DESIGN, SYNTHESIS AND EXPRESSION

IN DIFFERENT HOSTS

OF A GENE CODING FOR A SMALL

MULTIFUNCTIONAL PEPTIDE

by

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AND ABOVE ALL TO HIM, CREATOR OF US ALL.

THIS THESIS IS DEDICATED
TO MY PARENTS

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Platelets and coagulation play a crucial role in thrombosis, which is one of the major causes of death in the present-day Western society. During the past few years potent inhibitors have been developed to inhibit platelet function (Ruggeri *et al*, 1986; Dennis *et al*, 1989; Savage *et al*, 1990; Taylor and Gartner, 1992). Plow *et al* (1985) compared the effect of several Arg-Gly-Asp (RGD) containing peptides on binding of fibrinogen and von Willebrandt factor (vWF) to platelets. Hanson *et al* (1988) studied the effect of monoclonal antibodies against the platelet glycoprotein IIb/IIIa (Gp IIb/IIIa) complex on haemostasis in the baboon. Additionally, powerful inhibitors of thrombin are currently being studied in order to obtain the most effective inhibitor of coagulation. Dennis *et al* (1990) used hirudin fragments to investigate thrombin inhibition, while Maraganore *et al* (1989) and DiMaio *et al* (1990) studied the anticoagulant activity of synthetic hirudin C-terminal peptides. Native inhibitors of thrombin like hirudin have been manipulated to be powerful inhibitors of platelets as well (Knapp *et al*, 1992).

In this study we developed a multifunctional peptide that should inhibit thrombin, as well as prevent platelet aggregation. A 29 amino acid peptide, comprising three inhibitory regions, was designed (Fig. 1.1). The N-terminus consists of an amino acid sequence (arginine-glycine-aspartic acid) essential for binding to the fibrinogen receptor (Gp IIb/IIIa) on platelets to prevent fibrinogen binding (Ruoslahti and Pierschbacher, 1987). The N-terminus is followed by a part of fibrinopeptide A, which is a thrombin inhibitor (Martin *et al*, 1992). The C-terminus of the peptide comprises the C-terminal part of hirudin, a potent direct antithrombin produced by the leech, *Hirudo medicinales* (Markwardt, 1970).

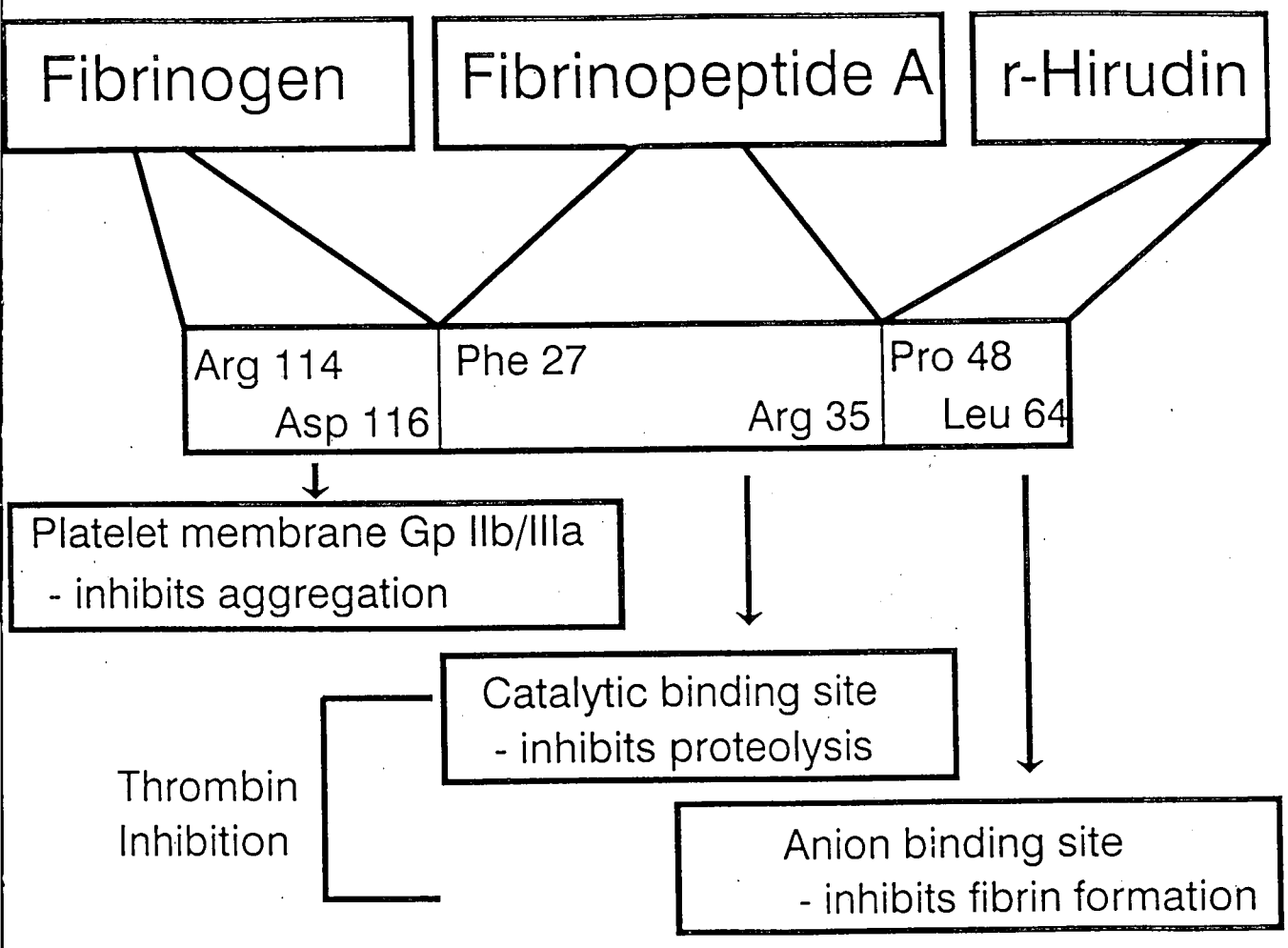

Fig. 1.1 Schematic representation of the synthetic peptide, comprising three inhibitory regions.

1.2 AIM AND SCOPE OF THE PROJECT

There are two ways of manufacturing a novel peptide. One possibility is to chemically synthesize it. This is a costly process especially when relatively large amounts of protein is required. Although it has the advantage that unconventional (e.g. D-isomers) amino acids can be used, each variant of the original must be synthesized from scratch.

The other approach is to design and synthesize a gene coding for the peptide. This could then be cloned into an appropriate vector and expressed in the host organism of choice. We chose this avenue because of lower initial expense and the ability to generate mutants relatively easily. As the reader will see the main disadvantage of this approach is to actually get the protein expressed in large enough amounts.

This project was thus aimed at synthesizing a gene coding for the composite peptide (provisionally named SAG) and expressing it in yeast and/or *Escherichia coli*. We chose these hosts because of their ease of use and the fact that we have some experience in dealing with them.

CHAPTER 2

LITERATURE REVIEW

2.1. INTRODUCTION

In the past decade, remarkable strides have been made to unravel the molecular principles that underlie thrombosis and haemostasis, especially with regard to the central role of thrombin and platelets. It is therefore not surprising that we have experienced a drastic increase in the development and design of new and potent antithrombotic agents that can effectively block the action of thrombin and that can inhibit platelet function. This review will briefly focus on the molecular events that govern the actions of thrombin and blood platelets. The development and actions of the new generation of antithrombotic agents will then be reviewed in more detail.

Escherichia coli is the pioneer of the genetic engineering revolution and has been used extensively for heterologous gene expression. It is still used comprehensively. Unfortunately, it is not ideal for secreting proteins into the medium or for correct folding and processing of some eukaryotic proteins. The yeast, *Saccharomyces cerevisiae,* is the favourite model organism to study aspects ranging from classical genetics to present-day biotechnology of eukaryotic cells. A well-documented combination of genetic, molecular and biochemical characteristics makes it the appropriate organism to study heterologous gene expression. Therefore, both organisms will be briefly discussed in relation to their suitability as expression hosts.

2.2. HAEMOSTASIS AND THROMBOSIS

Haemostasis is a defence mechanism that prevents excessive blood loss by maintaining the vascular integrity. Thrombosis, on the other hand, can be regarded as haemostasis gone wrong. Both the blood platelets and thrombin play a central role in these processes. When a blood vessel is damaged, excessive bleeding is prevented by an elegant cascade of interactions amongst several molecules, the coagulation factors and blood platelets to form a thrombus to arrest bleeding.

2.2.1 The role of platelets

Platelet aggregation is the process whereby platelets interact with one another to form a haemostatic plug (Colman & Walsh, 1987). Platelets do not normally adhere to any surface, which is essential for maintaining sufficient blood flow. Following endothelial damage, platelets adhere to the subendothelium. This adhesion can only be accomplished when vWF is bound to the subendothelium (Vicente *et al*, 1990), and most probably if bound to collagen (Pareti *et al*, 1987; Vicente *et al*, 1990). Once vWF is bound to collagen, it undergoes a conformational change to recognize its receptor in the platelet membrane, the glycoprotein Ib/IX-complex (Taylor and Gartner, 1992). Platelet adhesion leads to a change in shape of the platelets and they spread onto the surface. Platelet contents, including ADP, are released and thromboxane A_2 is synthesized and released. ADP and thromboxane A_2 activate those platelets that are in the proximity of adherent platelets by binding to specific receptors in the platelet membrane (Marcus, 1987). The activated platelet membrane also provides the negatively charged phospholipid surface to which the coagulation TENase and prothrombinase complexes can bind to accelerate activation of the coagulation cascade (Fig. 2.1).

Platelet activation converts Gp IIb/IIIa in the platelet membrane into fibrinogen receptors (Bennet and Vilaire, 1979; Marguerie *et al*, 1979). Aggregation is then accomplished by fibrinogen binding to Gp IIb/IIIa, resulting in formation of molecular bridges between adjacent platelets. Other adhesive proteins like von Willebrand factor, vitronectin and fibronectin are also able to recognize Gp IIb/IIIa and can also form aggregates (Ginsberg *et al*, 1988). Gp IIb/IIIa recognizes the highly conserved Arginine-Glycine-Aspartic acid (RGD) amino acid sequence found in fibrinogen and the other adhesive proteins (Ruoslahti and Pierschbacher, 1987).

2.2.2 The role of coagulation

The factors comprising the coagulation cascade are polypeptides that circulate as inert proenzymes. Activation of these proenzymes proceeds through conformational changes caused by interaction with another molecule or surface, or by cleavage by another enzyme, resulting in exposure of the proenzyme's active site (Kay, 1988). Ca-ions are essential in many of the activation reactions of the proenzymes (Scully, 1992). This is illustrated in Fig. 2.1. These enzymes are serine proteases, since serine is found in the active site (Kay, 1988).

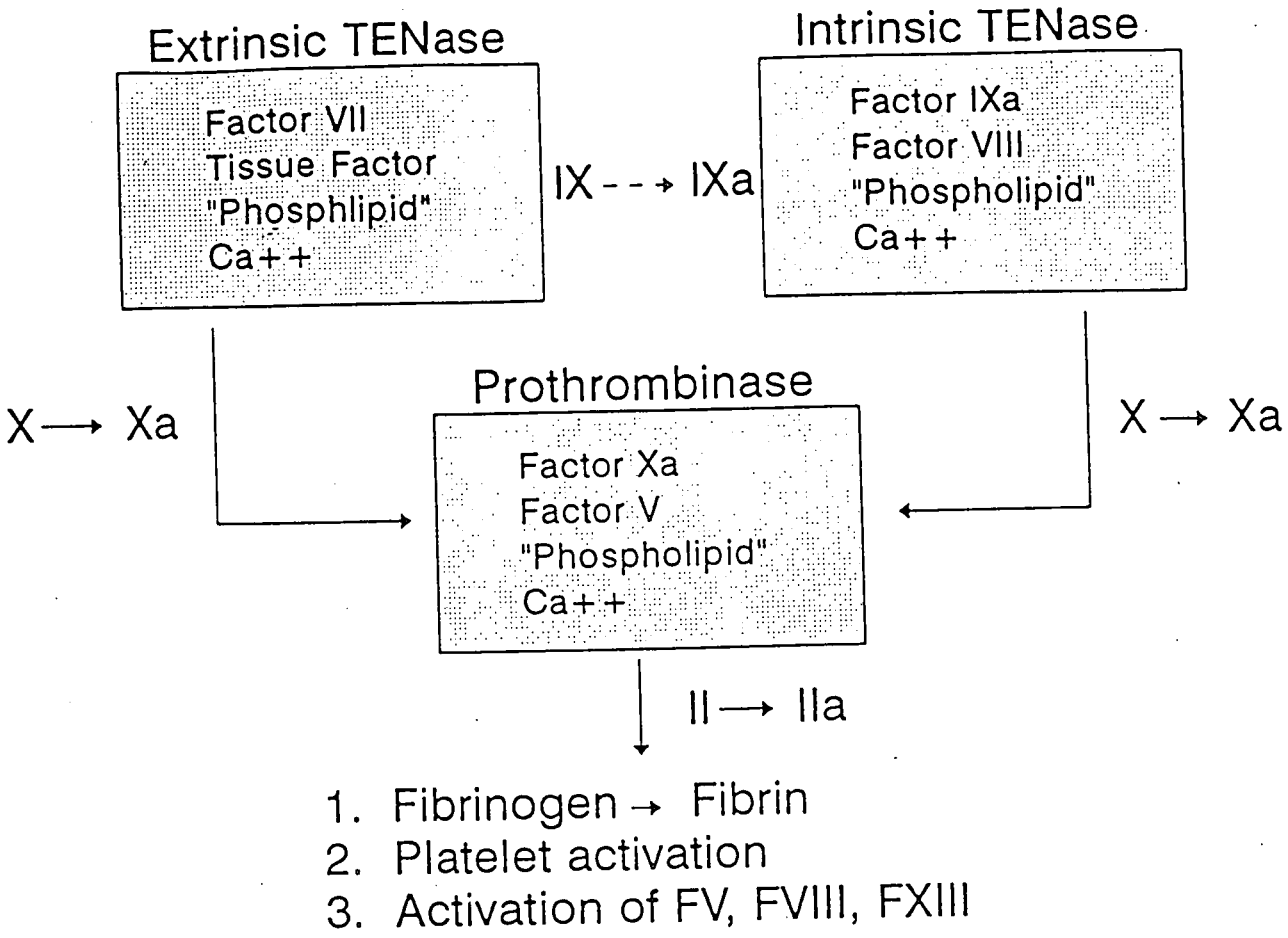

Fig. 2.1. Schematic representation of the vitamin K-dependent complexes of the coagulation cascade. Each enzymatic complex is represented by a box containing the necessary components that constitute the complete enzyme. The top component of each complex is the serine protease, with the second component the required cofactor protein. "Phospholipid" represent the appropriate membrane surface required for precise protein assembly. Calcium ions are essential for stabilization of several interactions and the arrows indicate proteolytic zymogen transformations. Both factor X activating complexes as well as the prothrombinase complex are procoagulant reactions, while activation of protein C (PC) is an anticoagulant reaction (Mann *et al*, 1987).

Prothrombin is cleaved by factor Xa to form thrombin A and B and to release α-thrombin. Activation leads to the exposure of the anion-binding exosite, which enables thrombin to bind to negatively charged molecules like heparin (Rosenberg and Damus, 1973), fibrinogen (Fenton *et al*, 1988) and the C-terminus of hirudin (Grütter *et al*, 1990; Naski *et al*, 1990; Rydel *et al*, 1990). Residues 7-16 of thrombin are essential for the catalytic efficiency of the molecule and represents the anionic exosite domain (De Cristofaro and Castagnola, 1991; Berliner *et al,* 1985; Lord *et al,* 1990). The active (catalytic) site is responsible for its amidolytic activity and results in hydrolysis of small substrates like tripeptide *p*-nitroanilide and binding of peptides like fibrinopeptide A (Martin *et al*, 1992). Additionally, thrombin has an apolar-binding site adjacent to its catalytic site in the fibrinopeptide groove, accounting for thrombin binding to compounds like proflavin (Sonder and Fenton, 1984).

Thrombin converts fibrinogen to fibrin and also activates platelets. Thrombin is the pivotal enzyme in the coagulation cascade. Regardless of how the cascade is triggered or which pathway of the coagulation cascade is followed, the amount of thrombin produced will eventually determine the amount of fibrin that is formed (Norrheim *et al*, 1991). This will also determine the extent of platelet activation (Norrheim *et al*, 1991). Thrombin mediates platelet activation and aggregation by binding to its receptor on the platelet membrane (Coughlin, 1993). In addition to the conversion of fibrinogen to fibrin, thrombin leads to the activation of factors V, VIII, XIII and proteins S and C. Protein C is activated when thrombin binds to thrombomodulin in endothelial cells (Esmon *et al*, 1982). Activated protein C inhibits factors Va and VIIIa, and so inhibits thrombin production (Scully, 1992).

2.3. INHIBITION OF THROMBIN

Inhibition of the formation and activities of thrombin is one of the major approaches to prevent thrombosis. Under normal circumstances, it is achieved by complex formation with antithrombin III (Rosenberg and Damus, 1973). The interaction between thrombin and antithrombin III is greatly facilitated by heparin. Direct inhibitors of thrombin include natural proteins isolated from bloodsucking animals and tailor-made synthetic peptides. Hirudin isolated from the medicinal leech, *Hirudo medicinales* (Markwardt, 1970), is the most potent known natural inhibitor of thrombin. Synthetic peptides include hirugen (Naski *et al*, 1990), the hirulogs

(Maraganore *et al*, 1990) and D-Phenyl-L-Prolyl-L-Arginyl-chloromethylketone (PPACK) (Kettner and Shaw, 1979). Inhibition of thrombin formation can be achieved by tick anticoagulant peptide (TAP) isolated from the soft tick, *Ornithodoros moubata* (Waxman *et al,* 1990). TAP effectively inhibits the actions of factor Xa and therefore the conversion of prothrombin to its active serine protease.

2.3.1 Antithrombin III and heparin

Antithrombin III is a potent inhibitor of thrombin (Rosenberg and Damus, 1973) and to a lesser extent of factors IXa, Xa (Kurachi *et al*, 1976), XIa (Damus *et al*, 1973) and XIIa (Stead *et al*, 1976). Binding of antithrombin III to thrombin is dependent on the active site serine of thrombin and the reactive site arginine on antithrombin III (Damus *et al*, 1973). Human antithrombin III is a 54 to 65 kDa single-chain glycoprotein (Rosenberg and Damus, 1973; Kurachi *et al*, 1976; Nordenman *et al*, 1977), which is synthesized in the liver and circulates the blood at a concentration of approximately 20 mg/dl (Collen *et al*, 1977). Nine percent of the protein weight consists of carbohydrates (Kurachi *et al*, 1976). Structural analysis of the carbohydrate moiety showed four identical N-glycosidically linked carbohydrate chains per molecule (Franzén *et al*, 1980). The domain of the antithrombin III-thrombin complex responsible for binding to cultured cells like bovine corneal endothelial cells, is located over amino acid residues 253 to 314 in the large loop close to the C-terminus of the molecule (Knoller and Savion, 1991). The heparins comprise a family of negatively charged glycosaminoglycans (which do not all have anticoagulant activity) and they act as cofactors of the inhibition of thrombin and factor Xa by antithrombin III (Dvorak, 1987). Inhibition is achieved when heparin binds to lysine residues on antithrombin III to cause a conformational change at the active site. Arginine in the active site then becomes more accessible for binding to the serine residue of thrombin (Rosenberg and Damus, 1973; Damus *et al*, 1973). Heparin can dissociate from the complex and catalyze other antithrombin III reactions (Rosenberg, 1987).

Commercial heparin is heterogeneous and the molecular weight ranges from 3 to 30 kDa, with an average of 15 kDa. The anticoagulant activity is situated in a pentasaccharide sequence with a very high affinity for antithrombin III. In order to achieve anticoagulant activity, heparin must form a molecular bridge between antithrombin III and thrombin (Björk *et al*, 1992). In contrast, inhibition of factor Xa requires only binding of heparin to antithrombin III (Weitz and Hirsh, 1992).

Low molecular weight heparins (LMWHs) are chemical or enzymatic depolymerized fragments of commercial heparin and have molecular weights ranging from 3 to 6 kDa. The anti-factor Xa activity of heparin is independent on molecular weight, while thrombin is only inhibited by molecules with longer chains (Weitz and Hirsh, 1992; Norrheim *et al*, 1991). Norrheim *et al* (1991) compared the involvement of the low molecular weight heparins Enoxaparine, Fragmin and Logiparin, and unfractionated heparin (UFH) in anticoagulation experiments. Whole blood evaluations revealed that UFH had greater inhibitory effects than LMWH, using fibrinopeptide A determinations as a criterion.

LMWHs have certain advantages over standard heparin. Firstly, LMWHs binds less to other plasma proteins and endothelial cells and thus have greater bio-availability than standard heparin. Furthermore, LMWHs are not neutralized by platelet factor 4. Since LMWHs have a longer dose-independent half-life, it can produce more predictable results than standard heparin (Weitz and Hirsh, 1992).

Platelets limit the inhibition of coagulation by heparin (Weitz and Hirsh, 1992). Firstly, factor Xa, which is activated on the platelet membrane is sheltered from heparin-antithrombin inhibition and secondly, platelets release platelet factor 4, which is a heparin-neutralizing protein. Additionally, thrombin bound to fibrin is protected from heparin-antithrombin inhibition. Binding of thrombin to subendothelial matrix proteins also leads to protection from heparin inhibition. The mechanism may be that thrombin binding to fibrin makes the heparin binding site on thrombin inaccessible to heparin. In contrast, Weitz *et al* (1990) discovered that clot-bound thrombin is susceptible to inhibition by heparin. The same results were also obtained by Okwusidi *et al* (1991). These observations may explain why heparin is less effective than hirudin in preventing arterial thrombosis in experimental animals. These findings may also suggest that antithrombin III-independent inhibitors, like hirudin, may be more effective in certain clinical settings (Weitz and Hirsh, 1992).

2.3.2 Heparinoids

Two heparinoids, dermatan sulphate and ORG 10172, are presently under investigation (Weitz and Hirsh, 1992). Dermatan sulphate acts as a catalizer for heparin cofactor II, a secondary inhibitor of thrombin. Since heparin cofactor II only inhibits thrombin, it has minimal anti-factor Xa activity. ORG 10172 consists of dermatan sulphate, chondroitin sulphate and heparan sulphate. In contrast to dermatan sulphate, it has both antithrombin and anti-factor Xa activity.

2.3.3 Hirudin

Hirudin is a small protein produced by the salivary glands of the medicinal leech, *Hirudo medicinalis*, and is the most potent and specific natural inhibitor of thrombin known (Markwardt, 1970). Hirudin has a low apparent molecular weight of 7000 Da and is a highly stable polypeptide. It consists of 65 amino acids and has a cysteine-rich amino terminus and an acidic carboxy-terminus. The carboxy-terminal part carries a stretch of homology with a thrombin cleavage site in prothrombin (Courtney *et al*, 1989). The amino acid sequence of three natural hirudin variants HV-1, HV-2 and HV-3 were analyzed by Dodt *et al* (1984), Harvey *et al* (1986) and Dodt *et al* (1986), respectively. The different sequences are shown in Fig. 2.2.

Hirudin inhibits thrombin by forming a tight stoichiometric complex. The dissociation constant of the complex is approximately 20 fM (Stone and Hofsteenge, 1986). The 48-amino acid globular N-terminal, which is stabilized by three disulphide bridges, binds within the active site of thrombin (Rydel *et al*, 1990; Rydel *et al*, 1991). The C-terminus, containing several acidic amino acids and a sulphated tyrosine at position 63, binds through ionic and hydrophobic interactions to the fibrinogen binding site of thrombin (Bode *et al*, 1989; Wallace *et al*, 1989; Grütter *et al*, 1990; Naski *et al*, 1990; Rydel *et al*, 1990). This results in inhibition of both the proteolytic and cellular activities of the enzyme (Fenton, 1989). Binding of hirudin within the active site region of thrombin is essential for optimal interaction (Stone *et al*, 1987). Kinetic studies performed by Stone and Hofsteenge (1986) indicated that complex formation is brought about by an ionic strength-dependent step; i.e. the interaction of the negatively charged C-terminal with the anion-binding site, followed by a second step in which hirudin becomes bound to the active site.

Sukumaran *et al* (1987) studied the conformation of hirudin in solution by [^{1}H]-nuclear magnetic resonance (NMR). Their studies showed that hirudin contains two β-sheets, but no α-helices. Furthermore, it is organized into three domains; a central core, which is stabilized by three disulphide bridges, and two exposed loops. The one loop contains the COOH-terminal and the other contains a protruding "finger", which consists of a beta-turn (Sukumaran *et al*, 1987). According to NMR studies performed by Clore *et al* (1987) the core region consists of residues 3-30, 37-46 and 56-57. The finger-like structure consists of residues 31-36 and the second loop of residues 47-55. Studies by Clore *et al* (1987) indicates that the N-terminal is a relatively compact region (residues 3-49), followed by a disordered C-terminal region (residues 50-56). These data were confirmed by solution studies of the thrombin-hirudin complex, which showed that hirudin acts as a bicovalent inhibitor with two binding regions (Stone and Hofsteenge, 1986; Braun *et al*, 1988;

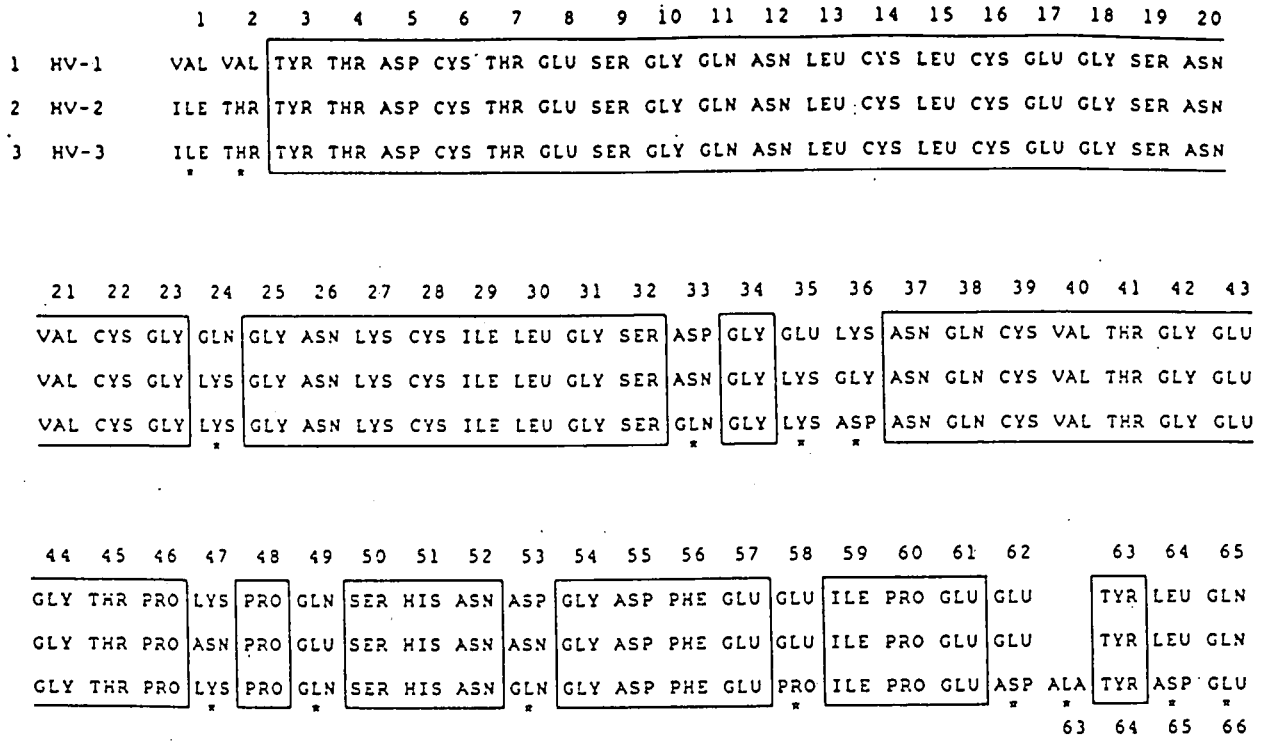

Fig. 2.2. Amino acid sequences of three natural hirudin variants, HV-1, HV-2 and HV-3 as described by Dodt *et al* (1984), Harvey *et al* (1986) and Dodt *et al* (1986), respectively. The boxes delineate regions of homology and the stars refer to variant residues.

Dodt *et al*, 1990; Ni *et al*, 1990). The crystallographic structure of the complex (Grütter *et al*, 1990; Rydel *et al*, 1990) also confirmed the structure. NMR studies done by Haruyama and Wüthrich (1989) showed that the secondary structure of natural hirudin (Sukumaran *et al*, 1987) is in essence identical to that of recombinant desulphatohirudin.

The three disulphide bonds play an important role in maintaining anticoagulant activity, since disrupting any of the disulphide bonds leads to inactivation of hirudin (Chang, 1990; Rydel *et al*, 1990). This was also confirmed by Rydel *et al* (1990). Chang (1991) observed that hirudin is stable even in the presence of strong denaturants like 6 M guanidinium chloride or 8 M urea. Additionally, thrombin inhibitory activity was retained even after cleavage of the Lys_{36}-Asn_{37} internal peptide bond (Chang, 1991).

Two unique characteristics of hirudin distinguish it from the conventional protease inhibitors. First, most other serine protease inhibitors contain a reactive site, which interacts with the active site of the target enzyme. In contrast, none of the three lysine residues of hirudin play such a crucial role (Braun *et al*, 1988). Second, hirudin contains a compact N-terminus but a disordered C-terminus, in contrast to most other serine protease inhibitors, which are compact molecules (Folkers *et al*, 1989).

Unlike native hirudin, recombinant hirudin lacks the sulphate group on Tyr_{63} (Dodt *et al*, 1984; Dodt *et al*, 1986; Harvey *et al*, 1986; Loison *et al*, 1988). As a result the desulphonated compound has a tenfold-reduced affinity for α-thrombin (Stone and Hofsteenge, 1986). Hofsteenge *et al* (1990) could, by introducing phosphotyrosine into the 63 position, restore the affinity of r-hirudin for α-thrombin to levels equivalent to that of wild-type hirudin. It does therefore appear that the negatively charged Tyr_{63} plays a role in determining the affinity of hirudin for thrombin.

The action of heparin is dependent on modulation by factors like antithrombin III, heparin cofactor II, platelet factor 4 and factor VIII (Dvorak, 1987; Talbot, 1989; Walenga *et al*, 1989). In contrast, hirudin acts independently of any other factors. A comparison of the characteristics and anticoagulant effects of hirudin and heparin is given in Table 2.1.

TABLE 2.1 Comparison of heparin and hirudin as anticoagulants (Markwardt, 1989).

Heparin	Hirudin
Heteropolysaccharide, family of sulphated glycosaminoglycuronans of diverse chain length	Single chain polypeptide, composed of 65 amino acids
Molecular weight: 5 000 to 25 000 Da	Molecular weight: 7 000 Da
Multiple sites of action	Selective tight-binding thrombin inhibition
Requires endogenous cofactors like antithrombin III and heparin cofactor II	Requires no endogenous cofactors
Neutralized by antiheparins	Not susceptible to blood peptides and enzymes
Affects platelets	No effect on platelets
Metabolized in the liver	Mainly excreted in unchanged form

2.3.4 Hirudin derivatives

Krstenansky and Mao (1987) chemically synthesized an unsulphated N^{α}-acetyl-hirudin$_{45\text{-}65}$ (Hir$_{45\text{-}65}$). They found that residues 45-65 were able to inhibit blood coagulation and the release of fibrinopeptide A by thrombin, but that it was unable to inhibit thrombin's amidolytic activity. This indicated that the C-terminus occupied a single binding site on thrombin, which were later confirmed by Dodt *et al* (1990), who showed that the C-terminus of hirudin bound to the anion-binding exosite of α-thrombin. Studies with Hir$_{45\text{-}65}$ showed inhibition of the interaction between thrombin and thrombomodulin (Schmitz *et al,* 1991). This results in inhibition of the activation of protein C, activation of platelets and endothelial cells, and interactions with blood clotting cofactors V and VIII, to prevent their activation. However, binding of hirudin to thrombin does not affect interaction between thrombin and antithrombin III, since antithrombin III does not recognize the anion-binding site (Dennis *et al*, 1990). Schmitz *et al* (1991) investigated the inhibition of α-thrombin by a hirudin-derived N-terminal fragment (Hir$_{1\text{-}47}$) and found that this fragment inhibited all enzymatic functions of thrombin.

In addition to the work done by Krstenansky and Mao (1987), Mao *et al* (1988) investigated the shortest C-terminal fragment with anticoagulation activity. The 10 amino acid peptide (Hir_{56-65}) NH_2-Phe-Glu-Glu-Ile-Pro-Glu-GLu-Tyr-Leu-Gln-COOH was the shortest fragment to show activity, while the 12 amino acid peptide (Hir_{54-65}) exhibited maximum inhibition. Furthermore, Phe_{56} appeared to be crucial for maintaining anticoagulant activity, since replacement of Phe_{56} with Glu or Leu led to complete loss of activity. Additionally, when Phe_{56} was replaced with D-Phe, to determine the conformational requirements of the residue, inhibition was again completely lost. Circular dichroism spectra showed that binding of the hirudin C-terminal peptides to thrombin led to significant conformational changes and that loss of thrombin activity might be due to the lack of conformational change taken place (Mao *et al*, 1988).

Maraganore *et al* (1989) studied synthetic peptides based on the 21 C-terminal residues of hirudin. The study was done to determine the role of this segment in thrombin inhibition, the shortest C-terminal segment capable of thrombin inhibition and the role of sulphation of Tyr_{63}. This synthetic peptide was able to inhibit the coagulation and fibrinogenolytic, but not amidolytic activities of thrombin. The minimal sequence with maximal anticoagulant activity was obtained from the peptide: NH_2-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-COOH (Hir_{53-64}). Additional residues to the N-terminus had no enhanced anticoagulation activities. The sequence contains six negative charges, which are proposed to contribute to the electrostatic interactions (Stone & Hofsteenge, 1986). According to Markwardt (1970) complex formation is a result of negative charges provided by hirudin and positive charges from thrombin. Compared to Hir_{53-64}, the Tyr-sulphated form (S-Hir_{53-64}) showed a 10-fold increase in anticoagulant activity. These results indicated that hirudin C-terminal derived peptides were involved in interaction with the anion-binding exosite (Maraganore *et al*, 1989). Furthermore, cross-linking studies performed by Bourdon *et al* (1990) showed that hirudin-derived peptides bind approximately 18-20 Å from thrombin's active site.

Jakubowski and Maraganore (1990) found that a synthetic, tyrosine-sulphated dodecapeptide (BG8865) based on residues 53-64 of hirudin, was able to inhibit thrombin activity and platelet aggregation. The data provided convincing *in vitro* evidence that the synthetic peptide had several advantages over heparin. The peptide showed effective inhibition of thrombin-induced platelet activation, no co-factor dependence, insensitivity to heparin-neutralizing factors and no direct or immune-mediated platelet stimulating properties were present.

Naski *et al* (1990) studied the inhibitory capacity of hirugen, the synthetic *N*-acetylated C-terminal of hirudin [Ac-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO_3)-Leu]. Their results showed that hirugen competitively inhibited the action of α-thrombin on fibrinogen, but with minimal inhibition of thrombin's amidolytic activity. Hirugen therefore binds to the exosite of thrombin to prevent the binding of fibrinogen (Maraganore *et al*, 1989; Hofsteenge *et al*, 1990; Naski *et al*, 1990; Niehrs *et al*, 1990). Additionally, hirugen's lack of thrombin inhibition towards synthetic substrates suggested that the active site was not blocked by hirugen (Naski *et al*, 1990). Their observations that heparin also inhibits thrombin by binding to the same exosite as fibrinogen and hirugen, were in agreement with results obtained by Fenton (1989) and Stone and Hofsteenge (1987).

In 1990 Maraganore *et al* designed a range of antithrombotic peptides called hirulogs. They consist of an active-site specificity sequence with an Arg-Pro scissile bond, a polymeric linker of glycyl residues (6 to 18 Å in length) and an anion-binding exosite (ABE) of α-thrombin, like the C-terminus of hirudin. Synthetic C-terminal hirudin peptides blocked the thrombin ABE and thus inhibited the fibrinogen clotting activity of the enzyme, but could not inhibit the hydrolysis of a tripeptide *p*-nitroanilide substrate. In sharp contrast, hirulog-1 [(D-Phe)-Pro-Arg-Pro-$(Gly)_4$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu] inhibited thrombin-catalyzed hydrolysis of *p*-nitroanilide at nanomolar concentrations (DiMaio *et al*, 1990; Maraganore *et al*, 1990). Hirulog-1 is specific for thrombin, and lacks inhibitory activities toward human factor Xa, human plasmin and bovine trypsin (Witting *et al*, 1992a). The interactions between hirulog-1 and thrombin are practically identical to that between PPACK and thrombin in the active site (Bode *et al*, 1989) and with that of the hirudin- or hirugen-thrombin complexes at the exosite (Skrzypczak-Jankun *et al*, 1991). The optimal length of the oligoglycyl spacer, forming a molecular bridge between the active-site and the ABE sequence, appeared to be at least three to four glycine residues (Maraganore *et al,* 1990). Fig. 2.3 is a schematic representation of thrombin inhibition by the hirulog and C-terminal hirudin peptide derivatives. Witting *et al* (1992b) investigated the thrombin-specific inhibition by hirulog-B2, which has D-cyclohexylalanine substituted in the first position. Their data demonstrated that hirulog-B2 was highly specific for binding to the catalytic site and adjacent regions, as well as the anion-binding exosite of thrombin. According to Dawson *et al* (1991) hirulog-B2 has potential pharmaceutical applications.

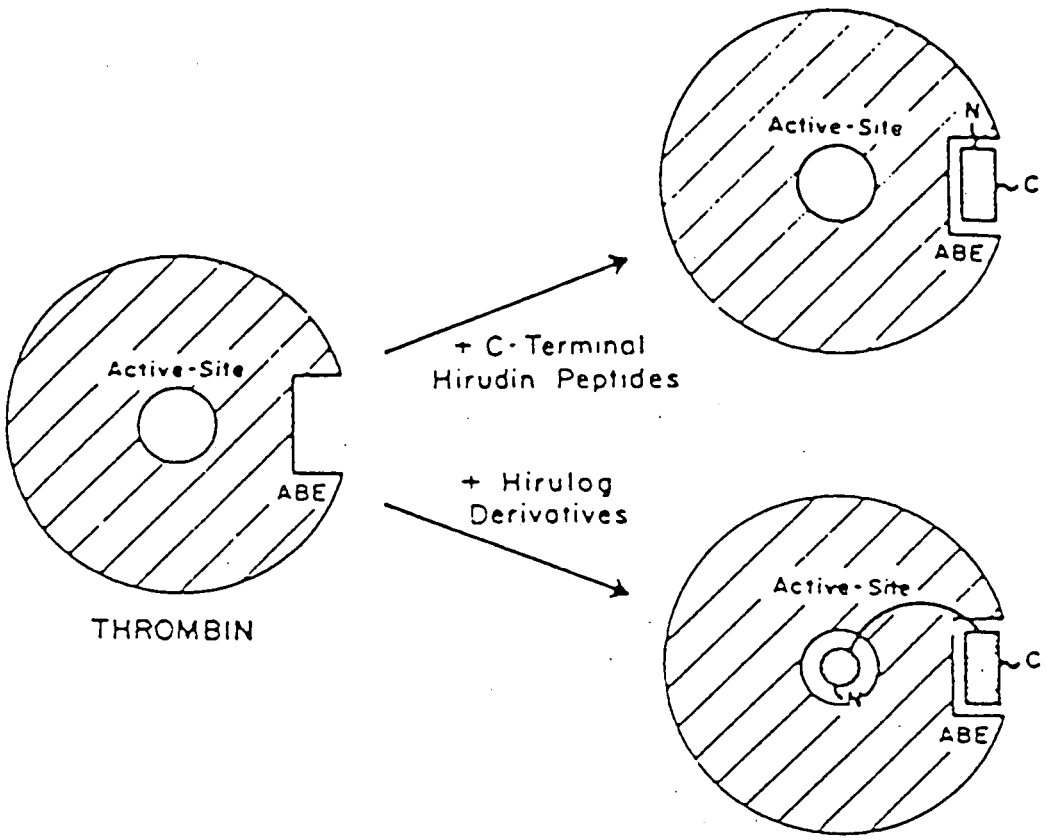

Fig. 2.3. Schematic representation of the action of hirulog and C-terminal peptide derivatives towards thrombin (Maraganore *et al*, 1990).

Dennis *et al* (1990) did site directed mutagenesis in order to identify the important areas of hirudin involved in its thrombin inhibitory capacity. Asn_{52} was replaced by methionine and the obtained mutant was expressed in *E. coli*. The purified polypeptide was cleaved with cyanogen bromide at this unique methionine residue, to produce two fragments. This study showed that binding of the C-terminal fragment (residues 53-65) of hirudin to thrombin caused a conformational change affecting the active site of thrombin. The binding of the rest of the hirudin molecule is slightly facilitated by this conformational change. The N-terminal fragment (residues 1-52) acts as a competitive inhibitor of thrombin. Hirudin and the C-terminus was able to protect α-thrombin from trypsin cleavage, whereas the N-terminus could not (Dennis *et al*, 1990). As a result, the use of synthetic hirudin C-terminal analogs became an accepted strategy to study the role of fibrinogen binding to the exosite of thrombin (Krstenansky and Mao, 1987; Bourdon *et al*, 1990; Chang, 1990; Jakubowski and Maraganore, 1990; Prescott *et al*, 1990).

2.3.5 PPACK

D-Phenyl-L-Prolyl-L-Arginyl-chloromethylketone (PPACK) is a specific inhibitor of the thrombin catalytic site. The effect is brought about by irreversible alkylation of the active-site histidine (Kettner and Shaw, 1979). Bode *et al* (1989) did crystallographic studies to investigate the formation of the stoichiometric complex between human α-thrombin and PPACK. They found that the exceptional specificity of PPACK could be explained by a hydrophobic cage formed by Ile_{174}, Trp_{215}, Leu_{99}, His_{57}, $Tyr_{60}A$ and $Trp_{60}D$. Furthermore, binding of PPACK to the active site results in only minor effects on the positions of the catalytic residues (Skrzypczak-Jankun *et al*, 1991). Studies done by Schmaier *et al* (1992) showed that human α-thrombin and PPACK-thrombin binds to different sites or binds differently to the same site on the platelets.

2.4. INHIBITION OF PLATELETS

Another important approach to inhibit thrombosis is to prevent platelet-platelet interactions. Platelet aggregation is mediated by fibrinogen that binds to Gp IIb/IIIa, situated on the platelet membrane (Marguerie *et al*, 1980). The recognition site is an RGD-motif situated in the carboxy-terminus of the gamma chain of fibrinogen (Kloczewiak *et al*, 1984; Coller *et al*, 1989).

Platelet activation leads to a change in the shape of platelets and subsequent conversion of Gp IIb/IIIa into receptors for fibrinogen (Bennet and Vilaire, 1979; Marguerie *et al*, 1979). Gp IIb/IIIa can also serve as a receptor for von Willebrand factor (Gartner and Bennett, 1985; Haverstick *et al*, 1985; Plow *et al*, 1985; Ginsberg *et al*, 1988), fibronectin, vitronectin and thrombospondin (Hynes, 1987). These adhesive proteins all contain the RGD-motif (Arg-Gly-Asp). Other adhesive proteins like type I collagen (Dedhar *et al*, 1987) and osteopontin (Oldberg *et al*, 1986) also contain the RGD-motif.

Peptides containing the RGD-sequence compete with fibrinogen, fibronectin and von Willebrandt factor for binding to Gp IIb/IIIa on activated platelets and so block platelet aggregation (Gartner & Bennett, 1985; Haverstick *et al*, 1985; Plow *et al*, 1985). Additionally, Gp IIb/IIIa binds directly to immobilized RGD-peptides (Pytela *et al*, 1986). Exchange of alanine for glycine or glutamic acid for aspartic acid, resulting in the addition of a methyl or a methylene group respectively, eliminated all adhesive activities of RGD-containing peptides (Pierschbacher and Ruoslahti, 1984). Synthetic peptides based on the sequences of 1) Arg-Gly-Asp, present in fibrinogen, vitronectin and von Willebrand factor and of 2) the fibrinogen γ-chain (γ 400-411) bind to platelets with the same affinity as intact fibrinogen and so lead to efficient inhibition of platelet function (Ruggeri *et al*, 1986).

Disintegrins isolated from snake venoms represent a new class of low molecular weight RGD-containing cysteine rich peptides (Niewiarowski *et al*, 1990). Isolation of these antiplatelet peptides from snake venoms is currently being explored. A novel platelet aggregation inhibitor from the southern copperhead snake venom, contortrostatin, has an apparent molecular weight of 9 kDa. It appears to inhibit aggregation by binding to the Gp IIb/IIIa membrane receptor (Trikha *et al*, 1990).

Dennis *et al* (1989) described the purification, complete amino acid sequence and biological activity of several snake venom proteins that are Gp IIb/IIIa antagonists and potent inhibitors of platelet aggregation. These proteins are kistrin from *Agkistrokon rhodostoma*, bitan from *Bitis arietans*, three isoforms of trigramin from *Trimeresusus gramineus* and an isoform of echistatin from *Echis carinatus.* These peptides consist of between 47 and 83 residues. All these proteins contain the RGD-motif, and inhibit platelet aggregation. They can therefore serve as potential antithrombotic agents (Dennis *et al*, 1989). Musial *et al* (1990) compared the action of Arg-Gly-Asp-Ser (RGDS) and four disintegrins from viper venoms (echistatin, flavoridin, albolabrin, bitistatin). Their results confirmed that disintegrins are potential candidates for antiplatelet agents. Shebuski *et al* (1990) successfully used echistatin in an animal model of thrombosis.

Savage *et al* (1990) studied the platelet-binding characteristics of snake venom-derived proteins applaggin and echistatin from *Agkistrodon piscivorus piscivorus* and *Echis carinatus*, respectively. Both proteins were able to inhibit platelet secretion and aggregation of platelets stimulated by ADP, collagen and human γ-thrombin. Monoclonal antibody LJ-CP3, which inhibits binding of RGD-containing proteins to Gp IIb/IIIa, also prevented applaggin binding to platelets. Consequently, applaggin and echistatin bind to Gp IIb/IIIa mediated by the RGD-motif (Savage *et al*, 1990).

Seymour *et al* (1990) purified a competitor for the fibrinogen receptor (Gp IIb/IIIa) decorsin, from the North American leech, *Microbdella decora*. Decorsin acts as a potent inhibitor of platelet aggregation. Connolly *et al* (1992) described a protein that inhibited collagen-induced platelet aggregation. It was isolated and purified from the leech *Haementeria officinalis* and the purified protein was called leech antiplatelet protein (LAPP). According to Keller *et al* (1992), LAPP also inhibited platelet adhesion to collagen. Waxman and Connolly (1993) purified a protein, moubatin, from the soft tick, *Ornithodoros moubata*. This protein inhibits aggregation of collagen-stimulated platelets.

The knowledge of the molecular principles that underlie thrombus formation makes it clear that the inhibition of platelets as well as inhibition of thrombin may be an effective way to inhibit thrombosis (Kotzé and Badenhorst, 1992). A recombinant variant of hirudin which inhibits thrombin and platelet aggregation was studied by Knapp *et al* (1992). The newly obtained disintegrin activity was obtained by introducing the RGD-motif to the finger-like tip of hirudin. Native hirudin contains Ser-Asp-Gly-Glu at the protruding finger (residues 32-35). The variants were designed by replacing the Ser-Asp-Gly-Glu by Arg-Gly-Asp-Ser to obtain hirudisin and Lys-Gly-Asp-Ser to obtain hirudisin-1. Thrombin inhibition studies showed that hirudisin is 2-fold more potent than hirudisin-1 and r-hirudin. Additionally, hirudisin was able to inhibit ADP-induced platelet aggregation due to the integrin-directed RGD-motif. Hirudisins are thus important proteins that combine potential antithrombotic and antiplatelet activities (Knapp *et al*, 1992).

2.5. TRANSGENIC PRODUCTION OF THERAPEUTIC PEPTIDES AND PROTEINS

Recombinant proteins can be used as tools in studying protein-protein interactions, as well as protein structure. Many theurapeutic proteins were traditionally isolated from animals, plants and micro-organisms with all the accompanying disadvantages. For instance, the large numbers of leeches required for supplying enough material for scientific purposes, as well as their endangered-species status, led to the development of recombinant desulphato-hirudin. Recombinant hirudin and hirudin mutants became popular to study thrombin-hirudin interactions (Braun *et al*, 1988; Dodt *et al*, 1988). Although many different expression systems are in use for the production of a large variety of theurapeutic proteins, the discussion that follows will concentrate on *E. coli*, yeast and selected antithrombins.

2.5.1 Production in *Escherichia coli*

Antithrombin III is a single-chain glycoprotein synthesized in the liver and it inhibits serine proteases, competing in the blood coagulation cascade (Rosenberg & Damus, 1973). Bock *et al* (1982) constructed a human cDNA library from liver RNA. They obtained overlapping cDNA clones encoding the protease inhibitor antithrombin III. The two clones confirmed the known DNA sequence for antithrombin III and was expressed in *E. coli*. However, the only biological activity was complex formation between thrombin and antithrombin III in the presence of heparin, as was detected by Western analysis. Recombinant antithrombin III had greater electrophoretic mobility than native antithrombin III, which could be due to absence of glycosylation of the bacterial product (Bock *et al*, 1982).

Hirudin is the most potent thrombin-specific inhibitor known and could thus have major pharmacological impact. Additionally, the thrombin-hirudin complex could act as an excellent model for studying protein-protein interactions (Dodt *et al*, 1986). Harvey *et al* (1986) constructed a cDNA library from mRNA isolated from leech salivary glands. The cDNA encoding a variant of hirudin, was subsequently isolated and cloned. It was expressed in *E. coli* under the control of the bacteriophage lambda P_L promoter. The amino acid sequence differed in nine positions from the sequence of HV-1, isolated from whole leech bodies. This variant was thereafter called HV-2.

Dodt *et al* (1986) cloned a synthetic hirudin gene into an expression vector and transferred it to *E. coli,* utilizing the alkaline phosphatase signal sequence to secrete recombinant hirudin into the periplasm. The recombinant hirudin was identical to desulphatohirudin and had similar biological properties.

Scacheri *et al* (1993) isolated mRNA from the Asian buffalo leech, *Hirudinaria manillensis*. The polymerase chain reaction (PCR) was utilized to isolate the gene coding for the hirudin variants HM1 and HM2 from the cDNA. The cloned gene was then expressed in *E. coli* (Scacheri *et al*, 1993). Both gene fragments code for polypeptides of 84 amino acids and are organized into four exons; the first one corresponding to a 20-amino acid signal peptide, while the remaining three share the primary structure of the peptides (Scacheri *et al*, 1993).

The main problem in using bacteria as expression hosts is their inability to perform necessary post-translational modifications which may be essential for the activity of the given protein (Bröker *et al*, 1987).

2.5.2 Production in yeast

Yeast has the ability to secrete extracellular proteins via an excellently organized multi-component secretory apparatus, where disulphide bond formation, N- and O-linked glycosylation and additional post-translational modifications occur (Buckholz and Gleeson, 1991). Futhermore, strong promoters have been isolated, auxotrophic markers are well characterized and stable plasmids have been identified. According to Linder (1992) a well-documented combination of genetic, molecular and biochemical approaches has made yeast a convenient organism to study translation and transcription.

Wiseman (1992) discussed the use of yeast as host organism for the production of recombinant human proteins (Table 2.2). These genetically-engineered proteins would have an immense influence on the future medical practice.

TABLE 2.2 Genetically-engineered human proteins from yeast for medical use (Wiseman, 1992).

Product	Intended use
Insulin	Anti-diabetic
Interferons	Anti-viral Anti-cancer
Tissue plasminogen activator (t-PA)	Thrombolytic properties
Serum albumin	Plasma substitute
Haemoglobin	Blood substitute
HIV antigens	Vaccine against HIV infection
β-Hepatitis antigens	Vaccine against β-Hepatitis
Cytochromes P-450	Drug-overdose therapy

Bröker *et al* (1987) studied the expression of antithrombin III in *Saccharomyces cerevisiae* and in *Schizosaccharomyces pombe*. The biologically active protein was expressed under the control of the *S. cerevisiae ADHI*, *CYCI* and *GAL1* promoters. The yield was relatively low, with no extraordinary change when using a variety of promoters. Interestingly, the production of biologically active antithrombin III was driven by *S. cerevisiae* promoters in *S. pombe*. In both species the protein was secreted under control of its human signal sequence. When replaced by ten random amino acids, the protein remained in the cell. Glycosylation experiments revealed that the protein was properly glycosylated by both species.

Loison *et al* (1988) studied the expression of recombinant hirudin (r-hirudin) in *S. cerevisiae* by using an auxotrophic strain with a complementing plasmid. The advantage of using yeast cells is the utilization of the secretory apparatus of the cell to transport proteins directly into the medium (Courtney *et al*, 1989). Loison *et al* (1988) constructed a cDNA library of mRNA isolated from leech heads. The cDNA encoding a variant of hirudin (HV-2) was fused to a native yeast promoter and region encoding the entire prepro secretion signal of MFα1. The prepro region is removed during export through the Golgi apparatus and the correctly synthesized r-hirudin is secreted into the medium. It is, however, crucial that the HV-2 sequence directly follows the *KEX2* gene product (yscF) cleavage site to ensure the correct processing of r-hirudin (Loison *et al*, 1988). The *KEX2* gene product (yscF) is a protease involved in the maturation of yeast α-factor.

Unlike natural hirudin obtained from *H. medicinalis,* r-hirudin produced by *S. cerevisiae* is not sulphated at tyrosine-63. Nevertheless, Loison *et al* (1988) found that the specific activity of the protein was 13 000 to 16 000 ATU/mg, which is similar to that of natural hirudin. According to Johnson *et al* (1989) one antithrombin unit (ATU) is the amount of hirudin required to neutralize 1 U NIH of thrombin at 37°C, using fibrinogen as substrate. Biological characterization experiments indicated that r-hirudin was most effective in inactivating thrombin (Courtney *et al*, 1989).

A non-medical application of hirudin was studied by Janes *et al* (1990). They investigated the influence of *GAP* promoter variants on r-hirudin production in *S. cerevisiae*. The variants are all shorter versions of the native *GAP* promoter, differing in their transcriptional efficiencies. Although relatively low secretion levels were observed for all the variants, hirudin production could be used to evaluate plasmid copy number and cell growth.

Waxman *et al* (1990) purified a tick anticoagulant peptide (TAP) from the soft tick, *Ornithodoros moubata*. Since only limited amounts of native TAP could be isolated from tick saliva, Neeper *et al* (1990) investigated the possibility of recombinant production of TAP in yeast. TAP was expressed in *S. cerevisiae* under the control of an inducible galactose promoter. Following the promoter were the sequences encoding the secretory preproleader of the yeast mating pheromone α-factor and a synthetic gene encoding TAP. The gene was constructed from eight synthetic overlapping oligodeoxynucleotides which were annealed and ligated. Successful production of recombinant TAP (rTAP) was achieved and biologically active rTAP was secreted to the medium at a concentration of 0.1-0.15 g/l. Native TAP and rTAP presented the same amino acid composition, primary structure, electrophoretic mobility and inhibition of factor Xa (Neeper *et al*, 1990).

Achstetter *et al* (1992) used hirudin as a reference protein in evaluation of the efficiency of different signal peptides. The *BGL2* gene from *S. cerevisiae* contains a 23-amino acid signal peptide which is responsible for directing ß-glucanase to the yeast cell wall. Achstetter *et al* (1992) compared the signal peptide derived from the *BGL2* gene to that of the MFα1 signal peptide in a series of gene fusions. In the different constructions both signals were followed by the native MFα1 propeptide or mutated forms of it. In both cases the maximum hirudin production was obtained when only the signal, without any pro sequence, was used. This is in agreement with the results of Loison *et al* (1988). The use of both the *MFα1* and the *BGL2* signals led to comparable concentrations of biologically active secreted hirudin (Achstetter *et al*, 1992).

2.6. SECRETORY PATHWAY OF EXTRACELLULAR YEAST PROTEINS

A well-documented combination of genetic, molecular and biochemical approaches has made yeast a convenient organism to study transcription and translation (Linder, 1992), as well as to study protein targeting (Reid, 1991). Eukaryotic cells are multicompartment structures with specialized transport systems for synthesized proteins. Most mitochondrial and chloroplast proteins, as well as extracellular proteins, are coded for in the nucleus and synthesized in the cytosol. These proteins must then be carefully distinguished from all other newly synthesized proteins and transported to the respective organelles (Mathews & Van Holde, 1990). A critical factor is protein sorting, which is a process of targeting each newly synthesized protein to the correct membrane (Darnell *et al*, 1990). Mitochondrial and chloroplast proteins contain specific signal sequences at their N-termini, which recognize the relevant membranes and are then assimilated into the respective organelles. Near the amino terminus one or more positively charged amino acids are found, followed by a stretch of 6 to 12 hydrophobic residues (Darnell *et al*, 1990). The signal, consisting of 13 to 36 residues, is subsequently removed (Voet & Voet, 1990).

Proteins destined to be secreted extracellularly, utilize a special transport system in which the rough endoplasmic reticulum (RER) and the Golgi-apparatus are involved (Larriba, 1993). The RER is a complex organelle of membrane-enclosed space, which is heavily coated with ribosomes. The Golgi-apparatus on the other hand consists of a stack of thin membrane-bound sacs, which are not interconnected, nor associated with any ribosomes (Mathews & Van Holde, 1990).

Synthesis of proteins due to be secreted extracellularly starts at translation of the 5' end of the mRNA (Fig 2.4, step 1). These proteins contain a short amino acid sequence at the amino terminus (Van Heijne, 1981). The amino terminal part of the signal sequence is followed by a highly hydrophobic region, which is followed by a short slightly hydrophilic chain (Van Heijne, 1985). In mammalian cells signal recognition particles (SRPs) recognize the signal sequence and bind to the ribosome as the signal sequence is being produced (Darnell *et al*, 1990). SRPs are also responsible for directing the signal sequence to the ER. The signal sequence is removed in the ER by signal peptidase and N-linked glycosylation is initiated. The yeast homologue (p54) of mammalian SRPs has been described recently (Hann and Walter, 1991). They have also shown that p54 forms part of a large particle (16S), of which one component is a small RNA, scR1. Disruption in both or either one of the genes led to impaired protein translocation (Hann and Walter, 1991).

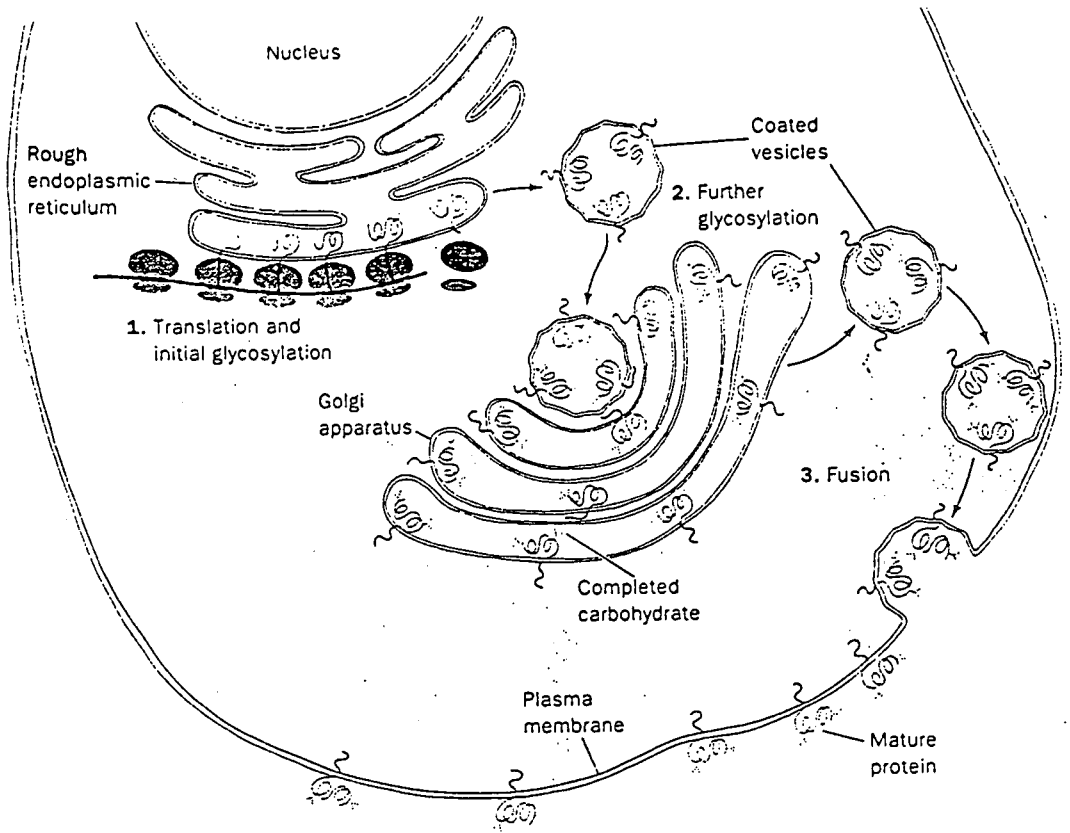

Fig. 2.4. Glycosylation of proteins during transport of vesicles from the rough endoplasmic reticulum to the Golgi apparatus. Glycosylation of proteins are completed and mature proteins secreted through the plasma membrane (Voet & Voet, 1990).

Novel genes (*SEC61, SEC62, SEC63*) involved in protein translocation has been identified (Deshaies and Schekman, 1987; Deshaies and Schekman, 1989; Feldheim *et al*, 1992). Genetic and biochemical evidence revealed that Sec61p, Sec62p and Sec63p act together and interact with other membrane proteins, with molecular weights of 31.5 kDa and 23 kDa, as well as with a luminal ER protein, Kar2p to result in efficient protein translocation (Sanders and Schekman, 1992).

The first stages of glycosylation occur in the RER, before the proteins are packaged into vesicles. Chang (1993) followed the disulphide folding pathway by using the hirudin N-terminal fragment (Hir1-27) as a marker protein. The vesicles bud off and move towards the *cis* end of the Golgi complex, where the vesicles fuse with the Golgi membranes (Fig 2.4, step 2). Kaiser and Schekman (1990) have shown that the products of *SEC12, SEC13, SEC16* and *SEC23* interact and are essential for transport of proteins from the ER to the Golgi. Other proteins encoded by *SEC19, SEC20, BET1, BET2* and *YPT1* are also involved in protein transport from ER to Golgi (Newman and Ferro-Novick, 1987; Segev *et al*, 1988). While the proteins are again packaged into small vesicles and moved to the successive intermediate layers of the Golgi, posttranslational modifications of the proteins are completed.

Posttranslational modifications include modifications of amino acid side chains, addition and modification of saccharide residues and specific proteolytic cleavages. Additionally, disulphide bonds can be formed and polypeptide chains may cluster to form multiprotein complexes (Darnell *et al*, 1990). Posttranslational modifications of the protein are essential stages, as only properly folded proteins can be excreted from the cell. Finally, the vesicles bud from the *trans* end of the Golgi and migrate to the plasma membrane (Mellman and Simons, 1992). These vesicles are then incorporated into the plasma membrane and the proteins are released into the extracellular fluid (Fig 2.4, step 3). Proteins forming the inner part of the vesicle will eventually form the outer surface of the plasma membrane and proteins forming the outer surface of the vesicle will end up facing toward the cytosol (Mathews & Van Holde, 1990).

The efficiency of protein secretion in yeast depends on the stability of the protein product, the efficiency of translation, the stability of the transcript as well as the plasmid copy number (Bröker *et al*, 1987). Additionally, the observation that some human proteins can be secreted by yeast led to the conclusion that the intrinsic properties of the proteins also have a significant effect on adequate protein secretion.

CHAPTER 3

MATERIALS AND METHODS

3.1 CHEMICALS: All chemicals used in this study were of analytical grade.

3.2 BACTERIAL AND YEAST STRAINS: The different strains used are summarized in Table 3.1.

TABLE 3.1 Summary of hosts and strains.

Host	Strain	Genotype
E. coli	M 15	*Nal^S, Str^S, rif^S, lac$^-$, ara$^-$, gal$^-$, mtl$^-$, F$^-$, recA$^+$, uvr$^+$*
E. coli	NM522	F' *lacI^{q}Δ(lacZ)M15 proAB /supE thiΔ(lac-proAB)Δ (hsdMS-mcrB)5*(r_k-m_k-McrB)
S. cerevisiae	DBY 746	*MATα, his3Δ1, leu2-3, leu2-112, ura3-52, trp1-289*
S. cerevisiae	BJ 5457	*MATα, ura3-52, trp1, lys2-801, leu2Δ1, his3Δ 200, pep4: : HIS3, prb1Δ1.6R, can1, GAL*
S. cerevisiae	BJ 5460	*MATa, ura3-52, trp1, lys2-801, leu2Δ1, his3Δ 200, pep4: : HIS3, prb1Δ1.6R, can1, GAL*
S. cerevisiae	20B-12	*MATα, pep4-3, trp1*
S. cerevisiae	AB 1380	*MATa, ade2-1, can1-100, lys2-1, trp1, ura3, his5[v+]*

3.3 VECTORS: The gene was cloned into pMFα8 and into pYES2 for expression in yeast, while pQE-32 was used as vector for expression in *E. coli*. The vector pUCBM21 was used as an intermediary plasmid to aid cloning of the gene into pQE-32. The plasmids and hosts used for expression are summarized in Table 3.2.

TABLE 3.2 Summary of plasmids and hosts used for expression of the synthetic anticoagulant gene.

Host	Vector	Type	Promoter	Leader sequence	Auxo-trophic marker	Antibiotic resistance gene
Yeast	pMFα8	Expression and secretion	MFα1	MFα1	*TRP1*	Ampicillin
Yeast	pYES2	Expression	GAL1	MFα1	*URA3*	Ampicillin
E. coli	pUCBM21	Cloning vector	_	_	_	Ampicillin
E. coli	pQE-32	Expression	*E. coli* phage T5	_	_	Ampicillin

3.4 GENERAL RECOMBINANT DNA METHODS: Methods that are not described in detail, were done as described by Sambrook *et al* (1989) and/or Ausubel *et al* (1990). That include agarose electrophoresis, restriction enzyme digestion and *E. coli* transformations.

3.5 PRIMERS: The primers used in this study are summarized in Table 3.3. SAG2 contains an additional *XhoI* restriction site and SAG4 contains an additional *HindIII* restriction site, both indicated in bold.

TABLE 3.3 Summary of primers.

Name	Sequence	Use
SAG1 (60-mer)	5'-AGAGGTGACTTCTTGGCTGAAGGTGGTGGT--GTTAGACCAGGTGGTGGTGGTAACGGTGAC-3'	Upper strand primer
SAG2 (60-mer)	5'-CG**CTCGAG**CTACAAGTATTCTTCTGGAATTT--CTTCGAAGTCACCGTTACCACCACCACCT-3'	Lower strand primer
SAG3 (19-mer)	5'-ATGAGAGGTGACTTCTTGC-3'	Upper strand primer + ATG-codon
SAG4 (33-mer)	5'-CG**AAGCTT**ATGAGATTTCCTTCAATTTTTACTGC-3'	*MFα1* signal sequence
FOR (17-mer)	5'-GTAAAACGACGGCCAGT-3'	pUC18 forward sequencing primer
REV (17-mer)	5'-CAGGAAACAGCTATGAC-3'	pUC18 reverse sequencing primer

3.6 GENE SYNTHESIS: Both chemically synthesized primers (SAG1 and SAG2) were diluted to a concentration of 1 μg/μl. One μg of each primer was denatured at 92°C and the two primers were annealed at 65°C. Elongation was accomplished by adding dNTPs and Sequenase and the reaction was incubated for 30 minutes at 37°C. Alternatively, gene synthesis was performed using PCR. After denaturation at 96°C for 5 minutes, annealing and elongation were performed at 72°C for 10 minutes. Using different conditions and DNA concentrations, the PCR was optimized. In the optimized reaction 1 μg of primer SAG1 and 10 μg of primer SAG2 were used. The concentration of SAG2 was increased due to its secondary structure. Twenty cycles of one minute at 96°C and one minute at 72°C were performed.

3.7 GENE PURIFICATION: Following PCR synthesis, the gene was run on a 4% Nusieve gel. The distinct band of approximately 98 base pairs was electrophoretically transferred onto a NA45-membrane. The membrane was covered with 1 M NaCl-TE and incubated at 65°C for 60 minutes. The membrane was removed and the purified DNA was ethanol precipitated.

3.8 PCR CONDITIONS FOR SUBCLONING:

3.8.1 Yeast expression: 1 μg of pSAG1 or pSAG2, as well as the primers, was denatured at 92°C for 1 minute and annealed at 55°C for 1 minute. Elongation was performed at 72°C for 1 minute and the reaction was allowed for 20 cycles.

3.8.2 *E. coli* expression: 1 μg of pSAG1U, as well as the primers, was denatured at 92°C for 1 minute and annealed at 37°C for 1 minute. Elongation was performed at 72°C for 1 minute and the reaction was allowed for 20 cycles.

3.9 *E. coli* TRANSFORMATION AND COLONY HYBRIDIZATIONS: Competent cells were prepared using $CaCl_2$ and transformed as described by Sambrook *et al* (1989). Since pMFα8 and pYES2 do not have insert selection systems, colony hybridizations (Sambrook *et al,* 1989) had to be performed to select positive colonies. Colonies were transferred to Hybond N membranes and lysed with 0.5 M NaOH and 1.5 M NaCl. Denaturation was followed by neutralization with 0.5 M Tris-HCl (pH 7.4) and 1 M NaCl. The DNA was fixed to the membrane by exposing to UV-light for 3 minutes. Primer SAG1 was labelled radioactively with [γ-^{32}P]ATP and used as probe for hybridizations, which were performed at 68°C for 12 hours. The membranes were subsequently washed at room temperature in 2 X SSC [20 X SSC - 3 M NaCl; 0.3 M Sodium citrate (pH 7.0)] and 0.1% SDS for 2 X 5 minutes. Stringency was enhanced by washing at 68°C in 0.1 X SSC and 0.1% SDS for 2 X 10 minutes.

3.10 PLASMID ISOLATION AND SEQUENCING: Positive colonies, identified by colony hybridization, were picked and plasmids were isolated using alkaline lysis as described by Sambrook *et al* (1989). Since most restriction enzymes used resulted in blunt ends, sequencing had to be done to verify correct orientation of the constructs. The method of Sanger *et al* (1977) was used with the Sequenase kit from United States Biochemicals, using one of the gene primers to read the gene/vector junction.

3.11 YEAST TRANSFORMATION: Yeast cells were transformed with the constructs using a modified method of Ito *et al* (1983). Gietz *et al* (1992) developed an improved method for high efficiency transformation. Single stranded carrier DNA and plasmids were simultaneously added to the cells to reduce the rate of intracellular nucleic acid degradation. Transformants were grown at 30°C in a defined synthetic medium and supernatant was evaluated for recombinant protein production. No protease inhibitors were added because of fear of interference with the thrombin interactions.

3.12 INDUCTION OF YEAST TRANSFORMED WITH pYES2 VARIANTS: *Saccharomyces cerevisiae* strains BJ 5457, BJ 5460 and AB 1380 were transformed with pSAG1Y and pSAG2Y and transformants were grown on synthetic medium (2% glucose, 0.67% yeast nitrogen base without amino acids and 0.08% amino acid mix, lacking uracil). The 2% glucose serves as repressor of the *GAL1* promoter. The cells were grown till late logarithmic phase and were harvested by centrifugation. The cells were then transferred to fresh medium containing 5% glycerol instead of 2% glucose as carbon source, for 4 hours. Induction was achieved by addition of 2% galactose and 5 ml cell samples were withdrawn at 30 minute intervals. The cells were harvested by centrifugation and stored at -80°C.

3.13 ANTICOAGULANT ACTIVITY ASSAYS: Venous blood was collected using polystyrene syringes and anticoagulated with 10.9 mM tri-sodium citrate. The ratio of blood to anticoagulant was 9:1. Platelet rich plasma (PRP) was prepared by centrifuging the blood at 200 x g for 10 minutes at room temperature, while platelet poor plasma (PPP) was prepared by centrifuging at 2 000 x g for 10 minutes.

Platelet aggregation response was measured as described by Born (1970).. In the control assay the platelet count of the PRP was adjusted to 200 x 10^9/l with an appropriate volume of PPP. In the test assays the platelet count of the PRP was adjusted by adding an appropriate volume of the supernatants. The volume of the PRP in the cuvettes was 450 µl and 50 µl of 20 µM ADP or 0.05 g/l collagen was added per cuvette. Platelet aggregation was measured turbidimetrically with a Monitor IV Plus Platelet Aggregometer at 37°C and stirring was performed at 1000 r.p.m. The platelets were stirred continuously for 10 minutes and aggregation was monitored by measuring the change in the turbidity.

3.14 ANTITHROMBIN ASSAY: The thrombin time assay as described by Chanarin (1989) was used to test for antithrombin activity. This test measures the time taken for the formation of a fibrin clot by the action of thrombin on fibrinogen. The control thrombin time is 25-30 seconds. 0.1 ml plasma was mixed with 0.1 ml sample at 37°C. 0.1 ml diluted thrombin was added and the time taken for the formation of a visible clot was recorded.

3.15 AMIDOLYTIC ASSAY OF THROMBIN ACTIVITY: Thrombin assays were performed at 30°C as described by Dodt *et al* (1990). The reactions were done in 0.1 M Tris-HCl, pH 8.3, containing 0.2 M NaCl and 0.05% Triton-X with 1 nM enzyme and 125 µg Tos-Gly-Pro-Arg-*p*-nitroanilide (Chromozyme TH) to a total volume of 1 ml. The reaction was followed over 60 minutes at 405 nm either in a Beckman DU 7500 spectrophotometer or in scaled down form in an Anthos microplate reader.

3.16 SDS-PAGE ANALYSIS: A modified method of Laemmli (1970) was used to analyze the extracellular proteins. The proteins were separated on 20% SDS-PAGE gels, containing 10% glycerol. The presence of the glycerol leads to retardation of peptide migration and it also decreases diffusion. Additionally, the pH of the separating gel buffer of this system is 9.3, compared to 8.8 of the traditional system. The increased pH is probably responsible for slowing down the peptides relative to the dye front (Giulian and Graham, 1990). 100 µl of the supernatants of the transformed yeast cultures were loaded on 20% SDS-PAGE gels. After the gels were run at 100V for 16 hours, the proteins were visualized by silver staining either with a Quick-silver kit (Amersham) or with the traditional method of Merrel *et al* (1983).

3.17 TOTAL RNA ISOLATION: The hot phenol method of Köhrer and Domdey (1991) was used for the isolation of total RNA. Approximately one gram of yeast cells was broken by means of vigorous shaking for 4 minutes in phenol at 65°C. A second phenol step was followed by vortexing in phenol/chloroform. The aqueous phase was further purified by a chloroform extraction, after which the purified total RNA was ethanol precipitated. The RNA concentration was quantified by spectrophotometric determinations and the quality of the isolated RNA was tested by agarose electrophoresis. All buffers were DEPC treated and glassware were baked overnight at 160°C.

3.18 NORTHERN ANALYSIS: Total RNA was used in Northern blotting according to the method of Perbal (1988). Denaturation of RNA samples (10 µg) was performed by the addition of formaldehyde and deionized formamide. The RNA was subsequently separated on denaturing gels containing formaldehyde. The gels were run for approximately 4 hours at 60 V and washed several times in DEPC treated water to remove any remaining formaldehyde from the gels. The RNA was subsequently transferred to Hybond N membranes using capillary transfer. After blotting for 4 hours at room temperature, the RNA was fixed to the membrane by baking at 80°C for 2 hours. Hybridizations were performed using the non-radioactive DIG system (Boehringer Mannheim) and the probe was labelled using asymmetric PCR. Detection of all Northern blots was done with AMPPD, a chemilluminescent substrate for alkaline phosphatase.

3.19 PCR-LABELLING: Asymmetric PCR-labelling with DIG-dUTPs was accomplished by using 1 μg of primer SAG1 and 10 μg of primer SAG2. Denaturation was performed at 96°C for one minute and labelling was achieved at 72°C for one minute. Twenty cycles were done and the labelled PCR-product was purified with two chloroform extractions, followed by ethanol precipitation. Preferential labelling of SAG2 was verified by dot blot hybridization.

3.20 EXPRESSION IN *E. coli*: Gene expression in *E. coli* was performed using the Qiaexpress system. The synthetic anticoagulant gene was cloned into the *E. coli* expression vector pQE-32 and transformed to strain M15. M15 contains another vector, pREP4, containing the *lacI* gene, encoding the *lac* repressor. To aid purification, the promoter region is followed by a histidine tag containing six consecutive histidine residues. The histidine residues have a high affinity for Ni-ions attached to the NTA resin. A convenient cloning site is fused directly downstream of the histidine tag. Since the M15 contains two plasmids, cultures had to be grown in Bacto LB-medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract and 5 g NaCl per liter), containing both ampicillin and kanamycin. Gene expression was rapidly induced by the addition of 2 mM IPTG and 1 ml samples were taken at time intervals. Cells were harvested and lysed by vortexing in the presence of 8 M urea, 0.1 M Na-phosphate and 0.01 M Tris-HCl (pH 8.0). The cell debris was spun down and the supernatant was added to Ni-NTA resin. Binding was obtained by gentle mixing for 30 minutes at room temperature. The resin was subsequently spun down and 10 μl of the supernatant of each sample was stored on ice. The resin was washed several times in 8 M urea, 0.1 M Na-phosphate and 0.01 M Tris-HCl (pH 6.3) to remove all unbound protein. Following washing, the protein was eluted by adding 20 μl of the washing buffer, containing 100mM EDTA. The EDTA chelates the Ni-ions from the resin and the protein was eluted. The eluted proteins were separated from the Ni-NTA resin by centrifugation and analysed on SDS-PAGE gels.

CHAPTER 4

RESULTS AND DISCUSSION

The amino acid sequence of the peptide as designed was reverse translated to nucleic acid sequence, keeping the optimal codon use for yeast in mind (Fig. 4.1). The choice of codons was such that those used preferentially in highly expressed genes were incorporated (Sharp *et al*, 1988). Although non-optimal codon use does not seem to be a problem for high expression of a foreign gene in yeast, we wanted to eliminate codon bias as a source of problems. The 3'-end of the gene contains an additional *XhoI* restriction site. As a first attempt, gene synthesis was done according to the method of Ausubel *et al* (1990), using modified T7 DNA polymerase (Sequenase) to fill in protruding single strands. The obtained DNA was run on a 4% Nusieve gel (Fig. 4.2). SAG2 (Lane 3) revealed some extent of secondary structure and gene synthesis appeared to have produced strands of heterogeneous length (Lane 5). This could be due to inadequate time allowed for elongation or because of a range of different products formed as a result of the secondary structure of SAG2. The reaction products were ligated into the *StuI* site of pMFα8, which would fuse the peptide directly downstream of a *KEX2* cleavage site. No clones containing the gene were found after transforming *E. coli* with these ligation products.

Alternatively, gene synthesis was performed using Taq polymerase and PCR. Following gene synthesis, the DNA was run on a 15% PAGE gel and silver stained. The gel (Fig. 4.3) revealed that Taq polymerase resulted in a more distinct band (Lane 2), than Sequenase (Lane 1). The optimized PCR reaction resulted in a distinct band of the correct size on a 4% Nusieve gel (Fig. 4.4, Lane 2). Although PCR artifacts were still produced, the band could be recovered and purified from the gel for cloning purposes.

The purified PCR products were ligated into *StuI* digested pMFα8 and used for *E. coli* transformation (Fig 4.5). Colony hybridization revealed the positive clones, which were subsequently sequenced to determine the orientation and fidelity of the vector-insert junctions. One clone (pSAG1) which met all the relevant criteria was isolated at large scale and used for further work.

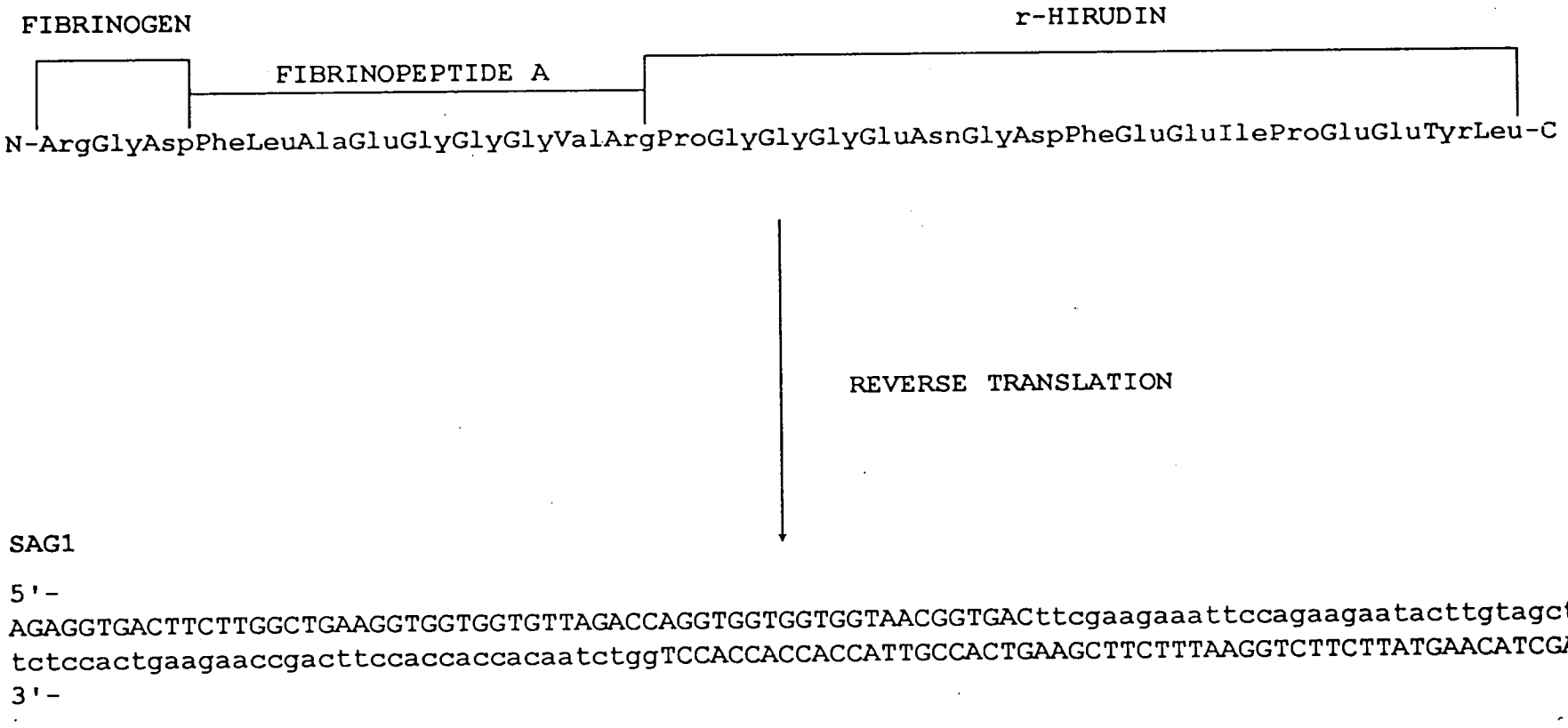

Fig. 4.1 Schematic representation of gene design and construction from SAG1 and SAG2 (two 60-mers) overlapping 22 bases.

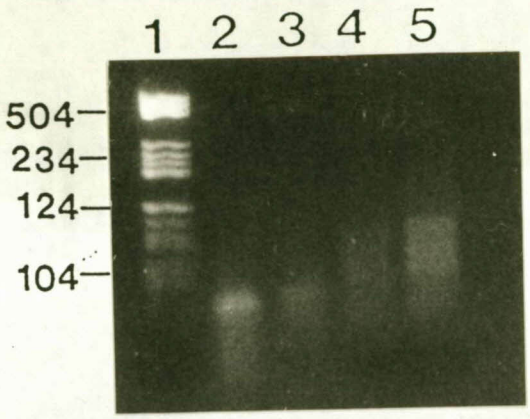

Fig. 4.2 Electrophoresis of reaction products on a 4% Nusieve gel. Modified T7 DNA polymerase (Sequenase) were used to fill in recessed ends.
Lane 1: Molecular weight markers, indicated in base pairs.
Lane 2: 2 μg of SAG1.
Lane 3. 2 μg of SAG2.
Lane 4: Following annealing of the two strands, before elongation.
Lane 5: The synthesized gene with the approximate size of 98 base pairs.

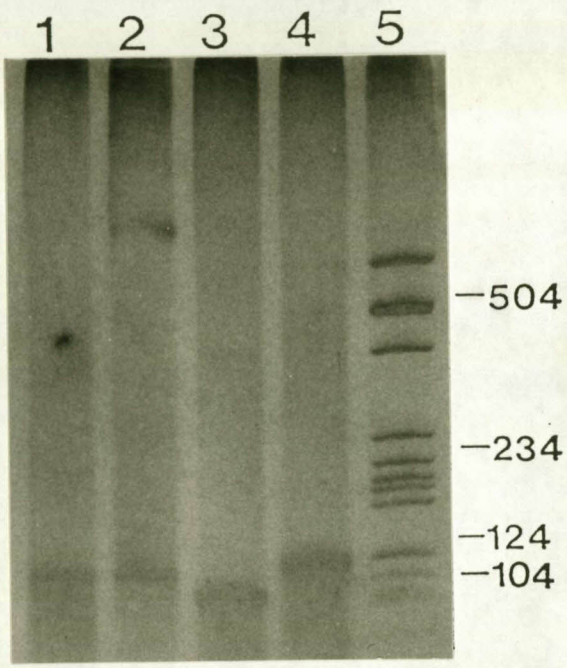

Fig. 4.3 Electrophoresis of reaction products on a 15% PAGE gel.
Lane 1: Synthesized gene, using Sequenase.
Lane 2: Synthesized gene, using Taq polymerase and 20 PCR cycles.
Lane 3. 1 μg of SAG1.
Lane 4: 1 μg of SAG2.
Lane 5: Molecular weight markers, indicated in base pairs.

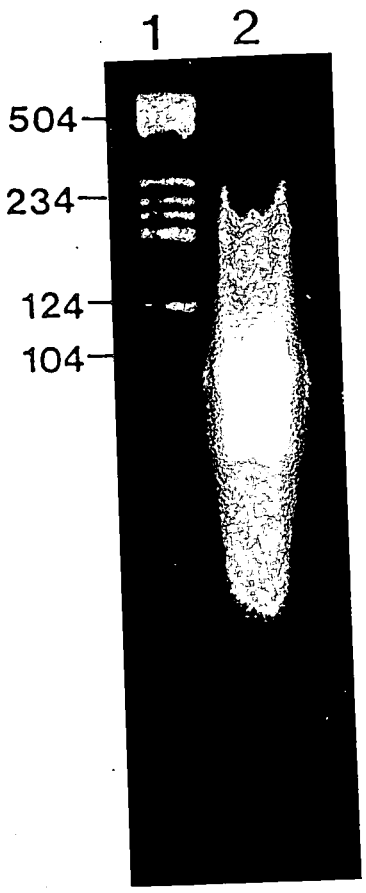

Fig. 4.4 Electrophoresis of gene synthesis on a 4% Nusieve gel, using optimal PCR conditions.

Lane 1: Molecular weight markers, indicated in base pairs.

Lane 2: The synthesized gene of 98 base pairs.

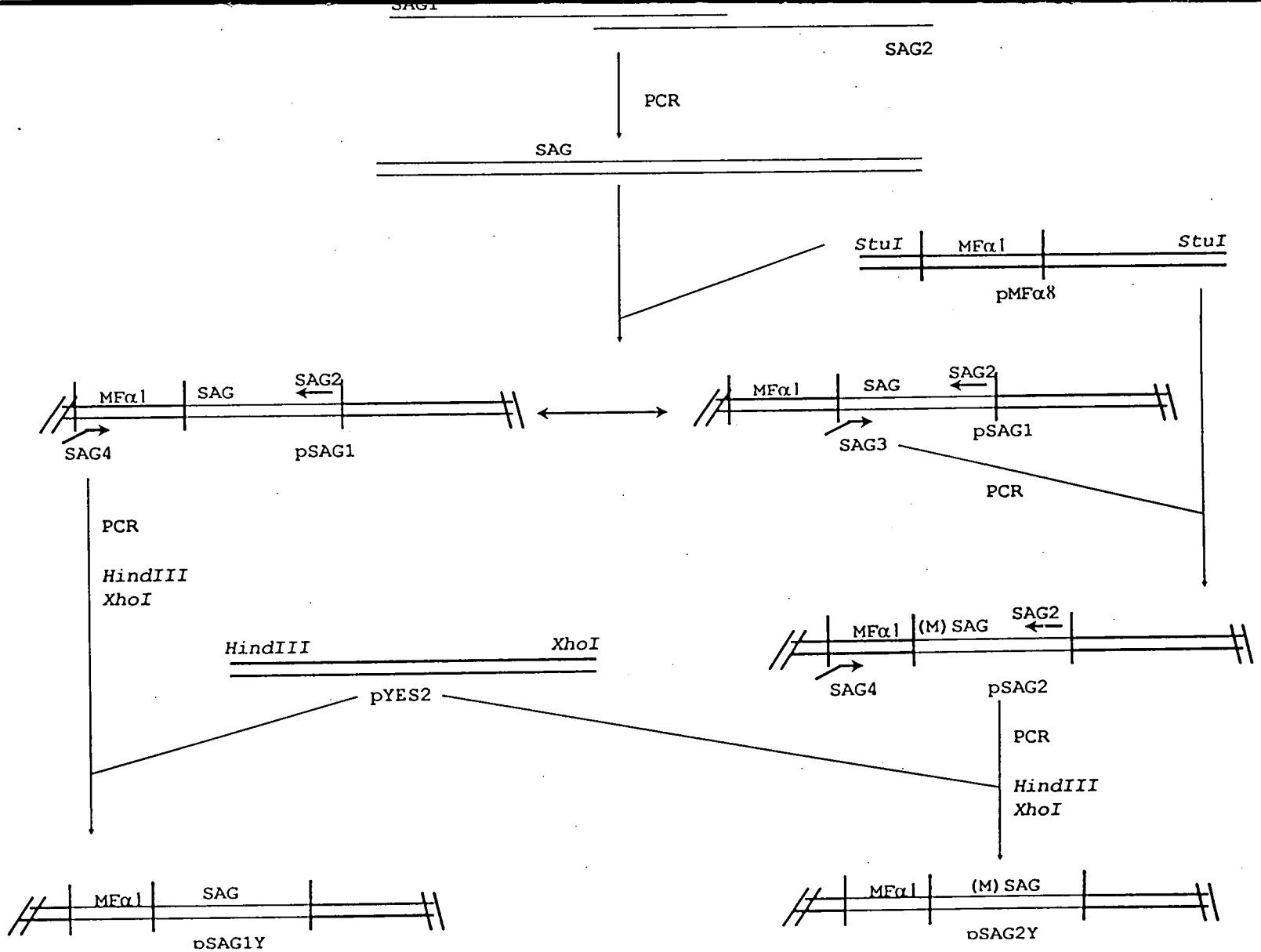

Fig. 4.5 The cloning strategy followed for extracelluar expression by yeast, using pMFα8 and pYES2 as expression vectors.

S. cerevisiae DBY 746 was transformed with pSAG1. To serve as a control, the same strain was transformed with pMFα8. Cells were grown until they reached late logarithmic phase and harvested by centrifugation. The supernatant was subsequently analyzed for anticoagulant and antithrombin properties, but no such activities could be detected (Fig 4.6). Very small amounts of hirudin was successful in inhibiting the activity of thrombin against Chromozyme TH. It was expected that SAG should show thrombin inhibition due to its binding to the active site or to the anion-binding exosite. When the supernatants were tested in a platelet aggregation assay and a thrombin time test, no difference could be seen between untransformed and transformed yeast (results not shown).

Aliquots of the supernatants were also analyzed on 20% SDS-PAGE gels. No difference in extracellular protein composition could be seen among strains transformed with pSAG1, pMFα8 and untransformed supernatants (results not shown, but similar to Fig 4.8). The N-terminal amino acid of SAG is arginine. According to the N-end rule (Varshavsky, 1992), peptides with an N-terminal arginine are rapidly degraded in yeast. Another PCR primer (SAG3) was designed and used to add a methionine in front of the arginine (Fig 4.5).

In order to further minimize the effect of proteases on peptide production, two protease-deficient *S. cerevisiae* strains, 20B-12 and BJ 5457, were transformed with pSAG1 and pSAG2. Transformants were cultivated as described and the same enzyme activity assays and SDS-PAGE analysis were performed with the supernatant. Although these strains are defective in producing certain proteases, they were still unable to produce both variants of the anticoagulant peptide at detectable concentrations.

Miyajima *et al* (1985) utilized the pMFα8 expression vector for the production of mouse interleukin-2 by *S. cerevisiae*. Mature mouse interleukin-2 was secreted extracellularly at a relatively low concentration (10 μg/l). Chen *et al* (1993) evaluated *MFα1* promoter-directed secretion of a small protease inhibitor, elafin, in *S. cerevisiae* batch fermentation. Elafin was produced at a concentration of 120 μg/l. In contrast, Brake *et al* (1984) used essentially the same *MFα1* promoter and leader sequence to secrete biologically active mature human epidermal growth factor at a much higher concentration (5 mg/ml). It is thus tempting to speculate that the sequence fused to the α-factor leader could influence translation efficiency.

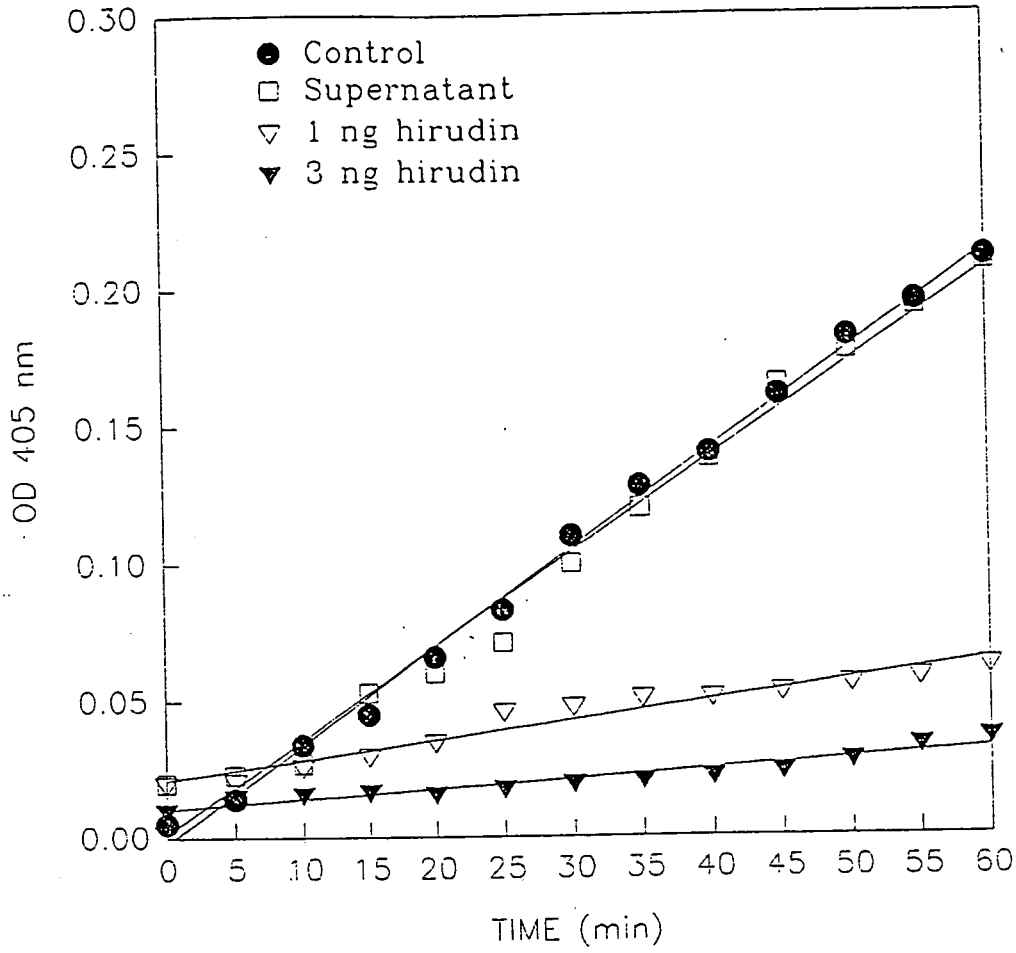

Fig. 4.6 Graphic representation of the amidolytic thrombin inhibition assay. All assays were performed in duplicate. Although the supernatants were concentrated 10-fold, no detectable inhibition of the amidolytic activity of thrombin could be shown.

We wanted to investigate the mRNA levels attained for our constructs and decided on non-radioactive Northern hybridization analysis. Total RNA was isolated as described by Köhrer and Domdey (1991). The Northern blot revealed a band of approximately 900 base pairs (Fig. 4.7). The band consists of the mRNA of the anticoagulant gene fused to that of the α-factor (Lane 1). Lane 2 reveals no band where total RNA, isolated from the cells transformed with pMFα8, was run. Although a band of the correct size was visible, it was very faint and we decided to evaluate another expression vector with a regulatable promoter.

The vector pYES2, containing the *GAL1* promoter, was employed in producing the anticoagulant peptide. It does not contain any leader sequence to secrete the recombinant peptide. To overcome this problem, PCR was used to amplify the leader sequence of *MFα1* from pMFα8, using primers SAG2 and SAG4 (Fig 4.5). The PCR products were ligated into *HindIII* and *XhoI* digested pYES2. Positive clones were identified by colony hybridization and were sequenced. Both variants were cloned into yeast and tested for expression in different yeast strains. In order to circumvent glucose repression of the *GAL1* promoter the yeasts were grown on glycerol as carbon source. The *GAL1* promoter was induced by adding galactose to the medium and samples were taken as a function of time.

SDS-PAGE analysis was done on 100 μl of supernatants from the time samples. Time 0 and 60 minutes samples were run next to each other for each construct and strain in order to facilitate comparison between induced and non-induced protein profiles. Fig 4.8 is representative of the results obtained in all cases. To investigate the possibility of the peptide not being secreted efficiently, we also ran samples of intracellular proteins on SDS-PAGE gels. Fig. 4.9 reveals that no detectable peptide was produced intracellularly by the induced cells (Lanes 1,3,5,7), in comparison with the uninduced control cells (Lanes 2,4,6,8). The peptide band could be masked by all the other proteins present, but the various tests showed no activity attributable to the SAG peptide (results not shown).

The absence of detectable levels of the peptide could be due to failure of the *GAL1* promoter to initiate a high level of mRNA synthesis. Therefore, the efficiency of induction of transcription was tested by Northern hybridization, using the same DIG labelled probe as in Fig. 4.7. Fig. 4.10 shows strong induction of the *GAL1* promoter after 30 minutes in galactose. In some lanes, e.g. lane 10, a doublet can be seen which is due to heterogeneity of the initiation site of the *GAL1* promoter (Yocum *et al*, 1984).

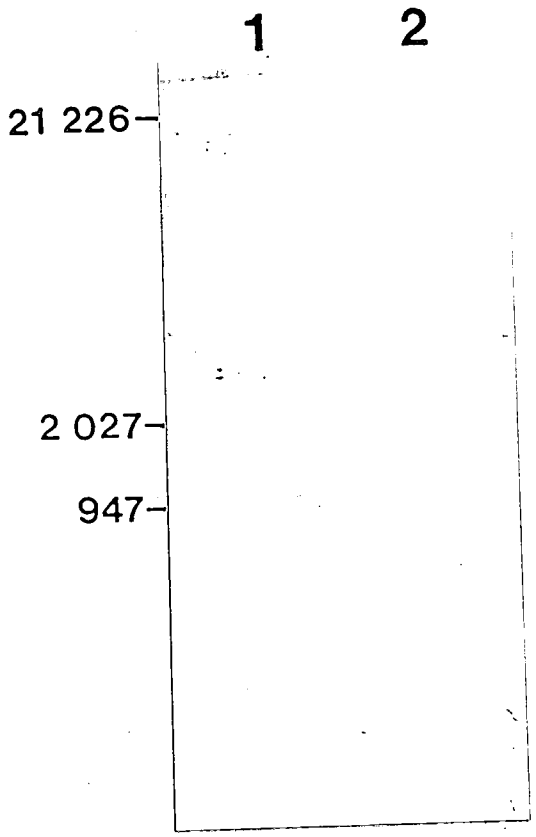

Fig. 4.7 Northern blot analysis of total RNA isolated from *S. cerevisiae* 20B-12.
Lane 2: Total RNA isolated from yeast transformed with pSAG1M.
Lane 3: Total RNA isolated from yeast transformed with pMFα8.

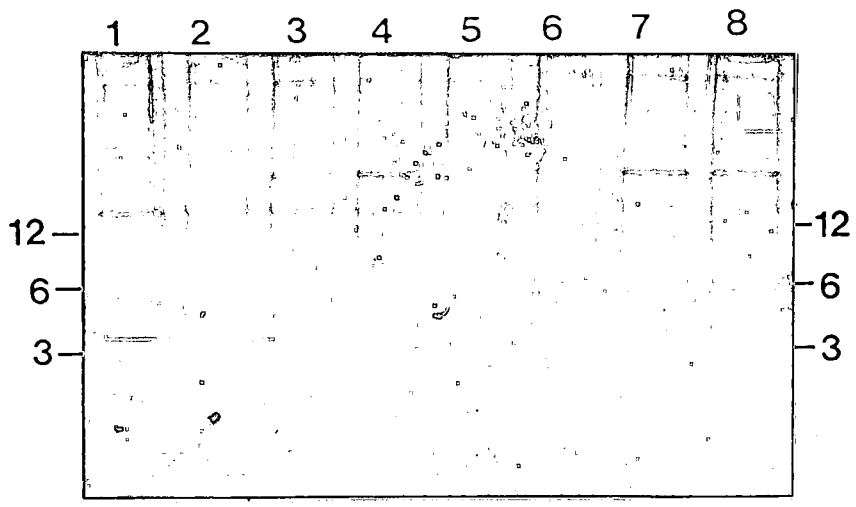

Fig. 4.8 SDS-PAGE analysis of induced peptide production, using pSAG1Yand pSAG2Y as expression vectors. 100 µl of supernatant samples taken at zero time and 60 minutes following galactose induction. Molecular weights are indicated in kDa.

Lane 1: Time 0', *S. cerevisiae* BJ 5457 - pSAG1Y.
Lane 2: Time 60', *S. cerevisiae* BJ 5457 - pSAG1Y.
Lane 3: Time 0', *S. cerevisiae* BJ 5457 - pSAG2Y.
Lane 4: Time 60', *S. cerevisiae* BJ 5457 - pSAG2Y.
Lane 5: Time 0', *S. cerevisiae* BJ 5460 - pYES2.
Lane 6: Time 60', *S. cerevisiae* BJ 5460 - pYES2.
Lane 7: Time 0', *S. cerevisiae* BJ 5460 - pSAG1Y.
Lane 8: Time 60', *S. cerevisiae* BJ 5460 - pSAG1Y.

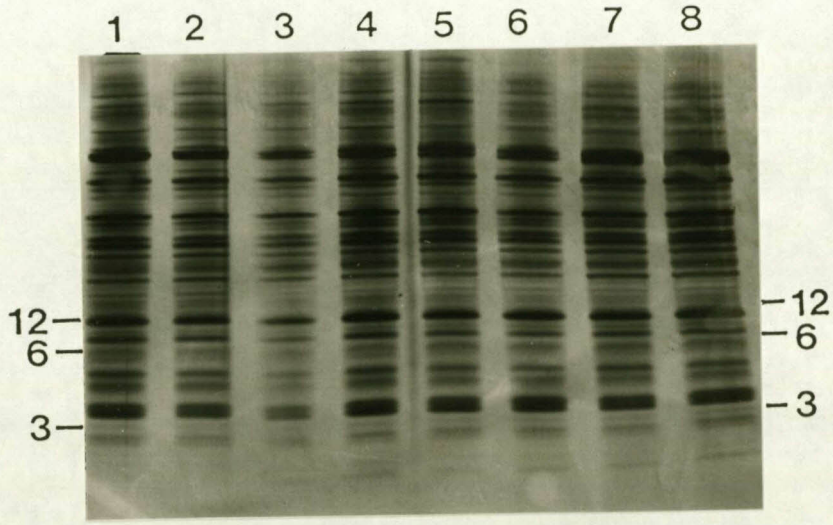

Fig. 4.9 SDS-PAGE analysis of induced peptide production, using pSAG1Y and pSAG2Y as expression vectors. 100 µl of the harvested cells were disrupted and the intracellular proteins of samples, taken at zero time and 60 minutes following galactose induction, were analyzed. Molecular weights are indicated in kDa.

Lane 1: Time 0', *S. cerevisiae* BJ 5457 - pSAG1Y.
Lane 2: Time 60', *S. cerevisiae* BJ 5457 - pSAG1Y.
Lane 3: Time 0', *S. cerevisiae* BJ 5457 - pSAG2Y.
Lane 4: Time 60', *S. cerevisiae* BJ 5457 - pSAG2Y.
Lane 5: Time 0', *S. cerevisiae* BJ 5460 - pYES2.
Lane 6: Time 60', *S. cerevisiae* BJ 5460 - pYES2.
Lane 7: Time 0', *S. cerevisiae* BJ 5460 - pSAG1Y.
Lane 8: Time 60', *S. cerevisiae* BJ 5460 - pSAG1Y.

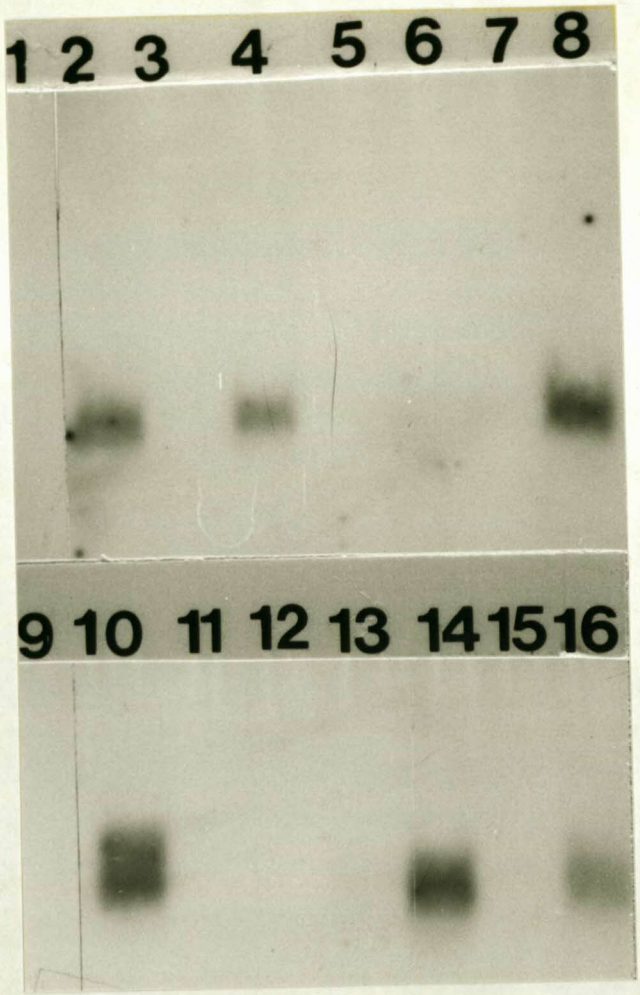

Fig. 4.10 Northern blot analysis of total RNA isolated from cells induced with galactose. Samples taken at time zero minutes and 30 minutes, following galactose induction, were utilized in Northern blot analysis.

Lane 1: Time 0', *S. cerevisiae* BJ 5457 - pSAG1Y.
Lane 2: Time 30', *S. cerevisiae* BJ 5457 - pSAG1Y.
Lane 3: Time 0', *S. cerevisiae* BJ 5457 - pSAG2Y.
Lane 4: Time 30', *S. cerevisiae* BJ 5457 - pSAG2Y.
Lane 5: Time 0', *S. cerevisiae* BJ 5460 - pYES2.
Lane 6: Time 30', *S. cerevisiae* BJ 5460 - pYES2.
Lane 7: Time 0', *S. cerevisiae* BJ 5460 - pSAG1Y.
Lane 8: Time 30', *S. cerevisiae* BJ 5460 - pSAG1Y.
Lane 9: Time 0', *S. cerevisiae* BJ 5460 - pSAG2Y.
Lane 10: Time 30', *S. cerevisiae* BJ 5460 - pSAG2Y.
Lane 11: Time 0', *S. cerevisiae* AB 1380 - pYES2.
Lane 12: Time 30', *S. cerevisiae* AB 1380 - pYES2.
Lane 13: Time 0', *S. cerevisiae* AB 1380 - pSAG1Y.
Lane 14: Time 30', *S. cerevisiae* AB 1380 - pSAG1Y.
Lane 15: Time 0', *S. cerevisiae* AB 1380 - pSAG2Y.
Lane 16: Time 30', *S. cerevisiae* AB 1380 - pSAG2Y.

In this laboratory successful expression of various proteins, e.g. a lipase from *Geothrichum candidum*, has been achieved in yeast. In this case better yield was achieved with pMFα8 than with pYES2, while the protease-deficient strains gave higher levels of active extracellular enzyme. Neeper *et al* (1990) expressed rTAP at a concentration of 0.1-0.15 g/l by yeast, using a very similar construct.

The failure to produce a detectable amount of the peptide in yeast is not unique. Three examples of such proteins were encountered in the literature. Ammerer (1983) reported high expression of human α-interferon and undetectable levels for hepatitis B surface antigen, bovine parathyriod hormone and rat growth hormone. Stepien *et al* (1983) were unable to detect proinsulin synthesis driven by the *ADH1* promoter, while Tuite (1991) mentioned the case of the *Drosophila hsp70* gene. In all three cases no single cause could be identified unambiquously, the best possibilities being mRNA stability, mRNA processing, translational efficiency of the mRNA, stability of the protein and/or posttranslational modifications. These recalcitrant proteins clearly identify weaknesses in our understanding of the complete mechanism of gene expression in yeast.

Having no success using yeast as expression system, we decided to explore the possibilities of *E. coli*. An aliquot of PCR synthesized gene was cloned into the *SmaI* site of the cloning vector pUCBM21 and the resultant plasmid was designated pSAG1U. The correct insert was verified by sequencing and the fragment was recovered by PCR amplification using primers For and Rev, which flanks the multiple cloning site. After digestion it was ligated into the *BamHI* and *SalI* sites of the *E. coli* expression vector pQE-32 (Fig. 4.11). Expression from this plasmid would result in the addition of 20 amino acids to the N-terminus, effectively doubling the size of the anticoagulant peptide. In extending the peptide, the risk was taken that one or more of its functions could have been interfered with. However, since nothing was changed at the C-terminus, we still expected antithrombin activity.

Intracellular gene expression in *E. coli* was performed using the Qiaexpress system where gene expression is under tight control of a regulatable *E. coli* phage T5 promoter. Transformants were grown overnight in medium containing ampicillin and kanamycin. After induction with IPTG, samples were taken and spun down. The eluted proteins were loaded onto a 20% SDS-PAGE gel. The results presented in Fig. 4.12 testify to the efficiency of purification by histidine affinity chromatography. Very few proteins bound to the Ni-NTA resin, but no extra band of approximately 6 kDa was visible after 5 hours of induction. An untransformed *E. coli* control (results

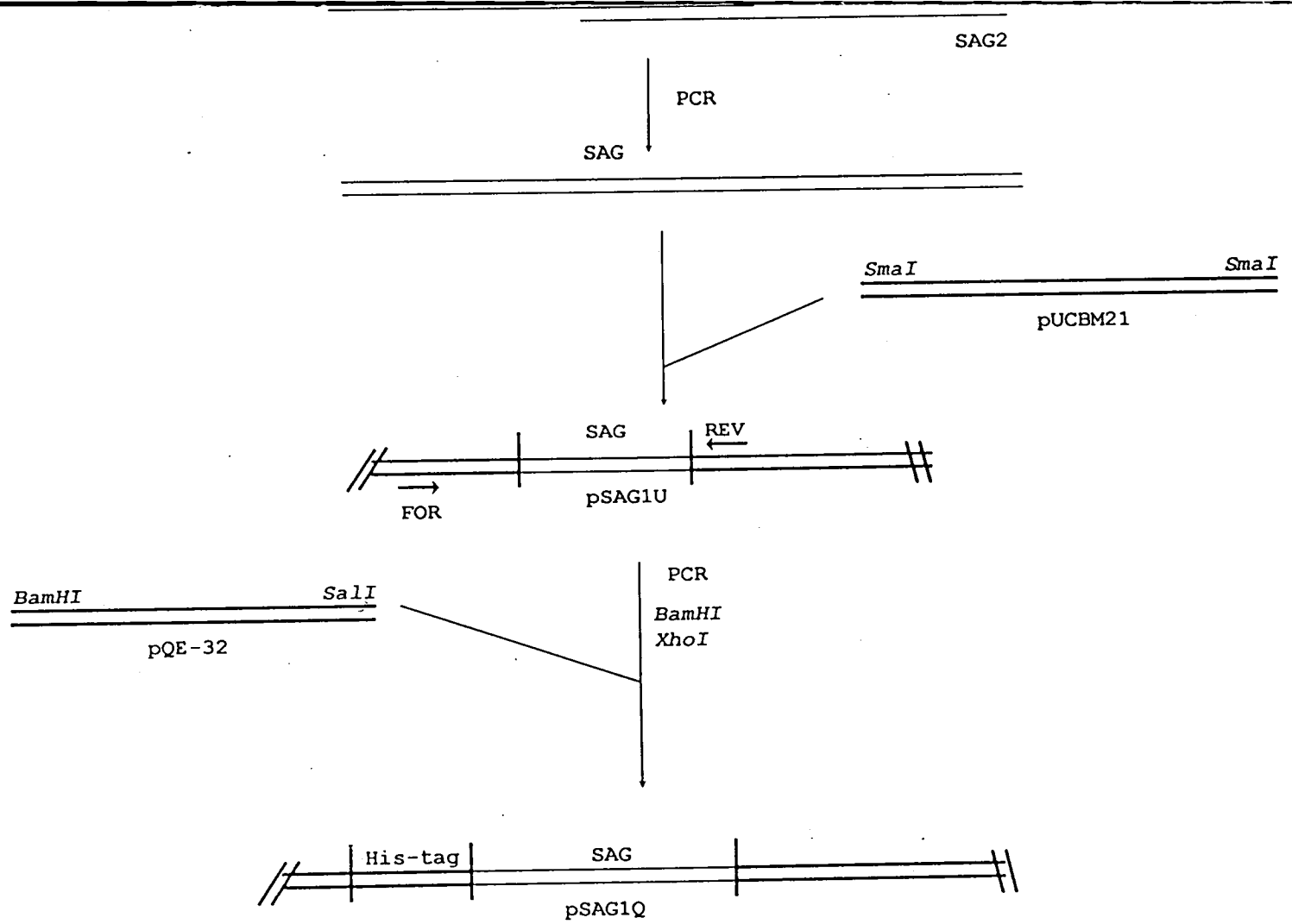

Fig. 4.11 The cloning strategy followed for intracellular expression in *E. coli*, using pQE-32 as expression vector.

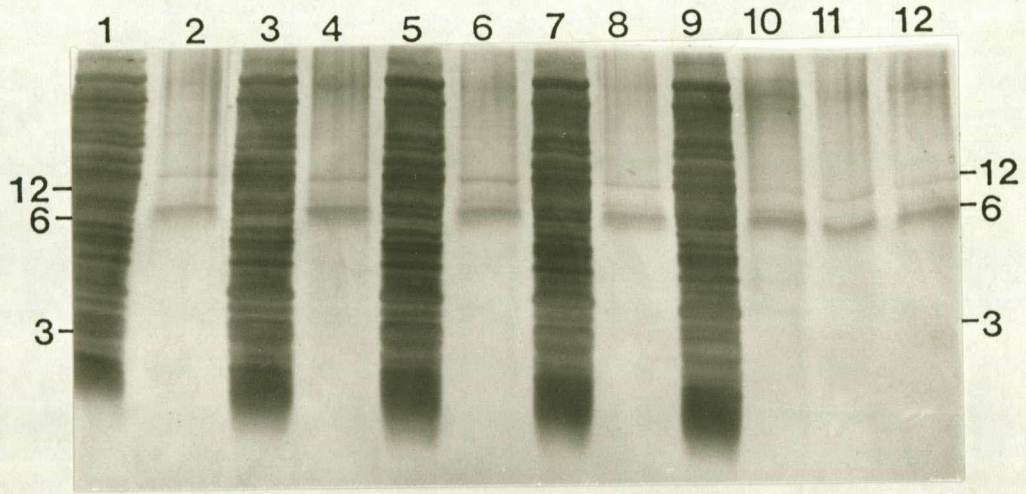

Fig. 4.12 SDS-PAGE analysis of intracellular *E. coli* proteins. Cells were induced with 2 mM IPTG and samples were taken at one hour intervals. The cells were lysed as described in Materials and Methods. Molecular weights are indicated in kDa.

Lane 1: Time 1h, supernatant of *E. coli* M15 - pSAG1Q.
Lane 2: Time 1h, His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 3: Time 2h, supernatant of *E. coli* M15 - pSAG1Q.
Lane 4: Time 2h, His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 5: Time 3h, supernatant of *E. coli* M15 - pSAG1Q.
Lane 6: Time 3h, His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 7: Time 4h, supernatant of *E. coli* M15 - pSAG1Q.
Lane 8: Time 4h, His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 9: Time 5h, supernatant of *E. coli* M15 - pSAG1Q.
Lane 10: Time 5h, His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 11: Time 0h, His-tag proteins of *E. coli* M15 - pQE-32.
Lane 12: Time 5h, His-tag proteins of *E. coli* M15 - pQE-32.

not shown) showed the same profile, eliminating the possibility of a leaky promoter leading to constitutive synthesis of the band of about 4-6 kDa visible in all lanes. We were curious as to the identity of the proteins that bound to the Ni-NTA resin. A computer search was conducted for *E. coli* proteins containing four or more consecutive histidines. The only protein containing seven histidines is the attenuator peptide synthesized from a short open reading frame upstream of the His-operon (Verde *et al*, 1981; Carlomagno *et al*, 1988). It is synthesized constitutively and would thus appear in all lanes. It is also a contaminant of all proteins purified from *E. coli* lysates by Ni-NTA affinity chromatography. In most cases it would not be a problem, but it is possible that it may interfere with further analyses in some way.

Samples were also taken at shorter time intervals following induction. Cells were removed every five minutes and extracted as described. The protein was run on a 20% SDS-PAGE gel and silver stained. Lanes 4 and 5 (Fig. 4.13) contain proteins extracted from cells ten and fifteen minutes after induction, respectively, and reveals a band of the correct size. However, untransformed *E. coli* control cells showed the same band (results not shown). We thus concluded that we were unsuccessful at producing the multifunctional peptide in yeast or *E. coli* with the plasmids and expression strategies employed.

E. coli is known for the rapid degradation of small peptides (Miller, 1987). Since an exact minimum size has not yet been determined, we hoped that this fusion construct would be more stable than the peptide alone. The absence of the peptide from the *E. coli* extracts could mean that the fusion was still too small for stability. Fusing the peptide with a cleavable linker to a larger carrier protein could be a solution in this case.

Two problems emerged from this study, namely our inability to synthesize the anticoagulant peptide at detectable levels and uncertainty about its activity. The first problem could be resolved by having the peptide synthesized chemically and testing it in the various assays. Transgenic expression of the peptide could be undertaken by using fusion constructs in *E. coli* or by testing *Aspergillus niger,* an expression system successfully in use in our laboratory.

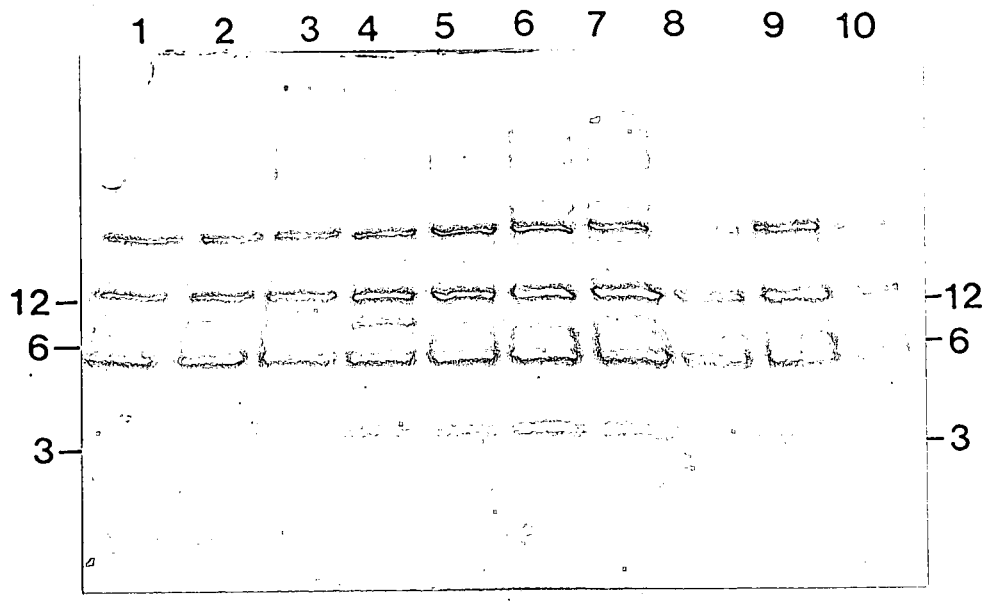

Fig. 4.13 SDS-PAGE analysis of intracellular *E. coli* proteins. IPTG induction was performed on untransformed *E. coli* M15 cells, *E. coli* M15 transformed with pQE-32 and *E. coli* M15 transformed with pSAG1Q. Molecular weights are indicated in kDa.

Lane 1: Time 0', His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 2: Time 5', His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 3: Time 10', His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 4: Time 15', His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 5: Time 20', His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 6: Time 25', His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 7: Time 30', His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 8: Time 35', His-tag proteins of *E. coli* M15 - pSAG1Q.
Lane 9: Time 35', His-tag proteins of *E. coli* M15 - pQE-32.
Lane 10: Time 35', His-tag proteins of *E. coli* M15.

This study was successful as an exercise in the design of a novel peptide and getting the gene expressed up to the level of mRNA. Choices had to be made regarding vectors and hosts for expression and it may be argued that some choices were better than others. Having gone through the learning curve we at least have a much better idea of which expression strategies to use in future.

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SUMMARY

Platelets and coagulation both play a pivotal role in thrombosis, one of the major causes of death in Western society. It is therefore not surprising that potent inhibitors are being developed to inhibit platelet function or coagulation. In this study we developed a multifunctional peptide that would not only inhibit thrombin, but will also prevent platelet aggregation. A 29 amino acid peptide was designed comprising three inhibitory regions: 1, a part of hirudin, a potent direct antithrombin; 2, fibrinopeptide A, also an inhibitor of thrombin and 3, an amino acid sequence (arg-gly-asp) essential for binding to the fibrinogen receptor on platelets to prevent fibrinogen binding.

The protein sequence was reverse translated and two 60-mers with an overlap of 22 base pairs were synthesized. After filling in the ends, the gene was cloned into a yeast expression and secretion vector, pMFα8. The construct was sequenced to verify correct orientation of the gene and transformed into yeast. The expected peptide of approximately 3 kDa could not be seen on SDS-PAGE gels, nor could inhibition of thrombin or platelet aggregation be shown. However, Northern hybridization analysis revealed the presence of the mRNA. The introduction of a methionine residue at the N-terminus of the peptide and the use of protease-deficient yeast strains as expression hosts, did not improve peptide production.

Another expression system, the inducible yeast expression vector pYES2, was then employed. Although no peptide could be detected, Northern blotting showed the presence of high levels of the mRNA. Optimal codons for expression in yeast were selected in the design of the gene, but the absence of a detectable level of peptide could be due to a low translation rate or due to proteolysis. Alternatively, the gene was cloned into a regulatable *E. coli* vector, pQE-32, and expressed intracellularly in *E. coli*. No band of the correct size could be detected on SDS-PAGE gels, which might be due to the extremely small molecular size of the peptide.

ABBREVIATIONS

ABE - Anion-binding exosite
ADP - Adenosine diphosphate
AMPPD - 3-(2'-Spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetane
ATU - Antithrombin unit
DEPC - Diethyl pyrocarbonate
DIG - Digoxigenin
EDTA - Ethylene diamine tetra-acid
ER - Endoplasmic reticulum
Gp - Glycoprotein
HV - Hirudin variant
IPTG - Isopropyl-β-D-thiogalactopyranoside
LAPP - Leech antiplatelet protein
LMWHs - Low molecular weight heparins
(M)SAG - Synthetic anticoagulant gene, containing an ATG codon at its 5'-end
NMR - Nuclear magnetic resonance
NTA - Nitrilo-tri-acetic acid
PAGE - Polyacrylamide gel electrophoresis
PC - Protein C
PCR - Polymerase chain reaction
PPACK - D-Phenyl-L-Prolyl-L-Argenyl-chloromethylketone
PPP - Platelet poor plasma
PRP - Platelet rich plasma
r.p.m. - Revolutions per minute
RER - Rough endoplasmic reticulum
RGD - Arginine-Glycine-Aspartic acid
RGDS - Arginine-Glycine-Aspartic acid-Serine
rTAP - Recombinant tick anticoagulant peptide
SAG - Synthetic anticoagulant gene
SDS - Sodium docecyl sulphate
SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRPs - Signal recognition particle
TAP - Tick anticoagulant peptide
UFH - Unfractionated heparin
vWF - von Willebrand factor