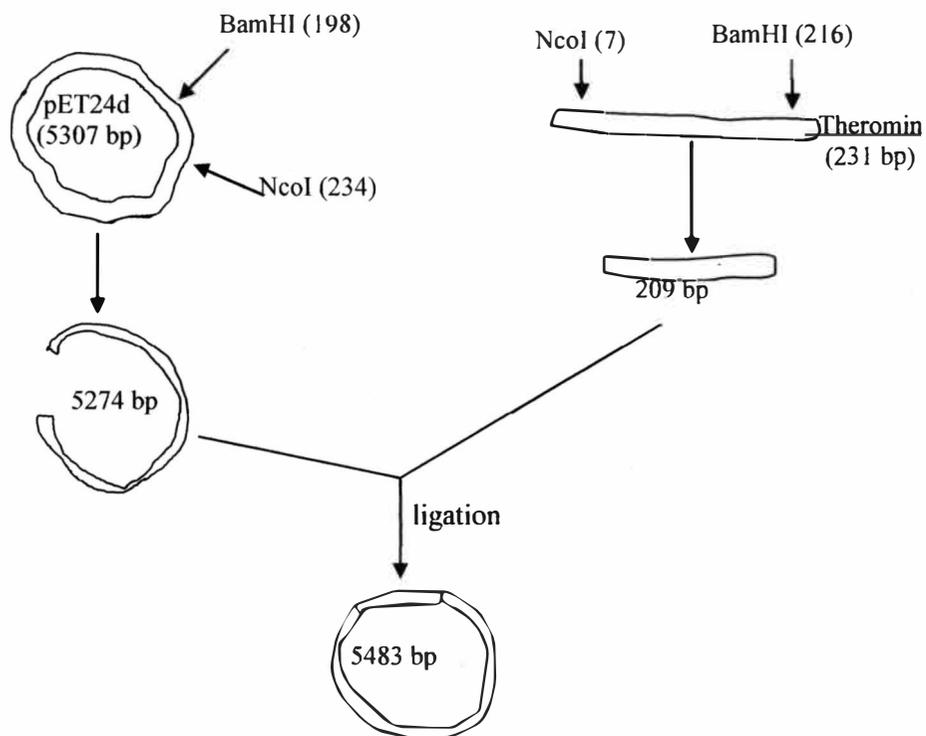


Fig. 9. A Schematic Describing the Ligation of Theromin into the Plasmid (pET24d).

The doubly digested insert and plasmid were each analyzed using 2% and 1% agarose gel electrophoresis respectively (Rickwood, 1990). The two different percentages of agarose used were necessary to ensure proper pore sizes in the gels for migration of different sizes of DNA. The digestion products were extracted from the gel using a kit from Qiagen Inc. Ligation (Sambrook et al., 1989) was done by adding 100 ng of the doubly digested theromin gene (insert) to the doubly digested plasmid (pET24d) (Figure 9) in three different molar ratios of 1:1, 1:3 and 3:1. 6 μ L of T4 DNA ligase buffer (10x) was used together with 1.5 μ L of T4 DNA ligase (20 U/ μ L).

Sterile deionized H₂O was added to bring the total reaction volume to 60 μ L. The ligation reaction was performed at 16°C for 12 hours.

Preparation of Chemically Competent Cells

A Lurie Broth (LB) (10 g/L Bacto Tryptone, 5 g/L Bacto Yeast Extract, and 10 g/L NaCl) plate was streaked with the DH5 α (*E. coli*) cells then incubated at 37°C for 14 hours. A single colony from the 14 hour growth was used to inoculate 5 mL of liquid LB medium. The inoculated medium was incubated at 37°C for 12 hours with shaking (250 rpm) in a New Brunswick Scientific shaker. 1 mL of the bacteria culture was drawn and added to 100 mL of LB medium that was then incubated at 37°C with shaking until the OD₆₀₀ of between 0.45 to 0.55 was attained. The bacteria culture was then chilled on ice for 2 hours followed by centrifugation at 5500 rpm for 20 minutes on an SS-34 rotor. The bacterial pellet (cells) was resuspended in 2 mL of Trituration buffer (100 mM CaCl₂, 70 mM MgCl₂, 40 mM Acetic acid). The same buffer was used to dilute the resuspended cells to 100 mL followed by chilling on ice for 45 minutes. After a 10 minute centrifugation at 4500 rpm on an SS-34 rotor, the supernatant was discarded leaving the pellet that was then resuspended into 10 mL of Trituration buffer. 80% glycerol was added dropwise with gentle swirling to a final concentration of 15% (V/V). The cells were aliquoted in 0.2 mL quantities then frozen on dry ice followed by storage at -80°C.

Transformation

Four aliquots of frozen (-80°C) DH5 α chemically competent cells (200 μ L each) were thawed on ice followed by addition of 3 μ L of DMSO. Each was mixed briefly then 20 ng of the purified ligation product was added to one of the aliquots. A similar amount of undigested pET24d plasmid was added to the second aliquot to form the positive control while 20 ng of doubly digested plasmid (pET24d) was added to the third aliquot to form the negative control. The fourth aliquot had nothing added and this formed the second negative control. All four aliquots were incubated on ice for 30 minutes after which they were heat-shocked at 42°C for 2 minutes followed by incubation on ice for 1 minute. One mL of Lurie Broth (LB) medium was added to each aliquot then cells were incubated at 37°C for 1 hour with shaking (250 rpm) in a New Brunswick Scientific shaker. The bacterial broth culture were pelleted by centrifugation at 14500 rpm for 1 minute in a microcentrifuge. The supernatant from each tube was discarded and the pellet (cells) were each resuspended in 200 μ L of LB medium then plated on different agar plates, each containing kanamycin sulfate (30 μ g/mL). The plates were incubated at 37°C for 12 hours.

Two different bacterial colonies were picked from the experimental plate (bacteria transformed with ligation product) and used to inoculate liquid LB cultures (5 ml each) containing kanamycin sulfate (30 μ g/mL) each in a different culture tube. Cultures were grown in a New Brunswick Scientific shaker (250 rpm) at 37°C for 12 hours. The recombinant gene was isolated from bacteria using the QIAGEN

plasmid mini kit (Qiagen Inc.), after which (1%) agarose gel electrophoresis was used to analyze the the isolate.

Two different methods were used to establish whether ligation worked. In the first method, the isolated DNA was digested using BamHI and BglII in a method similar to the one described earlier. BglII was used instead of NcoI to give a bigger DNA cut out (317 bp) for easy visibility in the gel. The digestion product was analyzed using 2% agarose gel electrophoresis. In the second method, 800 ng of the isolated DNA was submitted for DNA sequencing (dideoxy method). A deletion of one base pair (bp number 93) in the theromin gene was detected following the results from sequencing. The mutation was corrected using the QuikChange[®] XL Site-Directed Mutagenesis Kit (Stratagene) following instructions from the manufacturer. The correction was also performed based on a previously described method (Kajimoto, 1993). The corrected gene product was verified by sequencing.

Protein Expression and Purification

Induction of Protein Expression Using IPTG

IPTG (isopropyl thiogalactoside) is an artificial inducer of the Lac operon, but unlike the physiological inducer of the lactose system (the lactose isomer 1,6-allolactose), IPTG is not degraded by β -galactosidase. IPTG induces the activity of β -galactosidase by strongly binding and inhibiting the lac repressor. Being a synthetic analog of galactose, IPTG cannot be hydrolyzed and broken down by the cell. Hence, its concentration does not change during the induction test. The gene of interest is

cloned into an expression cassette downstream of a T7 promoter, synthesis of T7 polymerase (not usually expressed in bacteria) leads to transcription of the RNA of interest and hopefully protein synthesis. The DE3-derivatives of BL21 contain a λ prophage that encodes the T7 RNA polymerase gene under the control of the lacUV5 promoter. As such, addition of IPTG to medium induces expression of T7 polymerase with consequent expression of the gene of interest.

The recombinant gene was transformed into BL21 (DE3) chemically competent cells using the same transformation method described earlier. The bacterial pellet was resuspended in 200 μ L of LB medium then plated on an agar plate, containing kanamycin sulfate (30 μ g/mL).

To perform an induction test, a single colony was picked from the plate with the recombinant gene (experimental plate) and used to inoculate 50 mL of LB medium containing kanamycin sulfate (30 μ g/mL). The bacterial culture was incubated at 37°C with shaking to an OD₆₀₀ of approximately 0.6 (Smith and Johnson, 1988). One aliquot of 500 μ L was drawn from this culture and pelleted by centrifugation at maximum speed (14500 rpm) for 2 minutes in a microcentrifuge. After discarding the supernatant, the pellet was frozen at -20°C. Isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the remaining culture to a final concentration of 1.0 mM. The OD₆₀₀ reading was taken every 30 minutes for 4 hours, each time a 500 μ L aliquot being drawn, centrifuged and the pellet frozen at -20°C. A 15% tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was used to analyze the induction test.

Freeze-Thaw Protein Extraction Method

The remaining culture (45.5 mL) was pelleted by centrifugation followed by freeze-thaw protein extraction method in which the pellet was frozen in liquid nitrogen (77 K) for 5 minutes, and then thawed on ice for 35 minutes. This cycle was repeated two more times followed by resuspension of the freeze-thawed pellet in 1.08 mL of protein extraction buffer (20 mM MES/Na, pH 5.5, 1 mM EDTA). To ensure the protein remained in a reduced state, 5 mM dithiothreitol (DTT) was added to the protein extraction buffer. It was incubated on ice for 1 hour with periodic agitation followed by centrifugation at 6000 g for 15 minutes at 4°C. The supernatant containing the protein was carefully removed and filtered through the 0.22 µm PES (Polyether sulfone) filters (Corning Inc.). Some of the newly extracted protein was analyzed by running a 15% tricine SDS-PAGE. The remaining small scale extracted protein sample (approximately 1.08 mL) was concentrated 5.4 fold in preparation for high resolution size-exclusion chromatography (Superdex 75 HR 10/30) purification method. The protein was loaded and purified on the high resolution superdex column (Amersham Pharmacia Biotech) using the AKTA[®] FPLC instrument at a wavelength of 254 nm followed by a 15% tricine SDS-PAGE electrophoresis analysis of all the elution fractions.

Large Scale Protein Expression

Based on the induction test results from the small scale method described above, a 10 L fermentation was prepared. Two hundred and fifty mL of LB medium containing kanamycin sulfate (30 $\mu\text{g}/\text{mL}$) was inoculated with a single colony containing the recombinant gene. The culture was incubated at 37°C with shaking to attain an OD_{600} reading of approximately 0.6. The culture was pelleted by centrifugation using a SLA-3000 rotor at 6000 xg for 15 minutes at 4°C on a SORVALL® RC5B PLUS centrifuge (Sorvall Centrifuges). The supernatant was discarded while the pellet was resuspended in 10 mL of LB then added to 10 L of LB medium containing kanamycin sulfate (30 $\mu\text{g}/\text{mL}$). It was grown with a MICROFERM FERMENTOR (New Brunswick Scientific Co. Inc.) at 37°C until the OD_{600} of the culture reached approximately 0.6. Protein expression was induced by addition of IPTG to a final concentration of 1.0 mM. The fermentor continued running for 4 more hours after which the culture was pelleted by centrifugation at 6000 xg for 15 minutes using a SLA-3000 rotor at 4°C on a SORVALL® RC5B PLUS centrifuge (Sorvall Centrifuges). The pellet was frozen at -20°C.

Protein was extracted from the frozen pellet by the freeze-thaw method explained above. The freeze-thawed pellet was resuspended in 240 ml of protein extraction buffer (20 mM MES/Na, pH 5.5, 1 mM EDTA). It was incubated on ice for 1 hour with periodic agitation followed by centrifugation at 6000 xg for 15 minutes at 4°C using the SLA-3000 rotor. The supernatant containing the protein was carefully removed and filtered through a 0.22 μm PES (Polyether Sulfone) filter.

Protein Purification

The first purification step was the DEAE-Sepharose (anion exchange chromatography) on a Fast Performance Liquid Chromatography (FPLC) system (Amersham Pharmacia Biotech). The column was first washed using 2 column volumes of 100% buffer B (20 mM MES/Na, pH 5.5, 0.1 mM EDTA, 1 M NaCl) to release any bound protein from previous experiments. The column was then equilibrated using 1.5 column volumes of buffer A (20 mM MES/Na, pH 5.5, 0.1 mM EDTA). After loading the filtered protein extract onto the column, the unbound protein was washed off the column using 1.5 column volumes of buffer A. Elution was done using both the A and B buffers by an automated program at a flow rate of 0.5 mL/min. A total of 80 fractions were collected with each having a total volume of 3 mL. A 15% tricine SDS-PAGE electrophoresis method was used to analyze the DEAE-Sepharose elution fractions. Following determination by the thrombin inhibition assays and SDS-PAGE electrophoresis, all fractions that had the protein (theromin) were pulled together and concentrated by a factor of 65 using the Stirred Ultra-filtration Cells (Amicon). This was further purified on a high resolution size-exclusion chromatography column (Sephadex 75 HR 10/30) (Amersham Pharmacia Biotech) on the same AKTA[®] FPLC instrument. on which equilibration and elution was done using the Superdex 75 buffer (20 mM MES/Na, pH 5.5, 150 mM NaCl). The buffer also contained 10 mM DTT to maintain the protein in a reduced state. A total of 70 fractions were eluted and subjected to a 15% tricine SDS-PAGE gel electrophoresis analysis.

Protein concentration was determined at every purification step using the Bio-Rad Protein Assay (Bio-Rad Laboratories) method explained above. Theromin containing fractions from the high resolution size-exclusion chromatography column (Sephadex 75 HR 10/30) were submitted for Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS). Thrombin inhibition assays were carried out using the fractions containing theromin from the high resolution size-exclusion chromatography as described below.

The Bio-Rad Protein Assay Method

Protein concentration in the elution fractions was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories) method. The Bio-Rad standard curve was made by addition of increasing volumes of 1.51 mg/mL Immunoglobulin G (IgG) protein (from 0 μ L to 18 μ L) to 10 different cuvettes. Sterile deionized H₂O was added to each cuvette to bring the total volume to 800 μ L. To each cuvette was added 200 μ L of Bio-Rad dye followed by thorough mixing and incubation at room temperature for 15 minutes. Absorbance of each of the 10 mixtures was read at 595 nm on a DU[®] 7400 Nucleic Acid and Protein Analyzer (Beckman). The readings were used to construct a standard curve of protein concentration (mg/mL) versus absorbance. Samples were mixed and incubated the same way and absorbance read at the same wavelength. The standard curve was then used to determine the protein concentration of each sample based on its absorbance.

Thrombin Inhibition Assay

Thrombin inhibition properties of thrombin were studied (Salzet et al., 2000) by designing an assay (Figure 10) that involved addition of 961 μL of Tris buffer (50 mM Tris, pH 8.3, 227 mM NaCl), 5.3 μL of 343 nM α -thrombin and varying concentrations of thrombin purification fractions to different cuvettes. The mixtures were incubated at room temperature for 15 minutes before starting the reaction by addition of 100 μL of 1.9 mM Chromozyme TH (chromogenic substrate). They were thoroughly mixed before reading the absorbance at 405 nm for a total of 180 seconds on a DU[®] 7400 Nucleic Acid and Protein Analyzer (Beckman). A control was run the same way, but without any thrombin purification fractions. The rates of reactions for both the sample and control were determined as the change in absorbance per minute then a comparison was made.

Glutathione-S-Transferase Tag

Due to low protein yields, a new protein expression vector with a Glutathione-S-Transferase Tag (GST Tag) was used. The GST-Tag increases the size of the protein being expressed hence reducing some of the difficulties that would otherwise be experienced without it. The solubility of the tagged protein is also increased. The GST-Tag kit comes with a GST specific column enabling the use of affinity chromatography to purify the tagged protein. The gene for the tag is present on the pET42a plasmid along with a Factor Xa cleavage site. Following expression and purification of the tagged protein, the tag is cleaved off the protein using Factor Xa.

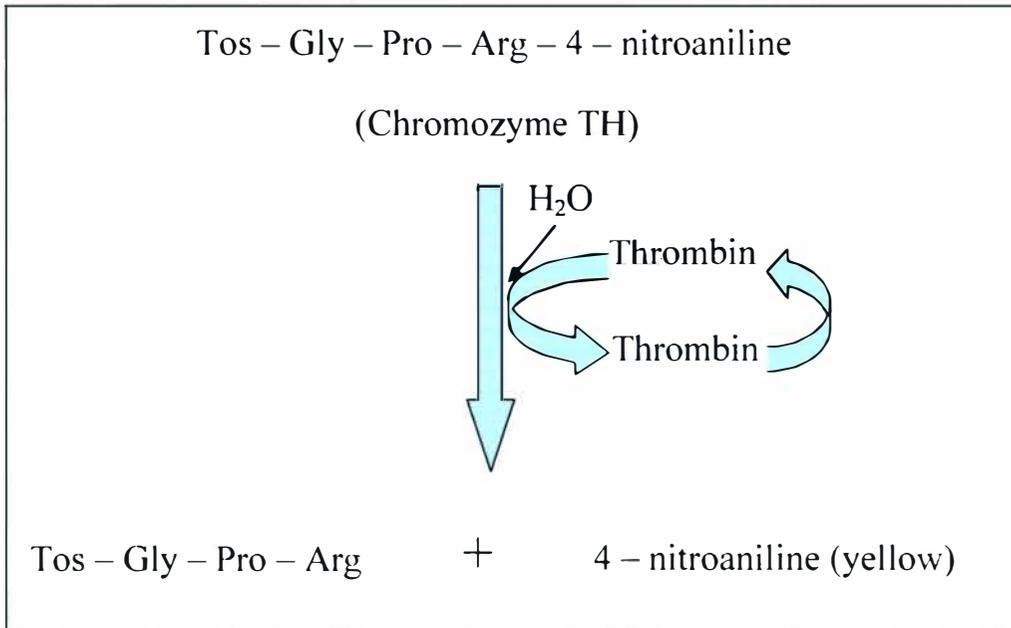


Fig. 10. A Schematic of the Thrombin Inhibition Assay. The chromogenic substrate Tos – Gly – Pro – Arg – 4 – nitroaniline (chromozyme TH) is broken down into two products in a reaction catalyzed by thrombin in presence of H₂O. The two products are: Tos – Gly – Pro – Arg and 4 – nitroaniline, which is yellow in color.

Both the synthesized thrombin gene and pET42a plasmid were subjected to double digestion using BamHI and NcoI restriction endonucleases. Purification of the digests and subsequent ligation were done according to methods described herein earlier. The ligation product was transformed into DH5 α competent cells, plated, grown and recombinant plasmid isolated from bacteria culture using the same methods and conditions described herein earlier. The gene was sequenced to confirm proper ligation after which the QuikChange[®] XL Site-Directed Mutagenesis Kit (Stratagene) was used to delete 9 DNA base pairs occurring between the factor Xa cleavage site and the insert. This was to ensure that the protein is pure once the tag is

cleaved off by Factor Xa, otherwise there would be an additional 3 amino acids that are not part of theromin. The insertion site was 9 DNA base pairs away from the Factor Xa cleavage site. The Site-Directed Mutagenesis product was transformed into DH5 α competent cells, plated, grown and recombinant plasmid isolated from bacteria culture the same way as described herein. After confirming the proper sequence by dideoxy sequencing method, the recombinant plasmid was transformed into BL21(DE3) competent cells for protein expression.

A similar induction test (as described earlier) was carried out using IPTG on both the recombinant gene and a control having pET42a plasmid without the insert. The induction results were analyzed using 15% tricine SDS-PAGE gel electrophoresis. A large scale (10 L) protein expression was done and then the culture was pelleted by centrifugation at 10,000 g for 10 minutes at 4°C using a pre-weighed centrifuge tube on a SLA-3000 rotor (Sorvall Centrifuges). After discarding the supernatant, the pellet was allowed to drain, removing as much liquid as possible. The wet weight of the pellet was determined. Protein extraction from the pellet, cleavage of theromin from the GST Tag and subsequent purification of the protein was done using the GST•Bind™ Kits and Factor Xa kits (Novagen) following the instructions from the manufacturer. The factor Xa-cleaved protein was analyzed by running a 15% tricine SDS-PAGE gel. Theromin was then purified further on a HiLoad Superdex 75 size exclusion chromatography column (Amersham Pharmacia Biotech) using the AKTA® FPLC instrument. The elution fractions were analyzed by the 15% tricine SDS-PAGE gel method after which purification fractions showing bands near the

expected thrombin size were submitted for Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) to determine the actual mass. Thrombin inhibition assays were done using thrombin containing fractions as described herein earlier.

Determination of Thrombin's Inhibitory Constant (K_I)

A thrombin inhibition assay was done as described earlier in which the change in absorbance/minute was read at 405 nm wavelength versus the substrate concentration, [S] in the presence of different concentrations of the dimeric form of thrombin. Assays were done in both the absence and presence of the inhibitor.

A Lineweaver-Burk plot ($1/V$ versus $1/[S]$) was constructed and used to calculate the K_I of the dimeric thrombin. The curves were extrapolated and their respective equations were used together with relevant enzyme kinetic equations (Figure 11) to calculate the inhibitory constant (K_I) and the substrate concentration (K_M) of the dimeric form of thrombin.

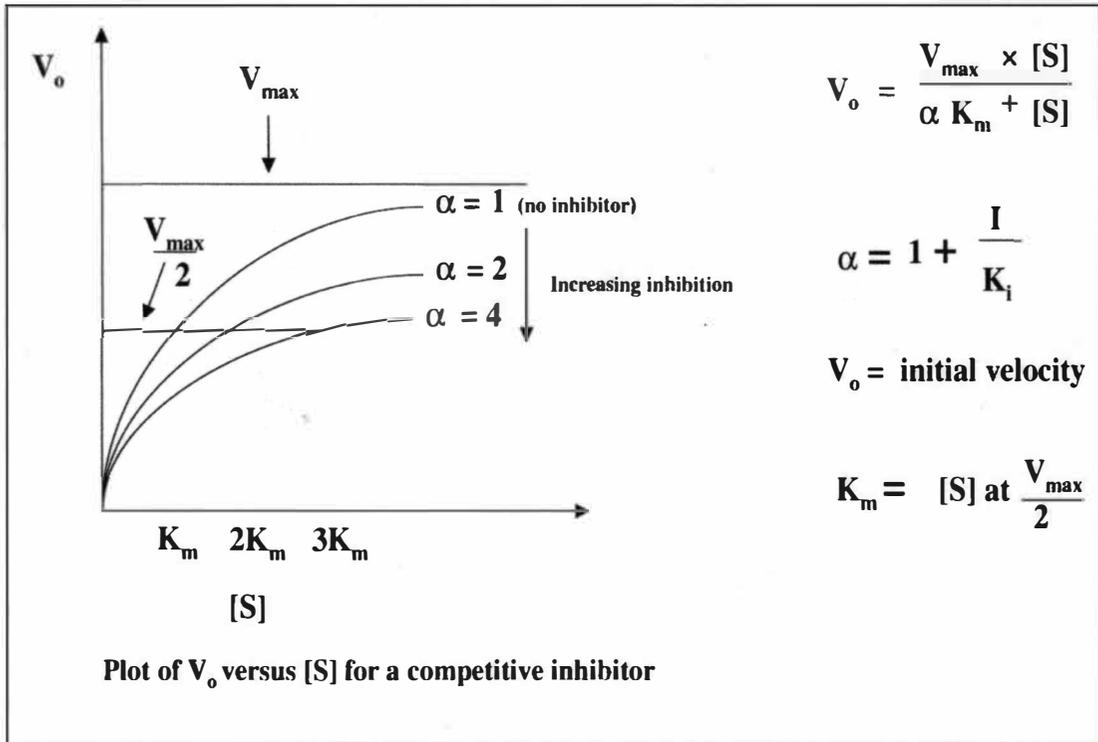


Fig. 11. A Schematic Showing a Plot of the Initial Velocity V_o of a Simple Michaelis-Menten Reaction Versus the Substrate Concentration $[S]$ in the Presence of Different Concentrations of a Competitive Inhibitor. The equations can be used to calculate the inhibitory constant, K_i and the K_m of a given inhibitor.

CHAPTER III

RESULTS AND DISCUSSION

Synthesis and Cloning of Theromin Gene

To synthesize the theromin gene, the Polymerase Chain Reaction (PCR) method was used in several steps (see Materials and Methods) utilizing the designed 10 overlapping primers. The first step gave rise to 5 different pieces of double strand DNA (Figure 12) all varying in size depending on the sizes of the starting primers and the number of overlapping base pairs between each pair of primers. The reaction products were analyzed by running a 2% agarose gel electrophoresis where the samples were compared to the 100 base pair ladder DNA standard (not shown in the picture). Other subsequent steps followed in which the five DNA pieces from step one were joined using the same method to get the final theromin gene. The PCR melting and annealing temperatures were adjusted according to the size of the DNA pieces being joined. The synthesis and subsequent amplification using the designed 10 primers resulted in a 234 base pair double strand DNA piece analyzed by running a 2% agarose gel in comparison with the 100 base pair ladder DNA standard (Figure 13). The gene had two restriction endonuclease sites, NcoI and BamHI, at positions 7 and 216 respectively.

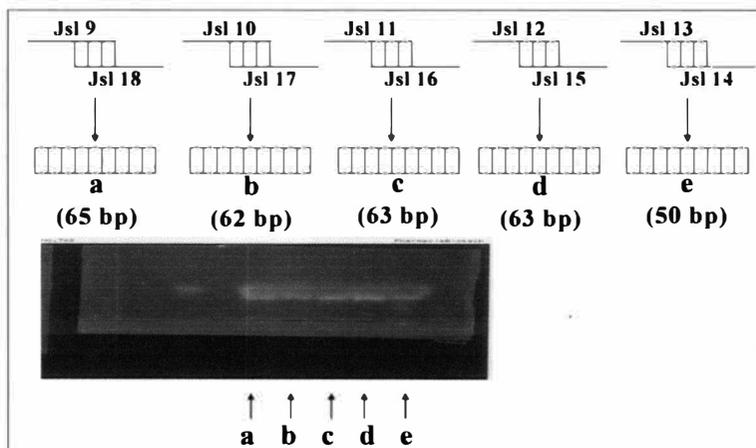


Fig. 12. The First Step in Theromin Gene Synthesis. PCR reactions were done in 5 different reaction tubes using 10 overlapping primers resulting in five double strand DNA fragments labeled a (65 bp), b (62 bp), c (63 bp), d (63 bp), and e (50 bp). 4 μ L (~ 20 ng/ μ L) of each fragment was mixed with 1 μ L of the loading dye and 1 μ L of H₂O then loaded into each lane followed by electrophoresis on 2% agarose gel.

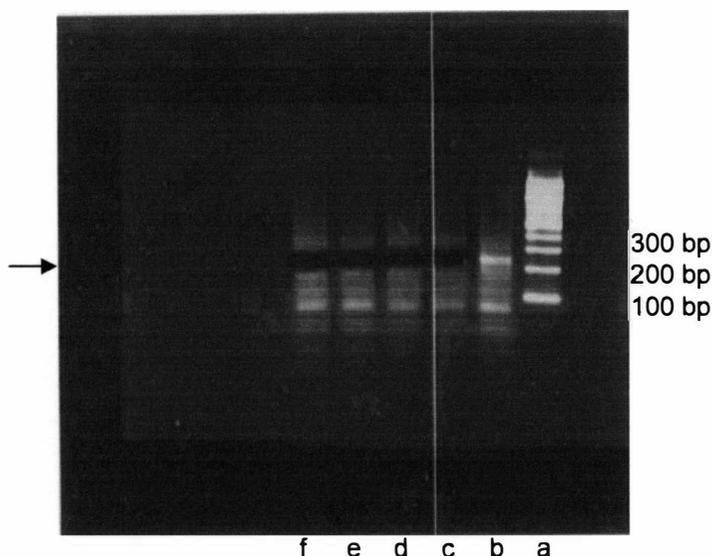


Fig. 13. Theromin Gene After Synthesis by PCR Method. Results show a 234 bp fragment (lane b) indicated by the arrow on a 2% agarose gel following electrophoresis. Theromin was extracted from the gel using a kit from Qiagen. Lane a is the 100 bp standard ladder DNA marker, lane b is the theromin lane, and lanes c, d, e and f show gaps left after theromin was cut out of the gel for purification.

Following restriction endonuclease digestion of both the plasmid (pET24d) and the *therom* gene (insert) using BamHI and NcoI, a ligation reaction was carried out resulting in a product that was used to transform chemically competent DH5 α (*Escherichia coli*) cells. A total of 17 colonies were observed. The transformation process included two controls, with the first one being a positive control in which the undigested pET24d was used to transform DH5 α cells and it resulted in 180 colonies. The second control was negative involving the use of double digested pET24d (BamHI and NcoI) to transform DH5 α cells. No bacteria colonies were observed on the negative control plate (Table 5). As expected, bacteria cells transformed with the undigested plasmid (pET24d) were able to resist the antibiotic while the double digested plasmid was unable to confer antibiotic resistance.

Table 5. Number of Bacterial Colonies Found on LB Plates Containing Kanamycin Sulfate after Incubation at 37°C for 12 Hours. The experimental plate had the chemically competent DH5 α cells transformed with the ligation product. Positive control plate had the same chemically competent cells transformed with undigested pET24d, while the negative control cells were transformed with double digested pET24d.

	Experimental plate	Positive control Plate	Negative control plate
No. of bacteria colonies	17	180	0

Two colonies were picked from the experimental plate and used to inoculate 10 mL of LB medium containing kanamycin sulfate (30 μ g/mL) each in a different tube. After growing the culture (see Materials and Methods), the recombinant plasmid was isolated as mentioned earlier. To confirm that the ligation process worked, BamHI (position 198) and BglII (position 515) restriction endonucleases were used to

digest the isolated recombinant plasmid and as expected, a 317 base pair insert and a 5166 base pair plasmid digest was observed (Figure 14) demonstrating that the clone did contain an insert of the correct size (209 base pairs) and orientation. The BamHI (position 198) and BglII (position 515) combination was used instead of BamHI (position 198) and NcoI (position 407) to get a bigger piece cut out to make it more visible in the gel for easy analysis. BglII is also one of the restriction sites in the pET24d multiple cloning site.

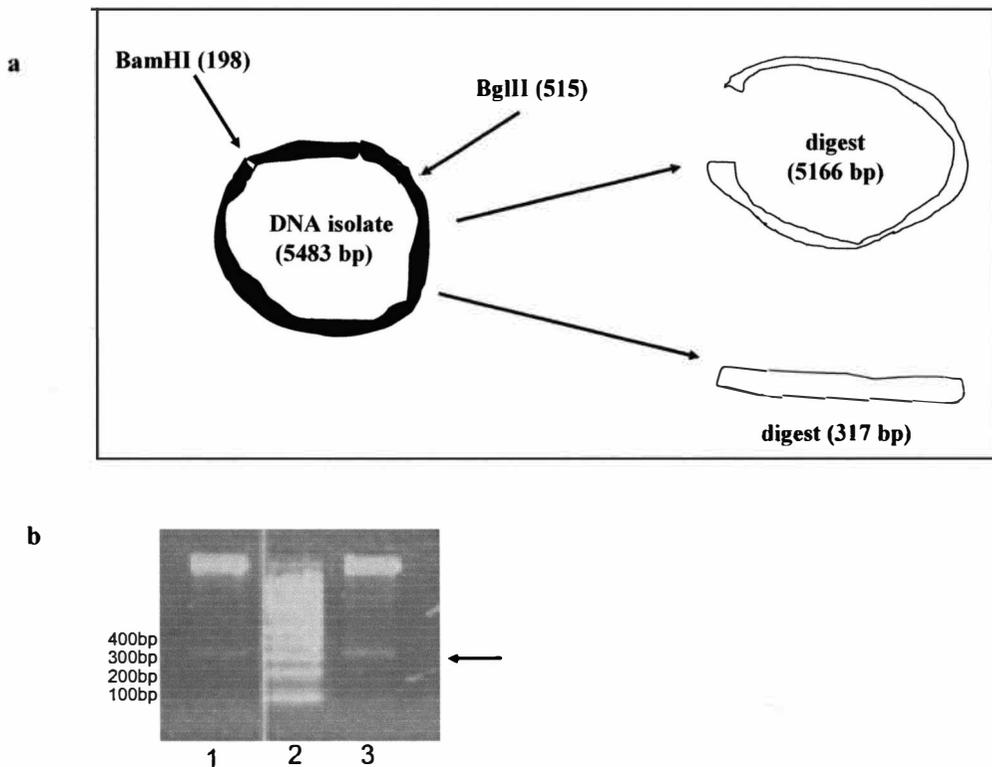


Fig. 14. Confirmation of Ligation. a) A schematic of the restriction endonuclease (BamHI and BglII) digestion of the recombinant plasmid. b) A 2% agarose gel picture showing the 317 base pair digest in both isolates (lane 1 and 3) and the 100 base pair ladder DNA marker (lane 2).

The recombinant plasmid was sequenced using the Chain Terminator sequencing method (dideoxy method) which revealed a deletion of one base pair (bp number 93) from the expected theromin gene. The mutation was corrected using a QuikChange[®] XL Site-Directed Mutagenesis Kit (Stratagene) following instructions from the manufacturer. The gene was re-sequenced after the correction to confirm the proper sequence and orientation.

Protein Expression and Purification

Following the transformation of chemically competent BL21(DE3) cells with the recombinant plasmid (see Materials and Methods), more than 200 bacteria colonies were observed after a 12 hour incubation period at 37°C. To carry out an induction test, a single colony was picked from the plate with the recombinant gene (experimental plate) and used to inoculate 50 mL of LB medium containing kanamycin sulfate (30 µg/mL). The bacteria culture was incubated at 37°C with shaking to an OD₆₀₀ of approximately 0.6. IPTG was used to induce protein expression as explained in Materials and Methods. Results obtained after running a 15% tricine SDS-PAGE show an increase in theromin expression 30 minutes after addition of IPTG (Figure 16). There was a steady increase in theromin expression for the first 120 minutes after which the amount of theromin remained constant. The amount of protein loaded in each well was normalized according to the formula (Figure 15) in which time X ranged from 0 minutes (uninduced) to 240 minutes at an

interval of 30 minutes. The largest volume loaded was 15 μL corresponding to time zero (uninduced sample).

$$\frac{\text{OD}_{600} \text{ at time zero}}{\text{OD}_{600} \text{ at time X}} \times 15 \mu\text{L}$$

Fig. 15. Loading Normalization Method. X is time in minutes ranging from 0 minutes to 240 minutes at an interval of 30 minutes. The largest volume to be loaded was 15 μL corresponding to time zero (uninduced sample).

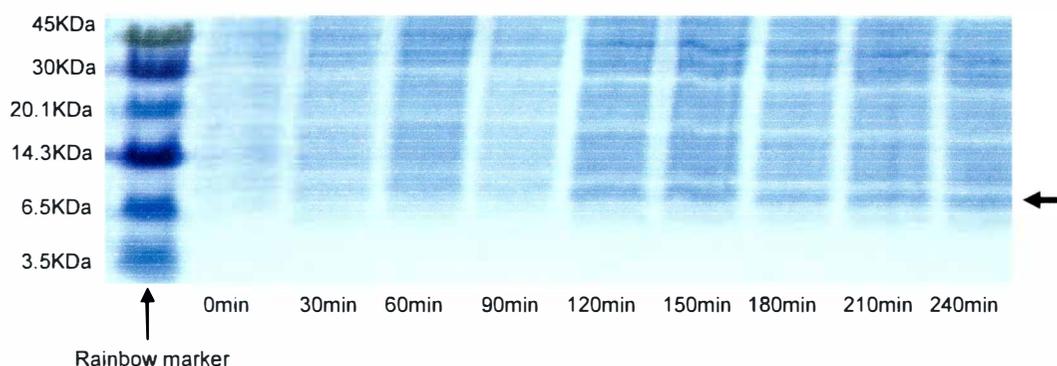


Fig. 16. A 15% Tricine SDS-PAGE Gel Showing the Protein Induction Test Results. The bold arrow to the right points to the theromin band which increases steadily in intensity from almost nothing at time zero (before addition of IPTG) to much higher concentrations after 4 hours.

The remaining 45.5 mL culture was pelleted by centrifugation followed by freeze-thaw protein extraction using the same method (see Materials and Methods). To ensure the protein remained in a reduced state, 5 mM dithiothreitol (DTT) was added to the buffer. The newly extracted protein was analyzed the same way as explained (Materials and Methods) using 15% tricine SDS-PAGE electrophoresis. A

protein band was visible between 6.5 kDa and 14.3 kDa standard markers, and it was around the expected mass of reduced theromin (approximately 7256) (Figure 17). Lanes b and c have less protein compared to lane d which represents TCA precipitation. This is because loading was not normalized and so different amounts of protein were loaded in each well. Purification was done to get rid of the other proteins and leave the pure theromin.

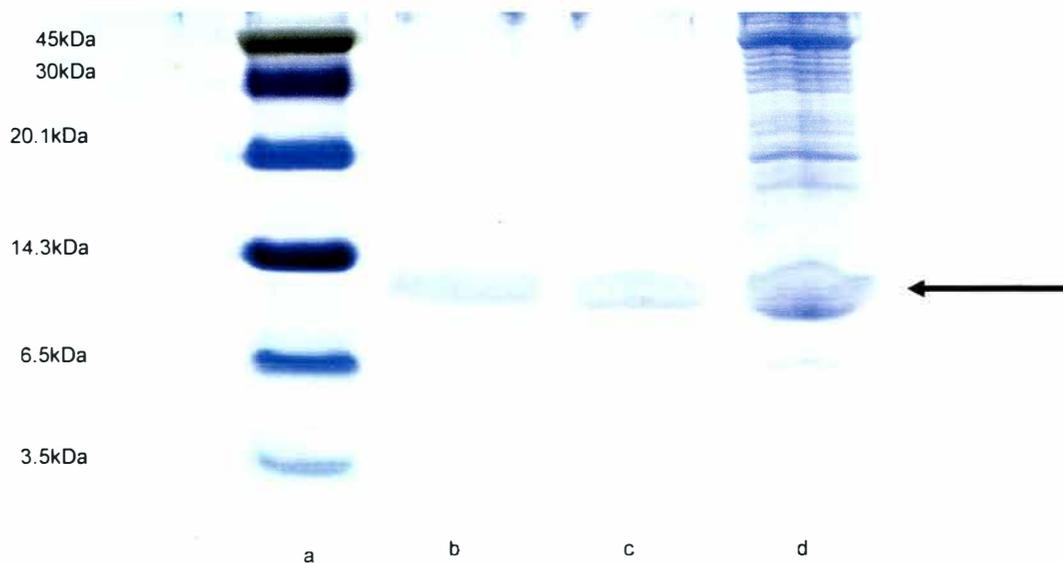


Fig. 17. Theromin Freeze-thaw Extraction Results Following a 15% Tricine SDS-PAGE Gel. The arrow points to the theromin band. Lane a shows the standard marker (Rainbow) while lanes b and c are freeze-thaw protein extracts. Lane d is the freeze-thaw extract following trichloroacetic acid precipitation.

The small scale extracted protein sample was concentrated 5.4 fold in preparation for high resolution size-exclusion chromatography (Superdex 75 HR 10/30) purification method. A total of 70 elution fractions were obtained, each with a volume of 0.5 mL. The fractions containing theromin did not absorb strongly at 254

nm wavelength and there were other stronger peaks (UV profile not shown). The theromin band was visible in fractions 19, 20, 22, and 24 (Figure 18), but a new protein band emerged between the standard markers of 14.3 kDa and 20.1 kDa. This band is visible in fractions 20 all the way to 27, with fraction 26 having the highest concentration. The same band does not exist in the freeze-thaw extract (Figure 17), meaning that theromin might have dimerized to form a homodimer of approximately 15 kDa despite the fact that 10 mM DTT was added in the elution buffer. This means that theromin was eluted both as a monomer and a homodimer. These results are in agreement with Salzet *et al.*, (2000) who reported the homodimeric form of theromin.

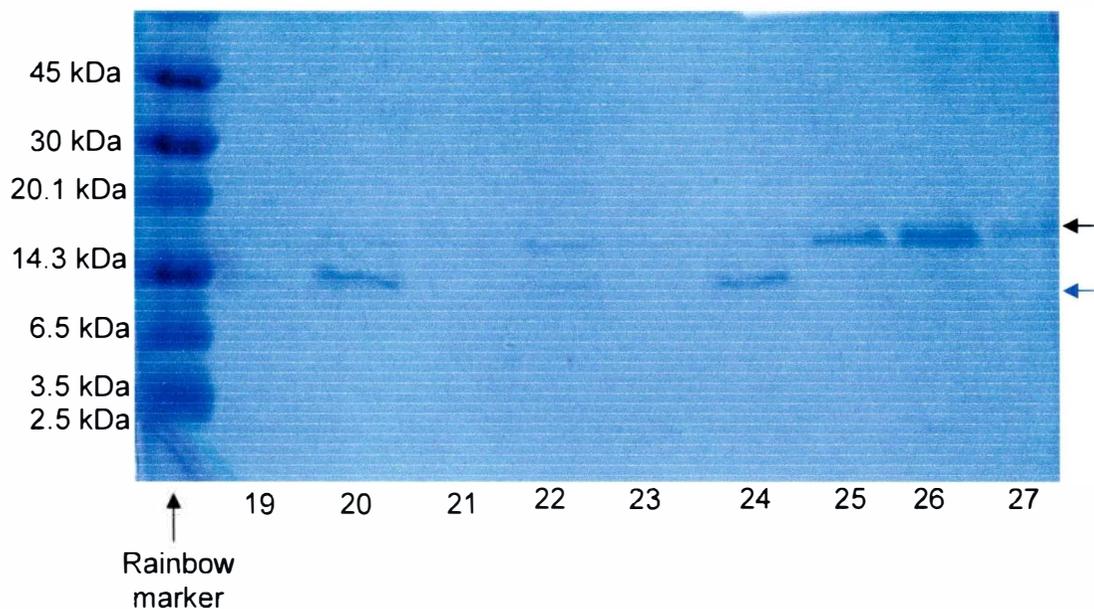


Fig. 18. High Resolution Size-exclusion Chromatography (Superdex 75 HR 10/30) Elution Fractions. Fractions 19, 20, 22, and 24 have theromin monomer (blue arrow) , but a new band is also visible in fractions 20 to 27 (black arrow). The new band is thought to correspond to the homodimeric form of theromin.

The protein concentration was determined in accordance with the Bio-Rad Protein Assay (Bio-Rad Laboratories) method explained in the Materials and Methods

whereby a standard curve (Figure 19) was constructed and used to determine the concentration of the crude freeze-thaw extract and each size exclusion chromatography fraction (Table 6).

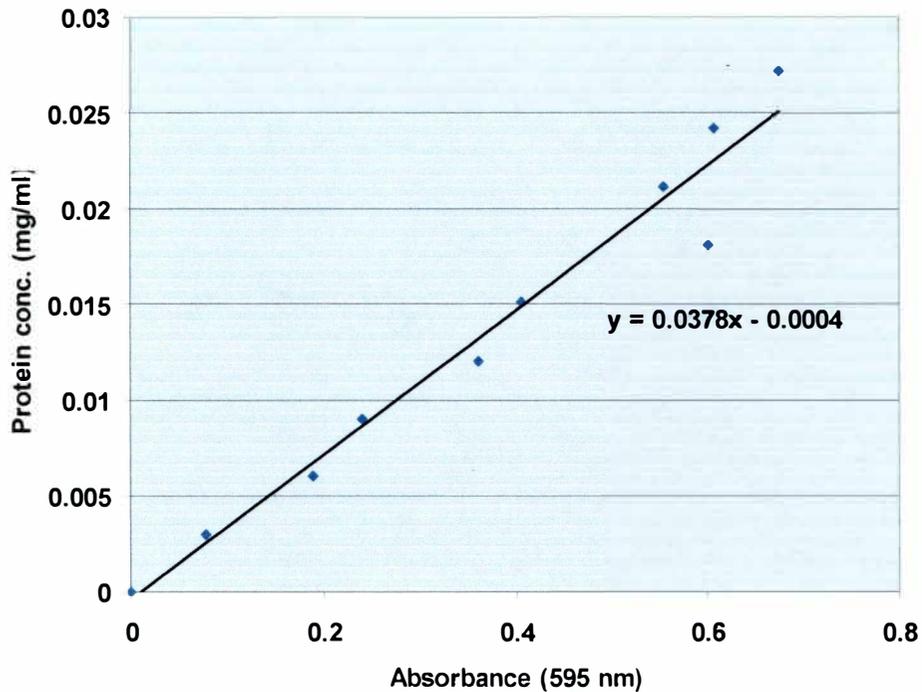


Fig. 19. The Bio-Rad Protein Assay Standard Curve. The protein concentration standard curve showing protein concentration (mg/mL) versus absorbance at 595 nm wavelength.

Fractions 20, 24 and 26 were submitted for MALDI-MS analysis, but the spectra was not well completed due to interference from salts that were said to be present in the samples, probably resulting from the size exclusion chromatography elution buffer (150 mM NaCl).

Table 6. Protein Concentration Based on the Bio-Rad Protein Assay Method. The freeze-thaw extract had the highest protein concentration.

Sample	Concentration
Crude freeze-thaw extract	2.100 mg/mL
Fraction 19	0.010 mg/mL
Fraction 20	0.080 mg/mL
Fraction 21	0.003 mg/mL
Fraction 22	0.010 mg/mL
Fraction 24	0.010 mg/mL
Fraction 23	0.030 mg/mL
Fraction 25	0.100 mg/mL
Fraction 26	0.050 mg/mL
Fraction 27	0.010 mg/mL

Thrombin Inhibition Assays

Thrombin inhibition assays were carried out as explained elsewhere (Materials and Methods) in order to confirm whether the size exclusion chromatography fractions had thrombin inhibition properties. Fraction 24 was used in this assay and results show a concentration dependent increase in thrombin inhibition as more inhibitor (fraction 24) is added. This is based on the results (Figure 20) that show a decrease in the change in absorbance per minute as more inhibitor is added. The assay was done at 405 nm wavelength and 343 nM of thrombin was used in the assay. Fraction 24 was chosen because it had high concentrations of the monomeric form of thrombin and less of the homodimer. It would have been good to use a pure monomer in order to draw a comparison with the results from Salzet *et al.*, (2000) in which they reported the homodimer to have higher inhibition than the monomer.

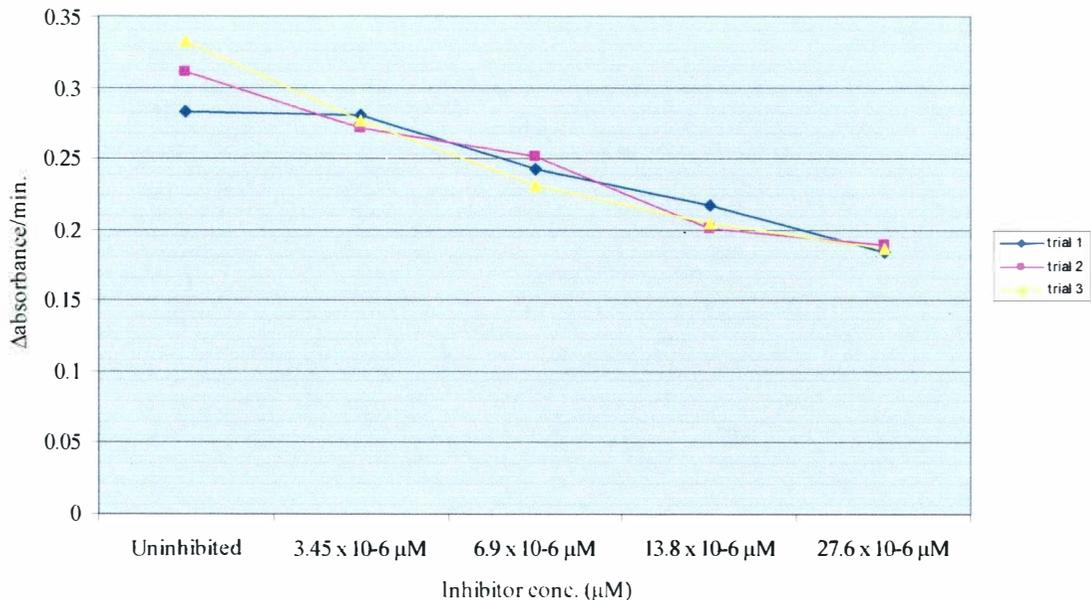


Fig. 20. Thrombin Inhibition Assay. The assay was done at 405 nm wavelength and 343 nM of thrombin was used. Increasing concentrations of the inhibitor (fraction 24) were added and corresponding values of change in absorbance per minute were recorded.

Based upon the induction test results obtained from the small scale level, a 10 L protein expression batch was prepared by inoculating 250 mL of kanamycin (30 μg/mL) containing LB medium with a single colony containing the recombinant gene. The culture was incubated at 37°C with shaking to attain an OD₆₀₀ reading of approximately 0.6. The same expression method (Materials and Methods) was applied followed by protein extraction. The freeze-thaw protein extraction method used in extracting the protein is effective, but limited to proteins less than 30 kDa. The extraction buffer contained 1 mM EDTA to chelate any metal ions that might be present. 5 mM Dithiothreitol (DTT) was added to the same buffer to ensure the

protein remained in its reduced form with expected theoretical mass of approximately 7256. The extracted protein was analyzed based upon the same method used at an earlier smaller scale study. The expression of theromin at a larger scale was not as pronounced as it had been at a smaller scale (results not shown).

In an effort to purify the extracted protein, the first purification step was the DEAE-Sepharose (anion exchange chromatography) on a Fast Performance Liquid Chromatography (FPLC) system (Amersham Pharmacia Biotech). Following equilibration of the column as explained elsewhere (see Materials and Methods), the filtered protein extract was loaded and elution done. A total of 80 fractions were obtained with each having a total volume of 3 mL. The UV absorbance profile was done at 254 nm wavelength because theromin was not expected to absorb strongly at 280 nm (profile not shown) Analysis of the DEAE-sepharose elution fractions was done on a 15% tricine SDS-PAGE gel.

Fractions 39, 40, 41 and 42 had the theromin band with fraction 40 having the highest concentration, but the purification was not very effective given the number of other bands present in these fractions as can be seen from the SDS-PAGE gel results (Figure 21). This can be attributed to the fact that many prokaryotic proteins are acidic just like theromin which has a theoretical isoelectric point (pI) of 4.17. This pI value falls within the pI range of many prokaryotic proteins hence having almost similar anionic binding properties. The four fractions presumed to contain theromin were pulled together and concentrated by a factor of 65 using the Stirred Ultra-filtration Cells (Amicon) as explained elsewhere (Materials and Methods).

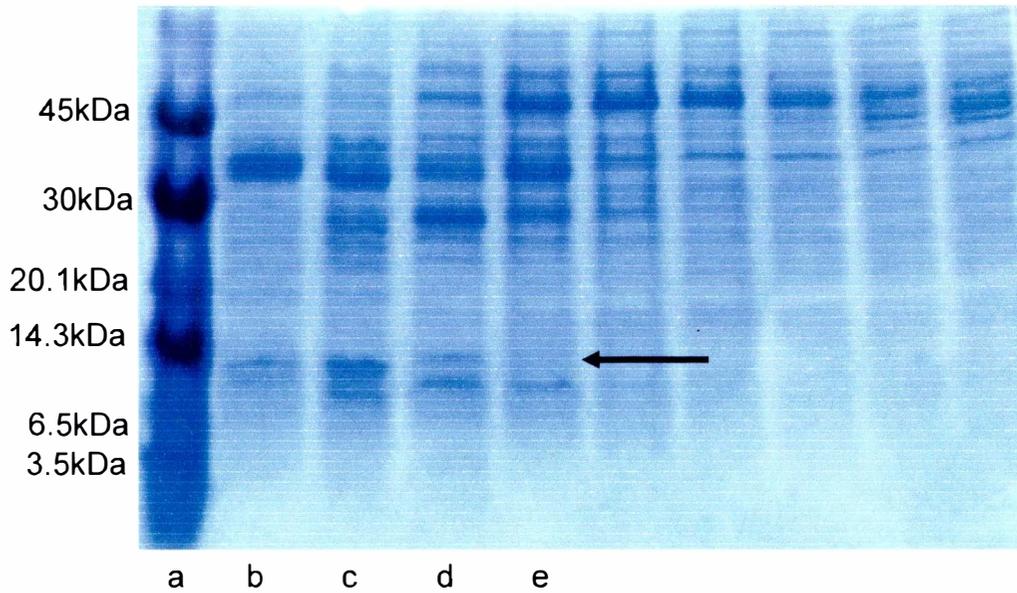


Fig. 21. DEAE Sepharose (Anion Exchange Chromatography). 15% tricine SDS-PAGE gel. The arrow points to the theromin band while the standard marker is in lane a and elution fractions containing theromin in lanes b, c, d, and e corresponding to elution fractions 39, 40, 41, and 42 respectively. The elution was done at 254 nm wavelength (UV profile not shown)

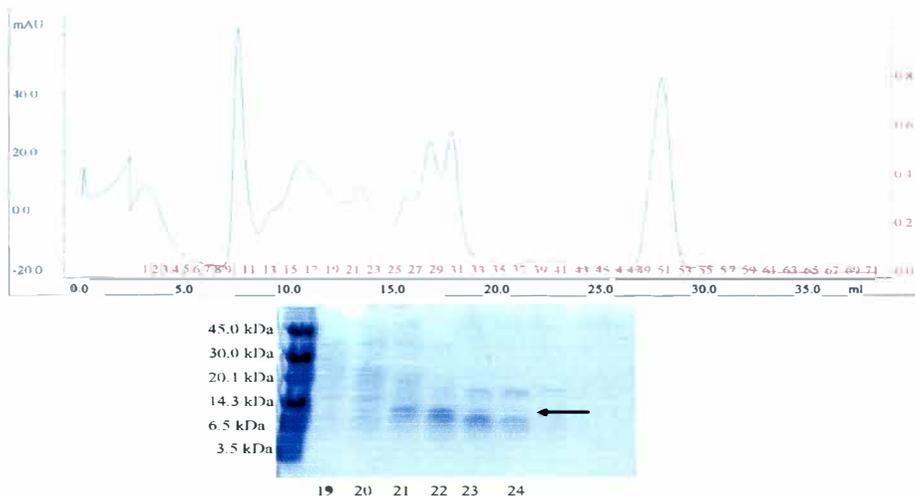


Fig. 22. Superdex 75 (Size Exclusion Chromatography). Top: the elution UV profile at 254 nm wavelength. Below: The 15% SDS-PAGE gel with the arrow pointing at the theromin band. Fractions 20, 21, 22, and 23 show theromin band. Another band appears in fractions 22, 23 and 24 very close to theromin but is slightly smaller than theromin

The concentrated protein was further purified on a high resolution size-exclusion chromatography column (Superdex 75 HR 10/30) (Amersham Pharmacia Biotech). The protein was not completely purified even after the high resolution size-exclusion chromatography (Figure 22). There seemed to be other proteins migrating with theromin, hence the difficulty in purification. Theromin did not absorb strongly at 254 nm wavelength according to the elution UV profile in the same figure. The elution buffer had 10 mM DTT to maintain theromin in its reduced state. Protein concentration was determined at every purification step using the Bio-Rad Protein Assay (Bio-Rad Laboratories) method (see Materials and Methods). Protein recovery was not high as evident from the results in Table 7. It was necessary to improve on the methods used or try alternative methods.

Table 7. Protein Concentration at Every Purification Step. Freeze-thaw extract has the highest concentration followed by the DEAE elution fractions pulled together. Fractions 21, 22, and 23 are from the size exclusion chromatography.

Purification step	Protein concentration	Total Protein
Freeze-thaw extract	2.29688 mg/mL	551.2512 mg
DEAE anion exchange	1.92938 mg/mL	23.1526 mg
Fraction 21	0.182608 mg/mL	0.0913 mg
Fraction 22	0.273328 mg/mL	0.1367 mg
Fraction 23	0.273328 mg/mL	0.1367 mg

Following the determination of protein concentration in various fractions, a thrombin inhibition assay was carried out using the fractions from size exclusion chromatography that were thought to contain thrombin (fractions 21, 22 and 23). A mixture of all DEAE anion exchange elution fractions thought to contain thrombin (a mixture of fractions 39, 40, 41, and 42) were also studied. The freeze-thaw extract was also tested in the same study. The results (Figure 23) show that the three fractions from size exclusion chromatography (F21, F22 and F23) inhibit thrombin activity. This is evident given that change in absorbance per minute is highest in absence of inhibitor. When the inhibitor is added, the rate of change in absorbance decreases, meaning that thrombin activity is inhibited. The freeze-thaw extract and the mixture of DEAE elution fractions (E39-E52) also show thrombin inhibition properties. The thrombin inhibition activities would have been expected to increase with increase in inhibitor concentration, but this is evident only in fractions F22 and F23 from size exclusion chromatography (Figure 23). The rest of the fractions don't show a consistent decrease in the rate of change in absorbance as inhibitor concentration is increased. There is a high probability that these assay results are affected by the purity of each fraction used. The fractions are not pure enough (see Figure 22) hence there could be interference from other proteins present. Another possible reason is that too much inhibitor was added at the beginning, and therefore any further addition of inhibitor does not result in significant change in inhibition.

Some fractions from the size exclusion chromatography believed to contain thrombin were submitted for the Matrix Assisted Laser Desorption Ionization Mass

Spectrometry (MALDI-MS) analysis. From the analysis of fractions 19, 22, 24 and 26 from the size exclusion column, a peak at 7236 was found in fractions 22 and 24, with the highest concentration being found in fraction 22 as seen from the strength of the peak (Figure 24). Although fractions 21 and 23 were not submitted for the same analysis, they seem to contain thrombin as can be seen in the SDS-PAGE gel picture of Figure 22. Although the mass of the band on the gel is near thrombin's theoretical mass of 7256 Da, there is a peptide with a mass slightly smaller than, but close to the mass of thrombin in both fractions.

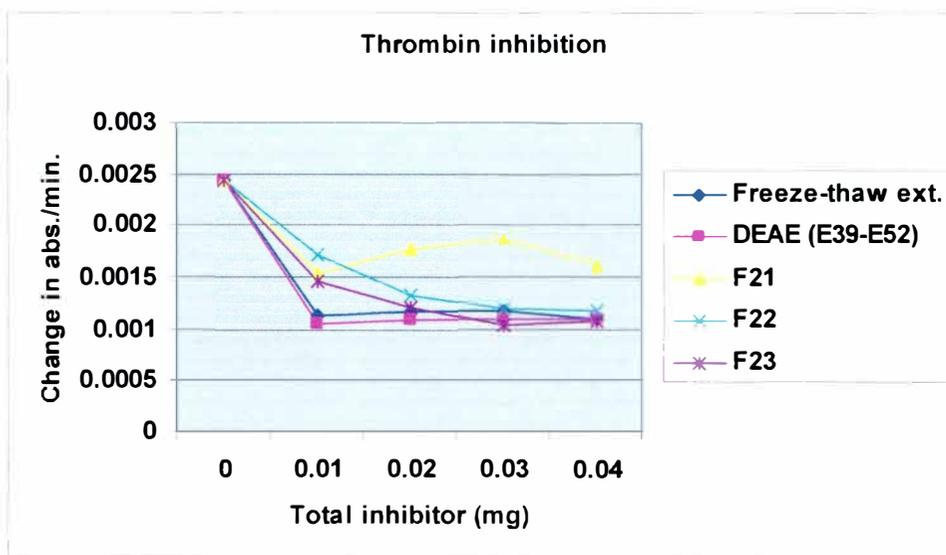


Fig. 23. Thrombin Inhibition Assay. The absorbance readings were taken at 405 nm wavelength and 343 nM of thrombin was used in a reaction volume of 1 mL. 0.1636 mM of the chromogenic substrate was used in the reaction. Increasing quantities of each fraction were added and change in absorbance per minute recorded. F21, F22 and F23 are elution fractions from the size exclusion chromatography.

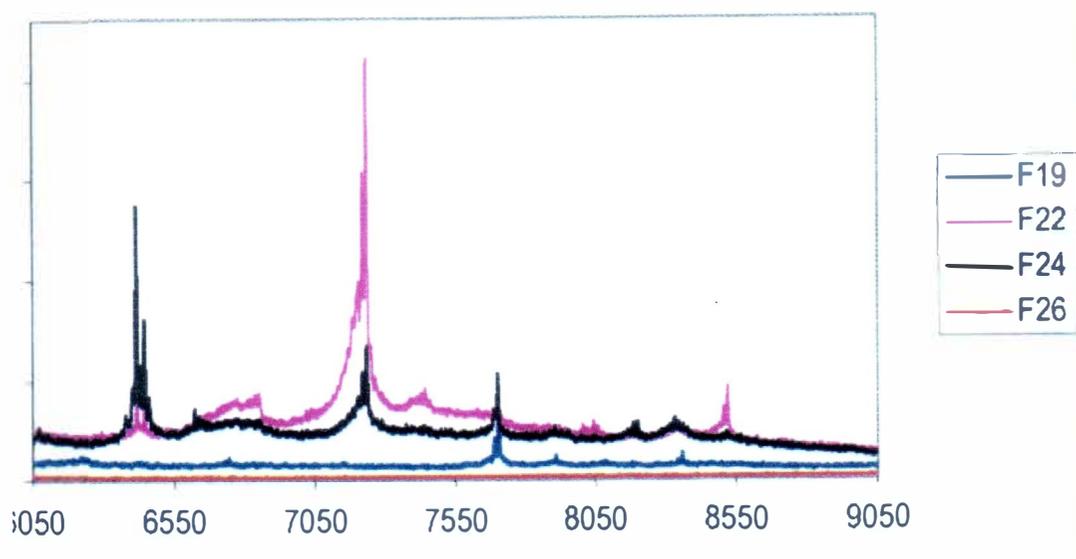


Fig. 24. The MALDI-MS Analysis Results of Fractions 19, 22, 24 and 26. Theromin was found in fractions 22 and 24 (7236 Da).

It was later discovered that the MALDI-MS instrument was not properly calibrated, so new samples were re-submitted for another analysis following proper calibration. The new Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) results showed a mass of 7259.74 Da (Figure 25), a value that is in close agreement with the expected mass of 7256 Da. The accuracy of MALDI-MS is 0.1% which is ± 7 Da. However, there were many other peaks in the spectrum that ionized much better than the peak of 7259.74 (Figure 25).

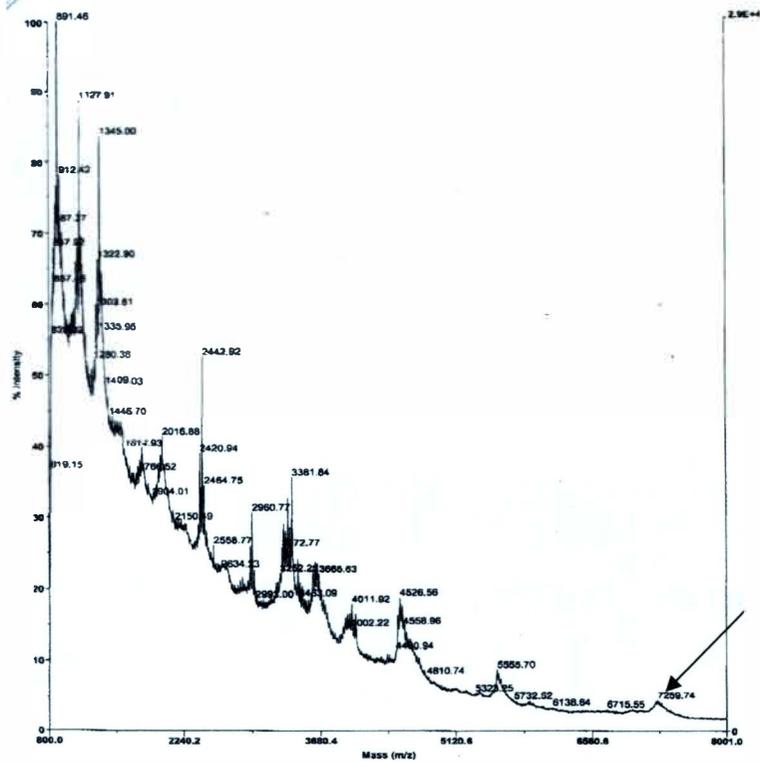


Fig. 25. MALDI-TOF MS Results of a Purification Fraction Containing Theromin. The arrow points to a peak corresponding to 7259.74 Da, a value that is in close agreement with the expected mass of 7256 Da. The accuracy of MALDI is 0.1% which is ± 7 Da.

Glutathione-S-Transferase Tag (GST-TAG)

In order to improve the protein yield, the synthesized gene for theromin was digested using BamHI and NcoI restriction endonucleases. A similar digestion was done on the pET-42a plasmid in preparation for the ligation process that followed. The Glutathione-S-Transferase Tag (GST-TAG) gene is part of the pET-42a plasmid and it stretches from base pair number 433 to 1092. Tagging theromin results in a bigger and more soluble protein. The digested theromin and pET-42a plasmid were analyzed by running a 2% and 1% agarose gel electrophoresis respectively. Both

digested products were excised from the gel and purified using a gel extraction kit from Qiagen before doing ligation.

Transformation of the ligation product into DH5 α competent cells resulted in 6 colonies that were each used to inoculate 10 mL of LB medium. Recombinant plasmid was isolated from the bacteria culture. The 6 recombinant plasmid isolates were digested using BamHI and NcoI restriction endonucleases. Results from the analysis of the digested recombinant isolates show presence of theromin gene (insert) in isolate number 6 only (Figure 26a). A 209 DNA base pair cut out was visible in isolate number 6, although it was very faint. A 100 base pair DNA ladder marker was used in the analysis, but the bands are not well defined because the level of brightness had to be increased in order to see the faint theromin cut out.

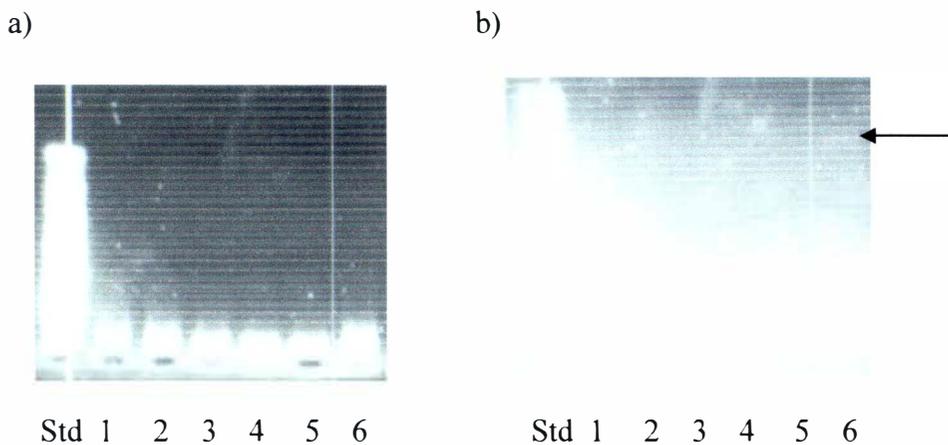


Fig. 26. Confirmation of Ligation. Both a) and b) are pictures of the same 2% agarose gel showing the 100 base pair DNA standard marker (Std) and the BamHI and NcoI restriction endonuclease digestion of the six recombinant plasmid isolates (1 to 6). The arrow in b) points to a very faint 209 DNA base pair digestion product in lane 6.

The isolated recombinant plasmid that had theromin (lane 6, figure 26) was submitted for sequencing. After confirming the proper sequence, a site directed

mutagenesis kit was used (see Materials and Methods) to delete 9 DNA base pairs occurring between factor Xa cleavage site and theromin insert. This was transformed into BL21(DE3) competent cells and then followed by an induction test (see Materials and Methods) in which a protein was expressed between the 30.0 and the 45.0 kDa markers (Figure 27). The protein ran at a mass close to the GST alone. This could be due to the fact that theromin is acidic, hence it contributes to the faster movement of the GST tag-theromin fusion protein.

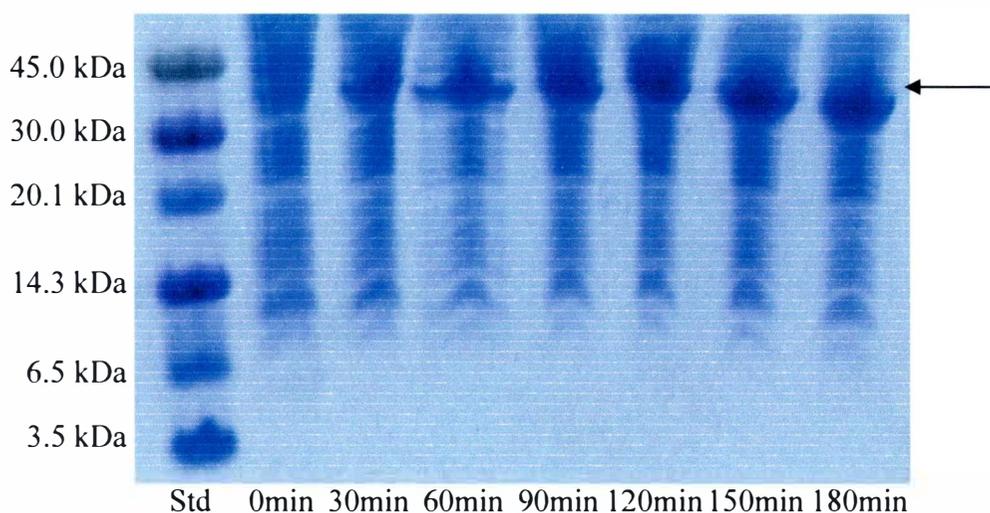


Fig. 27. 15% Tricine SDS-PAGE Gel Showing the Protein Induction Test Results for a GST Tag-theromin Protein. The arrow points to the GST tag-theromin protein band which increases steadily in intensity from almost nothing at time zero minutes (before addition of IPTG) to much higher concentrations after 3 hours.

A control study was done in which the plasmid (pET-42a) without insert was transformed into BL21 competent cells. A similar induction study was done using these cells transformed with pET-42a alone (Figure 28) to try and compare the GST tag protein alone (Figure 28) to the GST-tag-theromin protein (Figure 27). Both results (Figure 27 and 28) show expression of a protein between the 30.0 kDa and

45.0 kDa standard markers. The GST tag alone ran at the expected mass of ~ 35.6. Addition of theromin to the tag was expected to give a bigger protein of ~ 42.8 kDa, but the results in figure 27 above show a mass similar to that of the mass of GST tag alone.

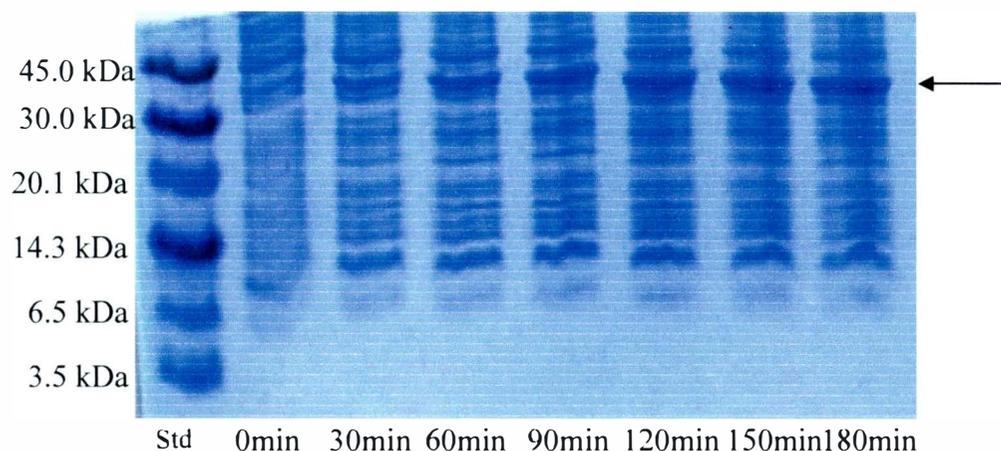


Fig. 28. 15% Tricine SDS-PAGE Gel Showing the Protein Induction Test Results for a GST Tag Protein. The arrow points to the GST tag protein band which increases steadily in intensity from almost nothing at time zero minutes (before addition of IPTG) to much higher concentrations after 3 hours.

The fusion peptide was purified by affinity chromatography and cleaved by Factor Xa (see Materials and Methods) after which it was purified by the size exclusion chromatography (HiLoad Superdex 75) method. Analysis was done using an SDS-PAGE gel and results show both the monomeric and dimeric form of theromin (Figure 29). Both forms of theromin were evident even though 10 mM DTT was added in the elution buffer. These results are consistent with the findings reported by Salzet et al., (2000), that theromin is a homodimer. We also don't have enough evidence to show that dimerization is only due to disulfide bond formation. The

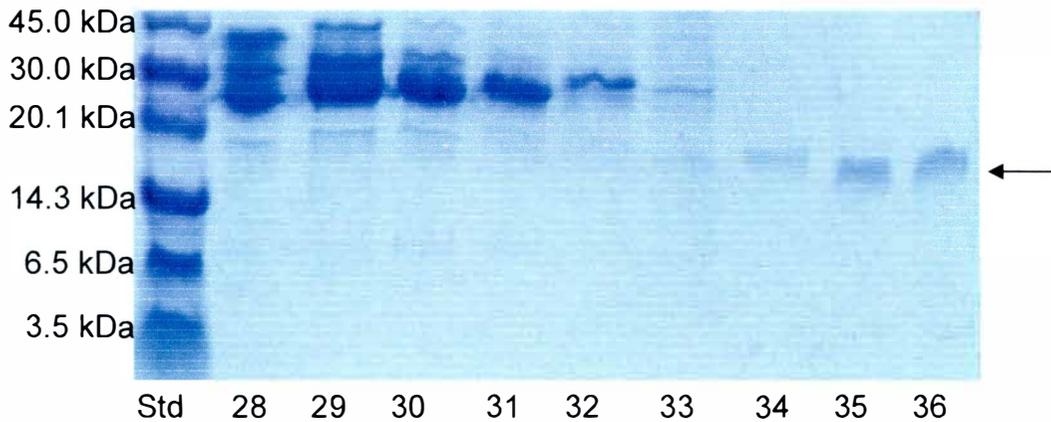
elution profile (Figure 30) shows peaks for both the dimeric (fractions 34, 35 and 36) and monomeric forms of theromin (fractions 40, 41 and 42). The corresponding bands are visible in figures 29a and 29b, with the monomeric form (Figure 29b) showing very faint bands. These bands are faint because the amount of protein was not normalized prior to loading. Another possible explanation is that the monomer is smaller than the dimer and therefore it will have a lower resolution.

Theromin Inhibition Assay

Following the size exclusion chromatography results above, the fractions containing the dimeric form of theromin (fractions 34, 35 and 36) were pulled together, mixed thoroughly and then used in the thrombin inhibition assays as described herein (see Materials and Methods). A similar assay was done using a mixture of fractions containing the monomeric form of theromin (fractions 40, 41 and 42). This enabled a comparison to be drawn between the monomeric and dimeric form of theromin. In order to properly assess the inhibition results, hirudin, a well studied thrombin inhibitor was also used in the thrombin inhibition assays to enable a comparison (Figure 31). Each reading is a result of calculated mean from three trials for which standard deviation and corresponding standard error were determined. The results from thrombin inhibition assay show both the monomeric and dimeric theromin to be stronger inhibitors of thrombin than hirudin. There was no major thrombin inhibition difference between the monomeric and dimeric forms of theromin. This differs from the results published by Salzet et al., (2000) showing that

the dimeric form of theromin inhibits more than the monomeric form. According to their findings, each theromin monomer acts in synergy to block the thrombin, a finding that is not consistent with these results because both curves representing the two forms of theromin in Figure 30 are almost super-imposed on each other.

a)



b)

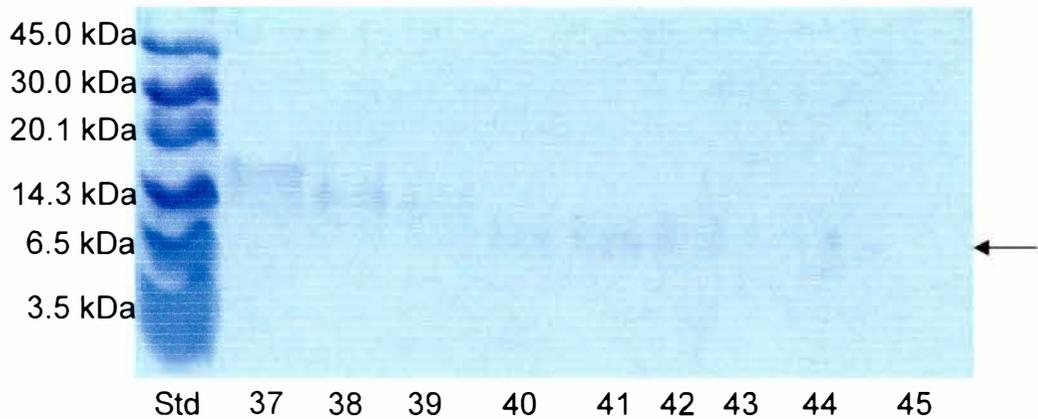


Fig. 29. 15% Tricine SDS-PAGE Gels Showing Size Exclusion Chromatography Elution Fractions: a) Fractions 28 to 36 with the protein standard marker in the first lane. The arrow points to the theromin dimers present in fractions 34, 35 and 36. b) Fractions 37 to 45 with the protein standard marker in the first lane. The arrow points to the theromin monomer present in fractions 40, 41 and 42.

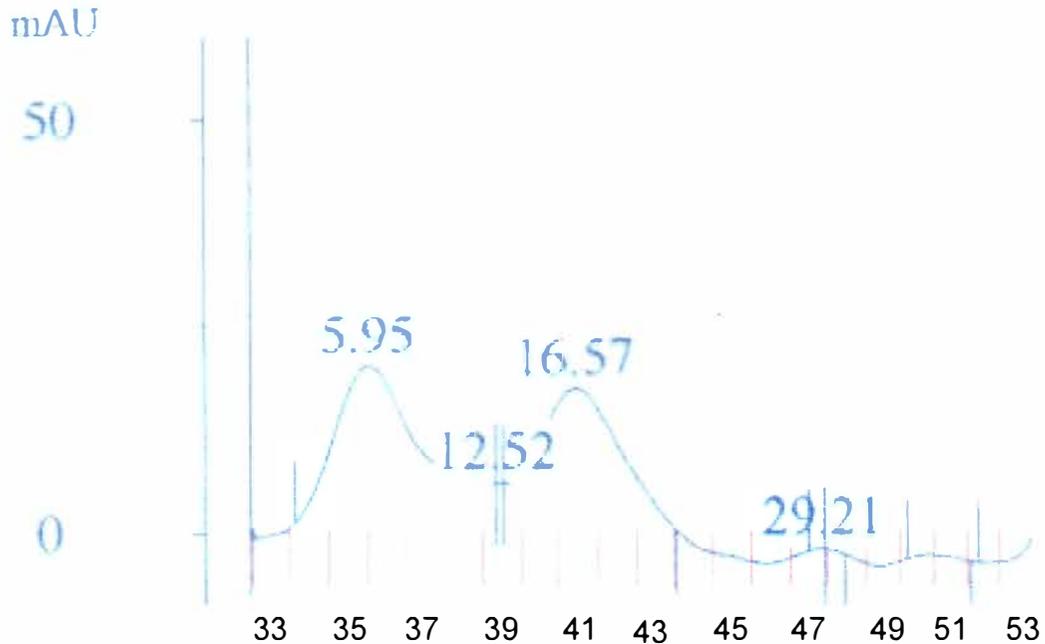


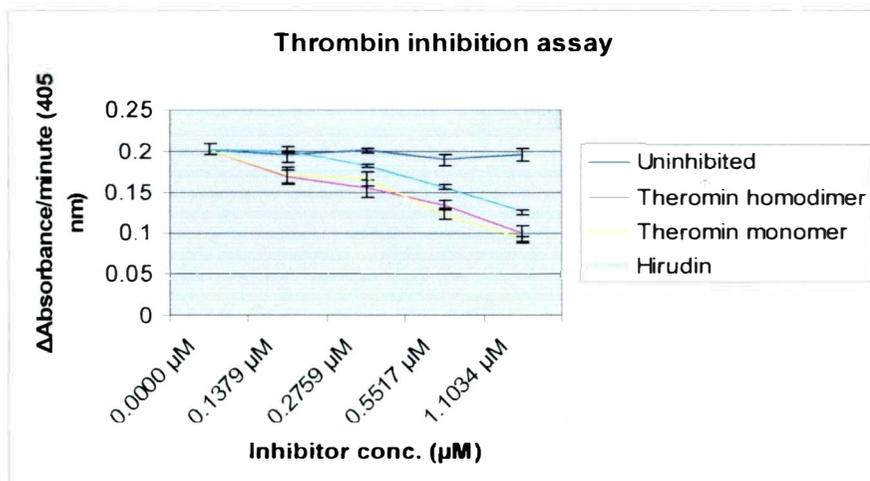
Fig. 30. UV (254 nm) Elution Profile for Size Exclusion Chromatography Showing Fractions 33 to 53. The first peak (fractions 34, 35 and 36) corresponds to the dimeric form of theromin, while the second peak (fractions 40, 41 and 42) corresponds to the monomeric form of theromin.

Determination of Theromin's Inhibitory Constant (K_i)

The purpose of this set of experiments was to carry out an assay that resulted in a set of values that were used to determine the K_i value of the purified recombinant theromin. A thrombin inhibition assay was done in which the change in absorbance/minute was read at 405 nm wavelength versus the substrate concentration, [S] in the presence of different concentrations of the dimeric form of theromin (Figure 32). The change in absorbance/minute values recorded were converted to change in concentration/minute in order to have both the V_o and [S] in the same concentration

units. The conversion was done using Beer's Law ($A = \epsilon bc$) in which each value was

a)



b)

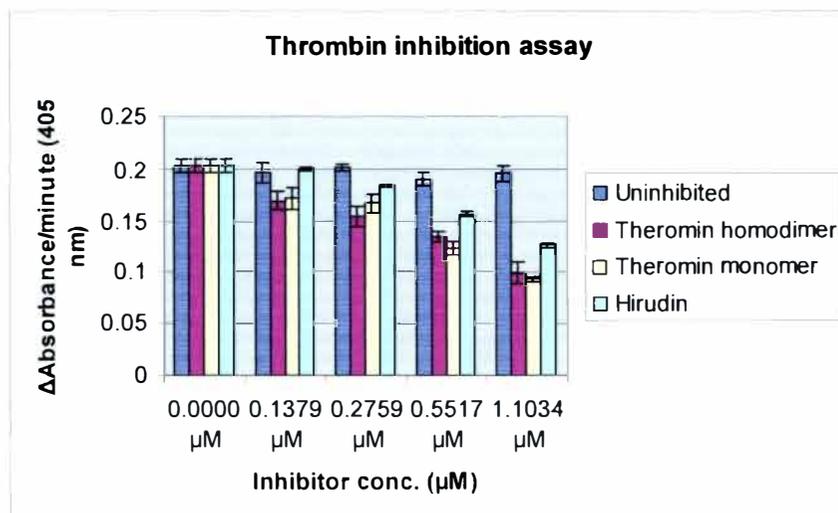


Fig. 31. Thrombin Inhibition Assay Results. The absorbance readings were taken at 405 nm wavelength and 343 nM of thrombin was used. 0.1636 mM of the chromogenic substrate was used in a reaction volume of 1 mL. a) Both the monomeric and dimeric forms of theromin inhibit thrombin activities more than hirudin, but do not show major thrombin inhibition differences between them. The uninhibited curve does not show a decrease in change in absorbance/minute. b) A presentation of the same results in bar graph form.

divided by the extinction coefficient (ϵ) value of chromozyme TH at 405 nm. The chromogenic substrate has $\epsilon_{405\text{nm}}$ of $10.4 \text{ [mmol}^{-1} \times \text{l} \times \text{cm}^{-1}]$. The assay results (Figure 32) show that the uninhibited reaction is faster, but the reaction speed drops as $0.138 \text{ }\mu\text{M}$ of the inhibitor is added. The K_M value drops further as the inhibitor concentration is doubled ($0.276 \text{ }\mu\text{M}$). The inhibitor makes the K_M appear larger than it really is.

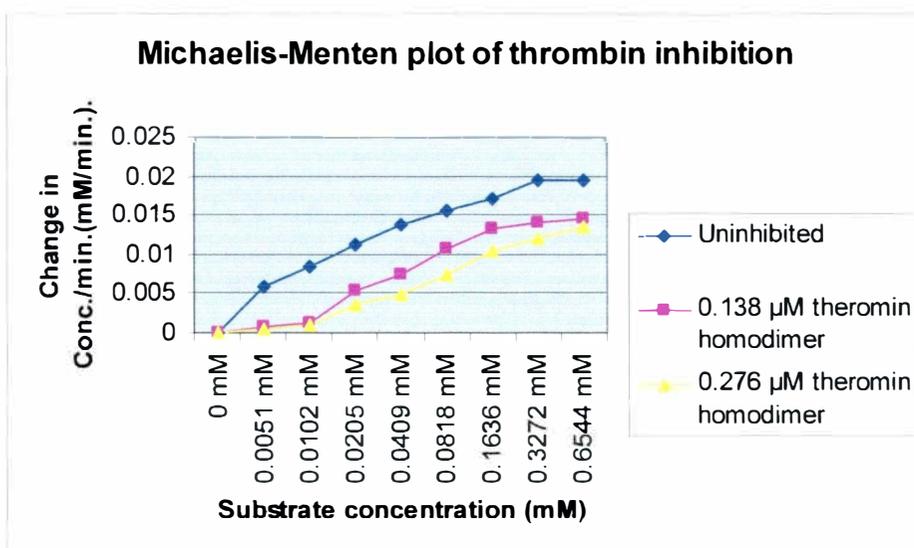


Fig. 32. A Plot of the Initial Velocity V_o (Change in conc./minute) Versus the Substrate Concentration $[S]$ in the Presence of Different Concentrations of the Inhibitor Thrombin. The change in concentration/minute increases with increase in substrate concentration.

A Lineweaver-Burk plot ($1/V$ versus $1/[S]$) of the above results was constructed and used to calculate the K_i of the dimeric thrombin. The gradients of the curves (Figure 33) become steeper with increasing inhibitor (thrombin) concentration with the $0.276 \text{ }\mu\text{M}$ thrombin homodimer having the steepest gradient. After extrapolating the three curves, their respective equations (given below) were used

together with the other relevant enzyme kinetic equations (see Materials and Methods) to calculate the inhibitory constant (K_i). The calculations resulted in a K_i of 355 pM. The resulting K_i value is higher than the reported value of 12 fM for the theromin dimer isolated from the leech (Table 8) (Salzet et al., 2000). The difference in K_i values could be attributed to the fact that these results are from preliminary kinetic studies, and therefore there is need for a more advanced study that can give a more accurate K_i value. It is possible to use less substrate than the lowest amount used in this assay (0.0051 mM). The ability to record very low changes in absorbance was limited by availability of equipment. The changes caused by variations in small amounts of substrate lead to a significant effect on the slope of the curve which in turn affects the calculations. The following equations resulted from the curves in figure 33:

Uninhibited	$y = 0.6104x + 54.939$
0.138 μ M theromin homodimer	$y = 7.4974x + 2.448$
0.276 μ M theromin homodimer	$y = 8.635x + 47.227$

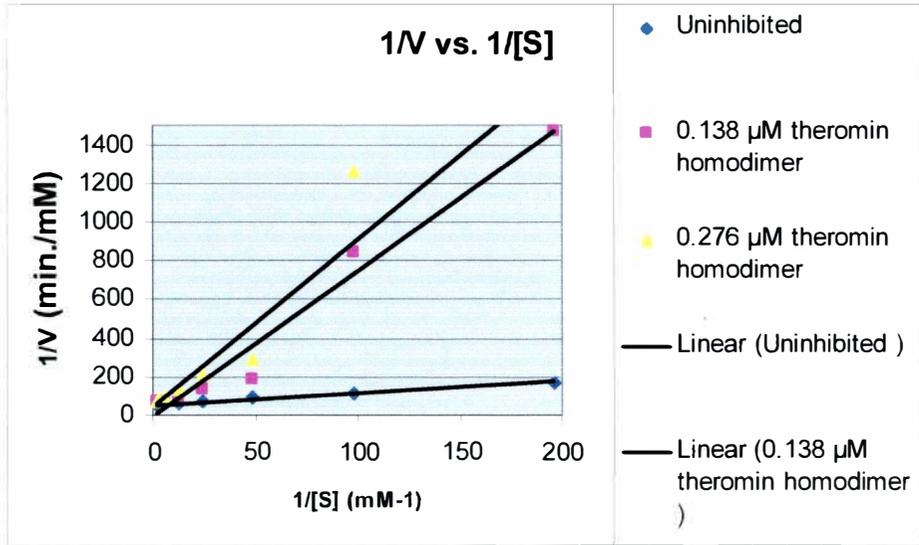


Fig. 33. A Lineweaver-Burk Plot of the Inhibited Thrombin Resulting from the Results in Figure 32. The three curves were extrapolated and their equations used to calculate the K_i of the dimeric form of theromin.

Table 8. Comparison of Inhibitory Constants (K_i) from Different Thrombin Inhibitors

Enzyme	Recombinant theromin (homodimer)	Theromin	Hirudin	Hemadin
Thrombin	355 pM	12 fM	21 fM	100 fM

CHAPTER IV

CONCLUSION

The aim of this study was to synthesize and characterize a recombinant thrombin inhibitor. The theromin (thrombin inhibitor) gene was successfully synthesized by a process that also involved incorporating the most highly expressed *E. coli* codons based on the *E. coli* high-frequency codon database. The synthesized gene was then integrated into an expression vector to produce the recombinant theromin gene. The recombinant gene was transformed into *E. coli* for protein over-expression after which the protein was extracted and purified. Purification methodologies were developed to purify recombinant theromin. For the native-like, non-fusion peptide, purification steps involved protein extraction by freeze/thaw, DEAE-Sepharose anion exchange, and gel filtration. SDS-PAGE, high resolution gel filtration, mass spectrometry, and thrombin activity assays verified the presence of recombinant theromin. In order to increase yield of recombinant theromin, the synthetic gene was fused to that of GST using pET42a (Novagen). SDS-PAGE revealed high expression level of GST fusion peptide and the fusion peptide was purified by affinity chromatography and cleaved by Factor X_a. Separation of cleaved GST away from the recombinant theromin was performed by gel filtration chromatography. The identity of the purified recombinant theromin was verified by SDS-PAGE and thrombin activity assays. Comparison of the recombinant theromin with the inhibitor hirudin, reveals that the former is a stronger inhibitor than hirudin,

consistent with the results of Salzet et al. Thrombin inhibition assays were carried out in the presence of different inhibitor concentrations in order to determine the kinetic parameters of this inhibitor. The Lineweaver-Burk plot of the thrombin inhibition assay results from the use of 0.138 μM and 0.276 μM theromin were used to determine the K_i .

From this study, results show that the recombinant theromin inhibits thrombin activities and the K_i was found to be 355 pM. The resulting K_i value from this study turned out to be different from the 12 nM reported for the isolated theromin from the leech (Salzet et al., 2000). The disparity in K_i values can be attributed to the fact that these were preliminary kinetic studies of the recombinant theromin and there is need for more advanced studies to be carried out. It is necessary for further assays to be carried out on the recombinant theromin using equipment that will permit the use of very low amounts of substrate. This is likely to have an effect on the gradient of the respective curves and hence the K_i .

In the previous study (Salzet et al., 2000) reported that theromin is a strict tight binding thrombin inhibitor. They also reported that the reduced and S- β -pyridylethylated theromin had little thrombin activity, although they did not mention how much activity. They suggested that dimerization is necessary to give the protein an active folded configuration for complete binding to thrombin. They concluded that each monomer acts in synergy to block thrombin. In this study, both the dimer and the monomer seemed to behave almost the same way in terms of thrombin inhibition assays (Figure 31). There is also a possibility that some fraction of the monomer

might have been oxidized to form the dimer despite the fact that DTT (a reducing agent) was present during the assay. This was evident during HPLC analysis in which two peaks appeared, one for the dimer and another one for the monomer resulting from analysis of the monomer. Theromin monomer seems to have a high tendency to oxidize into its dimeric form. This is the main reason why the dimer and not the monomer was used to determine the K_i .

Future studies should be carried out to determine the K_i of the monomer so that a proper thrombin activity comparison can be drawn between the monomeric and the dimeric forms of theromin. This will involve the use of a method mentioned by Salzet et al., 2000, in which theromin is reduced and S- β -pyridylethylated, but measures should be taken to account for any arising differences in activity of the reduced theromin in presence and absence of S- β -pyridylethylation. These two states of the reduced theromin are likely to give different thrombin inhibition results. Such studies need to be carried out in an oxygen free chamber.

Theromin is a potential anticoagulant and more studies need to be done in order to characterize it further. Theromin is composed of 67 amino acid residues, out of which 16 are cysteines, and our laboratory noted this peptide has homology to the metallothionein family of peptides. It is possible that metal ions are essential to the function of theromin. This calls for need to carry out studies that will establish if theromin binds metal ions. Such studies are possible with results from this study now that large amounts of theromin can be recombinantly synthesized. Such studies might open a new area of anticoagulant research.

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