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Disintegrin Purification and Characterization by Biological Activity and Specificity

by

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ABSTRACT

Platelets play a critical role in haemostasis both in the cell and factor mediated events. Platelets serve to initiate clot formation and are often involved in pathogenic states of haemostasis. As such, platelets are becoming targets for treatment of cardiovascular disease.

Disintegrins are proteins from animal venom that prevent the interaction of platelets and fibrinogen, preventing platelet aggregates. Disintegrins vary in their specificity for the platelet receptor-fibrinogen interaction; some disintegrins interfere with enclothelial cell-matrix interactions. The present study concentrates on two disintegrins, Mambin and Barbourin.

Mambin and Barbourin were purified from snake venoms and tested using three assay systems refined in this study. The assay systems were designed to determine disintegrin biological activity (platelet aggregation) and specificity (platelet and HUVEC adhesion). It was demonstrated that Barbourin carries a higher degree of specificity for the platelet-fibrinogen interaction than does Mambin, which confirms published data.

Recombinant Mambin and two derivatives were produced using the bacterium Bacillus subtilis, however instability of these proteins prevented further analysis.

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ABBREVIATIONS

BSA	Bovine Serum Albumin
bp	Base Pair(s)
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetate
ADP	Adenosine Diphosphate
g	Gram
HPLC	High Pressure Liquid Chromatography
HUVEC	Human Umbilical Vein Endothelial Cells
kb	Kilobase
ddH_2O	Distilled, Deionized Water
LB	Luria Bertani
l	Liter
mg	Milligram
ml	Milliliter
ul	Microliter
mM	Millimolar
uM	Micromolar
nm	Nanometer
nM	Nanomolar
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl Fluoride
rpm	Revolutions Per Minute
SDS	Sodium Dodecyl Sulfate
TFA	Trifluoroacetic Acid
Tris	Tris(hydroxymethyl)aminomethane

LITERATURE REVIEW

A. Platelets

1. Cardiovascular Disease and Platelets

Diseases of the cardiovascular and cerebrovascular systems comprise the leading causes of mortality and morbidity in the Western world. Current approaches to the treatment of vascular diseases are concentrating on prevention and on current treatment improvements. This study addresses the area of improvement to current treatments, and focuses on the role of platelets in the manifestation of vascular disease.

a) Myocardial Infarction

Platelets play a significant role in cardiovascular disease and are targets of both preventative and therapeutic approaches to thrombosis (the formation of a pathologic blood clot)(Schror, 1995). Currently, the antiplatelet agent aspirin, is used in this way for prevention of myocardial infarction, or heart attack. Myocardial infarction is a condition whereby a pathologic clot, or thrombus, occludes and stops blood flow in a coronary artery. The cardiac tissue fed by the artery becomes ischemic as oxygen is not being delivered and within a short period of time the tissue is irreversibly damaged. The current approach to treatment of myocardial infarction is to introduce a thombolytic, or clot-dissolving, agent. As a result of thrombolytic treatment, which cleaves the fibrin meshwork of the thrombus, platelets can be released. These platelets are often in an activated state, and are capable of initiating the formation of a second thrombus and potentially result in an embolus. The use of aspirin in such a

case helps prevent the activated platelet from creating this thrombus, but is not always successful (Becker, 1993).

b) Atherosclerotic Disease

Atherosclerotic disease is a condition in which a plaque is deposited on a blood vessel in response to injury to the vessel. This plaque contains lipoproteins and fibrinogen, the latter of which is a plasma glycoprotein that functions to crosslink platelets in a clot. Fibrinogen in the atherosclerotic plaque acts as a ligand for adhesion of circulating platelets. The adherent platelets may attract further platelets to this site, and eventually occlude the vessel (Sixma et al. 1995). Atherosclerotic plaques are often observed with high concentrations of platelet aggregates.

The potential clinical benefits of antiplatelet agents is indicated by the success of aspirin (Lefkovits et al. 1995). Although the efficacy of aspirin has been proven, it only effectively blocks one of several pathways that can result in activation of platelets and subsequent thrombus formation. Current research is focusing on other agents that inhibit activated platelets from interacting with one another to form a thrombus, irrespective of the method of platelet activation.

2. Biology of Platelets

Platelets are the anucleate cytoplasmic fragments of megakaryocytes. As such, they carry out all cellular functions in the absence of DNA (Ruggeri, 1994). Using proteins and cytoplasmic RNA synthesized from the parent megakaryocyte, platelets depend on cell metabolic processes.

Under normal circumstances, blood flows through the endothelium-lined veins and arteries without coagulation and

without haemorrhage. Coagulation and haemorrhage represent the two extremes of the heamostatic system, and the two are kept in balance by processes of cellular- and factor-mediated events. Platelets play a role in both processes but are most critical in the primary haemostatic response (cellular-mediated response). Platelets survey the vascular endothelial lining searching for exposed components of the subendothelium: these disruptions may include surgical wounds, abrasions, or ruptured atherosclerotic plaques. Once a vascular injury is detected, platelets enter a cascade of events which prevent haemorrhage: adhesion, activation, and aggregation.

Platelets normally circulate freely in the blood in a resting, or unactivated, state. This is characterized by a smooth disk shape and the inability of the platelets to bind many serum proteins. Platelets display glycoprotein receptors from different families on their surface. These include integrins, immunoglobulins, and selectins (Shattil et al. 1994). The most predominant are those of the integrin family of cell adhesion receptors, so termed for their ability to integrate the extracellular matrix of a cell with the cytoskeleton (D'Souza et al. 1991). This group of receptors recognize ligands through a tripeptide arginine-glycine-aspartic acid (RGD) sequence that is present on the ligand. However RGD is not of equal importance in all integrin-ligand interactions: some receptors contact their ligands at several points, not all of which are RGD, and the contribution of these contacts varies.

Integrins are non-covalently linked heterodimers of an α and a β subunit. Both subunits traverse the cell membrane, giving the integrin an extracellular domain that binds to ligands and an intracellular domain that interacts with the cytoskeleton (D'Souza et al. 1991). Different combinations of the α and β subunits

provide unique ligand specificities. The ligands for integrins include fibrinogen, fibronectin, vitronectin, and von Willebrand Factor (vWF), all of which function to mediate cell adhesion.

a) Platelet Adhesion

Platelets adhere to damaged endothelium through different interactions. Adhesion of cells in circulation to endothelium is set apart from other cell adhesions in the body, as platelets and other blood cells adhere under conditions of high shear; high shear is caused by the rapid flow of blood through the narrow vessels of the vascular system. The key interactions that platelets have with the subendothelium are through collagen, vWF, and fibronectin (Sixma et al. 1995). It is thought that this apparent redundancy is necessary to compensate for the variation in subendothelial components that a platelet may encounter (Ruggeri, 1994).

Collagen types I, III, IV, V, VI, VIII are present in the subendothelium. Platelets adhere to types I, III, and IV strongly and under conditions of high shear and to types V, VI, and VIII at lower shear rates. The adhesion of platelets to collagen is through integrin $\alpha_2\beta_1$ (Saelman et al. 1994). This integrin-ligand interaction is not dependent on an RGD motif (D'Souza et al, 1991).

Adhesion to vWF is mediated through GPIb and is not dependent on shear rates. vWF may also bind GPIIb/IIIa; the proposed function of GPIIb/IIIa is discussed later. At high shear rates, it is reported that the interaction of vWF and GPIb is responsible for the initial contact of platelets with the subendothelium (Shattil et al. 1994).

The interaction of fibronectin with platelets is currently under investigation. It had been previously thought that fibronectin bound through $\alpha_5\beta_1$ and GPIIb/IIIa (Sixma et al. 1995), however the latter has been questioned. Platelets from patients

with Glanzmann's thrombasthenia are not diminished in their capacity for adhesion to fibronectin (Nievelstein and Sixma, 1988). This condition was first noted in families with high intermarriages in Swizerland and was characterized by prolonged bleeding times (Caen and Rosa, 1995). It is now known that Glanzmann's thrombasthenia is due to dysfunctional or absent GPIIb/IIIa. Therefore, the receptor responsible for fibronectin-mediated adhesion is thought to be either $\alpha_5\beta_1$ or a yet undiscovered platelet receptor.

Although not a large component of the subendothelium, surface bound fibrinogen serves as a good ligand for platelet adhesion. Physiologically, this is only observed when fibrinogen is laid down at the site of an atherosclerotic plaque. GPIIb/IIIa is the receptor responsible for surface-bound fibrinogen binding for resting platelets (Savage and Ruggeri, 1991).

After initial adhesion, platelets have been observed to spread to cover the site of injury; this spreading lays down a monolayer of cells to prevent haemorrhaging from the damaged vessel. It has been proposed that this spreading is dependent on the interaction of vWF with GPIIb/IIIa (Joubert and Taijard, 1979).

b) Platelet Activation

Following platelet adhesion, platelets become activated. This is characterized by a change of shape from a smooth disk to a spiny sphere. This shape change may be due to a change in lipid structure that is observed upon activation in conjunction with changes in the cytoskeleton (Caiman et al. 1987). Activation also results in the release of storage granules from platelets. Platelets have two types of such granules: alpha (α) and dense. Depending on the type of stimulant used, platelets may release one or both types of granules. Dense granules contain serotonin, ADP,

ATP, pyrophosphate, and calcium and α granules contain fibrinogen, vWF, fibronectin, Factor V, and platelet-derived growth factor. Secreted components such as ADP serve to activate surrounding platelets and other secreted components such as fibrinogen provide the means for platelet-platelet adhesion. Upon activation, cytoskeletal rearrangement and an increase in cytoplasmic calcium results in the fusion of these granules with the call membrane, thus releasing the granular contents to the extracellular space.

Another consequence of platelet activation is the exposure of receptors that bind plasma proteins. These receptors are present in α granules and in the invaginations on the cell surface referred to as canalicular pits. Upon activation, the receptors previously hidden in the platelet canalicular pits and granules come to the cell surface. The ligand binding properties of the receptors are also changed upon platelet activation (Ruggeri, 1994).

As a result of the release of ADP from the activated platelets, surrounding (unactivated) platelets are activated and the platelets begin to cross-link. The activation process functions to attract and adhere more platelets from the circulating blood to the site of vascular damage. This increased platelet to platelet contact results in growth of a platelet aggregate (Caiman et al. 1987).

c) *In vitro* Platelet Agonists

Platelets may be activated by several agonists in vitro. These include ADP, thrombin, collagen, platelet-activating factor, epinephrine, and arachidonic acid (Wallace and Woodman, 1995). Although these agonists all activate platelets, they do not all do so in the same manner or to the same extent. The precise mechanisms of activation are not completely understood, however

some aspects have been elucidated. For the purposes of this study, arachidonic acid, thrombin, and ADP mediated platelet activation will be discussed.

(1) Arachidonic Acid

Arachidonic acid activates platelets via its conversion to thromboxane A_2. This metabolism consists of two steps, the first of which is catalyzed by cyclo-oxygenase. This enzyme is irreversibly inhibited through acetylation by aspirin. Thromboxane A_2 is an ionophore which facilitates calcium mobilization from storage granules to the cytoplasm. The rise in intracellular calcium, through a signal transduction pathway, effects release of granular contents to the extracellular environment. This includes both dense and α granules. As mentioned, release of contents of both types of granules results in the secondary phase of activation and aggregation through the contents of the dense (ADP and serotonin) and α granules (fibrinogen, vWF, and fibronectin). The contents of the dense granules further stimulate adjacent platelets and the contents of the α granules provide additional material for cross-linking platelets into an aggregate. Arachidonic acid is considered a 'strong' agonist.

(2) Thrombin

Thrombin is considered a 'strong' agonist for platelets: thrombin causes release of both dense and α granules and causes irreversible platelet aggregation. The precise mechanism of signal transduction involved with thrombin mediated activation is still under investigation, however, it is known that thrombin induces phosphorylation of at least thirteen proteins in platelets (Bachelot et al. 1992). It is suggested that these

phosphorylations are a result of activated protein kinase C. This pattern of phosphorylation is seen with other 'strong' agonists, such as arachidonic acid, but not with 'weak' agonists.

(3) ADP

ADP is considered a 'weak' platelet agonist: ADP stimulates platelets to a lesser extent than thrombin and arachidonic acid. Again, the precise mechanism is not fully understood, however it has been shown that ADP induces phosphorylation of only two proteins compared to the thirteen induced by thrombin (Bachelot et al. 1992). It has also been determined that aspirin has no effect on ADP-mediated stimulation of platelets (Rink and Hallam, 1984), suggesting that ADP stimulation is mediated by a pathway other than that one involving arachidonic acid. In addition, different concentration of ADP have different effects on platelets and this in turn is dependent on the extracellular calcium concentration (Born, 1962). The effects range from platelet shape change to release of dense granules.

d) Platelet Aggregation

Platelet aggregation is the result of binding of fibrinogen to platelets through the receptor GPIIb/IIIa (Hawiger, 1987). Fibrinogen can cross-link platelets through its dimeric conformation. It is composed of two α chains, two β chains, and two γ chains. The nature of the interaction of fibrinogen with platelets will be discussed in detail. Platelets aggregate as fibrinogen cross-links platelets. As platelets have many copies of the GPIIb/IIIa receptor, many fibrinogen molecules are able to bind one platelet, and in turn many platelets can be bound to each other through fibrinogen.

B. Integrin Receptors

1. Platelet GPII/IIIa and Fibrinogen

The GPIIb/IIIa is restricted to megakaryocyte cells and their platelet lineage. The receptor is present in approximately 100,000 copies per platelet (Calvete et al. 1986), 20% of which are not exposed until the platelet is stimulated. This population is proposed to reside in the surface connected canalicular system and within the α granules (Calvete, 1994).

GPIIb/IIIa is a member of the integrin family of cell adhesion receptors. It is composed of an α subunit, GPIIb, which is a dimer of a 136 kD subunit disulfide linked to a 23 kD subunit. The β subunit, GPIIIa, is a monomer of 90 kD (Bennett, 1996). Both GPIIb and GPIIIa have a cytoplasmic, transmembrane, and extracellular domain. It is speculated that GPIIb/IIIa binds several calcium ions and GPIIb contains four regions with calcium binding loops.

The physiological importance of GPIIb/IIIa is indicated by the genetic disease Glanzmann's thrombasthenia, discussed earlier. Platelets isolated from patients with this disease are unable to aggregate with all tested agonists (Lefkovits et al. 1995).

The precise interaction of fibrinogen with GPIIb/IIIa has been characterized (Bennett et al. 1988). Fibrinogen is a dimer of three chains: α, β, and γ. The α chain contains two RGD sequences and peptides corresponding to either of these regions inhibits interaction of GPIIb/IIIa with fibrinogen. The region of RGD interaction is between amino acids 109 and 171 on GPIIb (Bennett, 1996). Another site on fibrinogen, which does not contain an RGD motif, has been implicated in binding to GPIIb/IIIa; peptides corresponding to the C-terminus of the γ chain also inhibit the

GPIIb/IIIa-fibrinogen interaction. This region of the γ chain binds to GPIIb between amino acids 294 and 314.

Recent work has indicated that the C terminus of the γ chain is more important in the interaction than the RGD sites: mutation of these RGD and non-RGD sites in fibrinogen indicated that the GPIIb/IIIa interaction with fibrinogen is through the γ chain (Farrel et al. 1992). However, RGD based peptides are efficient in preventing the GPIIb/IIIa-fibrinogen interaction. It is therefore evident that this interaction is complex, requires more investigation, and may involve a combination of interactions with both the α and γ chains of fibrinogen.

GPIIb/IIIa interacts with soluble fibrinogen, but only after platelet activation. Prior to activation, GPIIb/IIIa exists in a low-affinity state for these soluble proteins. However, resting platelets are capable of binding fibrinogen that is bound to a solid surface. Using monoclonal antibodies against GPIIb/IIIa in a competitive reaction, it has been determined that surface-bound fibrinogen interacts with GPIIb/IIIa (Savage and Ruggeri, 1991). It is apparent that GPIIb/IIIa has a different ligand specificity, depending on the state of the platelet. If a platelet is resting, then it can bind to surface bound fibrinogen, but not soluble fibrinogen; if a platelet is stimulated, then it can bind to surface bound and soluble fibrinogen. It is yet not understood how GPIIb/IIIa is able to distinguish surface bound and soluble fibrinogen, but two theories exist: (1) surface bound fibrinogen is at a high local concentration, which may increase the possibility of interaction with GPIIb/IIIa; (2) the process of binding fibrinogen to a solid surface may induce conformational changes in the protein, making it more accessible for binding GPIIb/IIIa (Savage and Ruggeri, 1991).

2. Other Platelet Integrin Interactions

GPIIb/IIIa is the most studied integrin receptor to date. Other platelet integrins are more difficult to study, partially due to their low numbers relative to GPIIb/IIIa in platelets. The study of platelet integrins in general is complicated by the very nature of platelets: any protein found in a platelet may be functionally significant in two different parts of the life of the cell: (1) in megakaryocytopoiesis (the generation of the megakaryocyte cell), in which case the protein is a remnant of the parent cell; (2) in the platelet function.

GPIIb/IIIa is considered a 'promiscuous' receptor as it is capable of forming integrin-ligand interactions with several glycoproteins in addition to fibrinogen; these glycoproteins include fibronectin, vitronectin, and vWF (Shattil et al. 1994). These interactions are mutually exclusive and suggested to be through an RGD motif (Calvete, 1994).

The integrin $\alpha_v\beta_3$ is present in platelets, with 250 copies per cell (Shattil et al. 1994). As it shares the β subunit with GPIIb/IIIa, it shares specificity for fibrinogen, fibronectin, vitronectin, and vWF. This apparent redundancy has been questioned (Ginsberg et al. 1995) and one explanation is that the presence of a $\alpha_v\beta_3$ on the platelet is a residue from megakaryocytopoiesis. This is supported by the low number of $\alpha_v\beta_3$ receptors remaining on the platelet. Another suggestion is that $\alpha_v\beta_3$ may function as a means of transport of calcium signals, as this function has been attributed to α_v, (Schwartz
and Denninghoff, 1994).

$\alpha_2\beta_1$ is the integrin receptor for collagen on platelets. It is present in at least 1000 copies per cell. $\alpha_2\beta_1$ is required

for collagen induced activation and adhesion of platelets (Shattil et al. 1994).

The integrin $\alpha_5\beta_1$ is present in at least 1000 copies on the platelet and is the receptor for fibronectin. It is not believed that this integrin binds any other ligand except fibronectin.

3. Endothelial Cell Integrins

Endothelial cells line blood vessels, providing a continuous monolayer on the subendothelial extracellular matrix. The endothelial cells adhere to this matrix through several basal contacts including fibronectin, vitronectin, vWF, collagen, and laminin. A number of these contacts are a result of integrin-ligand interactions. Like platelet integrins, endothelial cell integrin interactions are mediated by the RGD motif. The RGD type interactions are attributed primarily to the $\alpha_v\beta_3$ receptor, which binds fibronectin, vitronectin, and vWF. Additionally, $\alpha_5\beta_1$ binds fibronectin, $\alpha_2\beta_1$ binds collagen, $\alpha_3\beta_1$ binds fibronectin, collagen, and laminin (Albelda et al. 1989), but these interactions are not considered as essential as those involving $\alpha_v\beta_3$ (Satoh et al. 1994).

Understanding of endothelial cell adhesion is required for the development of therapeutic agents that inhibit platelet GPIIb/IIIa as many such agents have been demonstrated to lack specificity for GPIIb/IIIa and will therefore interfere with other integrin-ligand interactions like those adhering endothelial cells to blood vessels (Chen and Hawiger, 1991). In a clinical setting, a nonspecific GPIIb/IIIa inhibitor would be exposed to endothelial cells as it passed through the vascular system. One concern is that the inhibitor could interfere with endothelial cell adhesion and would cause disruption of the continuous cell monolayer; this situation would result in a risk for vascular haemorrhage (Schror, 1995).

Another concern is that endothelial cells expose integrin receptors on their apical surface, which exposes the integrin receptors to circulation. If a GPIIb/IIIa inhibitor was introduced that also bound to these exposed integrins, the agent would be effectively removed from circulation as it bound to the endothelial cells of the vascular system. The dose of the inhibitor would have to be increased to overcome this cross-reaction in order to reach the platelet GPIIb/IIIa receptor.

C. Disintegrins

1. General

Animal venoms and saliva have been looked to for proteins that effect neurological and haematological systems. Disintegrins are a family of proteins isolated from these sources that affect haemotology through interactions with platelets. Disintegrins are named for their function in disrupting the integrin-ligand interaction, specifically at the GPIIb/IIIa site.

For the most part, disintegrins are found in venoms of the snake family Viperidae (Niewiarowski et al. 1994). The first disintegrin isolated from snake venom (Trimeresurus gramineus)was Trigramin, and the first isolated from another source was Decorsin from the leech Macrobdella decora (Lazarus and McDowell, 1993). Since that time, over forty disintegrins have been identified (Niewiarowski et al. 1994).

Disintegrins are small proteins, ranging from 39 to 83 amino acids. They are cysteine rich and average 17% cysteine content. All disintegrins but one contain the tripeptide RGD, also seen in integrin ligands. It has been proposed that it is through RGD that disintegrins bind to the integrin receptors (Lazarus and McDowell, 1993).

Most disintegrins have conserved cysteine positions, however it is becoming evident that the disulfide pairing varies (Lazarus and McDowell, 1993). One common feature of the disulfide pairing is that it results in presentation of the RGD tripeptide at the apex of a loop, held together at its base by disulfide bonds.

2. Mambin

One disintegrin with a unique secondary structure is Mambin, also known as Dendroaspin, and herein referred to as Mambin. The structure of Mambin resembles that of the short chain neurotoxins from the Elapidae snake family, sharing 40% identity in amino acids (McDowell et al. 1992). This type of structure that Mambin shows is based on that of Erabutoxin b and is characterized by a central core that is held together by four disulfide bonds, and three loops which extend from the core (M.J.Dufton and R.C.Hider, 1992). The loops are numbered 1, 2, and 3 as they occur sequentially in the amino acid sequence (ie. loop 1 is at the N terminus). Mambin has this three loop structure however it does not retain any neurotoxic activity (M.J.Dufton and R.C.Hider, 1992). Instead, it has only disintegrin activity due to the presence of the RGD tripeptide at the apex of loop 3.

Mambin is isolated from the venom of Dendroaspis jamesonii (McDowell et al. 1992). It is interesting that this snake is a member of the snake Elapidae family, as no other disintegrin has been identified in Elapidae snakes. All other snake venom disintegrins are from the Viperidae family. As mentioned, Mambin shares amino acid sequence homology with short chain neurotoxins from this family. Mambin, therefore, has the structure of an Elapidae protein with the function of a Viperidae protein. Mambin may represent a protein that has

evolutionary significatlce in terms of snake families. That is, Mambin could represent the evolutionary protein link between the short chain neurotoxins of Elapidae snakes and the disintegrins of Viperidae snakes.

Mambin was identified first by Joubert and Taljard (1979), however they did not recognize its disintegrin properties. Later, two independent groups simultaneously reported the isolation of the protein: one group named it Mambin (McDowell et al. 1992) and the other named it Dendroaspin (Williams et al. 1992). The latter group have since analyzed Mambin further (Lu et al. 1994) and produced a recombinant form of the protein in Escherichia coli (Lu et al. 1996).

3. Biological Specificity

Different disintegrins have specificity for different integrin receptors, and some disintegrins will bind to several integrins. In the interest of developing a GPIIb/IIIa antagonist, specificity for this receptor is desired. As mentioned, GPIIb/IIIa specificity is needed to reduce the risk of vascular haemorrhage by cross-reacting with endothelial cell integrin-ligand interactions.

Barbourin, a disintegrin from the venom of Sistrurus m. barbouri, has been identified as a disintegrin with high specificity for GPIIb/IIIa (Scarborough et al. 1991). Studying the direct interactions of GPIIb/IIIa with fibrinogen, $\alpha_v\beta_3$ with vitronectin, and $\alpha_5\beta_1$ with fibronectin, it was determined that Barbourin only inhibited the first. This high specificity had not previously been observed with any other disintegrin. Barbourin was tested in this assay with another disintegrin, Tergeminin. These two proteins both blocked the GPIIb/IIIa-fibrinogen interaction, but only Tergeminin blocked the a $\alpha_v\beta_3$-vitronectin interaction.

Interestingly, these two proteins are identical, with the exception of two amino acids. One difference is at position 11 where Barbourin has glutamic acid and Tergeminin has alanine. As glutamic acid is seen in other disintegrins at this point, this substitution seems to be trivial. However, the second amino acid substitution is in the RGD tripeptide. In fact, Barbourin is the only disintegrin lacking the RGD tripeptide. In its place is KGD. So it has been suggested that the change of arginine to lysine alters the specificity of the disintegrin (Scarborough et al. 1991).

Aside from this example, another speculation as to contributing factors for disintegrin specificity is the role of the amino acids flanking the RGD sequence. Of the most interest is the residue beside the aspartic acid in RGD. It has been noticed that Barbourin and Tergeminin have a negligible effect on the interaction of $\alpha_5\beta_1$ with fibronectin (Scarborough et al. 1991). These disintegrins possess a tryptophan residue beside the aspartic acid: R(K)GDW. Other disintegrins possess alternate amino acids at this site. So it has been speculated that tryptophan may reduce the affinity for $\alpha_5\beta_1$, while maintaining the interaction with GPIIb/IIIa.

4. Studying Disintegrins

Many groups are studying disintegrins and although the assay systems used are similar, several key differences exist. The first assay systems address the biological activity of the disintegrins. Biological activity was approached in two different ways. The first was inhibition of platelet aggregation and the second was inhibition of GPIIb/IIIa binding to fibrinogen.

The assay for platelet aggregation has long been developed (Born, 1962) and is based on the principle that light transmission

through a suspension of platelets increases as the platelets aggregate (Eratantoni and Paindexter, 1990). The instrumentation for such spectrophotometric measurements vary from microplate readers (Fratantoni and Poindexter, 1990) to electronic particle size analysis (Yukio Ozaki et al. 1994) to instruments designed specifically for platelet aggregation measurements termed aggregometers (Born, 1962). However, the current trend in the literature is the use of aggregometers.

When comparing current reports, two key differences in investigative methodology are evident. The first is that the method of platelet preparation varies. Some investigators have chosen to use whole blood to monitor platelet aggregation (Born,. 1962), while some use platelet-rich plasma (Scarborough et al. 1991; McDowell et al. 1992), and still others use washed platelets (Lu et al. 1996). It is apparent that data generated from one type of preparation cannot be directly compared to another. For example, when the disintegrin Mambin was assessed for its ability to inhibit platelet aggregation for platelet rich plasma, an IC_{50} value (that concentration of Mambin that will inhibit platelet aggregation 50%) of 95 nM was determined. For the same concentration of washed platelets, however, the IC_{50} value was 20 nM (Willliams et al. 1992). This discrepancy is not accounted for in the literature.

The second difference in methodology is the type of agonist used. Many agonists are available, including ADP, thrombin, collagen, platelet-activating factor, epinephrine, and arachidonic acid (Wallace and Woodman, 1995). Most studies concerning disintegins currently use ADP or thrombin. As mentioned, these agonists activate platelets via different pathways and the resulting cellular changes can also differ. For example, aggregation of platelets by ADP requires exogenous fibrinogen,

whereas this is not the case for thrombin (Hantgan, 1985). Fibrinogen is required to cross-link the platelets in order to form an aggregate, however in the activation by thrombin cellular signaling results in mobilization of stored fibrinogen from storage granules to the extracellular space (Shattil et al. 1994). This local concentration of fibrinogen is sufficient to support aggregation. So if platelet rich plasma is used, which contains exogenous fibrinogen, either ADP or thrombin may serve as agonists. If washed platelets are used, which lacks exogenous fibrinogen, only thrombin may serve as as agonist without supplementation of fibrinogen.

Another test for biological activity that is commonly used is a solid-phase assay for GPIIb/IIIa binding to fibrinogen (Scarborough et al. 1991). This assay is similar to an enzyme-linked immunosorbant assay (ELISA) in setup. The integrin GPIIb/IIIa is bound to wells. A mixture of biotinylated fibrinogen and different concentrations of the disintegrin to be tested is added to the wells and incubated. After washing, the amount of biotinylated fibrinogen bound to GPIIb/IIIa is quantitated by anti-biotin antibody conjugated to alkaline phosphatase. This assay appears to function well, however problems may lie in the preparation of the integrin receptor.

As mentioned, GPIIb/IIIa has a different ligand specificity, depending on the activation state of the platelet. The precise mechanism of this change in integrin affinity is not understood currently (Bennett, 1996). When GPIIb/IIIa is purified from platelets, the activation state of the platelets is uncertain. Therefore the state of the integrin receptor when it is removed from the platelet membrane is questionable. The receptor may be in one of several forms, from a low affinity for soluble fibrinogen to a high affinity form. In addition, the natural state for any

integrin receptor contains two hydrophilic domains separated by a transmembrane region. When the receptor is removed from an environment with a membrane, the question arises: is the receptor in a non-membranous environment behaving as it would in vivo?

Biological specificity of the disintegrins has been tested in several ways, each addressing the inhibition of several integrin-ligand interactions. The first is similar to the solid-phase assay described above, except that the integrin receptor used $\alpha_v\beta_3$ or $\alpha_5\beta_1$ and the ligand used is vitronectin or fibronectin. However, this assay system bears the same problems as the GPIIb/IIIa solid-phase assay.

Another approach to testing integrin specificity that addresses the problems associated with the solid-phase system is using whole cells and monitoring their interactions with different ligands. This is termed a cell adhesion assay. The main advantage to this assay is that the integrin receptors can interact with the ligands in their natural membrane environments: although not in vivo, this approach is considered ex vivo. This cell adhesion assay is not without its drawbacks, however. First, it requires a supply of a cell that carries the integrin of interest. For the purposes of studying agents with human pharmaceutical potential, it is preferred to use human cells as the integrin receptors vary from species to species (Hawiger, 1987). Second, interpretations are difficult from this assay system. The understanding of platelet and other integrin receptors is elementary and to determine whether or not a single ligand is binding to a single integrin in a cell system is difficult at this time. Third, the system currently developed uses ligands that are coated on solid surfaces. As mentioned, GPIIb/IIIa has a different specificity for soluble and surface bound fibrinogen. So if an indication is required for the

interaction of an integrin and its soluble ligand, this assay may not be suitable.

The cell adhesion assay system may use platelets for studying GPIIb/IIIa, however, the presence of $\alpha_5\beta_1$ and $\alpha_v\beta_3$, which share ligands with GPIIb/IIIa, complicates the interpretations. Other cells have been used to complement the data gained from platelets. These include M21 melanoma cells (Scarborough et al. 1993b) and human umbilical vein endothelial cells (HUVEC) (Foster et al. 1993). Regardless of the cell used, the assay system is the same in that ligand is bound to wells. A mixture of the cells and different concentrations of the disintegrin to be tested is added to the wells and incubated. After washing, the number of cells that have adhered to the ligand is quantitated. The method of quantitation varies from microscopic evaluation (Chen and Hawiger, 1991) to staining (Henrich, 1996) to assaying for an enzyme endogenous to the cell (Lu et al. 1994).

D. Research Objectives

The goals of this study are: (1) to produce a recombinant form of Mambin in addition to two Mambin variants, (2) to develop assay systems to test both the biological activity and biological specificity towards GPIIb/IIIa of the proteins, and (3) to purify Mambin and Barbourin from snake venom for analysis in the assay systems with the recombinant proteins.

1. *B. subtilis* Expression System

The recombinant forms of Mambin will be produced in a *B. subtilis* expression system. This expression system is different from the often used *E. coli*, which has several problems due to the intracellular expression of the foreign gene (Behnke, 1992): first, foreign proteins are limited to those that are not toxic to

the cell. Second, overproduction of proteins in E. coli often results in the formation of aggregates of denatured foreign protein; recovery of such protein requires harsh conditions to denature the proteins.

In B. subtilis, the foreign protein may be secreted to the extracellular space, which keeps the protein in a soluble form (Wang and Doi, 1992). This facilitates easier purification as the cells are simply removed by centrifugation and the foreign protein is then purified from the media. In the past, the main problem with this system is the presence of extracellular proteases, which degrade foreign secreted proteins (Behnke, 1992). This has been addressed by the engineering of a six extracellular protease deficient B. subtilis strain (Wu et al. 1991). This strain has since been improved with the removal of a seventh extracellular protease and is termed WB700.

2. Production of a Fusion Protein

As the gene for Mambin has yet to be cloned, the gene was synthesized using the method of dual-asymmetric PCR (Sandhu et al. 1992). The protein was expressed as a fusion protein. This ensured secretion and facilitate easier purification.

Mambin was fused to Staphylokinase. The latter protein was chosen for several reasons. First, Staphylokinase has been produced successfully in strain WB700. Second, Staphylokinase does not contain any disulfide bonds, so the interference with disulfide bond formation in Mambin will be minimal. Third, a rapid assay system has been developed for Staphylokinase activity, which eases purification.

Following purification of the fusion protein, Staphylokinase-Mambin, the protein must be cleaved to release Mambin for study. Several proteases are available for the purpose of cleaving fusion

proteins. Unfortunately many of these would leave one or more amino acids at the N terminus of Mambin, which may affect the function of the protein. One protease that would leave an intact N terminus is Factor Xa. The use of this protease is not ideal for Mambin. Factor X is isolated from bovine or human plasma, but must be treated with Russell's Viper venom to be activated. This addition of snake venom may contribute contaminating proteins that may complicate purification of Mambin.

One commercially available protease remains for the purposes of cleaving Mambin from Staphylokinase. Collagenase is a metalloprotease from the bacterium Clostridium histolyticum. It is available commercially, however contaminating amounts of Clostripain remain in the preparations. As Clostripain cleaves at arginine residues, this is clearly undesired with Mambin which contains the RGD tripeptide. The Collagenase gene was recently cloned (Yoshihara et al. 1994). The protein is currently expressed in B. subtilis strain DB104. Unfortunately, this strain is only two extracellular protease deficient, so a risk of contamination of the purified Collagenase with other extracellular proteases that could degrade Mambin exists.

3. Disintegrin Purification

Mambin and Barbourin have been purified using techniques of reverse-phase HPLC (Scarborough et al. 1991: McDowell et al. 1992; Willliams et al. 1992). Similar protocols will be used for the purification of the native venom proteins and the recombinant proteins.

4. Disintegrin Assay Systems

Three assay systems will be established to test the purified disintegrins. The first will measure biological activity and

monitors the ability of the disintegrin to inhibit human platelet aggregation.

The other two assay systems measure the biological specificity of the disintegrins. Both are cell adhesion assays, one uses human platelets and one uses human umbilical vein endothelial cells (HUVEC). The disintegrins will be tested for their ability to inhibit cell adhesion to fibrinogen, fibronectin, and vitronectin.

MATERIALS AND METHODS

A. Bacterial Strains Used

The E. coli and B. subtilis strains used in this study are listed in Table 1. All strains were maintained in a frozen stock in LB broth supplemented with 15% glycerol at -80°C. All strains were single colony isolated on LB agar (E. coli) or TBAB agar (B. subtilis) prior to use and appropriate antibiotics used where necessary.

B. Media

LB broth was prepared by mixing: 15 tryptone, 0.5% yeast extract, 171 mM NaCl, adjusted to pH 7.0 with 10 M NaOH. LB agar was made by the addition of 1.5% Bacto-agar (Difco) to the LB broth. TBAB (tryptose blood agar base) broth was prepared by adding 33 g of pre-mixed TBAB (Difco) to 1 liter of ddH_2O. TBAB agar was made by the addition of 1.5% agar to the TBAB broth. The antibiotics used in colony selection and plasmid maintenance were as follows: ampicillin (75 ug/ml), kanamycin (10 ug/ml).

SP1 and SP2 broth were used for all B. subtilis transformations. SP1 broth contained 15 mM $(NH_4)_2SO_4$, 61.3 mM K_2HPO_4, 44 mM KH_2PO_4, 3.4 mM sodium citrate:2 H_2O, 811 uM $MgSO_4$:7 H_2O, 28 mM glucose, 0.02% casamino acids, 0.1% yeast extract, 245 uM tryptophan. SP2 broth was prepared by adding 381 uM $CaCl_2$ and 5. 3 mM $MgCl_2$, to SP1 broth.

Super-rich broth was used for growth of B. subtilis strains for the purpose of protein purification. It was made as follows: 2.5% Bacto-tryptose, 2% yeast extract, 22 mM K_2HPO_4 was mixed in ddH_2O and the pH was adjusted to 7.0. Kanamycin (10 ug/ml) was added prior to inoculation.

Incubations for all E. coli were at 37°C and B. subtilis newly transformed cells were at 37°C. All other B. subtilis incubations were at 30°C.

C. Enzymes and Chemicals

Restriction endonucleases were obtained from Gibco-BRL (BamHI, EcoRI, HindIII) and New England Biolabs (SphI). T4 DNA ligase, Taq and Vent Polmerases were from Gibco-BRL. dNTPs were from Pharmacia. Wizard PCR Product Purification Kits were obtained from Promega. T7 sequencing kits were from Pharmacia. Radioisotopes for sequencing were purchased from Amersham. Most laboratory chemicals were obtained from Sigma Chemical Co. and BioRad. D. jamesonii snake venom was purchased from Sigma Chemical and S. m. barbouri snake venom was purchased from Miami Serpentarium. The Sephacryl S-200 gel filtration matrix was purchased from Pharmacia. The CM-52 ion exchange matrix was obtained through Whatmann. Sep-Pak cartridges and C-18 reverse-phase HPLC column were from Waters.

D. Mambin Proteins

Three Mambin proteins were used in this study. Mambin I is the wild type Mambin, expressed recombinantly from the mamI gene by B. subtilis. The amino acid sequence for Mambin I is represented in Figure 1, A (bold letters). Mambin II contained 3 amino acid substitutions in comparison to Mambin I, but otherwise retains the same sequence. The amino acid changes are shown in Figure 1, B. Mambin III had 9 amino acid substitutions in comparison to Mambin I, with the entire sequence between cysteine 39 and cysteine 51 (loop 3) changed from the Mambin I sequence to the KGD-containing loop from Barbourin. These amino acid changes are indicated in Figure 1, C.

E. Plasmids

1. pMAMI, pMAMII, pMAMIII

Plasmid pMAMI was constructed by inserting the PCR-synthesized mamI gene (Figure 1) into the plasmid pBM⁻ by HindIII (5′ end of gene) and SphI (3′ end of gene). The plasmid is presented in Figure 2. As with pBM⁺, pMAMI is a high copy number E. coli plasmid and confers ampicillin resistance. pMAMII and pMAMIII are identical to pMAMI, except that the synthetic genes mamII and mamIII, respectively, were inserted into pBM⁻.

2. pUP4SSAKLK

pUP4SSAKLK (Figure 3A) was constructed by Elaine Sihota. It is based on the B. subtilis high copy plasmid pUB18 can be used for expression of fusion proteins. As with pUB18, pUP4SSAKLK confers kanamycin resistance. The promotor chosen to direct expression of fused genes is P43, which is a strong vegetative B. subtilis promotor. Downstream from the promotor, the encoding region for the levansucrase signal peptide (sacBSP) is placed to direct secretion of the remainder of the protein. The signal peptide is cleaved from the final secreted protein product upon secretion, The staphylokinase gene (sak) is downstream of sacBSP, followed by 60 bp, which encodes a linker region in the fusion protein. This linker has been constructed to join the Staphylokinase domain with Mambin I without disturbing folding or function of either protein, while remaining resistant to cleavage by any B. subtilis proteases (Argos, 1990). The last component of the construct is the Kl domain, which is a remnant of the construction and does not impact on this study.

3. pSAKL

pSAKL (Figure 3B) is a plasmid that served as an intermediate in the construction of pSAKCMAMI. A 917 bp EcoRI/ BamHI fragment was removed from pUP4SSAKLK, that contained the P43 promotor, sacBSP, sak, and the region encoding the linker domain. The fragment was introduced into pUB18, and as such retained the properties af the high copy B. subtilis plasmid, conferring kanamycin resistance.

4. pSAKCMAMI, pSAKCMAMII, pSAKCMAMIII

These 3 plasmids are the constructions for the expression of the 3 fusion proteins, Staphylokinase-Mambin I, Staphylokinase-Marnbin II, Staphylokinase-Mambin III. The plasmids were constructed using pSAKL, introducing the mamI, mamII, or mamIII genes. The three mam genes had been PCR-amplified from pMAMI, pMAMII, and pMAMIII with the primers clmam and M13 reverse. pSAKCMAMI is represented in Figure 4. It retains all the features of pUP4SSAKLK, with the exception of the latter part of the fusion protein.

F. Plasmid Isolation by Alkaline Lysis

1. E. coli

E. coli plasmid DNA was isolated by the method of alkaline lysis, modified from Sambrook et al. (1989). 2 ml of LB broth (with appropriate antibiotics) was inoculated with a single colony and grown at 37°C for 16 hours with shaking. The cells were transferred to microfuge tubes and centrifuged for 1 minute. The supernatant was removed and the cells were resuspended in 1 ml of

SET buffer (584 mM sucrose, 50 mM EDTA, 50 mM Tris pH 7.6). The cells were centrifuged again for 1 minute, and the supernatant discarded. The cells were resuspended in 150 ul of SET and 5 ul of RNase (10 mg/ml RNase in 0.1 M sodium acetate, 0.3 mM EDTA pH 4.8; solution boiled for 10 minutes to remove DNase activity) was added. 350 ul of lytic buffer (1% SDS, 0.2 M NaOH) was added and the tube inverted several times, then placed on ice for 5 minutes. 250 ul of 1.5 M potassium acetate (pH 4.8) was added and the tube returned to ice for 20 minutes. The precipitated chromosomal DNA was removed by centrifugation for 10 minutes at 4°C. The supernatant was transfered to a clean tube and was phenol extracted three times using an equal volume of phenol:$CHCl_3$. The plasmid DNA, retained in the aqueous phase, was precipitated by the addition of one volume of isopropanol and centrifuged for 5 minutes. The DNA pellet was washed with cold 70% ethanol and vacuum dried before resuspension in 20 ul of TE (50 mM Tris pH 7.6, 50 mM EDTA).

2. B. subtilis

A TBAB agar plate (with appropriate antibiotics) was streaked to cover the plate with the single colony isolate of B. subtilis and incubated at 30°C for 16 hours. The cells were transferred to 2 ml of LB broth (with appropriate antibiotics) and grown at 37 °C for 2 hours with shaking. The cells were transferred to microfuge tubes and centrifuged for 1 minute. The supernatant was removed and the cells were resuspended in 1 ml of SET. The cells were centrifuged again for 1 minute, and the supernatant discarded. The cells were resuspended in 80 ul of SET and 20 ul of lysozyme (20 mg/ml lysozyme in SET buffer) and 5 ul of RNase was added and the solution was incubated at: 37°C for 10 minutes. 200 ul of lytic buffer was added and the tube inverted several times, then placed on ice for 5 minutes. 150 ul of 1.5 M potassium acetate (pH 4.8)

was added and the tube returned to ice for 20 minutes. The chromosomal DNA was removed by centrifugation for 10 minutes at 4°C. The supernatant, containing the plasmid DNA, was transfered to a clean tube and extracted three times with phenol:$CHCl_3$. The DNA was precipitated by the addition of one volume of isopropanol and centrifugation for 5 minutes. The pellet was washed with cold 70% ethanol and vacuum dried before resuspension in 10 ul of TE.

G. Preparation of Competent Cells

1. E. Coli

A single colony of E. coli DH5α was inoculated into 2 ml LB broth and grown at 37°C for 16 hours with shaking. The 2 ml of cells was then added to 30 ml LB broth and grown until the cell density reached 140 Klett units. The cells were centrifuged at 10,000 rpm for 5 minutes at 4°C, resuspended in 15 ml 10 mM NaCl, and centrifuged as before. The cells were resuspended in 15 ml 30 mM $CaCl_2$ and kept on ice for 20 minutes, then centrifuged at 5,000 rpm for 10 minutes at 4°C. The cells were gently resuspended in 3 ml 30 mM $CaCl_2$ with 15 % glycerol, aliquoted into 100 ul portions, and stored at -80°C for later use.

Prior to the cells being transformed, they were defrosted on ice. The transforming DNA (which was heat denatured at 65°C for 2 minutes) was added and the mixture left on ice for 1 hour. The cells were then heat shocked at 42 °C for 45 seconds. The cells added to 2 ml of LB broth and incubated at 37°C for 30 minutes with shaking. The cells were then plated onto LB agar, containing the appropriate antibiotics.

2. *B. subtilis*

A TBAB plate (with appropriate antibiotics) was streaked with the single colony isolate of *B. subtilis* and incubated at 30°C for 16 hours. The cells were then transferred to 2 ml of SP1 broth and grown at 37°C for 3 hours and 45 minutes with shaking. 500 ul of the cells was transferred to prewarmed SP2 broth and incubated at 37°C for 90 minutes with shaking. 50 ul of EGTA (100 mM, pH 7.0) was added and the cells further incubated for 10 minutes. The competent cells were used for transformation immediately.

To transform the cells, 500 ul of the cells was mixed with the transforming DNA (which was heat denatured at 65°C for 2 minutes). The mixture was incubated at 37°C for 90 minutes, shaking at 300 rpm. The cells were then plated onto TBAB agar containing the appropriate antibiotics.

H. Design of Primers

A list of the primers used in this study are presented in Table 2. The universal and reverse primers were used in the sequencing reactions for pMAMI, pMAMII, and pMAMIII. Primer sakintf was used in the sequencing of pSAKCMAMI, pSAKCMAMII, and pSAKCMAMIII. Primers maml, mam2, mam3, mam4, mam3(II), and mam3(III) were used in the construction of the *mam* genes by PCR; the complete DNA sequence is represented in Figure 1. The sequences that the primers encode for is shown by the dashed and solid lines above the sequence. Primer maml was designed to include the first 65 nucleotides of the *mamI* gene (in the sense direction), with an additional 15 nucleotides at the 5' end for introduction of a *Hin*dIII and *Sph*l site for subsequent cloning. Primer mam2 was designed to include the next 51 nucleotides of the *mamI* gene (in the antisense direction), with an additional 16 nucleotides at the 3′ end to overlap with maml. Primer mam3 was

designed to include the next 54 nucleotides of the mamI gene (in the sense direction), with an additional 17 nucleotides at the 5′ end to overlap with mam2. Primer mam4 was designed to include the last 7 nucleatides of the mamI gene (in the antisense direction), with an additional 21 nucleotides at the 5′ end to introduce two stop codons, a HindIII and SphI site for subsequent cloning and 18 nucleotides at the 3′ end to overlap with mam3. Primer mam3(II) is identical to mam3, with the nucleotide substitutions corresponding to the 3 amino acid changes for Mambin II (Figure 1B). Primer mam3(III) is identical to mam3, with the nucleotide substitutions corresponding to the 9 amino acid changes for Mambin III (Figure 1C). Primer clmam was designed to introduce the Collaganase cleavage site and a connection to the linker region in order for introduction to the plasmid pSAKL. The three Mambin proteins were amplified from pMAMI, pMAMII, and pMAMIII using clmam and reverse M13 primers. For purposes of amplification of the mamI, mamII, mamIII genes, clmam contained 23 nucleotides of the 5′ region of the mamI sequence.

I. Dual-Asymmetric PCR of mam Genes

The mamI gene, and the two derivatives, mamII and mamIII (Figure 1), were synthesized using dual-asymmetric PCR (Sandhu et al. 1992). Four primers (mam1, mam2, mam3, and mam4) were constructed to cover the entire mamI gene sequence with a minimum of 16 base pair overlap. HindIII and SphI recognition sites were introduced at the 5′ and 3′ ends of the gene for cloning. For all 3 amplicons containing mamI, mamII, and mamIII, primers mam1, mam2, and mam4 were used. For the mamI amplicon, the primer mam3 was used. For the mamII amplicon, the primer mam3(II) was used. For the mamIII amplicon, the primer mam3(III) was used. Primers mam1 and

mam4 were diluted to a 6.25 uM stock for PCR. Primers mam2, mam3, mam3(II), and mam3(III) were diluted to a 62.5 nM stock for PCR.

The PCR reaction mixture was as follows: In the Gibco-BRL Taq Polymerase Buffer (1X), 180 uM $MgCl_2$ 20 uM dNTPs, 500 nM mam1, 5 nM mam2, 5 nM mam3, 500 nM mam4, and 5 units/150 ul Taq polymerase. The reaction was carried out in a Techne Dry-Block thermocycler as follows: thirty cycles of 1 minute denaturation (94°C), 1 minute annealing (50°C), and 1 minute extension (72°C) were carried out. A 9.9 minute extension (72°C) was added as a final step to amplify any prematurely truncated amplicons.

J. Purification of PCR Products

PCR products were purified as follows: The completed PCR mixture was electrophoresed in TAE (40 mM Tris-acetate, 1 mM EDTA) 1.2% agarose. The PCR amplicon was identified under long-wavelength ultraviolet light and cut from the agarose. The DNA was further purified from the agarose using the Wizard PCR Preps DNA Purification System (Promega) according to the manufacturer's instructions. The DNA was resuspended in 50 ul of TE.

K. Cloning Mambin Genes

The purified PCR amplicons represented three mam genes: wild type mambin (mamI), and two modified forms of mamI, mamII and mamIII (Figure 1). All three genes and pBM^+ were digested by SphI: 10 ul of DNA was incubated with 5 ul 10X Buffer 6 (BRL), 1 ul SphI (5 units/ ml), and 34 ul sterile ddH_20 at 37°C for 2 hours. The cleaved DNA was purified with the Wizard Purification System to remove the SphI enzyme and buffer. The three genes and pBM^+ were further digested with HindIII: 10 ul of DNA was incubated with 5 ul 10X Buffer 2 (BRL), 1 ul HindIII (5 units/ ml), and 34 ul sterile ddH_20 at 37°C for 2 hours. The DNA was purified again with the Wizard Purification System. After overnight ligation with T4 DNA

ligase (10 ul of mamI, 3 ul of pBM', 12 ul of 5X Ligase Buffer (BRL), 4 ul of T4 DNA Ligase (1 unit/ul), 29 ul of sterile ddH_2O; 15°C), the mixture was used to transform competent DH5α cells. Successful transformants were selected for ampicillin resistance and confirmed by diagnostic digestion of isolated plasmids. All three constructions were confirmed by sequencing with both the M13 universal and M13 reverse primers (Table 2).

L. Construction of Fusion Proteins in a Secretion Vector

The genes encoding the fusion proteins Staphylokinase-Mambin I, Staphylokinase-Mambin II, Staphylokirlase-Mambin III were constructed in a B. subtilis vector for secretion of the proteins into the growth medium. This consisted of two procedures: preparation of the vector for introduction of the mam genes and PCR of the mam genes from pMAMI, pMAMII, and pMAMIII to introduce the correct restriction enzyme sites and the Collagenase recognition site. The vector pUP4SSAKLK (Figure 3A), was digested with EcoRI and BamHI to release a 918 bp fragment, containing the P43 promoter and sacB signal peptide - staphylokinase - linker coding region. This fragment was introduced into pUB18, that had also been digested with EcoRI and BamHI. The resulting plasmid was termed pSAKL (Figure 3B). The mambin genes were amplified using Vent polymerase, as Vent contains a proofreading activity that ensures sequence fidelity, from the plasmid templates pMAMI, pKAMII, and pMAMIII using primers clmam and M13 reverse (Table 2). The resulting PCR products had a BamHI site cut the 5′ end and an SphI site cut the 3′ end that enabled insertion into pSAKL. The resulting vectors were termed pSAKCMAMI, pSAKCMAMII, pSAKCMAMIII (Figure 4). All three constructions were confirmed by sequencing with the sakintf primer (Table 2).

M. Sequencing Protocol

Plasmids, isolated by alkaline lysis, were resuspended in 10 ul of TE, and denatured by addition of 15 ul of 1 M NaOH, 10 mM EDTA and 50 ul of sterile ddH_20. This mixture was incubated at 45°C for 30 minutes. 30 ul of 2M ammonium acetate (pH 4.5) and 300 ul of cold 95% ethanol were added and the mixture was placed at -80°C for 40 minutes. The DNA was centrifuged for 10 minutes, the pellet washed with Cold 70% ethanol, and then resuspended in 10 ul of sterile ddH_2O and sequenced according to the manufacturer's instructions for the T7 Sequencing Kit (Pharmacia). The reaction mixtures were heat denatured for 2 minutes at 75°C prior to electrophoresis. The acrylamide gel used for electrophoresis was made as follows: 38.4 g of urea, 4 g of acrylamide, 0.21 g of bis-acrylamide were dissolved in 80 ul of TBE (89.2 mM Tris, 89 mM boric acid, 3.2 mM EDTA) by heating. The mixture was filtered through a Wattman 2 filter and degassed for 10 minutes. Before pouring, 800 ul of 10% ammonium persulfate and 15 ul of N,N,N',N'-tetramethlyenediamine (TEMED) were added. Once poured, the gel was allowed to polymerize for 1 hour. The gel was electrophoresed in TBE buffer, and was pre-run at 75 Watts until the temperature of the gel reached 45°C. Samples were loaded and electrophoresed at 50 Watts; the time varied depending on the sequence desired. The gel was vacuum dried and placed in a film cassette with Kodak XAR film for 24 to 48 hours. Exposed films were developed according to the manufacturer's instructions.

N. Purification of Fusion Proteins

A single colony of B. subtilis WB700 carrying pSKCMAMi, pSKCMAMII, or pSKCMAMIII was streaked onto TBAB agar with selection for kanamycin and grown at 30°C for 16 hours. The cells were transferred to 500 ml super-rich broth containing kanamycin. The

cell density was between 2 and 4 Klett units. The cells were grown at 37 °C with shaking until the cell density was observed to be between 400 and 500 Klett units (measured as a 10 fold dilution at 40 to 50 Klett units) after approximately 5 hours. Phenylmethylsulfonyl fluoride (PMSF, made fresh), which was solubilized in isopropanol, was added to 10 uM to irreversibly inhibit any contaminating serine proteases. Ethylenediaminetetraacetate (EDTA) was added to 2 mM to inhibit any contaminatirlg metalloproteases. The cells were centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected and ammonium sulfate was added to 40% saturation (243 g/l). This solution was allowed to stir slowly at 4 °C for 1 hour and then centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was retained and ammonium sulfate was increased to 55% saturation (97 g/l). This solution was allowed to stir slowly at 4°C for 1 hour and centrifuged at 12,000 rpm for 15 minutes at 4 °C. The pellet was resuspended in 10 ml of phosphate buffer (81 mM Na_2HPO_4, 27.8 mM NaH_2PO_4, 250 mM NaCl). The pH of the phosphate buffer was adjusted to the pI of each fusion protein with NaOH: pH 7.8 for Staphylokinase-Mambin I, pH 8.1 for Staphylokinase-Mambin II, pH 7.5 for Staphylokinase-Mambin III. The protein suspension was applied directly to a Sephacryl S-200 gel filtration column (250 ml, height 80 cm). The column was run with 4 column volumes to elute the protein. The column was subsequently washed with 0.5 M NaOH and re-equilibrated with phosphate buffer. Fractions were assayed for the fusion protein by the staphylokinase assay. The active fractions were electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions that contained maximal fusion protein with activity were pooled and 10 uM PMSF and 2 mM EDTA were added. The protein mixture was dialyzed against 2 l of citrate buffer (5 mM citric acid, 14 mM Na_2HPO_4, pH

5.6) for 12 hours at 4°C. The buffer was changed twice, once after 4 hours and once after 8 hours. The protein mixture was applied directly to a CM-52 (45 ml) ion exchange column. The column was washed with 3 column volumes of citrate buffer and the protein was eluted in a NaCl gradient in citrate buffer from 0 to 0.4 M. The column was further washed with 1.0 M NaCl (in citrate buffer). Fractions were assayed for the fusion protein by the staphylokinase assay. The active fractions were run on 12% SDS-PAGE. Fractions were pooled on the basis of staphylokinase activity and 10 uM PMSF and 2 mM EDTA were added. The purified fusion protein was dialyzed against 2 l of 10 mM Tris pH 7.5 for 4 hours, then dialyzed against 20% PEG until the sample volume was between 500 ul and 1000 ul. The sample was further dialyzed against 2 l of 10 mM Tris pH 7.5 for 4 hours.

O. Protein Electrophoresis

Protein samples were prepared in equal volume with loading buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue, 10% mercaptoethanol) and boiled for 5 minutes. The SDS-PAGE gels of either 12% or 15% concentration were cast and electrophoresed according to Sambrook et al. (1989). The gels were stained in 0.25% coomassie blue, 10% acetic acid, 10% methanol for 1 hour and destained in 10% acetic acid, 10% methanol until the background staining was minimal.

P. Staphylokinase Assay

The fractions from the various Columns were assayed for staphylokinase activity. A master mixture containing the following was prepared: 75 ul of Tris (100 mM, pH 7.5), 30 ul of BSA (5%), 9 ul of plasminogen (1 uM), and 15 ul of Chromozym PL (21.8 mM). After mixing, 4.3 ul was aliquoted into microplate wells. 2.5 ul of each fraction to be tested was then added and mixed. The

microplate was then incubated at 37°C for 10 minutes. 90 ul of 5% acetic acid was added and the samples were read at 405 nm on a microplate reader (Ceres 900UV); fractions with a high absorbance at this reading had staphylokinase activity.

Q. Collagenase Cleavage of Fusion Proteins

The fusion proteins were cleaved by recombinant Collagenase. The reaction was conducted as follows: 0.04 units of Collagenase was added per ug of fusion protein in 50 mM tricine, 500 mM NaCl, 10 mM $CaCl_2$. The mixture was incubated at 37°C for 5 hours. The extent of cleavage of the fusion protein was assessed by electrophoresis through a 15% SDS-PAGE.

R. Purification of Disintegrins

Recombinant and native dispntegrin proteins were first applied to a Sep-Pak cartridge (Waters). The cartridge (volume of 0.7
ml) was first pre-conditioned: 7 ml of acetonitrile (0.1% TFA) was slowly passed through the cartridge by syringe followed by 7 ml of ddH_20 (0.1% TFA). The protein sample was applied and washed (by gravity) with 2.5 ml ddH_2O (0.1% TFA). The protein was then eluted with 2 ml of 60% acetonitrile (0.1% TFA) by gravity. The eluted sample was allowed to evaporate under a vacuum to remove the acetonitrile.

The Sep-Pak eluted protein was applied to a C18 reverse-phase HPLC via a 1 ml injection loop. The column was then washed with 10 ml ddH_20 (0.1% TFA) . The disintegrins were eluted using a gradient of 0% to 60% acetonitrile (0.1% TFA). The column was washed with 10 ml acetonitrile (0.1% TFA) and re-equilibrated with 10 ml ddH_20 (0.1% TEA). The proteins were analyzed by SDS-PAGE and by the ability to inhibit platelet aggregation. Selected fractions were evaporated under a vacuum and stored at -80°C. Prior to assaying,

the proteins were resuspended in PBS buffer (138 mM NaCl, 2.7 mM KCl, 16.2 mM
Na_2HPO_4, 1.47 mM KH_2PO_4).

S. Quantitation of Disintegrins

Protein samples were sent to the University of Victoria, Department of Biochemistry for quantitation. Quantitation was performed by method of mass spectroscopy and quantitation of the N-terminal amino acid.

T. Human Platelet Purification

Human platelets were prepared fresh as follows and used within 4 hours ex vivo. Human donors were chosen who did not smoke and had not consumed any medication within 10 days. The blood was drawn through a butterfly needle into a 3 ml syringe. The first 1 ml of blood was discarded to prevent contamination of the blood with tissue juice. A 50 ml syringe was then used to draw 30 ml of blood, which was added to 6 ml of ACD anticoagulant (140 mM citric acid, 200 mM sodium citrate, 220 mM glucose) and inverted to mix. The blood was centrifuged at 900 rpm for 10 minutes at 20°C. The top layer (platelet rich plasma) was removed to a new tube and 500 ul of ACD anticoagulant was added. The volume was brought up to 15 ml with PBS buffer and centrifuged at 2100 rpm for 10 minutes at 20°C. The supernatant was discarded and the cell pellet was resuspended in 5 ml of PBS buffer and 500 ul ACD. The volume was brought up to 15 ml with PBS buffer and centrifuged at 2100 rpm for 10 minutes at 20°C. The platelets were resuspended in 5 ml of PBS+ buffer (315 uM $CaCl_2$, 540 uM MgCl, and 7.5 mM glucose added to PBS buffer). These platelets were termed "washed platelets". The cells were stained with toluidine blue (0.15% in ddH_20) and counted on a haemocytometer.

U. Platelet Aggregation Assay

Purified human platelets were diluted to 3 X 10^8 cells/ml with PBS+ buffer. 135 ul of platelets were placed in microplate wells. If a disintegrin was to be tested, it was added to the platelets at this time and incubated for 10 minutes at room temperature. The platelets were stimulated with human thrombin to a final concentration 0.5 units/ml and the absorbance at 620 nm was measured by a Ceres UV 900HDI microplate reader (Bio-Tech Instruments) at 1 minute intervals, with shaking for 50 seconds between readings. Each condition was repeated a minimum of three times and each time point within the condition averaged to plot a platelet aggregation data (Figure 5). The IC_{50} value, the concentration of disintegrin which reduces platelet aggregation to 50% of the maximal, was determined by linear regression of the platelet aggregation data.

V. Platelet Adhesion Assay

The ligands for platelet adhesion were prepared as follows. Microplate wells were coated with various proteins (BSA, 2 mg/ml; fibrinogen, 50 ug/ml; fibronectin 25 ug/ml; vitronectin 4 ug/ml) in PBS buffer for 16 hours at room temperature. The wells were then blocked with BSA (2 mg/ml in PBS buffer) for 1 hour and subsequently washed three times with PBS buffer.

Purified human platelets were diluted to 1.5 X 10^7 cells/ml with PBS+ buffer. If a disintegrin or EDTA was to be tested for inhibition of platelet adhesion, it was added to the platelets aliquoted into polypropylene tubes and incubated for 10 minutes at room temperature. For non activated platelets, 90 ul of the cells were added directly to the protein coated plates. For activated platelets, 10 ul of ADP (200 uM, in PBS buffer) was added to the protein coated plates prior to addition of the 90 ul of platelets.

The platelets were incubated for 60 minutes at room temperature. Non adherent platelets were then removed by three washes with PBS buffer. 130 ul of developing buffer (100 mM sodium citrate, 10 mM p-nitrophenyl phosphate, 0.1% Triton X-100, pH 5.5) was added to each well and incubated for 45 minutes. 10 ul of 1 M NaOH was added to stop the reaction and incubated at room temperature for 10 minutes. The absorbance at 405 nm was read by a Ceres UV 900HDI microplate reader. A blank was prepared by measuring the absorbance of the developing buffer with the NaOH added.

W. HUVEC (Human Umbilical Vein Endothelial Cell) Adhesion Assay

The ligands for HUVEC adhesion were prepared as follows: 96-well culture plates were coated with various proteins (fibrinogen, 50 ug/ml: fibronectin 25 ug/ml; vitronectin 4 ug/ml) by aliquoting 100 ul onto the plate and removal after 1 minute. HUVEC were generously supplied by Dr. P. Kubes (University of Calgary) in culture flasks with HUVEC medium. The cells were washed three times with Versene, a buffered EDTA solution used to detach the cells from the wells, and incubated with 10 ml Versene at 37°C for 10 minutes. The culture flask was shaken to lift the cells from the plate. The cells were removed from the culture flask and centrifuged at 1200 rpm for 10 minutes. The cell pellet was resuspended in 10 ml HUVEC medium and centrifuged at 1200 rpm for 10 minutes. The cell pellet was resuspended in 5 ml HUVEC medium. The cells were stained with toluidine blue (0.15% in ddH_2O) and counted on a haemocytometer. The cells were diluted to 6 X 10^6 cells/ml with HUVEC medium. If a disintegrin was to be tested, it was diluted into HUVEC medium. 50 ul of the diluted disintegrin (or 50 ul of HUVEC medium for a control) was incubated with 50 ul HUVEC for 10 minutes at room temperature. The mixture was then

applied to the protein coated wells and incubated at 37°C for 2 hours. The media was removed and the wells were washed three times with PBS buffer to remove non-adherent cells. 100 ul formalin (10% in PBS buffer) was added to each well and incubated at room temperature for 30 minutes. 50 ul of toluidine blue (1% in formalin) was added to stain the adherent cells and was incubated at room temperature for 60 minutes. The wells were washed extensively with ddH_2O and allowed to dry for 16 hours. The stained wells were treated with 100 ul of 2% SDS for 15 minutes at 37°C to solubilize the dye. Absorbance readings at 620 nm were recorded by microplate reader (Ceres UV 900HDI). A blank was prepared by measuring the absorbance of a well treated with the stain and processed with the samples.

X. Computer Software

Data from Ceres UV 900HDI microplate reader was ported to Microsoft Excel version 6. All calculations for the data reported in this study were performed in this program.

RESULTS

A. Synthesis and Cloning of Mambin Genes

The mamI gene and the two derivatives, mamII and mamIII were synthesized by dual asymmetric PCR, based on a similar procedure reported for hirudin gene (Sandhu et al. 1992) (Figure 6). The amino acid sequence for Mambin published by McDowell et al. (1992) served as the template for design of the four primers: mam1, mam2, mam3, and mam4 (Figure 1). Each primer was optimized for codon usage in B. subtilis, as this organism has a preference for codons of some amino acids. Primer mam1 encoded a HindIII restriction site on its 5' end and primer mama encoded an SphI site on its 5' end. This introduction of the two sites facilitates cloning of the mamI gene into pBM$^-$. The overlap of primers mam1 and mam2 was 16 bp, of primers mam2 and mam3 was 18 bp, and of primers mam3 and mam4 was 17 bp. The primers mam1 and mam4 were used in a standard Taq polymerase reaction in 100 fold excess of primers mam2 and mam3. These differential primer concentrations favored initial base pairing of mam1 with mam2 and mam3 with mama (Figure 6A). As such, mam1 primed polymerization using mam2 as a template, and mam4 primed polymerization using mam3 as a template. This resulted in the amplification of two intermediate PCR amplicons: 131 and 99 bp (Figure 6B). Once the concentration of mam1 and mam4 were depleted and the concentrations of the intermediate amplicons increased, base pairing between these two intermediate amplicons at the overlap of mam2 and mam3 was favored. The intermediate 131 bp amplicon primed polymerization using the intermediate 99 bp amplicon as a template and the intermediate 99 bp amplicon primed polymerization using the intermediate 131 bp amplicon as a

template; this resulted in polymerization of the full-length mam gene of 213 bp (Figure 6C).

A time course of the PCR reaction is presented in Figure 7. In the first 15 cycles, the two intermediate amplicons can be detected at 99 and 131 bp. As the reaction proceeded, the full length amplicon containing the mamI gene was detected by increasing amounts of a 213 bp product. The same reaction was carried out, replacing primer mam3 with either mam3(II) and mam3(III); this allowed synthesis of the other two mam genes, mamII and mamIII (Figure 1). The results of the full-length PCR amplicons for all three reactions is depicted in Figure 8.
The three genes, mamI, mamII, and mamIII were cloned into pBM' for sequencing and subsequently cloned into the B. subtilis secretion vectors, pSAKCMAMI (Figure 4), pSAKCKAMII, and pSAKCMAMIII for production of the fusion proteins Staphylokinase-Mambin I, Staphylokinase-Mambin II, and Staphylokinase-Mambin III.

B. Fusion Protein Purification

1. Ammonium Sulfate Fractionation

The first step in the purification of Staphylokinase-Mambin I, Staphylokinase-Mambin II, and Staphylokinase-Mambin III was fractionation of the B. subtilis culture supernatant by differential ammonium sulfate precipitation. To choose the lower and upper fractionation limits, aliquots of the culture supernatant were subjected to different amounts of ammonium sulfate, to yield 30 to 65% saturation. Both the supernatant and pellet were analyzed by SDS-PAGE (data not shown) and it was found that at 40% ammonium sulfate saturation the majority of the fusion protein remained in the supernatant; this was set as the lower fractionation limit. At 55% ammonium sulfate saturation, the

majority of the fusion protein had moved to the pellet, and therefore this was set as the upper fractionation limit. Figure 9 depicts one of the purification schemes of Staphylokinase-Mambin I; the purification scheme for Staphylokinase-Mambin II and Staphylokinase-Mambin III were similar.

2. Gel Filtration Chromatography

The ammonium sulfate fractionated protein was applied to a S-200 gel filtration column. This facilitates purification of the fusion protein based on molecular size, as the gel filtration matrix retards smaller proteins as they enter the matrix beads whereas the larger proteins proceed through the column unretarded. The resultant purification is indicated in Figure 9 (lane 3).

3. Ion Exchange Chromatography

The fractions containing activity fro the gel filtration column were pooled, dialyzed, and applied to a CM-52 ion exchange column. This facilitates purification of the fusion protein based on charge. This matrix contains carboxymethyl groups and is negatively charged at the range of pH values (5.4 to 5.8) used. The pH of the buffer is set 2 pH units below the pI of the protein of interest, so that the protein carries a net positive charge. It can then bind to the carboxymethyl group when applied to the column. The protein is eluted by increasing the NaCl concentration of the buffer. The sodium ions compete with the protein for the carboxymethyl sites, therefore releasing the protein from the matrix. The resultant purification is indicated in Figure 9 (lane 4). The pooled fractions were dialyzed against Tris buffer and concentrated by PEG.

C. Collagenase Cleavage of Fusion Proteins

The recombinant Collagenase was generously provided by Dr. Matsushita. He had cloned the collagenase gene from Clostridium histolyticum (Yoshihara et al. 1994) and expressed the gene in a secretion vector through the B. subtilis strain DB104. This strain, unlike B. subtilis WB700, only has two extracellular proteases removed. Dr. Matsushita purified the recombinant Collagenase (Personal communication, 1996), however analysis by SDS-PAGE in this study revealed that at least three other protein bands were present (data not shown). Thus, the potential exists that these contaminants may be extracellular proteases.

Conditions for Collagenase cleavage were established on the basis of a buffer system to optimize the activity of this metalloprotease. The literature uses different buffer systems for the various commercially available Collagenases (Colman et al. 1987). Tris buffer is commonly used. The use of Tris at this stage of the study was not desired as the use of Tris-based buffers is cautioned against when working with platelets since Tris is able to chelate calcium ions which are required for platelet integrin function (Colman et al. 1987). In addition, the use of Tris may require additional calcuim to be added as Collagenase requires calcium for optimal function. Tricine (50 mM) was used instead of Tris as a buffer. The optimal salt concentration was 500 mM NaCl. As Collagenase is a metalloprotease, 10 mM $CaCl_2$ was included in the reaction.

A time course of Collagenase cleavage of Staphylokinase-Mambin I is presented in Figure 10. The cleavage is maximal at 4 hours, and at 6 hours the amount of mambin begins to decrease. This is in agreement with other trials performed in this study, where cleavage reactions beyond 5 hours resulted in degradation of Mambin (data not shown). To attempt to protect Mambin from potential

contaminating serine proteases, the same time course was repeated in the presence of 2 mM PMSF (Figure 11). The extent of cleavage was clearly less than without the PMSF and this established that PMSF would not be used in subsequent cleavages and the reaction time would be 5 hours. The protease inhibitor EDTA was not tested as it would inhibit the activity of Collagenase, due to its calcium dependence.

D. Purification of Recombinant Mambin Proteins

Following Collagenase cleavage, Mambin I, Mambin II, and Mambin III were further purified by reverse phase HPLC from the remainder of the fusion protein (Staphylokinase-linker) and Collagenase. A chromatogram of a successful purification of Mambin II is presented in Figure 12. The first peak (*) was Mambin II, as determined by SDS-PAGE and inhibition of platelet aggregation. The second peak contained the remainder of the fusion protein, Staphylokinase-linker, as determined by SDS-PAGE and the Staphylokinase assay (data not shown). An SDS-PAGE analysis of the three purified proteins, Mambin I, Mambin II, and Mambin III are shown in Figure 13.

E. Instability of Recombinant Mambin Proteins

Two main problems were encountered: protein instability during the HPLC purification and following HPLC purification. In the first case, each of Mambin I, Mambin II, and Mambin III had been cleaved by Collagenase activity and purified by HPLC, however the protein elution pattern from the chromatogram differed from previous purifications: Instead of one peak visible for the disintegrin, several smaller peaks were evident. An example of this is shown in Figure 14, which shows a chromatogram of Mambin II. Note that this is the same protein as depicted in Figure 12, but with a key difference: the single peak of Mambin II in Figure 12 was now

present as multiple peaks with reduced protein content, as evidenced by the reduction in absorbance at 280 nm (Figure 14). In addition, analysis by SDS-PAGE (Figure 15) revealed an unusual protein electrophoresis pattern as several bands were apparent in the size range of Mambin II. This indicated a heterogeneity of the Mambin II protein and accounts or the multiple peaks on the chromatogram; this heterogeneity may have been a result of degradation of the Mambin II protein.

Although Mambin I, Mambin II, and Mambin III was each successfully taken through HPLC (Figure 13), indications of instability were evident. The three preparations were analyzed by SDS-PAGE over a period of 12 days from frozen stock and the results are presented in Table 3. Each protein concentration was reduced more than 2 fold over the period observed. Unlike the heterogeneity observed in association with the multiple HPLC peaks (Figure 15), SDS-PAGE analysis of the proteins presented in Table 3 exhibited single bands, with no sign of degradation products (data not shown).

F. Purification of Native Mambin and Barbourin

Mambin was purified from Dendroaspis jamesonii venom as outlined in the Methods and Materials. The gradient was adjusted for maximal separation of mambin from the other venom proteins. A typical purification is presented in Figure 16. Mambin is indicated by the star. Barbourin was purified from Sistrurus m. barbouri venom as outlined in the Methods and Materials. The gradient was adjusted for maximal separation of Barbourin from the other venom proteins. A typical purification is presented in Figure 17. Barbourin is indicated by the star. A 15% SDS-PAGE of the purified venom proteins shows the relative sizes of Mambin (lane 1) and Barbourin (Figure 18). The theoretical molecular mass

Mambin is 6678 daltons and this gel indicates a value closer to 8000 daltons. The theoretical molecular mass of Barbourin is 7700 daltons and this gel indicates a value closer to 14400 daltons. The apparent molecular masses of Mambin and Barbourin by SDS - PAGE are higher than the actual, however analysis by this method is not accurate as many factors contribute to the electrophoretic mobility of proteins, including both molecular mass and net charge.

G. Assay for Biological Activity

Biological activity was determined by inhibition of human platelet aggregation. The assay system developed in this study reproduced aggregation curves as presented in the literature. However, several optimizations were made that included determination of the method of purification of human platelets, which agonist of platelet aggregation was to be used, and which instrumentation was best suited to monitor the aggregation.

1. Human Platelet Preparation

The published methods of platelet purification include simple preparation of platelet-rich plasma, gel filtered platelets, and washed platelets. Platelet-rich plasma was prepared, however the aggregation curves were not consistent from day to day (Figure 5A). Gel filtered platelets were not attempted. Washed platelets gave more consistent results with respect to aggregation (Figure 5B). For the purposes of testing the disintegrins, washed platelets were used.

2. Platelet Agonist

Using washed platelets for the assay system, the two agonists tested were 20 uM ADP and 0.5 units/ml human thrombin. These agents serve to activate the platelets and stimulate aggregation. As the

washed platelet suspension has had all plasma fibrinogen removed, the only fibrinogen remaining is that which is stored in the platelet granules. As such, only agonists that can stimulate platelets to release their granular contents will aggregate platelets. Thrombin is such an agonist, however ADP is not. ADP requires the addition of exogenous human fibrinogen for platelet aggregation. Figure 19 shows the aggregation attained by washed human platelets stimulated by thrombin and ADP. Thrombin induced aggregation without the addition of exogenous fibrinogen. To attain the same degree of aggregation with ADP required 320 ug/ml human fibrinogen. Concentrations of fibrinogen lower than this did not allow ADP to fully aggregate the platelets. For the purposes of testing disintegrin activity human thrombin was used as the agonist as there was no requirement for exogenous fibrinogen.

3. Aggregation Instrumentation

The standard instrumentation used for measuring platelet aggregation in the literature is a platelet aggregometer. This method follows changes in light transmission as the platelet suspension changes from individual cells to aggregates (Born, 1962). Aggregometers use cuvettes fitted with magnetic stir bars, which require between 250 and 500 ul of platelet suspensions to attain proper readings. Most aggregometers can read four samples at a time and provides readings as printouts. It has been recently suggested that microplate readers may be used in the place of aggregometers (Fratantoni and Poindexter, 1990) and has been applied in the literature (Douglas et al. 1992). Like aggregometers, microplate readers can follow changes in light transmission. Unlike aggregaometers, the wells cannot be fitted with stir bars. Fortunately, many microplate readers have the ability to mix the contents of the wells by shaking at various

speeds. Microplates contain 96 wells, which allows the monitoring of many more samples at once. This is a distinct advantage over aggregometers, considering the short time for experimentation with platelets of three to four hours. One final advantage over aggregometers is the interfacing of microplate readers with computers, which allows for storage of each absorbance value for each timepoint. This can further be anayzed with conventional spreadsheet programs. both aggregometer and microplate reader methods were tested. Figure 20A shows three typical human platelet aggregations, using washed platelets stimulated with 0.5 units/ml thrombin, in an aggregometer (Payton Scientific). These results were comparable to the results from the microplate reader (Bio-Tech Instruments) (Figure 20B). For the purposes of testing the disintegrins, a microplate reader was used as this method allows for the testing of many more samples with one preparation of human platelets and greatly facilitated post-experimental analysis.

H. Assay for Biological Specificity

1. Human Platelet Adhesion

The first assay for biological specificity, human platelet adhesion, has been reported in the literature using monoclonal antibodies to platelets that do not interfere with cell adhesion as a means to quantitate the adherent platelets. Without access to such antibodies, an alternate strategy for platelet detection was found. Lu et al. (1994) suggested the use of an acid phosphatase chromogenic substrate as platelets have this enzymatic activity. The platelets were allowed to adhere to different ligands which had previously been coated to microplate wells. After washing, the platelets were treated with the chromogenic substrate. The amount of substrate converted was monitored spectrophotometrically. As

this is the only example of such method, it had to be assessed with several controls.

a) Incubation of Chromogenic Substrate

First, the time of incubation of the chromogenic substrate with the platelets was analyzed to ensure that the enzymatic reaction had not passed the linear range. Using a range of incubation times of 6 to 70 minutes, the absorbance at 405 nm was determined (Figure 21). The results indicated that the enzymatic reaction was within the linear range for at least 70 minutes. For all subsequent assays, the time of incubation with the substrate was chosen to be 45 minutes as this time was within the linear range of detection by the chromogenic substrate for acid phosphatase.

b) Dose Response Control

The second control was to determine if acid phosphatase activity was a true determination of the number of platelets adherent to the wells. To test this, a known number of cells were introduced to the microplate wells and assayed for acid phosphatase activity (Figure 22). The result was a complex curve that contained a region of linear response (0.5×10^7 to 1.6×10^7 cells) range of detection, which indicates that the absorbance at 405 nm correlates to the number of platelets. Above 1.6×10^7 cells the response is non-linear, and as such the absorbance at 405 nm is not a reflection of the number of platelets. This maximal value of 1.6×10^7 platelets is corroborated by mathematical platelets that can adhere to the surface of the microplate well. Considering the average diameter of a human platelet is 2.5 um and the available surface area of the well is 94 mm^2, an estimated 1.5×10^7 platelets can adhere to the well. If more platelets are added, it is assumed

that all of the available binding sites on the well have been taken, so these excess platelets would be washed away.

For all assays, 1.5 X 10^7 platelets were added to the microplate wells as this is close to the maximal number of platelets that lies within the linear range of the dose response.

c) Platelet Agonists

Like the platelet aggreation assay, ADP and human thrombin were tested as agonists. As mentioned above, thrombin causes the release of fibrinogen from platelet granules. The purpose of the adhesion assay was to monitor the interactions of the platelets with the protein treated wells alone (platelet-ligand interaction). The release of endogenous fibrinogen may result in increased adherent platelets. For example, a thrombin stimulated platelet bound to a vitronectin treated well (platelet-ligand interaction) would have released fibrinogen from its storage granules. This fibrinogen would adhere free platelets to the vitronectin bound platelet in a manner similar to platelet aggregation (platelet-platelet interaction). In the case of ADP, however, endogenous fibrinogen is not released, so the number of adherent platelets is a more accurate indication of the platelet-ligand interaction alone, with no interference from platelet-platelet interactions. When the two agonists were tested (Figure 23), thrombin did induce an elevated response of platelet adhesion to BSA when compared to ADP. In the adherence of fibrinogen, this increase was minimal. According to the current understanding of platelet activation a more significant increase would be expected. In the adherence to BSA, indicating the background adherence, the platelets activated by thrombin gave a response 2.7 times that of ADP. This indicated that this measure of background adherence was unacceptably high for thrombin stimulation. For all subsequent assays, 20 uM ADP was

used to stimulate the platelets as the use of thrombin as an agoinst brings complications of fibrinogen release upon platelet stimulation.

d) Microplate Wells

For the purpose of protein coating the microplate wells, three types of wells were tested from Dynatech Laboratories. Immuno-1, Immuno-2, and Immuno-4 all bind proteins to varying degrees, so each was tested for its ability to bind BSA and fibrinogen. The optimal well should bind BSA and fibrinogen to a maximum. In order to determine the amount of protein binding, the platelet adhesion assay was performed in parallel using both stimulated and unstimulated platelets.

Human platelets theoretically should not bind BSA, and BSA is often used as a blocking agent in similar platelet assays (Lu et al. 1996; Lu et al. 1994). If the well has not been completely saturated with BSA, then the platelet will non-specifically bind to the well directly through platelet surface proteins (results not shown). Therefore, if a high number of platelets adhere to the well that was treated with BSA, this would indicate that the BSA did not coat the well entirely. This would be a concern because BSA is used to block the wells following treatment with fibrinogen, fibronectin or vitronectin. If these proteins did not saturate the well and the BSA did not bind, then the well may be exposed in places and platelets would bind. This would result in false positive results. Figure 24 shows that Immuno-2 wells best suit the assay system. Although the binding of BSA was not as low as Immuno-4, it was much lower than Immuno-1. The binding of fibrinogen was close to the maximal level that Immuno-1 exhibited. Immuno-2 gave the best combined results for BSA and fibrinogen binding so it was chosen for all subsequent assays.

e) Pre-Treatment of Platelets

In the study by Lu et al. (1994), prostaglandin E_1 (PGE_1) and apyrase were added to the platelets in their preparation to prevent activation during processing. As with other prostacyclins, PGE_1 activates adenylate cyclase, which in turn results in elevated intracellular cAMP. Such a rise in cAMP prevents the mobilization of granular calcium, thus retarding intracellular signaling that prevents platelet activation. Apyrase is an ADP scavenger and prevents platelet activation in the preparation process by removing any ADP that may accumulate (Mustard et al. 1972). However, if apyrase is present in the final platelet preparation, any ADP added to stimulate the platelets may be degraded. In this case, there is a question of whether the platelets are truly activated. Lu et al. (1994) used ADP as the agonist but did not demonstrate if the platelets that were tested were activated.

The platelet purification scheme in this study does not use either PGE_1 or apyrase and yet the platelets are not activated prior to use in the aggregation assay, as evidenced by the inability of the platelets to aggregate in the absence of agonists. In the presence of aDP, with the addition of fibrinogen, these platelets are stimulated and aggregate (Figure 19).

To compare the platelets purified in the absence and the presence of PGE_1 and apyrase, four platelet preparations were performed in parallel: (1) preparation included no addition, (2) included both PGE_1 and apyrase, (3) included only PGE_1 and (4) preparation included only apyrase. The four sets of platelets were assayed for the ability to adhere to fibrinogen, both unstimulated and stimulated with ADP (Figure 25). The addition of PGE_1 appeared to make the platelets less responsive to ADP stimulation. The addition of apyrase made the platelets unresponsive to ADP stimuation and appeared to either make the unstimulated platelets

bind fibrinogen to a stronger extent or interfere with the detection method, making the signal higher. The latter conclusion is more likely as the signal of two optical density units is the maximal possible considering the number of platelets added to the well. When the platelets were treated with both PGE_1 and apyrase the signal increased and exceeded the maximum of four absorbance units. For this reason, it was sifficult to ascertain if ther was any difference in unstimulated versus stimulated platelets. The extremely high absorbance values were above the maximum for the number of platelets in the well. It would seem that the addition of both PGE_1 and apyrase effected the detection of platelets by monitoring acid phosphatase activity with the chromogenic substrate. Thus in the purification of platelets for the adhesion assay, neither PGE_1 nor apyrase was used.

f) Control Values

To determine control values for platelet adhesion to fibrinogen, fibronectin, and vitronectin, platelets were tested both in unstimulated and stimulated forms, using adhesion to BSA as a control (Figure 26). Unstimulated platelets bound to all three proteins, with the highest observed adhesion being to fibrinogen. Stimulated platelets bound to all three proteins at elevated levels, with the highest observed adhesion being to fibrinogen. These values represented the control values for inhibition of adhesion by the disintegrins in subsequent assays.

g) Effect of EDTA

To determine the calcium and/or magnesium dependence of the interaction of platelets with fibrinogen, fibronectin, and vitronectin, platlets were treated with 5 mM EDTA prior to incubation in the protein coated wells. Figure 27 compares the

control values presented in Figure 26 to those of platelets treated with EDTA. EDTA had a minimal effect on the background platelet adhesion to BSA, both unstimulated stimulated. EDTA had a significant effect on the adhesion of both stimulated and unstimulated platelets to fibrinogen. This is expected as this interaction is through GPIIb/IIIa, which requires calcium to bind fibrinogen (Bennett, 1996). EDTA had a much less significant effect on the adhesion of stimulated and unstimulated platelets to fibronectin and vitronectin.

2. HUVEC Adhesion

The second assay for biological specificity, human umbilical vein endothelial cell (HUVEC) adhesion, was modified from various published endothelial cell adhesion assays.

a) HUVEC Staining

Methods for quantitating endothelial cells from the literature include microscopic analysis (Chen and Hawiger, 1991), and three different biological stains: Rose Bengall (Foster et al. 1993), crystal violet (Satoh et al. 1994), and toluidine blue (Henrich, 1996). Both of the methods that use crystal violet and toluidine blue staining were tested (Figure 28). In addition, two plating methods for the HUVEC were assessed using a 96 well culture plate and a 48 well culture plate (Corning). The 96 well plate has advantages over the 48 well plate in that more trials can be performed with the same number of cells and the final spectrophotometric readings can be performed on a microplate reader, which in turn facilitates further computer analysis. Upon microscopic analysis, the HUVEC grow equally well on 96 and 48 well plates. On the 96 well plate, toluidine blue is a superior stain as it has a high signal-to-background ratio (Figure 28). On the 48

well plate, the solubilized stain was removed to a cuvette for absorbance readings and the background sample was used to blank the spectrophotometer. Thus, a signal-to-noise ratio cannot be determined. However, both toluidine blue and crystal violet stained the cells to a similar extent with a higher variation in readings observed for crystal violet. Since the cells stained well with toluidine blue in both 96 well and 48 well plates, this stain was used for further assays. 96 well plates were chosen as the plating method.

b) Dose Response Control

As with the platelet adhesion assay, a control must be performed to determine if the detection method was a true representation of the number of cells adherent to the wells. To test this, a known number of HUVEC were introduced to a well and assayed by toluidine blue staining. Unlike the platelet assay, the HUVEC must be bound to the well in order to be properly fixed and stained. The cells were allowed to adhere to the three proteins, fibrinogen, fibronectin, and vitronectin for two hours then fixed and stained. Figure 29 depicts a linear trend of the number of cells according to the staining process for all three protiens. For all subsequent assays, 1.5×10^3 HUVEC were added to the wells, as this value was within the linear range of this detection method.

c) Time Course of HUVEC Adhesion

The HUVEC adhesion to fibrinogen, fibronectin, and vitronectin was monitored over four hours to determine the time required for incubation of the cells for the assay (figure 30). The time selected must be within the linear range of adhesion. The adhesion profile for fibrinogen was linear up to three hours of incubation, where after the rate of adhesion slowed. The profiles for

fibronectin and vitronectin were similar. These adhesions appeared to begin more rapidly than fibrinogen and had a linear adhesion profile between one and three hours. A two hour time of incubation of the HUVEC in the protein coated wells was chosen for further assays.

d) Control Values

To determine control values for HUVEC adhesion to fibrinogen, fibronectin, and vitronectin, the cells were incubated on the wells for two hours then fixed and stained (Figure 31). The cells appeared to bind to the three proteins with equal affininty, when the standard devation is accounted for. These values represented the control values for inhibition of adhesion by the disintegrins in subsequent assays.

I. Determination of Native Mambin and Barbourin Biological Activity

Platelet aggregation assays were performed at various concentrations of Mambin and Barbourin to determin IC_{50} values;an IC_{50} value is that concentration at which there is a 50% inhibition of platelet aggregation. Figure 32 presents the dose response curves for Mambin and Barbourin in terms of the nanomolar concentration of each disintegrin. The inhibition of platelet aggregation is similar for Mambin and Barbourin, with maximal inhibition around 50 nM for each protein. The IC_{50} values were determined by linear regression of the data in the linear range of the dose response curve and is presented in Table 4. The IC_{50} for Mambin was 29.5 nM and for Barbourin it was 31.1 nM. These values are similar and indicate that both proteins have a similar biological activity. Mambin and Barbourin inhibit the GPIIb/IIIa-fibrinogen interaction to the same extent.

The IC_{50} value of washed platelet aggregation for Mambin of 29.5 nM can be compared to the literature values presented by two different groups. McDowell et al. (1992) reported an IC_{50} value of platelet rich plasma aggregation for Mambin of 172 nM. This value is higher than that determined in this study, however it appears that the IC_{50} values for disintegrins are higher for platelet rich plasma than for washed platelet aggregation (Lu et al. 1996). Lu and coworkers have also reported IC_{50} values of platelet rich plasma aggregation for Mambin of 95 nM (Williams et al. 1992) and 160 nM (Lu et al. 1994). Although no explanation is provided for this discrepancy, these values are in the same range as the value reported by McDowell. IC_{50} values for washed platelet aggregation have also been reported by Lu et al. and these should be directly comparable to the IC_{50} value of 29.5 nM determined in this study. They reported values of 20 nM (Williams et al. 1992) and 75 nM (Lu et al. 1996). Again, no explanation wsa provided by Lu et al. for this difference in reported values. The former value of 20 nM determined by Lu et al. is very close to the IC_{50} value determined here. However, the point remains that a 1.68 fold variation for IC_{50} value (for platelet rich plasma) and 3.75 fold variation (for washed platelets) was reported for the same protein in the same assay by one group.

The IC_{50} value of washed platelet aggregation for Barbourin of 31.1 nM can be compared to the literature value presented by Scarbrough et al. of 309 nM (1991). However, this published IC_{50} value is for platelet rich plasma, so it is not directly comparable to that value determined in this study.

J. Determination of Native Mambin and Barbourin Biological Specificity

1. Human Platelet Adhesion

a) Unatimulated Platelet Adhesion to Fibrinogen

Platelet adhesion asseys were performed at various concentrations of Mambin AND Barbourin to determine the IC_{50} values. Figure 33 presents the dose response curves for Mambin and Barbourin in terms of the nanomolar concentration of each disintegrin. The inhibition of unstimulated platelet adhesion to fibrinogen is slightly stronger for Mambin than Barbourin. The IC_{50} values were determined by linear regression of the data and is presented in Table 4. The IC_{50} for Mambin was 15.8 nM and for Barbourin it was 27.5 nM. Although not as close as the values observed for inhibition of platelet aggregation, these IC_{50} values are considered to be in the same range. It is expected that the relative IC_{50} values for Mambin and Barbourin would be the same for platelet aggregation and unstimulated platelet adhesion to fibrinogen since both interactions are through GPIIb/IIIa. These results further suggest that Mambin and Barbourin inhibit the GPIIb/IIIa fibrinogen interaction to the same extent.

b) Stimulated Platelet Adhesion to Fibrinogen

Platelet adhesion assays were performed at various concentrations of Mambin and Barbourin to determine the IC_{50} values. Figure 34 presents the dose response curves for Mambin and Barbourin in terms of the nanomolar concentration of each disintegrin. The inhibition of stimulated platelet adhesion to fibrinogen is similar for Mambin and Barbourin. The IC_{50} values

were determined by linear regression of the data and is presented in Table 4. The IC_{50} for Mambin was 219.9 nM and for Barbourin it was 220.5 nM. These values are similar and indicate that both proteins have similar biological activities. It is expected that the relative IC_{50} values for Mambin and Barbourin would be the same for platelet aggregation and stimulated platelet adhesion to fibrinogen since both interactions are through GPIIb/IIIa. This further indicates that Mambin and Barbourin inhibit the GPIIb/IIIa-fibrinogen interaction to the same extent.

c) Stimulated Platelet Adhesion to Fibronectin

Platelet adhesion assays were performed at various concentrations of Mambin and Barbourin to determine the IC_{50} values. Figure 35 presents the dose response curves for Mambin and Barbourin in terms of the nanomolar concentration of each disintegrin. The inhibition of stimulated platelet adhesion to fibronectin is different for Mambin and Barbourin: that is, Mambin inhibits adhesion to a greater extent than Barbourin. The IC_{50} values were determined by linear regression of the data and is presented in Table 4. The IC_{50} for Mambin was 841.4 nM. An IC_{50} value for Barbourin could not be determined as the maximum amount of Barbourin tested was 2180 nM and at this concentration Barbourin had a negligable effect on adhesion. This difference in IC_{50} was expected: the biological specificity that Barbourin maintains indicates that the protein has little effect on the platelet-fibronectin interaction. The IC_{50} value for Mambin is 3.8 times that for stimuated platelet adhesion to fibrinogen. This implies some degree of specificity of Mambin to the GPIIb/IIIa fibrinogen interaction, although not as extreme as the specificity of Barbourin.

d) Stimulated Platelet Adhesion to Vitronectin

Platelet adheson assays were performed at various concentrations of Mambin and Barbourin to determine the IC_{50} values. Figure 36 presents the dose response curves for Mambin and Barbourin in terms of the nanomolar concentration of each disintegrin. The inhibition of stimulated platelet adhesion to vitronectin was similar for Mambin and Barbourin. The IC_{50} values were determined by linear regression of the data and are presented in Table 4. The IC_{50} for Mambin was 325.6 nM and for Barbourin was 1005.1 nM. The higher IC_{50} value for Barbourin demonstrates the specificity of Barbourin towards the GPIIb/IIIa-fibrinogen interaction, as observed for the stimulated platelet adhesion to fibronectin. This inhibition of platelet interaction with vitronectin may be accounted for by the ability of GPIIb/IIIa to bind vitronectin (Shattil et al. 1994). Mambin inhibits the platelet-vitronectin interaction to a similar level as the platelet-fibrinogen interaction, and therefore showed no specificity in this regard.

2. HUVEC Adhesion

a) HUVEC Adhesion to Fibrinogen

HUVEC adhesion assays were performed at various concentrations of Mambin and Barbourin to determine the IC_{50} values. Figure 37 presents the dose response curves for Mambin and Barbourin in terms of the nanomolar concentration of each disintegrin (nM). The inhibition of HUVEC adhesion to fibrinogen was different for Mambin and Barbourin. The IC_{50} values were determined by linear regression of the data and is presented in Table 4. The IC_{50} for Mambin was 102.2 nM, although the IC_{50} value for Barbourin could not be

determined since the maximum amount of Barbourin tested was 290 nM. At this concentration Barbourin had a negligible effect on HUVEC adhesion. This difference in the IC_{50} values for Mambin and Barbourin further demonstrates the specificity that Barbourin has for the GPIIb/IIIa-fibrinogen interaction that Mambin does not: Mambin has the ability to interfere with the adhesion of HUVEC to the ligand fibrinogen by binding to a receptor on the cell. As HUVEC do not have the GPIIb/IIIa receptor, Mambin must be interacting with another receptor, which facilitates fibrinogen binding to HUVEC.

b) HUVEC Adhesion to Fibronectin

HUVEC adhesion assays were performed at various concentrations of Mambin and Barbourin to determine the IC_{50} values. Figure 38 presents the dose response curves for Mambin and Barbourin in terms of the nanomolar concentration of each disintegrin. The inhibition of HUVEC adhesion to fibronectin shows a similar trend in both the Mambin and Barbourin assays, but Mambin is more effective in this inhibition. The IC_{50} values were determined by linear regression of the data and is presented in Table 4. The IC_{50} for Mambin was 299.6 nM and for Barbourin was 597.0 nM. These values had to be extrapolated from the data recorded and may not be as accurate as the other IC_{50} values determined in this study. Nevertheless, the trends for Mambin and Barbourin inhibition of HUVEC adhesion to fibronectin is clear: Mambin inhibits the HUVEC-fibronectin interaction to a greater extent than Barbourin. This demonstrates that Barbourin has more specificity for the GPIIb/IIIa-fibrinogen interaction than does Mambin.

c) HUVEC Adhesion to Vitronectin

HUVEC adhesion assays were performed at various concentrations of Mambin and Barbourin to determine the IC_{50} values. Figure 39 presents the dose response curves for Mambin and Barbourin in terms of the nanomolar concentration of each disintegrin. The inhibition of HUVEC adhesion to vitronectin is different for Mambin than for Barbourin and depicts a trend similar to that observed for HUVEC adhesion to fibrinogen. The IC_{50} values were determined by linear regression of the data and is presented in Table 4. The IC_{50} for Mambin was 34.8 nM although the IC_{50} value for Barbourin could not be determined as the maximum amount of Barbourin tested was 290 nM and at this concentration Barbourin had a negligible effect on adhesion. The IC_{50} value for Mambin in the inhibition of HUVEC adhesion to vitronectin of 34.8 nM is in the same range as the IC_{50} value for Mambin in platelet aggregation (29.5 nM). This demonstrates the lack of specificity that Mambin has for the GPIIb/IIIa-fibrinogen interaction, especially considering that Barbourin did not inhibit the HUVEC-vitronectin interaction at the concentrations tested.

DISCUSSION

A. Synthesis of Mambin Genes

The method of dual-asymmetric PCR was successful in synthesizing the mamI gene that encodes the native Mambin protein. This method is rapid in that the gene was completely synthesized in less than three hours. The nature of the Mambin protein lends itself to easy manipulation with this system; the region encoding the third loop of Mambin, which is responsible for disintegrin activity, is within the primer mam3. Any desirable mutations in this region can be easily introduced by substituting an appropriate primer in the place of mam3 in the PCR reaction. This primer substitution was successfully completed for the construction of the two Mambin derivatives in this study.

Dual-asymmetric PCR may ultimately be limited by the length of the DNA to be synthesized. Longer primers would be required; in current systems used to generate such primers the rate of incorporation of an incorrect nuclectide increases, and the yield of oligonucleotide decreases with the length of the primers (Barnett and Erfle, 1990).

B. Recombinant Nambin Proteins

1. Bacterial Expression System

Bacillus subtilis extracellular protease-deficient strain WB700 was employed to express the mam genes. The use of Staphylokinase as a fusion protein with Mambin yielded enough secreted protein to allow for efficient purification. Recently, Lu et al. (1996) successfully expressed the mam gene and purified Mambin in Escherichia coli. Several concerns in this study were

addressed by the investigators. The Mambin protein was produced as a fusion protein with glutathione 5-transferase (GST), which is commonly used as a fusion protein due to the ease in purification. The fusion protein was isolated from cell lysates and purified under reducing conditions: this ensured that no disulfide bonding would be formed. A protocol for refolding of Mambin was not described, however full biological activity was achieved following cleavage from GST. This is somewhat expected as many venom proteins that are produced recombinantly or chemically tend to fold in predictable ways that bring the correct cysteine residues together for proper disulfide linkages (P. Alewood, Personal communication, 1996).

It is interesting that Lu et al. (1996) used Factor Xa for cleavage of the GST-Mambin fusion protein. As discussed in the Literature Review, this approach was discounted in this study due to concerns of contaminating proteins from the Russel's Viper venom used to activate Factor X. This concern was not addressed by Lu et al., however the possibility of contamination exists.

2. Protein Purification

The fusion proteins Staphylokinase-Mambin I, Staphylokinase-Mambin II, and Staphylokinase-Mambin III were purified and subsequently cleaved with a recombinant Collagenase that was supplied by Dr. Matsushita. The Collagenase used in this study had protein contamination and suspected protease contamination. Interestingly, the fusion protein could not be incubated with Collagenase for longer than five hours without degradation of Mambin. Although this does not prove that the Collagenase was contaminated, such observed of protein degradation is consistent with protease contamination.

Purification of Mambin I, Mambin II, and Mambin III was attempted several times for each protein. Each protein was successfully purified once (Figure 13) and other purifications were unsuccessful. Analysis by RP-HPLC and SDS-PAGE indicated degradation of the Mambin proteins (Figures 14 and 15). This degradation may have occurred in the Collagenase digestion of the fusion proteins or in the purification through HPLC.

3. Protein Instability

The pure samples of the recombinant Mambin proteins displayed instability. Over a period of twelve days, a decreade in protein concentration for all three proteins was observed. The instability can be accounted for in several ways.

One explanation for the instability is that the proteins were contaminated with proteases. As the proteins were dry samples and rehydrated with sterile (autoclaved) buffer, the contamination would have to be present prior to storage. This could be accounted for in two ways: (1) It is possible that proteases which contaminated the Collagenase preparation were present in the HPLC purification of the Mambin proteins. As no contaminating protein bands appear on the SDS-PAGE gels of the purified proteins (Figure 13), these proteases would be in very low concentrations. If this was the case, then the proteases would not be easily detected in the HPLC chromatogram. Since the Mambin instability was observed after the HPLC step, the contaminating proteases would have co-eluted with Mambin in the HPLC. It is possible then that with time, these proteases degraded the Mambin proteins. (2) Proteases could contaminate the buffers used in the HPLC purification. Due to the volatile nature of the buffer components, acetonitrile and TFA, sterilization is difficult; it is unlikely, however that proteases would be stable in such a hydrophobic acidic environment.

The last component of the buffer is water, which was Milli-Q grade, but not sterilized; the water used was freshly prepared and used within 24 hours. Although it is unlikely that proteases could have been introduced to the water, it is conceivable that some proteases may be stable in this environment. The precise reasons for the instability of the recombinant Mambin proteins is not presently understood. However, it is suspected that proteases may have come from the Collagenase preparation.

C. Native Disintagrin Purification

Purification of Mambin and Barbourin from snake venoms was performed using a one step reverse-phase HPLC scheme. This type of purification is well documented in the literature (McDowell et al. 1992; Scarborough et al. 1993b) and was successfully repeated several times in this study (Figure 16, 17, 18).

D. Assay Systems

1. Platelet Aggregation

The assay developed for disintegrin biological activity was inhibition of human platelet aggregation. This type of assay has been present the literature for many years, however the details of the system differ from research group to research group. The aim of this study was to adapt aspects of published protocols to suit the analysis of disintegrins. This included the integration of a microplate reader in place of the more commonly used aggregomeler and determining a platelet purification protocol that yields platelets with reproducible responses.

2. Platelet Adhesion

The first assay developed for disintegrin biological specificity was inhibition of human platelet adhesion to fibrinogen, fibronectin, and vitronectin. The assay was modified from a published protocol that monitored inhibition of platelet adhesion to fibrinogen and fibronectin (Lu et al. 1994). To determine the validity of the assay several control experiments were performed, which included repetition of the conditions used by Lu (Lu et al. 1994). The conditions used in this study were established and differed from those of Lu et al., most significantly in the method of platelet purification.

3. HUVEC Adhesion

The second assay developed for disintegrin biological specificity was inhibition of human umbilical vein endothelial cell (HUVEC) adhesion to fibrinogen, fibronectin, and vitronectin. The assay was modified from several published protocols that monitored inhibition of HUVEC and other cell adhesion to different ligands (Satoh et al. 1994; Foster et al. 1993). As these studies did not report control values for the assay systems, direct comparisons of the controls established in this study are difficult. The controls determined for the HUVEC adhesion assay included determination of detection method by staining, dose responses and time courses of HUVEC adhesion to fibrinogen, fibronectin, and vitronectin.

E. Characterization of Disintegrins

Purified Mambin and Barbourin were characterized in parallel using three assays. The data was presented in terms of IC_{50} values and is shown in Table 4.

1. Biological Activity

The IC_{50} value for inhibition of washed human platelet aggregation for Mambin was 29.5 nM. This compares to published values of 20 nM (Willliams et al. 1992) and 75 nM (Lu et al. 1996), using the same assay, and 172 nM (McDowell et al. 1992), 95 nM (Willliams et al. 1992) and 160 nM (Lu et al. 1994) using platelet rich plasma. With the exception of the McDowell's determination (1992), all other determinations were made by the research group headed by Dr. X. Lu. It is interesting that one group should observe such variation in IC_{50} values for one protein.

This discrepancy in IC_{50} values determined (Willliams et al. 1992; Lu et al. 1994; Lu et al. 1996) may be accounted for in several ways. First, the problem could lie with the Mambin protein. If Lu et al. were using Mambin from the same preparation in all studies, one would assume that the IC_{50} values should remain the same. However, the data for platelet rich plasma (Willliams et al. 1992; Lu et al. 1994) were published two years apart and the data for washed platelets were published four years apart. It is interesting that both the IC_{50} values for platelet rich plasma and washed platelets should increase over time. If Lu et al. were using the same preparation of protein, it would appear that their protein had lost biological activity over time. If the protein used was from different preparations, it is possible that the method used for protein concentration determination was not accurate; Lu et al. did not mention how they determine these values, so it is very difficult to comment on this point. However, for the study presented here, amino acid sequencing was used to obtain the most accurate protein concentration possible.

A second view of the discrepancy observed by Lu et al. would be the assay system. As blood is isolated from different donors, variation in the responses of platelets is often seen (data not

shown). This variation may account for the differences in IC_{50} values.

Barbourin has an IC_{50} value of 31.1 nM for the inhibition of washed platelet aggregation. This value has not been determined in the literature, but an IC_{50} value for inhibition of platelet rich plasma aggregation is reported to be 309 nM (Scarborough et al. 1991). Although these values cannot be compared directly, the higher value for platelet rich plasma is consistent with other disintegrins as discussed.

Mambin and Barbourin have a similar biological activity as the IC_{50} values for platelet aggregation are in the same range (29.5 nM and 31.1 nM).

2. Biological Specificity

Biological specificity towards the platelet GPIIb/IIIa receptor was determined by two cell adhesion assays. The first was human platelet adhesion. The platelet adhesion to fibrinogen indicates the contribution of GPIIb/IIIa. The adhesion to both fibronectin and vitronectin indicates the contribution of $\alpha_5\beta_1$ and $\alpha_v\beta_3$; inhibition of platelet adhesion to either ligand would indicate lack of specificity to GPIIb/IIIa.

The second assay used was HUVEC adhesion. These cells were tested on the same ligands as the platelets. As HUVEC do not possess the GPIIb/IIIa receptor, any inhibition of HUVEC adhesion to the ligands indicates a lack of specificity to GPIIb/IIIa.

a) Platelet Adhesion

Mambin inhibited all platelet adhesions, but to varying extents; the IC_{50} value for fibrinogen (unstimulated platelets) was 15.8 nM, for fibrinogen (stimulated platelets) was 219.9 nM, for

fibronectin was 841.4 nM, and for vitronectin was 325.6 nM. Mambin was most effective at inhibiting the fibrinogen interactions, which are mediated through GPIIb/IIIa.

The data for Mambin can be compared to those reported by Lu (Lu et al. 1994), keeping in mind that there are some differences in the methodology; Lu determined the IC_{50} value for fibrinogen to be 260 nM, which is in the range of that determined in this study for activated platelets. Their second determination was for fibronectin at 20 uM. This value is different from that determined here (841.1 nM). One explanation for this discrepancy could be in the differences in methodology.

Barbourin inhibited platelet adhesion to fibrinogen and vitronectin, but to varying extents; the IC_{50} value for fibrinogen (unstimulated platelets) was 27.5 nM, for fibrinogen (stimulated platelets) was 220.5 nM and for vitronectin was 1005.4 nM. Barbourin was most effective at inhibiting the fibrinogen interactions, which are mediated through GPIIb/IIIa. Barbourin had not noticeable effect on the fibronectin adhesion and a minimal effect on the vitronectin adhesion.

The data for Barbourin can not be compared to a published result, as Barbouin has not been tested in a platelet adhesion assay in the literature. However, solid phase assays, which test the interference of Barbourin in integrin-ligand interactions directly, can provide some information. In one study, it was determined that Barbourin had an IC_{50} value of 15 nM for the GPIIb/IIIa-fibrinogen interaction, yet IC_{50} values for $\alpha_5\beta_1$-fibronectin and a $\alpha_v\beta_3$-vitronectin could not be determined as Barbourin had no effect on these interactions at the concentrations tested (Scarborough et al. 1991). These observations are consistent with the IC_{50} values determined here, since Barbourin

interfered with platelet adhesion to fibrinogen to the greatest extent, and fibronectin and vitronectin to a much lesser extent.

Mambin and Barbourin have IC_{50} values for both unstimulated and stimulated platelet adhesion to fibrinogen in the same range (15.8 nM and 27.5 nM; 219.9 nM and 220 nM). This indicates that the two disintegrins inhibit the platelet-fibrinogen interaction to a similar extent. This interaction can be viewed in two manners: GPIIb/IIIa-fibrinogen and $\alpha_v\beta_3$-fibrinogen. Assuming that the data presented by Scarborough et al. (1991) is valid (Barbourin interacts with fibrinogen but has no detectable interaction with fibronectin or vitronectin), the four platelet adhesion curves can be interpreted with respect to the integrin receptor interaction. Any effect that Barbourin has on platelet adhesion can be viewed as through GPIIb/IIIa. Increased inhibition of platelet adhesion caused by Mambin would therefore be through other integrin receptors. This increase in IC_{50} could be accompanied by a change in the nature of the dose response curve: if one receptor-ligand interaction is involved, a simple dose response curve would be anticipated, if two receptor-ligand ineractions are involved, a more complex curve would be expected.

On platelets, fibrinogen can be bound by both GPIIb/IIIa and $\alpha_v\beta_3$ (Kishimoto and Anderson, 1992). Comparing unstimulated and stimulated adhesion of platelets to fibrinogen, Barbourin inhibits the unstimulated to a greater extent. This reflects the increased number and affinity of GPIIb/IIIa to fibrinogen. In both cases, Mambin exhibits both an increased IC_{50} value and different dose response curve than Barbourin. This reflects the additonal interaction of Mambin with $\alpha_v\beta_3$.

The difference seen between IC_{50} values for the platelet-fibronectin interaction for Mambin (841.1 nM) and Barbourin (>2180 nM) indicates that Mambin interacts with $\alpha_5\beta_1$, thus preventing the

fibroectin interaction with the platelets. Barbourin does not appear to interact with $\alpha_5\beta_1$ and this shows the specificity that Barbourin has for GPIIb/IIIa that Mambin does not.

Fibronectin is thought to interact with platelets through GPIIb/IIIa, $\alpha_v\beta_3$, and $\alpha_5\beta_1$ (Kishimoto and Anderson, 1992). Barbourin was seen here to have little effect on activated platelet adheison to fibronectin (Figure 35). It would follow that platelets interact with fibronectin through $\alpha_v\beta_3$ and/or $\alpha_5\beta_1$ and not through GPIIb/IIIa. Mambin is observed to inhibit this interaction, presumably by its interaction with $\alpha_v\beta_3$ and/or $\alpha_5\beta_1$.

The difference seen between IC_{50} values for the platelet-vitronectin interaction for Mambin (325.6 nM) and Barbourin (1005.4 nM) indicates that Mambin interacts with $\alpha_v\beta_3$, thus preventing the vitronectin interaction with the platelets. Barbourin interacts with $\alpha_v\beta_3$ to a lesser extent as evidenced by the high IC_{50} value. This further shows the greater specificity that Barbourin has for GPIIb/IIIa than Mambin.

Platelets bind vitronectin through GPIIb/IIIa and $\alpha_v\beta_3$ (Kishimoto and Anderson, 1992). Barbourin was seen here to inhibit activated platelet adheison to fibronectin (Figure 35). It would follow that platelets do interact with vitronectin through GPIIb/IIIa, however this does not eliminate a role for $\alpha_v\beta_3$. Mambin too is observed to inhibit platelet adhesion to vitronectin, presumably via GPIIb/IIIa and possibly $\alpha_v\beta_3$.

b) HUVEC Adhesion

Mambin inhibited all HUVEC adhesion assays, but to different extents. For adhesion to fibrinogen the IC_{50} value was 102.2 nM, for adhesion to fibronectin the IC_{50} value was 299.6 nM, and for

adhesion to vitronectin the IC_{50} value was 34.8 nM. These values indicate the lack of specificity that Mambin has for the GPIIb/IIIa receptor.

Barbourin had little effect on all HUVEC adhesion assays. The IC_{50} value for adhesion to fibrinogen could not be determined since Barbourin did not have any effect over the concentrations tested, for adhesion to fibronectin, the IC_{50} value was 597 nM, and the IC_{50} value for adhesion to vitronectin could not be determined since Barbourin did not have any effect over the concentrations tested. These values indicate the high degree of specificity that Barbourin has for GPIIb/IIa, since the inhibition was minimal for all ligands tested. Overall, the data presented in this study demonstrate the difference in biological specificity that two disintegrins, Mambin and Barbourin, have using two cell assay systems.

HUVEC adhere to fibrinogen through $\alpha_v\beta_3$ (Kishimoto and Anderson, 1992). Barbourin has no effect on this interaction as seen in Figure 37. This indicates that Barbourin does not bind this integrin in HUVEC. The inhibition of this interaction by Mambin therefore reflects Mambin's affinity for $\alpha_v\beta_3$.

Although HUVEC are known to bind fibronectin through three integrins ($\alpha_v\beta_3$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$), the most important of these is $\alpha_v\beta_3$ (Kishimoto and Anderson, 1992). In the present results (Figure 38) Barbourin had a lesser effect on HUVEC adhesion to fibronectin than Mambin. It was observed by Scarborough (1991) that Barbourin does not interact with $\alpha_v\beta_3$ or $\alpha_5\beta_1$ and the lack of Barbourin interaction with $\alpha_v\beta_3$ is demonstrated with HUVEC adhesion to both fibrinogen and vitronectin. It can be therefore be said that the inhibition observed with Barbourin reflects the interaction of HUVEC $\alpha_3\beta_1$-fibronectin. The increased inhibition observed with Mambin could reflect Mambin binding $\alpha_v\beta_3$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$.

HUVEC adhere to vitronectin through $\alpha_v\beta_3$ (Kishimoto and Anderson, 1992). The absence of Barbourin effect on this interaction (Figure 39) demonstrates further that Barbourin does not bind this integrin. Again, Mambin interferes with HUVEC adhesion to vitronectin and confirms the observation with fibrinogen that Mambin binds $\alpha_v\beta_3$.

F. Future Studies

Future studies could take two directions. One aspect of the work that could be pursued is the instability of the recombinant Mambin proteins. The cause of the instability could be determined in the hopes of improving protein stability for further studies involving the production of disintegrins in B. subtilis. It might be of interest to attempt expression of the mam genes in E. coli as Lu et al. were successful in this regard (1996). It remains a question which protease would be best used to cleave the fusion protein.

It has been suggested that a more efficient means to study the contribution of amino acids to disintegrin specificity is via chemical synthesis. This approach has been applied to the study of disintegrins (Scarborough et al. 1993a) and has several advantages over recombinant expression: (1) the synthesis of a protein is rapid; a synthetic protein can take several hours to construct whereas the expression of recombinant protein requires days to construct the genes in a plasmid and days to purify; (2) unusual amino acids, which may be useful in structure-function studies, can be incorporated readily in chemical synthesis; (3) recombinant expression is not guaranteed as the protein may be toxic to the cell or may be susceptible to protease degradation, whereas these concerns do not exist for chemical synthesis of proteins. One drawback of chemical synthesis is by the limitation to small

proteins. Disintegrins, however, are sufficiently small that chemical synthesis is a viable alternative to recombinant production. Another drawback of chemical synthesis is the low yields; recombinant production of proteins has unlimited yield. For the purposes of studying disiategrins, only nanomolar concentrations are required, so in the early stages of investigation small amounts of protein are required. Should a protein be found to have desirable properties, it could then be expressed recombinantly and much time would have been save through chemical synthesis. A final concern with chemical synthesis is the potential heterogeneity of the product of synthesis. Fortunately high resolution HPLC purification of the products negate this concern.

Another avenue of further studies would be with regard to the disintegrin assays. The assays developed in this study could be applied to a variety of disintegrins. As discussed, it is desirable, therefore, to test disintegrins in parallel to enable comparisons between disintegrins

TABLE 1. List of Bacterial Strains Used in This Study

Strain	Species	Description	Reference/Source
DH5α	*E. coli*	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	(Hanahan, 1983)
WB700	*B. subtilis*	*nprE aprE epr bpf mpr::ble nprB::bsr vpr::ery*	S-L. Wong

TABLE 2. Primers Used in This Study

Primer	Sequence (5′ TO 3′)	Source
M13 universal	GTAAAACGACGGCCAGT	Pharmacia
M13 reverse	AACAGCTATGACCATG	UC DNA
mam1	GGGAAGCTTTTGCACGGATCTGCTATAATCATCTTGGCACAAAACCGCCTACAACAGAAACATGTCAAGAAGACAGCTG	UC DNA
mam2	CAGCCGCAGCCTCTGCGAATAATATTATCAAATGTCCAAATATTTTTATAGCAGCTGACTTCTTGAC	UC DNA
mam3	CGCAGAGGCTGCGGCTGCTTTACACCACGTGGCGATATGCCGGGACCTTATTGCTGCGAATCTGATAAATG	UC DNA
mam3(II)	CGCAGAGGCTGCGGCTGCAGAACACCAAAAGGCGATTGGCCGGGACCTTATTGCTGCGAATCTGATAAATG	UC DNA
mam3(III)	CGCAGAGGCTGCGGCTGCAGAGTCGCGAAAGGCGATTGGAATGATGATACGTGCTGCGAATCTGATAAATG	UC DNA
mam4	GGGAAGCTTGCATGCTTATTAAAGATTGCATTTATCAGATTCGCAGCAATAAGGTCCCGGCATATCGCCACGTGGTGTAAAG CAGCCGCAGCCTCTGCG	UC DNA
sakintf	GAATACTATGTCGAATGGGC	UC DNA
clmam	GGGGATCCGGACCTGGAGGACCGCGGCGGATCTGCTATAATCATCTTGG	UC DNA

TABLE 3. Relative Concentrations of Recombinant Mambin Proteins

	Relative Protein Concentration		
Protein	Day 1	Day 9	Day 12
mambinI	1.0	0.522	0.385
mambinII	1.0	0.711	0.379
mambinIII	1.0	ND	0.369

* determined by quantitation on MacBas software (Fuji), relative to that concentration determined on day 1

ND - not determined

TABLE 4. IC_{50} Values for Mambin and Barbourin

	IC_{50} (nM)[a, b]	
	mambin	barbourin
platelet aggregation	29.5	31.1
unstimulated platelet adhesion to fibrinogen	15.8	27.5
stimulated platelet adhesion to fibrinogen	219.9	220.5
stimulated platelet adhesion to fibronectin	841.4	> 2180[c]
stimulated platelet adhesion to vitronectin	325.6	1005.4
HUVEC adhesion to fibrinogen	102.2	> 290 [c]
HUVEC adhesion to fibronectin	299.6[d]	597.0[d]
HUVEC adhesion to vitronectin	34.8	> 290 [c]

[a] the IC_{50} values were determined by linear regression

[b] the correlation coefficients for the linear regression were between 0.937 and 0.999

[c] no correlation coefficient was determined

[d] value determined by linear regression was extrapolated from the data

TABLE 5. Single Letter Code for Amino Acids

Single Letter	Amino Acid
A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

Figure 1. Mambin Genes.

A. mamI

The complete nucleotide sequence for mamI is shown. Below the nucleotides, the corresponding amino acids for the protein Mambin I are shown using the single letter amino acid code (Table 5). Above the nucleotides, the primers mam1, mam2, mam3, and mam4 are shown. The position of the lines indicate that nucleotide sequence that the primer covers. The direction of the arrows on the primers indicates the direction of the primer: mam1 and mam3 are in the sense direction, mam2 and mam4 are in the antisense direction. The stop codon is indicated by ".".

B. mamII

The nucleotide sequence for the primer mam3(II) that is identical to mam3. Only those nucleotides that differ from mam3 are noted. The corresponding amino acid changes of Mambin I to Mambin II are shown and all other amino acids of Mambin II are identical to Mambin I.

C. mamIII

The nucleotide sequence for the primer mam3(III) is shown. The dashed line indicates that region of mam3(III) that is identical to mam3. Only those nucleotides that differ from mam3 are presented. The corresponding amino acid changes of Mambin I to Mambin III are shown and all other amino acids of Mambin III are identical to Mambin I.

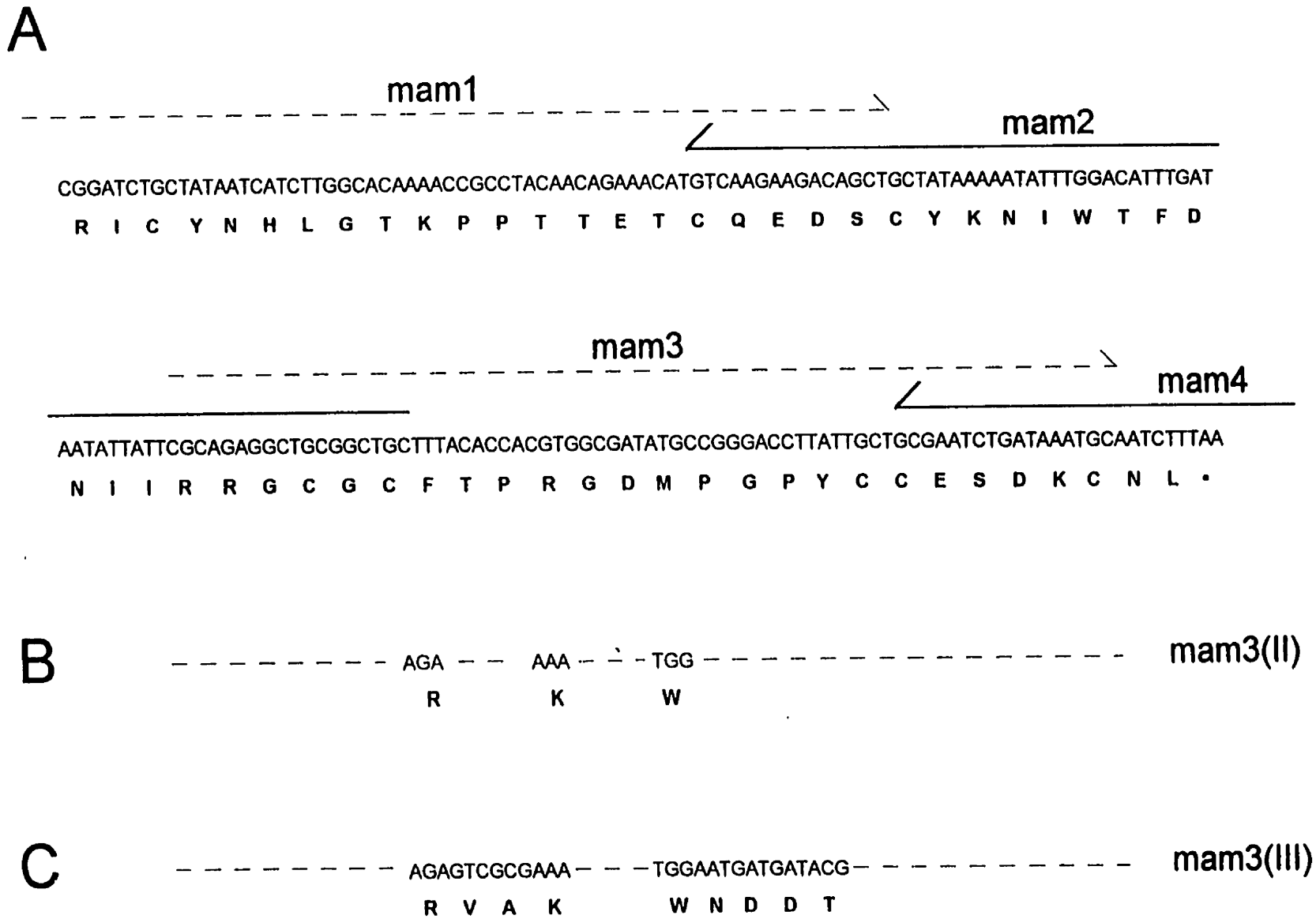

A
mam1
mam2
CGGATCTGCTATAATCATCTTGGCACAAAACCGCCTACAACAGAAACATGTCAAGAAGACAGCTGCTATAAAAATATTTGGACATTTGAT
R I C Y N H L G T K P P T T E T C Q E D S C Y K N I W T F D
mam3
mam4
AATATTATTCGCAGAGGCTGCGGCTGCTTTACACCACGTGGCGATATGCCGGGACCTTATTGCTGCGAATCTGATAAATGCAATCTTTAA
N I I R R G C G C F T P R G D M P G P Y C C E S D K C N L •
B
AGA
AAA
TGG
R
K
W
mam3(II)
C
AGAGTCGCGAAA
TGGAATGATGATACG
R V A K
W N D D T
mam3(III)

Figure 2. pMAMI.

pMAMI is based on the E. coli plasmid pBM*. As such it is a high copy number plasmid, encoding a gene for ampicillin resistance (Amp). The plasmid shown is that for the mamI gene. It was introduced into pBM* using SphI and HindIII. This plasmid and the series of plasmids, pMAMII and pMAMIII, were designed to facilitate sequenceing of the PCR-synthesized mam genes. The primers and their positions are indicated in the figure by the circles with letters (R-reverse, U-universal). The arrows indicate the direction of sequenceing with each primer.

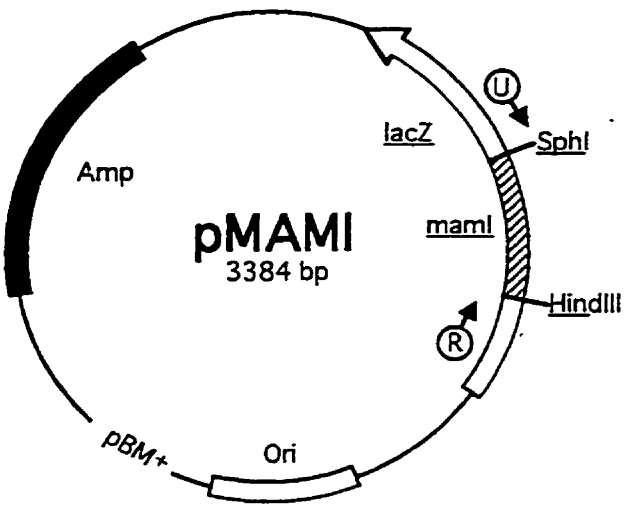

pMAMI
3384 bp
Amp
lacZ
SphI
maml
HindIII
U
R
pBM+
Ori

Figure 3. Plasmids Involved in the Construction of pSAKCMAMI.

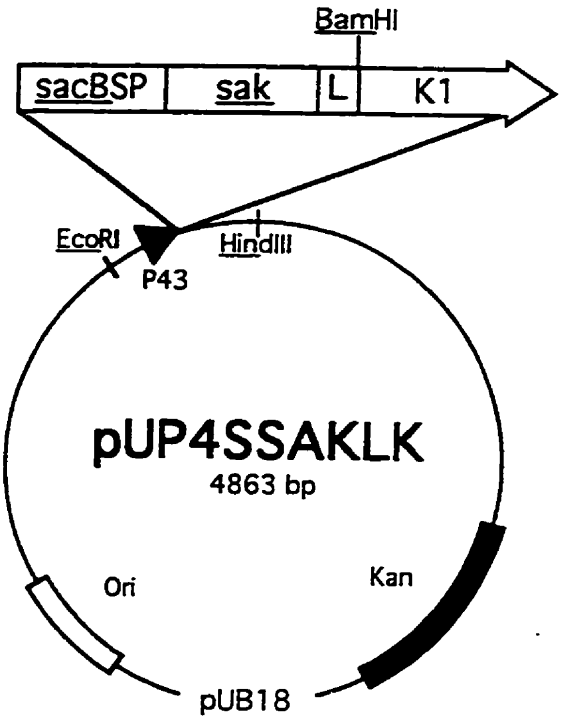
A. pUP4SSAKLK

This plasmid was constructed by E. Sihota (Personal communication, 1995). pUP4SSAKLK is based on the B. subtilis plasmid pUB18. As such it is a high copy number plasmid, encoding a gene for kanamycin resistance (Kan). It directs the expression of a foreign fusion protein. The genes in the fusion protein encode Staphylokinase (sak), a linker domain (L), and a K1 domain (K1). These genes are expressed under the control of the P43 promoter, followed by a gene encoding a signal peptide (sacBSP) which directs secretion of the fusion protein.

B. pSAKL

pSAKL was constructed by removing the EcoRI BamHI fragment from pUP4SSAKLK. This fragment contains the P43 promoter, sacBSP, sak, and L. The fragment was inserted into pUB18 and as such retains the properties of pUB18 described above.

A


BamHI
sacBSP
sak
L
K1
EcoRI
HindIII
P43
pUP4SSAKLK
4863 bp
Ori
Kan
pUB18

B

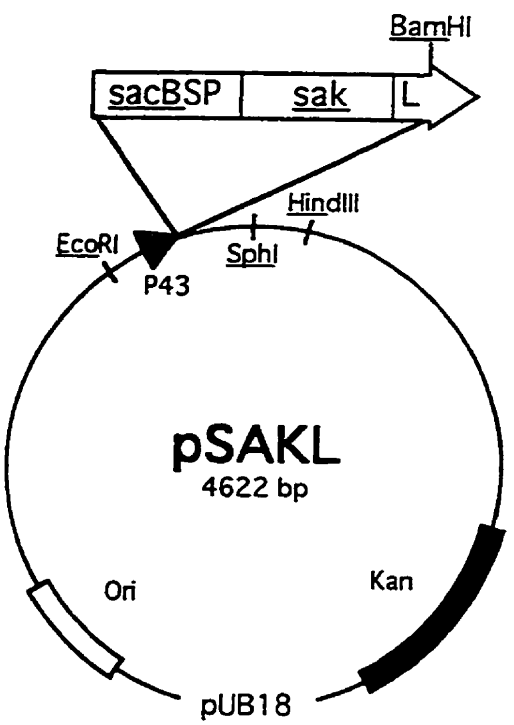

BamHI
sacBSP
sak
L
HindIII
EcoRI
SphI
P43
pSAKL
4622 bp
Ori
Kan
pUB18

Figure 4. pSAKCMAMI.

pSAKCMAM is based on the B. subtilis plasmid pUB18. As such it is a high copy number plasmid, encoding a gene for kanamycin resistance (Kan). The plasmid was condtructed as follows: A PCR amplicon containing the gene mamI was introduced by BamHI and SphI into the plasmid pSAKL. pSAKCMAMI directs the expression of a foreign fusion protein. the genes for the fusion protein are Staphylokinase (sak), a linker domain (L), the Collagenase cleavage site (C), and Mambin I (mamI). These genes are expressed under the control of the P43 promoter, followed by a gene encoding a signal peptide (sacBSP) which directs secretion of the fusion protein.

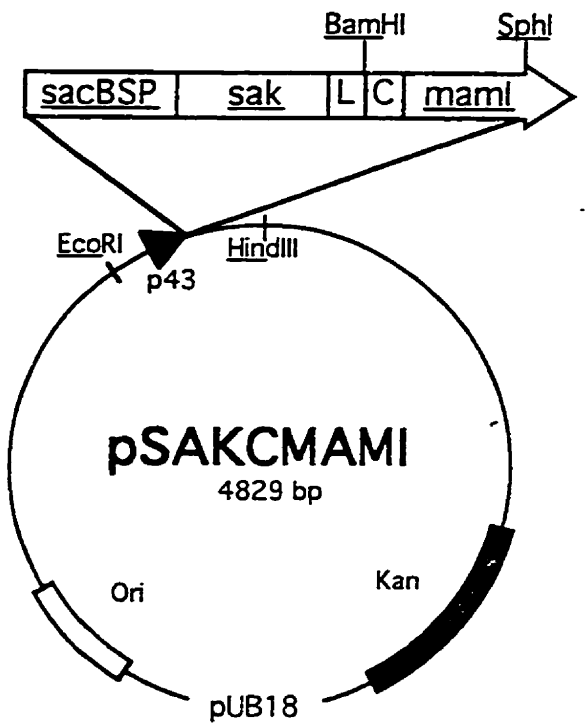

BamHI
SphI
sacBSP
sak
L
C
maml
EcoRI
HindIII
p43
pSAKCMAMI
4829 bp
Ori
Kan
pUB18

Figure 5. Platelet Aggregation.

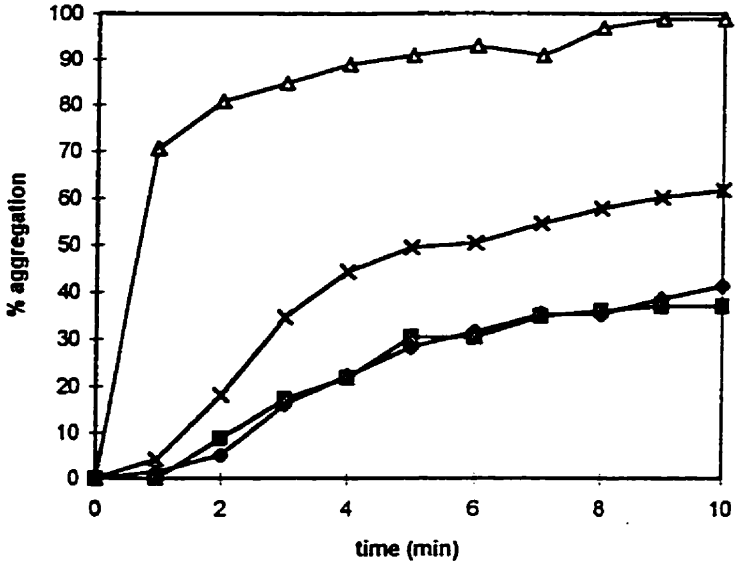
A. Platelet-Rich Plasma

This time course depicts the high variation seen in four different platelet-rich plasma preparations. Platelet-rich plasma prepared from human blood and diluted to 3 X 10′ cells/ml. 135 ul of platelets were aliquoted into microplate wells and human thrombin was added to 0.5 units/ml. The course of aggregation was monitored by a microplate reader (620 nm). Readings were taken every minute for 10 minutes. 0% aggregation was set as the absorbance of the platelets prior to addition of the agonist. Each set of data represents at least three replicates of the same platelet preparation.

B. Washed Platelets

This time course depicts the low variation seen in four different washed platelet preparations. Washed platelets were prepared from human blood and diluted to 3 X 10^{e} cells/ml. 135 ul of platelets were aliquoted into microplate wells and human thrombin was added to 0.5 units/ml. The course of aggregation was monitored by a microplate reader (620 nm). Readings were taken every minute for 10 minutes. 0% aggregation was set as the absorbance of the platelets prior to addition of the agonist. Each set of data represents at least three replicates of the same platelet preparation.

A


100
90
80
70
60
50
40
30
20
10
0
% aggregation
0
2
4
6
8
10
time (min)

B

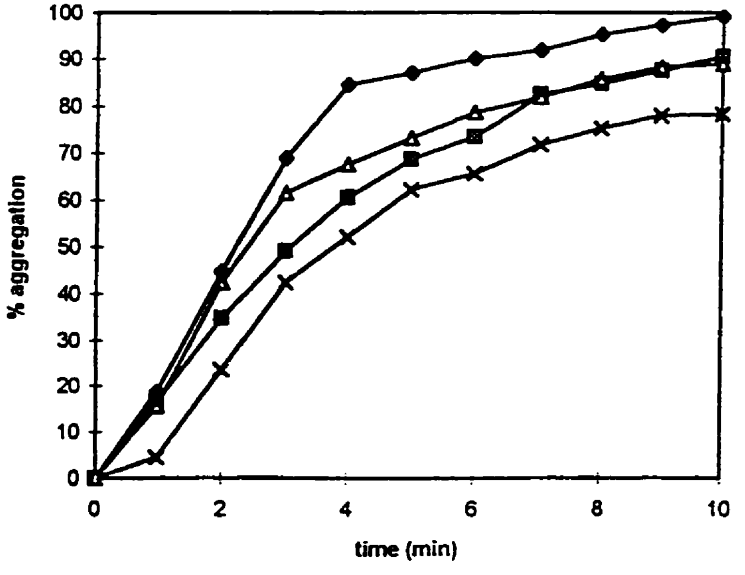

100
90
80
70
60
50
40
30
20
10
0
% aggregation
0
2
4
6
8
10
time (min)

Figure 6. Schematic of Dual-Asymmetric PCR.

Presented is an overview of the theory of dual-asymmetric PCR as it was applied to the synthesis of the genes, mamI, mamII, and mamIII. **(A)** 4 primers were constructed to cover the gene sequence. Primers maml and mam3 are in the sense direction and mam2 and mam4 are in the antisense direction. The primers were designed with a minimum of 16 bp overlap, so that they can anneal as shown. For the PCR reaction, maml and mam4 are introduced at concentrations 10-fold higher than mam2 and mam3. This differential primer concentration favors annealing of maml with mam2 and mam4 with mam3. In the first 10 cycles, two intermediate products are predicted to be synthesized **(B)**. The 131 bp amplicon results from maml priming the synthesis of mam2. The 99 bp results from mam4 priming the synthesis of mam3. When the concentration of these intermediate products increases over the first 10 cycles, they will begin anneal. In the next 10 cycles, the full length product of 213 bp is formed **(C)**. This is a result of the reciprocal priming and synthesis of the 131 and 99 bp intermediate products. The final 10 cycles of the PCR reaction facilitate synthesis of any incomplete PCR amplicons.

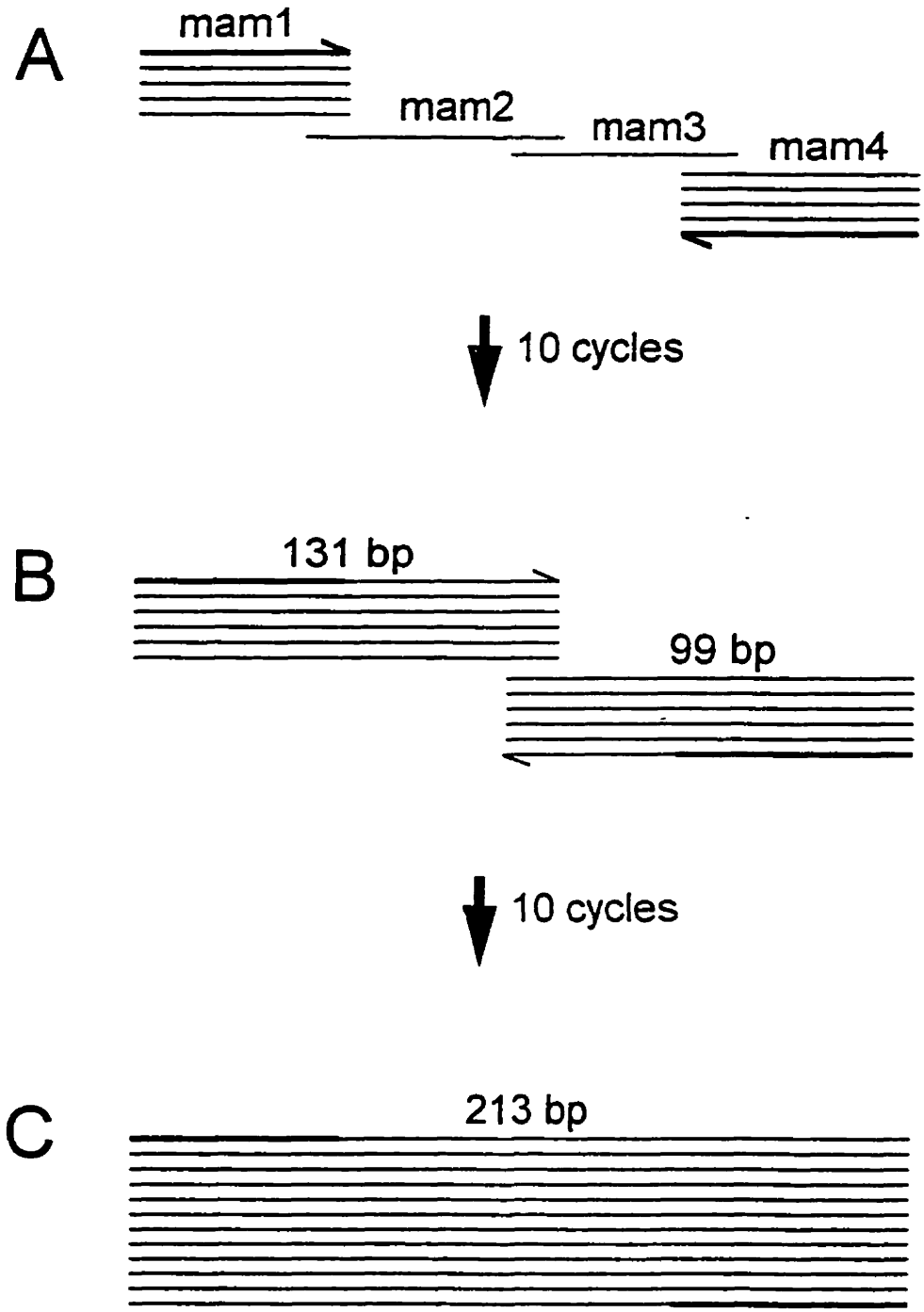

A
mam1
mam2
mam3
mam4
10 cycles
B
131 bp
99 bp
10 cycles
C
213 bp

Figure 7. Time Course of Dual Asymmetric PCR.

DNA amplicons were electrophoresed in 1.2% agarose gel. The sizes of the DNA fragments are noted on the right. The numbers shown above the gel indicate the number of cycles of dual-asymmetric PCR that have elapsed. For each lane, 10 ul was removed from 150 ul reaction at the cycle indicated and electrophoresed. The PCR-amplified DNA was first evident at 5 cycles with a broad band slightly above the 75 bp marker. Two bands reside here, one being 99 bp and one being 131 bp; both representing the PCR intermediate amplicons. At 20 cycles a larger DNA band was visible slightly below the 247 bp marker. It is proposed that this band is the full amplicon of 213 bp. This band increases in intensity until the 30 cycle. The molecular weight marker used (M) was HinfI digested pBM'.

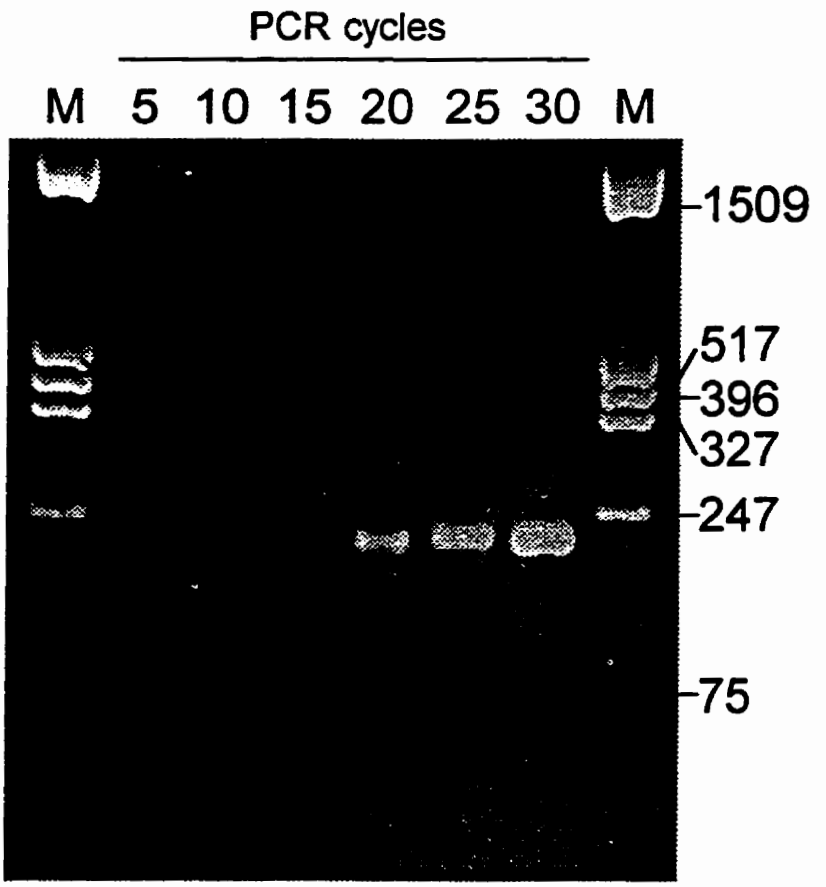

PCR cycles
M
5
10
15
20
25
30
M
1509
517
396
327
247
75

Figure 8. Dual Asymmetric PCR of Mambin Genes.

DNA amplicons were electrophoresed in 1.2% agarose gel. The sizes of the DNA fragments are noted on the left. The labeling above the gel represent the three mam genes (mamI, mamII, and mamIII), synthesized by dual-asymmetric PCR. For each lane, 10 ul of the 150 ul completed PCR reaction was mixed with running dye and electrophoresed. All three lanes show a major DNA band slightly below 247 bp at approximately 213 bp, the size of the complete amplicon. Further cloning and sequencing determined that these bands were the mamI, mamII, and mamIII. The molecular weight marker used (M) was HinfI digested pBM^{-}.

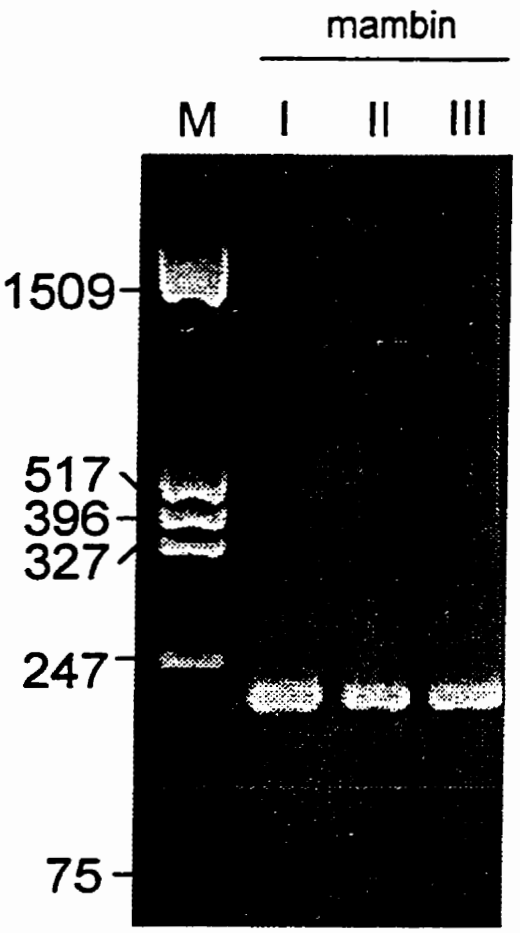

mambin
M
I
II
III
1509
517
396
327
247
75

Figure 9. Purification of Fusion Protein.

Proteins were electrophoresed in a 12% SDS-PAGE gel to show the course of purification of the fusion protein Staphylokinase-Mambin I (sakcmamI). The sizes of the proteins are indicated to the right. Lane 1 is an aliquot of the culture supernatant of the B. subtilis strain expressing the fusion protein. sakcmamI can be observed in the mixture of proteins and is indicated on the right. Lane 2 is an aliquot of the ammonium sulfate treated fraction. Many of the contaminating proteins have been removed by this treatment. Lane 3 is an aliquot of the protein eluted and pooled from the gel filtration column. Most of the proteins in the large molecular weight range (35 kD and above) have been removed by this separation by molecular size. Lane 4 is an aliquot of the protein eluted and pooled from the ion exchange column. At this point, sakcmamI has been removed from almost all of the contaminating proteins. The molecular weight marker used (M) was the Low-Range Marker
(BioRad).

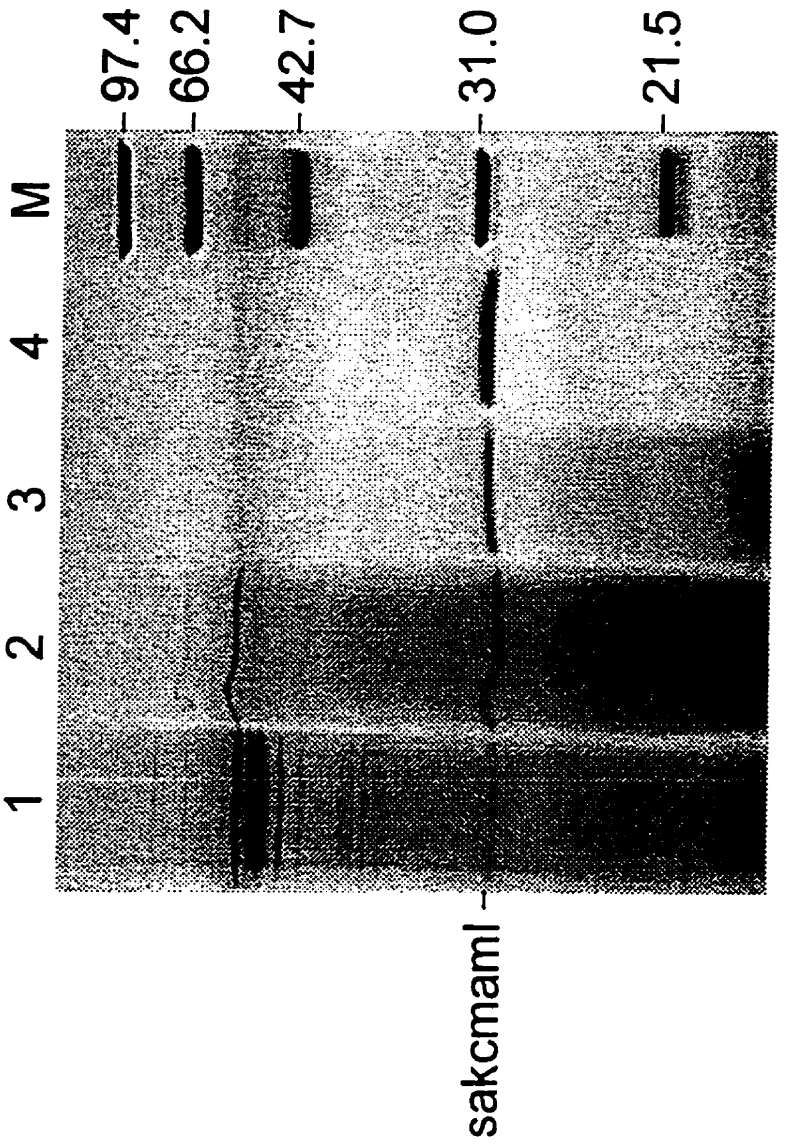

97.4
66.2
42.7
31.0
21.5
M
4
3
2
1
sakcmaml

Figure 10. Time Course of Collagenase Cleavage.

Proteins were electrophoresed ia a 12% SDS-PAGE gel to show the time course of Collagenase cleavage of the fusion protein sakcmamI from 0 to 6 hours. The sizes of the proteins are indicated on the left. The three proteins of concern in this figure are indicated on the right. sakcmamI is the full-length fusion protein, Staphylokinase-Mambin I. "sak" represents Staphylokinase and the linker and "mamI" represents Mambin I. Lane 1 contains 5 ul the undigested fusion protein, sakcmamI. Lanes 2 through 7 contain the same amount of sakcmamI, which was incubated with Collagenase for different times. Lane 2 was incubated for 1 hour, lane 3 was incubated for 2 hours, lane 4 was incubated for 3 hours, lane 5 was incubated for 4 hours, lane 6 was incubated for 5 hours, and lane 7 was incubated for 6 hours. The cleavage of Staphylokinase-Mambin I is evident over the times indicated. Conversely, Staphylokinase appears over the times indicated, with the maximal amount evident at 6 hours. The Mambin I band increases in intensity over the times indicated, however it seems to reach a maximum at 5 hours. The molecular weight marker used (M) was the 2,850 - 43,000 Molecular Weight Range Standards (Gibco-BRL).

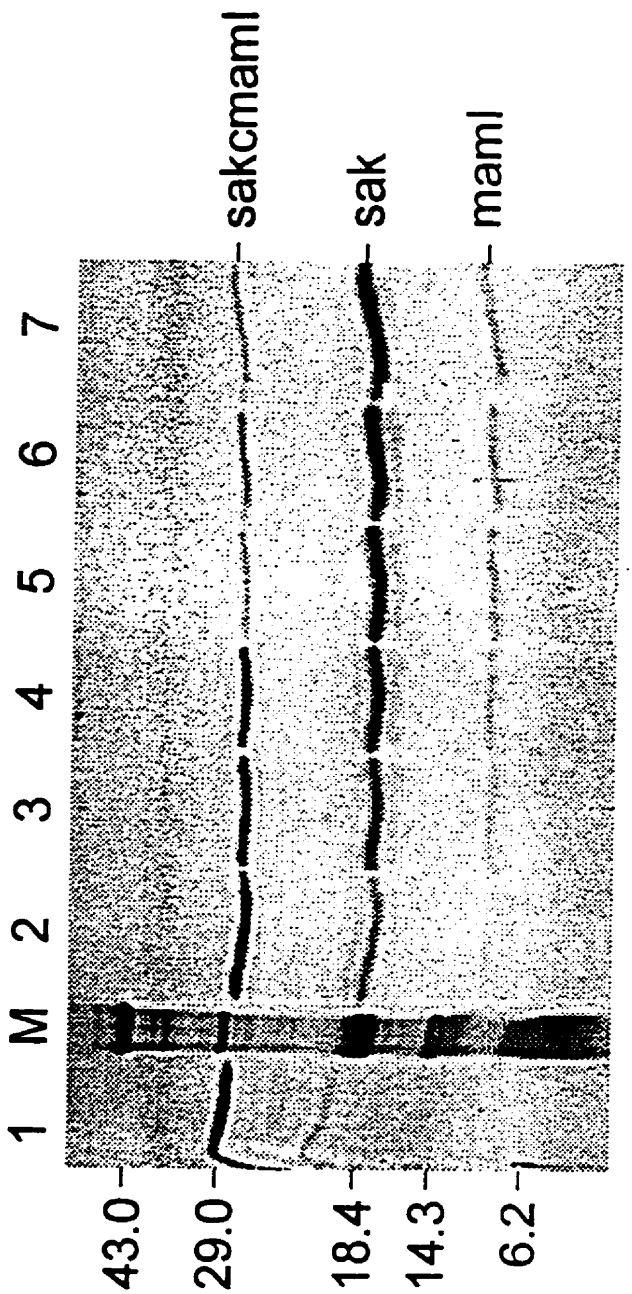

1
M
2
3
4
5
6
7
sakcmaml
sak
maml
43.0
29.0
18.4
14.3
6.2

Figure 11. Time Course of Collagenase Cleavage (PMSF).

Proteins were electrophoresed in a 12% SDS-PAGE gel to show the effect of PMSF on the time course of Collagenase cleavage of sakcmamI from 0 to 6 hours. The sizes of the proteins are indicated on the left. The three proteins of concern in this figure are indicated on the right. sakcmamI is the full-length fusion protein, Staphylokinase-Mambin I. "sak" represents Staphylokinase and the linker and "mamI" represents Mambin I. Lane 1 contains 5 ul the undigested fusion protein, Staphylokinase-Mambin I. Lanes 2 through 7 contain the same amount of Staphylokinase-Mambin I, which was incubated with Collagenase and PMSF for different times. Lane 2 was incubated for 1 hour, lane 3 was incubated for 2 hours, lane 4 was incubated for 3 hours, lane 5 was incubated for 4 hours, lane 6 was incubated for 5 hours, and lane 7 was incubated for 6 hours. Staphylokinase-Mambin I cleavage is not as evident as in Figure C over the times indicated. Staphylokinase appears over the times indicated, with the maximal amount evident at 6 hours, but not to the extent as in Figure C. The Mambin I band has very low intensity over the times indicated, however it seems to reach a maximum at 6 hours. Overall, it is evident that PMSF slows the Collagenase cleavage reaction. The molecular weight marker used (M) was the 2,850 - 43,000 Molecular Weight Range Standards (Gibco-BRL).

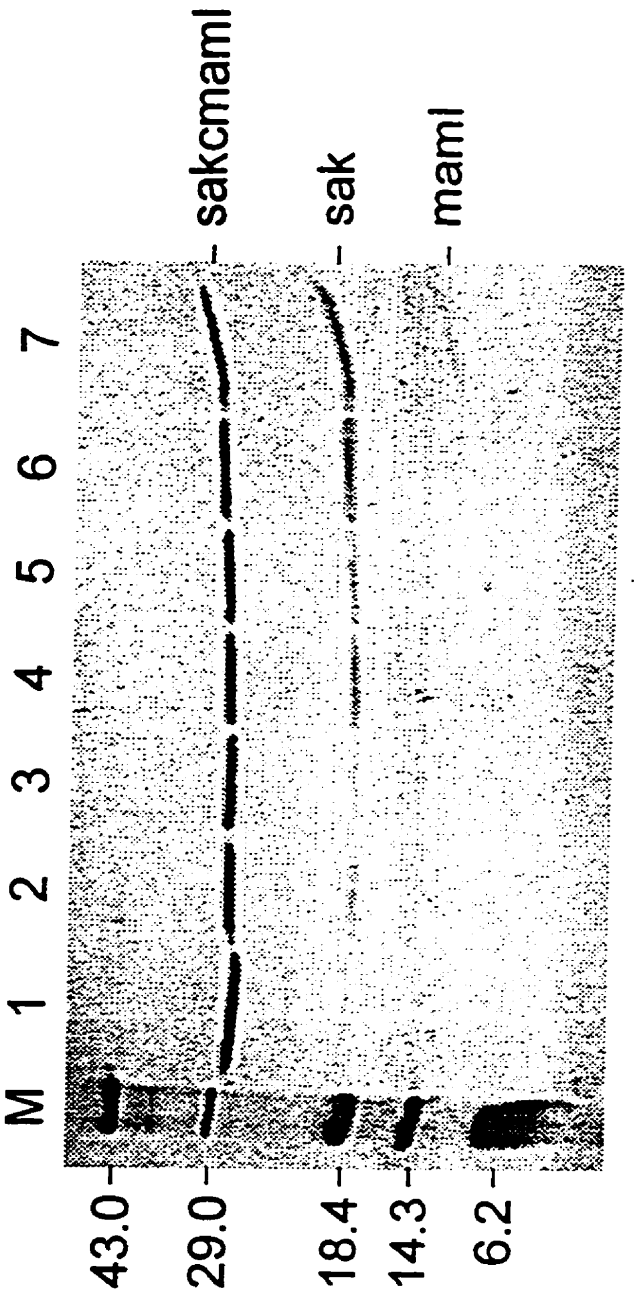

M
1
2
3
4
5
6
7
sakcmaml
sak
maml
43.0
29.0
18.4
14.3
6.2

Figure 12. HPLC Chromatogram of Recombinant Mambin Purification (I).

A completed Collagenase cleavage reaction of sakcmamII was applied and eluted from a Sep-Pak cartridge as outlined in the Methods and Materials section. The sample was applied to a C18 reverse-phase HPLC column. The resultant chromatogram is presented. The solid line represents the absorbance at 280 nm. The dashed line represents the buffer composition that was applied to the column: Buffer A was acetonitrile (0.1% TFA) and Buffer B was H_2O (0.1% TFA). After injection of the protein to the column, it was washed with 10 ml H 0 (0.1% TFA). Mambin II was eluted using a gradient of 0% to 60% acetonitrile (0.1% TFA) that was broken down as follows: 0 to 20% (1%/min), 20 to 40% (0.33%/min), and 40 to 60% (1%/min). The colimn was washed with 10 ml acetonitrile (0.1% TFA) and re-equilibrated with 10 ml H 0 (0.1% TFA). Mambin II eluted at 21% acetonitrile. The peak corresponding to Mambin II is indicated by *.

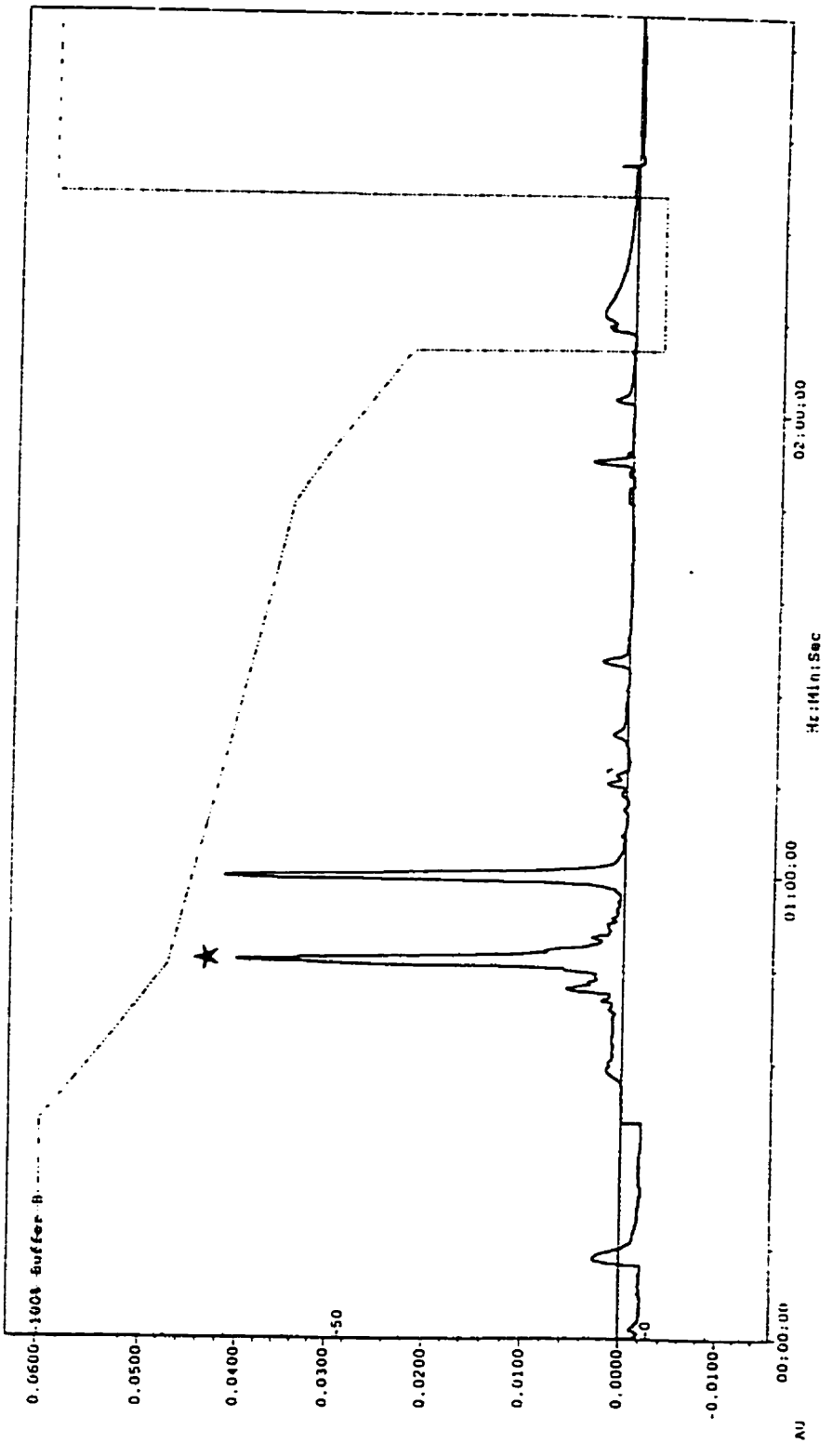

0.0600
0.0500
0.0400
0.0300
0.0200
0.0100
0.0000
-0.0100
AU
100% Buffer B
50
0
00:00:00
01:00:00
02:00:00
Hr:Min:Sec

Figure 13. Purified MambinI, MambinII, and MambinIII.

Proteins were electrophoresed in a 15% SDS-PAGE gel to show the purified forms of Mambin I, Mambin II, and Mambin III. The sizes of the proteins are indicated to the right. Lane I is approximately 0.5 ug of Mambin I. The theoretical size of Mambin I is 6.678 kD and in this gel it runs closer to 11 kD. Lane II is approximately 0.5 ug of Mambin II. The theoretical size of Mambin II is 6.790 kDa and in this gel it runs close to the size of Mambin I. Lane III is approximately 0.5 ug of Mambin III. The theoretical size of Mambin III is 6.793 kD and in this gel it runs closer to 12 kD. The molecular weight marker used (M) was the 2,850 - 43,000 Molecular Weight Range Standards (Gibco-BRL).

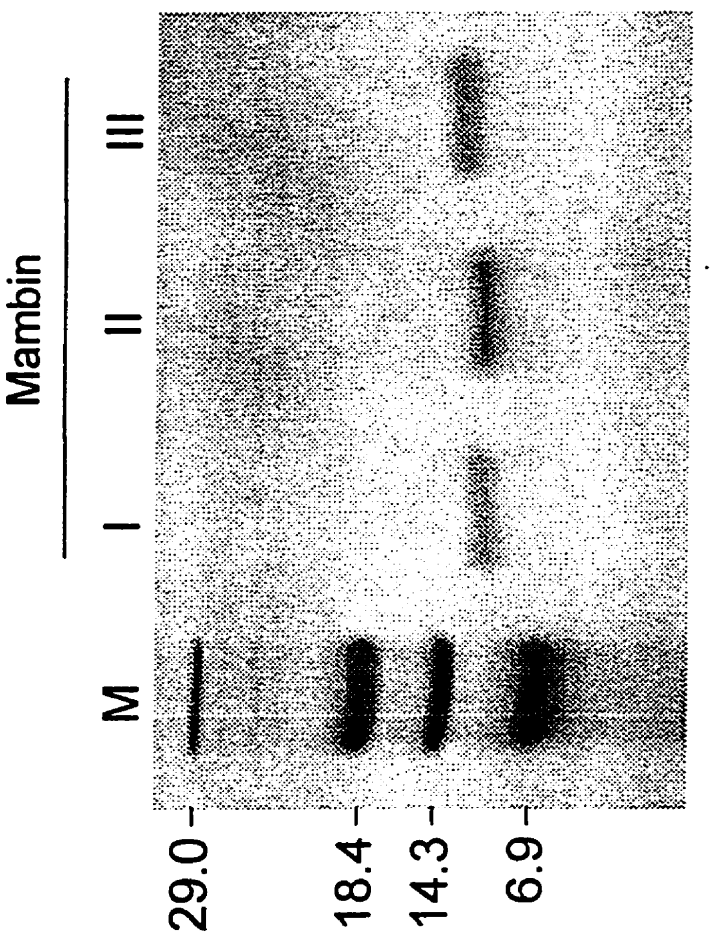

Mambin
M
I
II
III
29.0
18.4
14.3
6.9

Figure 14. HPLC Chromatogram of Recombinant Mambin Purification (II).

A completed Collagenase cleavage reaction of the fusion protein sakcmamII was applied and eluted from a Sep-Pak cartridge as outlined in the Methods and Materials section. The sample was applied to a C18 reverse-phase HPLC column. The resultant chromatogram is presented. The solid line represents the absorbance at 280 nm. The dashed line represents the buffer composition that was applied to the column: Buffer A was acetonitrile (0.1% TFA) and Buffer B was H_2O (0.1% TFA). After injection of the protein to the column, it was washed with 10 ml H_2O (0.1% TFA). Mambin II was eluted using a gradient of 0% to 60% acetonitrile (0.1% TFA) that was broken down as follows: 0 to 25% (1%/min) and 25 to 60% (0.5%/min). The column was washed with 10 ml acetonitrile (0.1% TFA) and re-equilibrated with 10 ml H_2O (0.1% TFA) . Mambin II eluted as a complex set of peaks between 30 and 35% acetonitrile. The set of peaks that correspond to Mambin II are indicated by *.

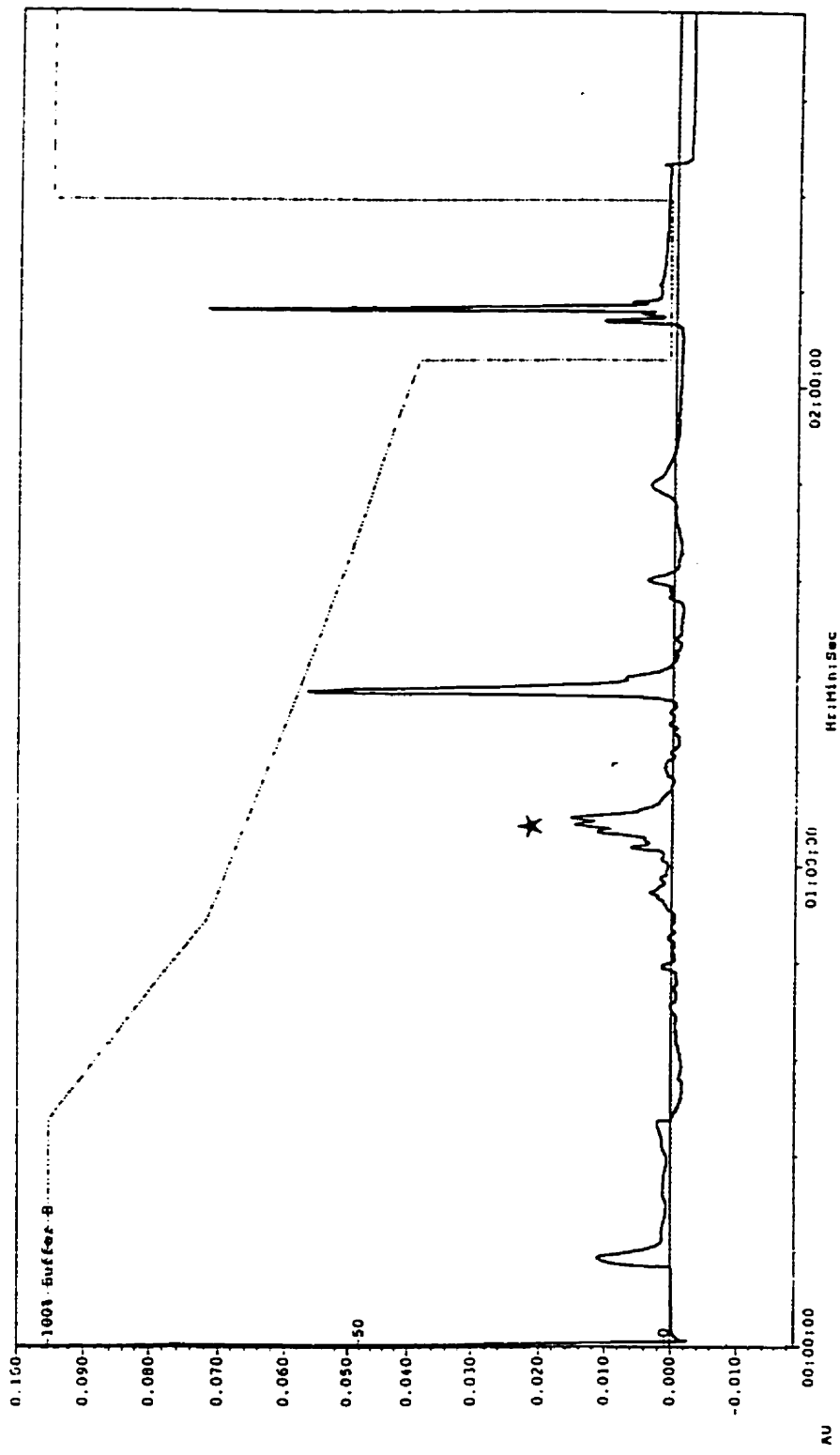

100% Buffer B
50
0.100
0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
-0.010
AU
00:00:00
01:00:30
02:00:00
Hr:Min:Sec

Figure 15. Protein Profile of Mambin II Purification.

Proteins were electrophoresed in a 15% SDS-PAGE gel to show the fractions collected from the RP-HPLC purification of Mambin II presented in Figure 13. The sizes of the proteins are indicated on the left. Lanes 1 through 9 are aliquots from the fractions indicated on Figure 13 by "*". The fractions contain several sizes of proteins. It is presumed that these bands are different forms of Mambin II, in various stages of degradation. A larger band is evident in lanes 1 through 4 and a smaller band in lanes 5 through 8. Although the resolution on this SDS-PAGE is not optimal, it clearly shows a pattern of degradation of Mambin II. The molecular weight marker used (M) was the Low-Range Marker (BioRad).

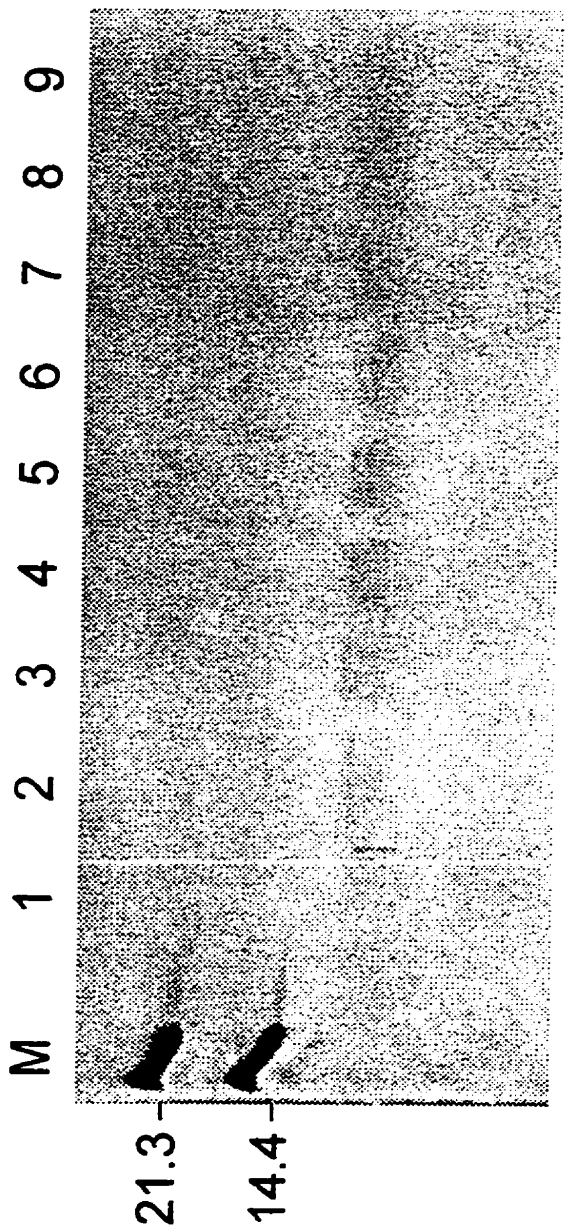

M
1
2
3
4
5
6
7
8
9
21.3
14.4

Figure 16. HPLC Chromatogram of Mambin Purification.

5 mg of lyophilized D. jamesonii venom was rehydrated in 1 ml of sterile ddH_2O, applied and eluted from a Sep-Pak cartridge as outlined in the Methods and Materials section. The sample was applied to a C18 reverse-phase HPLC column. The resulting chromatogram is presented. The solid line represents the absorbance at 280 nm. The dashed line represents the buffer composition that was applied to the column: Buffer A was acetonitrile (0.1% TFA) and Buffer B was H_2O (0.1% TFA). After injection of the venom to the column, it was washed with 10 ml H_2O (0.1% TEA). Mambin was eluted using a gradient of 0% to 60% acetonitrile (0.1% TFA) which was broken down as follows: 0 to 20% (1%/min), 20 to 30% (0.167%/min), and 30 to 60% (2%/min). The column was washed with 10 ml acetonitrile (0.1% TFA) and re-equilibrated with 10 ml H_2O (0.1% TFA). Mambin eluted at 23% acetonitrile. The peak corresponding to Mambin is indicated by "*".

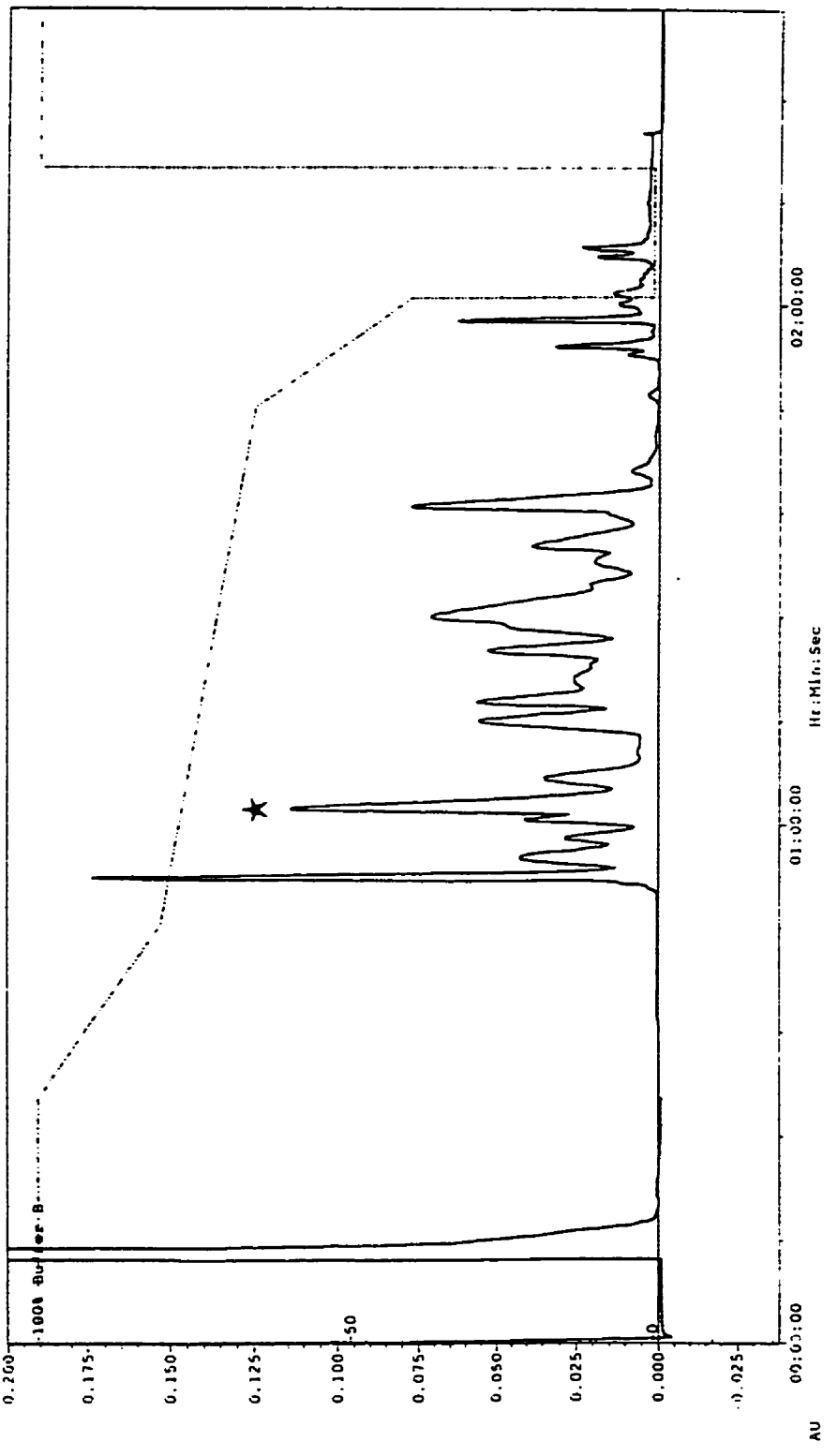

100% Buffer B
0.200
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
-0.025
AU
00:00:00
01:00:00
02:00:00
Hr:Min:Sec

Figure 17. HPLC Chromatogram of Barbourin Purification.

10 mg of lyophilized S. m. barbouri venom was rehydrated in 1 ml of sterile ddH_2O applied and eluted from a Sep-Pak cartridge as outlined in the Methods and Materials section. The sample was applied to a C18 reverse-phase HPLC column. The resulting chromatogram is presented. The solid line represents the absorbance at 280 nm. The dashed line represents the buffer composition that was applied to the column: Buffer A was acetonitrile (0.1% TEA) and Buffer B was H_2D (0.1% TEA). After injection of the venom to the column, it was washed with 10 ml H_2O (0.1% TFA). Barbourin was eluted using a gradient of 0% to 100% acetonitrile (0.15 TFA) which was broken down as follows: 0 to 60% (0.5%/min) and 60 to 100% (1%/min). The column was washed with 10 ml acetonitrile (0.1% TFA) and re-equilibrated with 10 ml H_2O (0.1% TFA). Barbourin eluted at 20% acetonitrile. The peak corresponding to Barbourin is indicated by "*".

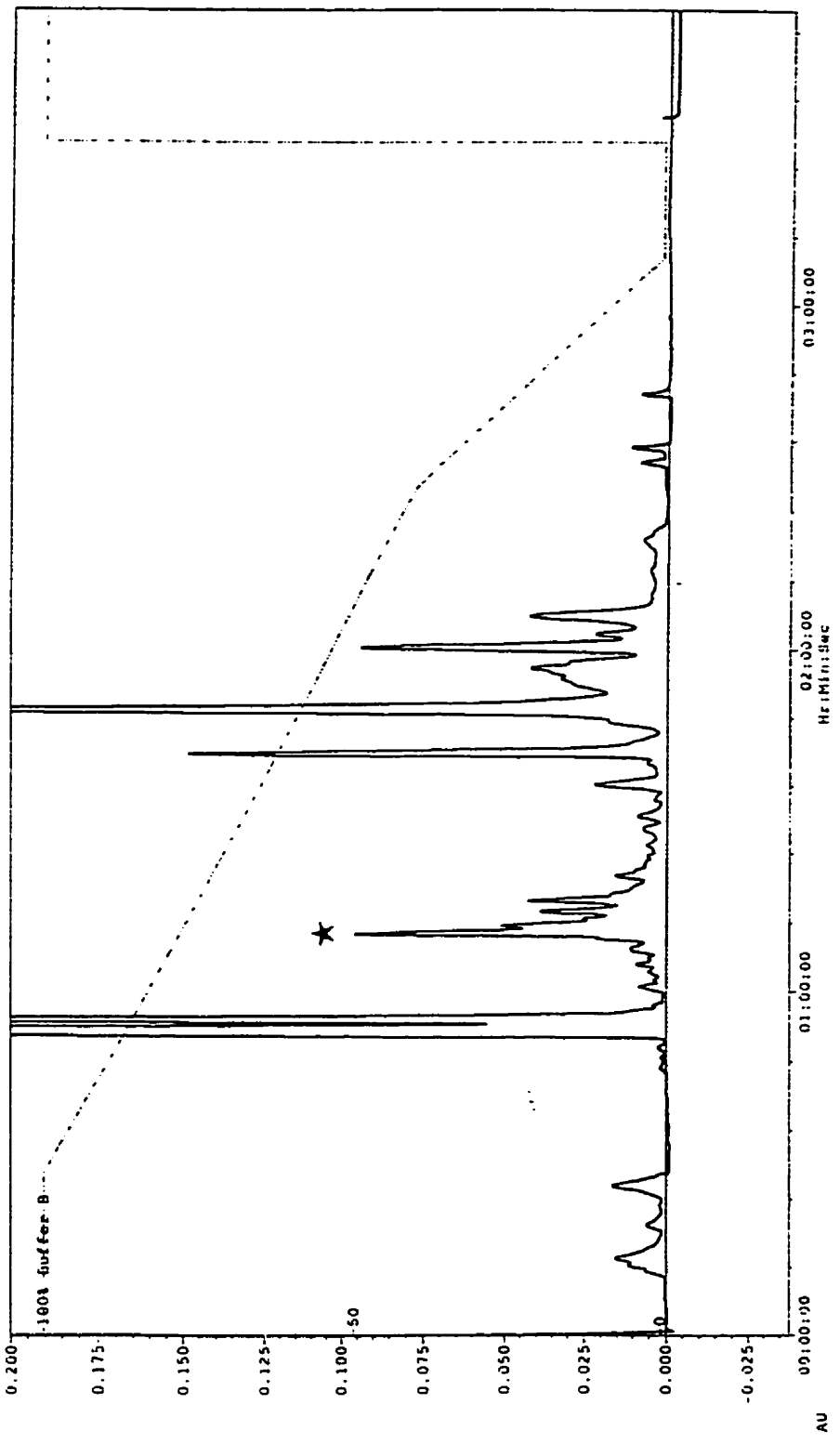

100% buffer B
50
0
0.200
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
-0.025
AU
00:00:00
01:00:00
02:00:00
03:00:00
Hr:Min:Sec

Figure 18. Purified Mambin and Barbourin.

Proteins were electrophoresed in a reducing 15% SDS-PAGE to show the purified forms of Mambin and Barbourin. The sizes of the proteins are indicated on the right. Lane 1 is 0.29 ug of Mambin, purified from D. jamesonii snake venom. The theoretical size of Mambin is 6.678 kD and in this gel it runs closer to 8 kD. Lane 2 is 0.56 ug of Barbourin, purified from S. m. barbouri venom. The theoretical size of Barbourin is 7.700 kD and in this gel it runs closer to 15 kD. The smearing seen below the protein bands is an artifact of the gel, as it was seen in many such gels of small proteins. The molecular weight marker used (M) was 2,850 - 43,000 Molecular Weight Range Standards (Gibco-BRL).

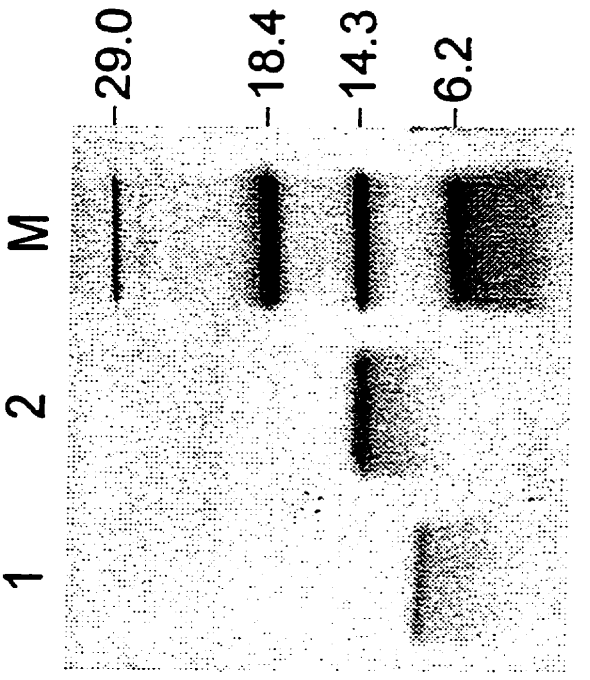

29.0
18.4
14.3
6.2
M
2
1

Figure 19. Platelet Aggregation - ADP vs. Thrombin.

The maximal aggregation attained with the use of the agonists thrombin and ADP (the latter with varying amounts of exogenous fibrinogen added) is presented. Washed platelets were prepared from human blood and diluted to 3 X 10^8 cells/ml. Some of the platelets were incubated with varied amounts of human fibrinogen, which was resuspended in PBS, for 10 minutes at room temperature prior to stimulation. 135 ul of platelets were aliquoted into microplate wells and either ADP (final concentration 20 uM) or human thrombin (final concentration 0.5 units/ml) was added. 0% aggregation was set as the absorbance of the platelets prior to addition of the agonist. Each set of data represents at least three replicates of the same platelet preparation and the standard error is indicated (1 standard deviation unit).

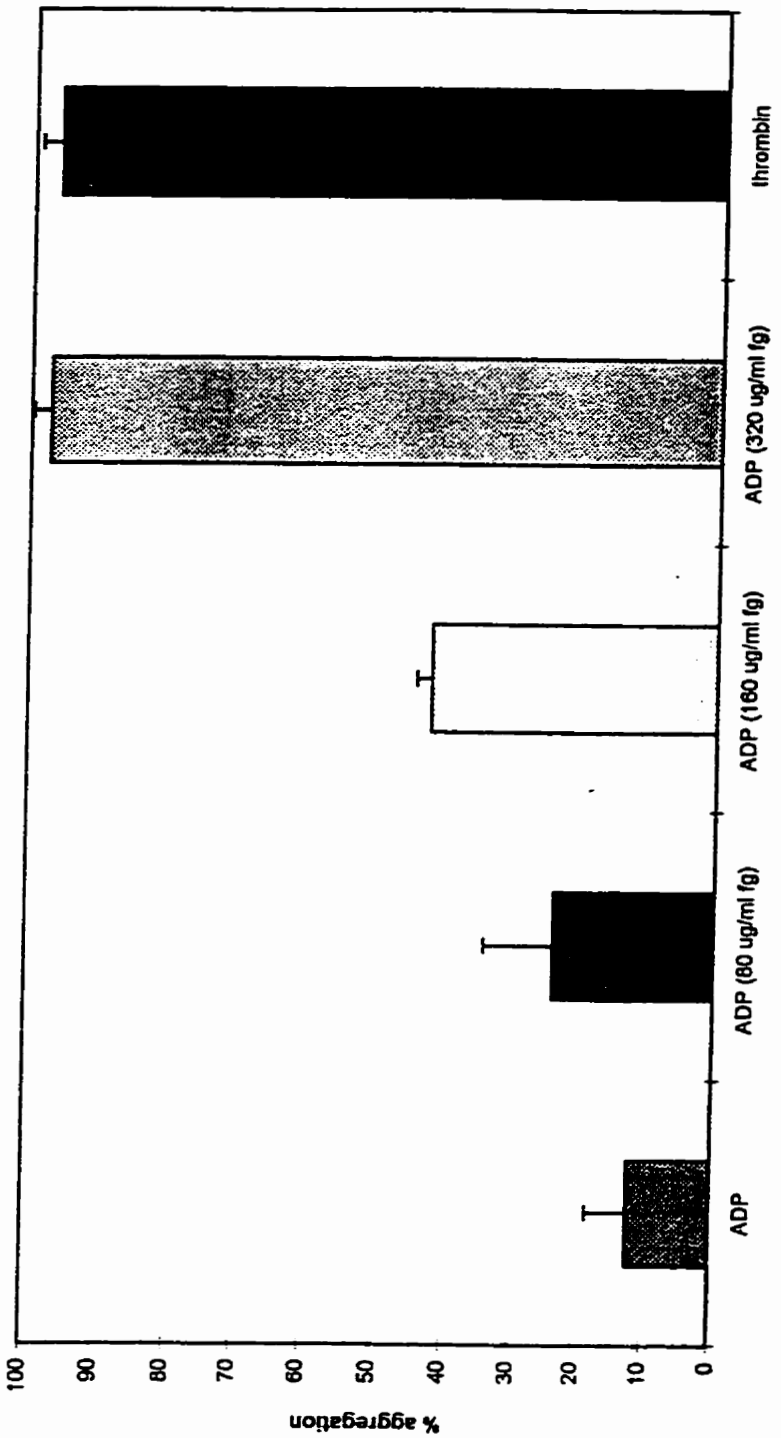

% aggregation
100
90
80
70
60
50
40
30
20
10
0
ADP
ADP (80 ug/ml fg)
ADP (160 ug/ml fg)
ADP (320 ug/ml fg)
thrombin

Figure 20. Platelet Aggregation.

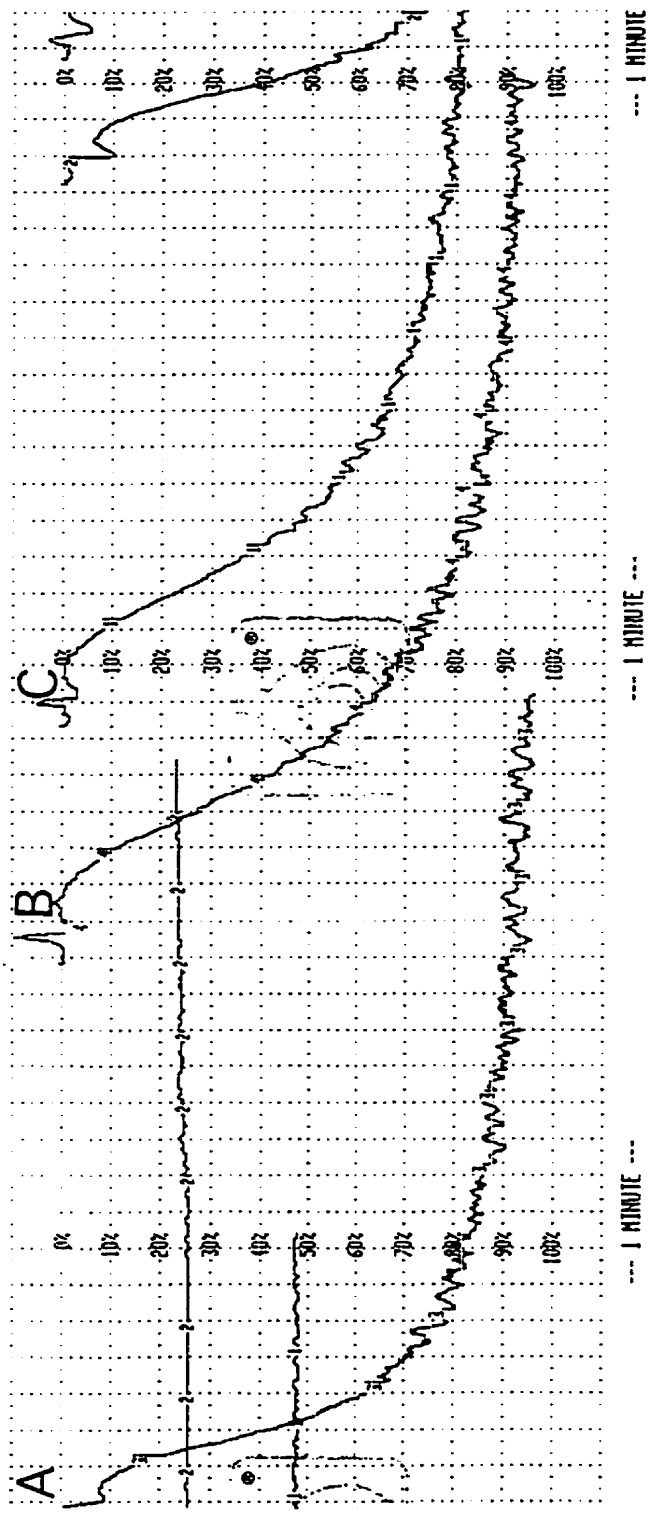
A. Aggregometer

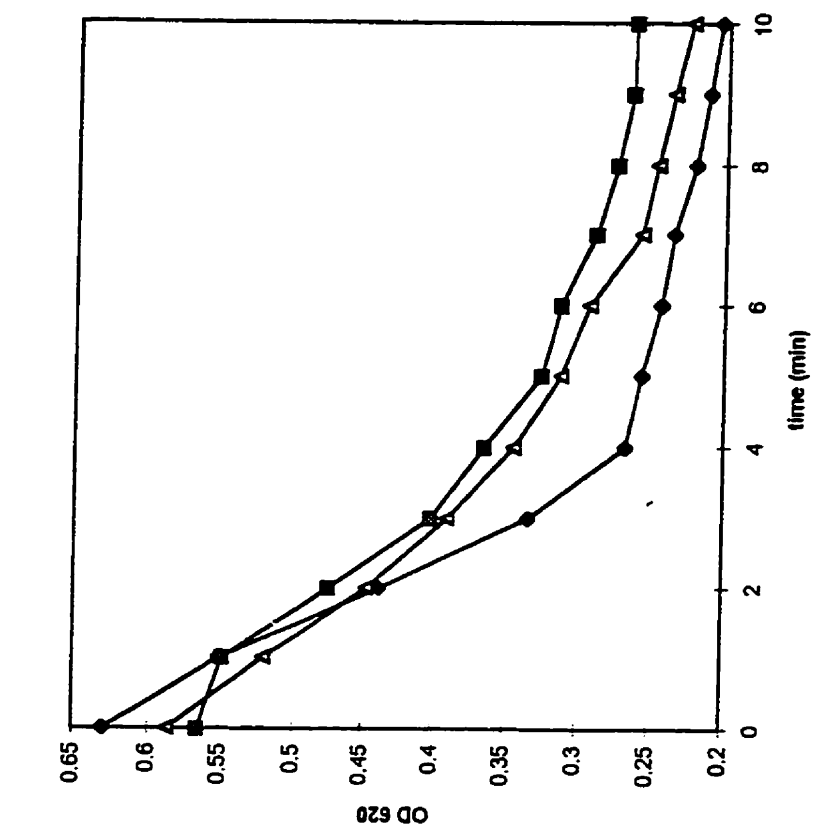
This time course depicts three aggregation curves generated from a platelet aggregometer (Payton Scientific). Washed platelets were prepared from human blood and diluted to 3 X 10^8 cells/ml. 230 ul of platelets were aliquoted into cuvettes and human thrombin was added to 0.5 units/ml. 0% aggregation was set as the absorbance of the platelets prior to addition of the agonist. 100% aggregation was set with the PBS buffer. Each curve represents one sample.

B. Microplate Reader

This time course depicts three aggregation curves generated from a microplate reader (Bio-Tech Instruments). Washed platelets were prepared from human blood and diluted to 3 X 10^8 cells/ml. 135 ul of platelets were aliquoted into microplate wells and human thrombin was added to 0.5 units/ml. 0% aggregation was set as the absorbance of the platelets prior to addition of the agoinst. Each curve represents three replicates. Standard deviation was negligable.

A





B

Figure 21. Platelet Adhesion – Time Course of Chromogenic Substrate Incubation.

This time course indicates the linear trend of the time of incubation of the platelets with the acid phosphatase substrate and the detection method. Mashed platelets were prepared from human blood and diluted to 2.7 X 10^9 cells/ml. 5 ul of platelets were aliquoted into microplate wells and incubated with 130 ul of developing buffer (100 mM sodium citrate, 10 mM p-nitrophenyl phosphate, 0.1% TritonX-100, pH 5.5) for the times indicated. 10 ul of 1 M NaOH was added to stop the reaction and incubated at room temperature for 10 minutes. The absorbance at 405 nm was recorded. Each point represents one replicate.

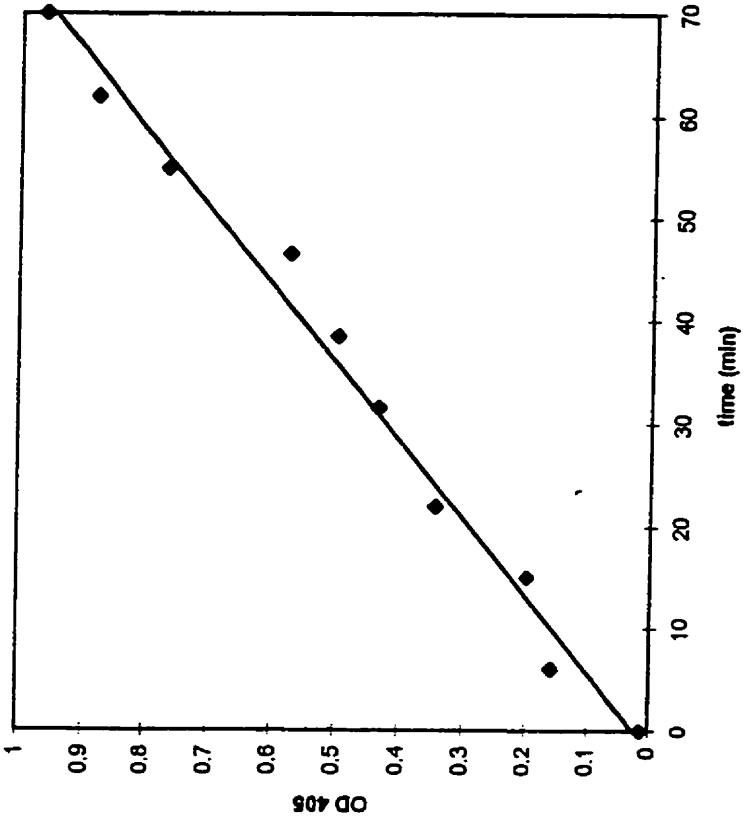

OD 405
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
time (min)

Figure 22. Platelet Adhesion – Dose Response of Number of Cells.

This dose response curve indicates the trend of the number of platelets plated to the microplate wells and the detection method. Washed platelets were prepared from human blood and diluted to 2.7 X 10^9 cells/ml. The indicated number of platelets were aliquoted into microplate wells and incubated with 130 ul of developing buffer (100 mM sodium citrate, 10 mM p-nitrophenyl phosphate, 0.1% TritonX-100, pH 5.5). 10 ul of 1 M NaOH was added to stop the reaction and incubated at room temperature for 10 minutes. The absorbance at 405 nm was recorded. Each point represents three replicates and the standard error is indicated (1 standard deviation).

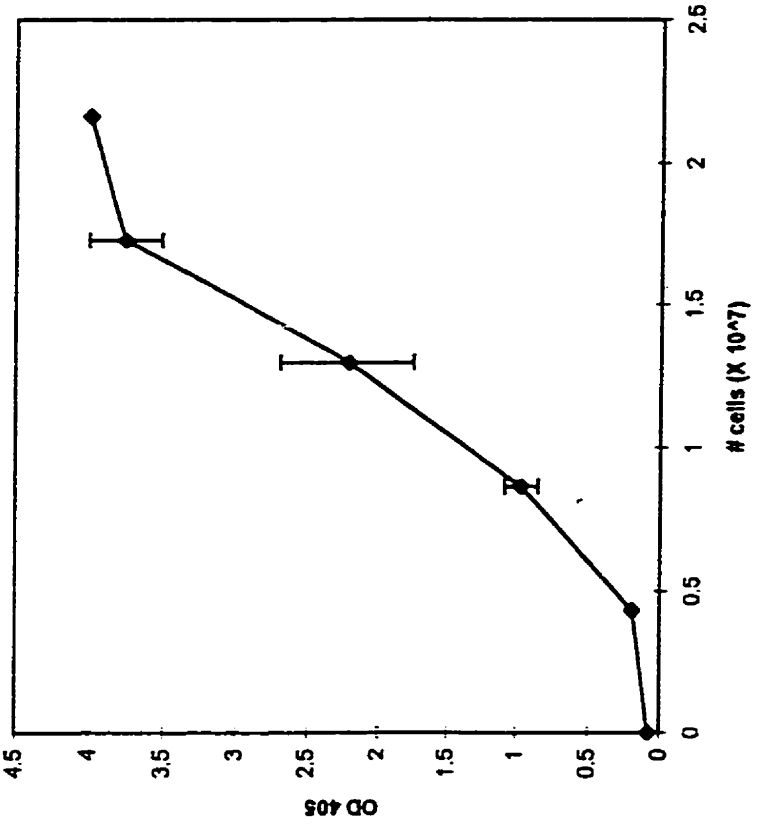

OD 405
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
cells (X 10^7)
2.5
2
1.5
1
0.5
0

Figure 23. Platelet Adhesion - ADP vs. Thrombin.

Presented is the adhesion of unstimulated and stimulated platelets to BSA and fibrinogen to compare the method of stimulation, ADP and thrombin. 1.5 X 10^{-} platelets were aliquoted onto either BSA treated or fibrinogen treated microplate wells. To stimulate the platelets, either ADP (final concentration 20 uM) or thrombin (final concentration 0.5 units/ml) was added to the wells prior to the platelets. The cells were then treated as outlined in the Methods and Materials section. Thrombin stimulated platelets adhered to the BSA more than ADP stimulated platelets. However, thrombin and ADP stimulated platelets appear to adhere equally to fibrinogen. Each point is representative of three replicates and the standard error is indicated (1 standard deviation unit).

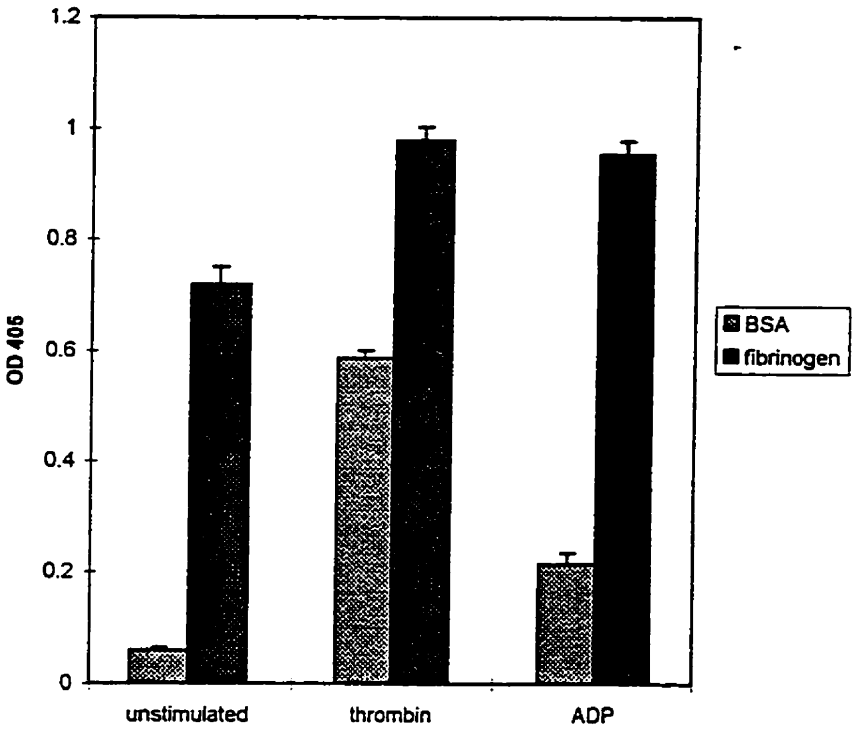

1.2
1
0.8
0.6
0.4
0.2
0
OD 405
unstimulated
thrombin
ADP
BSA
fibrinogen

Figure 24. Platelet Adhesion - Testing Microplates.

Presented is the adhesion of unstimulated and stimulated platelets to BSA and fibrinogen to compare three different microplate wells. 1.5 X 10^7 platelets were aliquoted onto either BSA treated or fibrinogen treated microplate wells. To stimulate the cells ADP (final concentration 20 uM) was added to the wells prior to the platelets. The cells were then treated as outlined in the Methods and Materials section. For all three plates, the trend is for a higher adhesion to fibrinogen than BSA, both for unstimulated and stimulated platelets. The data for the Immuno-2 plates shows the greatest difference between the adhesion to BSA and fibrinogen. Each point is representative of three replicates and the standard error is indicated (1 standard deviation unit).

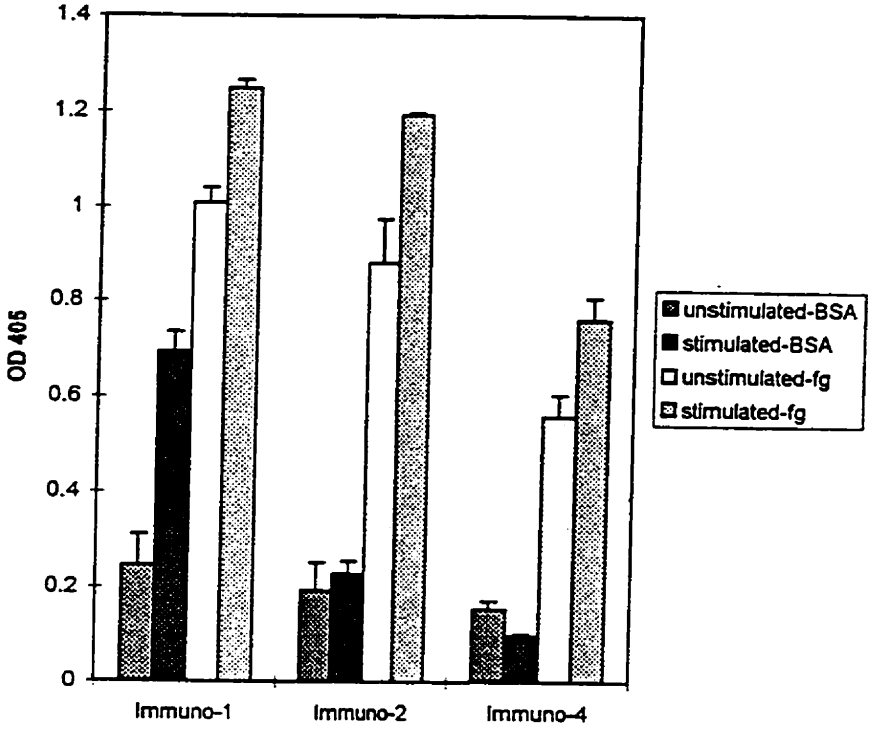

1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD 405
Immuno-1
Immuno-2
Immuno-4
unstimulated-BSA
stimulated-BSA
unstimulated-fg
stimulated-fg

Figure 25. Platelet Adhesion - Different Platelet Treatments.

Presented is the adhesion of unstimulated and stimulated platelets fibrinogen to compare 4 different treatments of the platelets. Platelets were prepared as outlined in the Methods and Materials section, except that following the initial centrifugation the cells were divided into four groups, given different treatments, and carried through the remainder of the washed platelet purification. One group had no additions and represented the washed platelets (WP). Potato apyrase (1 unit/ml) was added the second group (WP + A). Prostaglandin E_1 (1 uM) was added to the third group (WP + P). The third group was treated with both apyrase and prostaglandin E_1 (WP + A + P). 1.5 X 10^7 platelets were aliquoted fibrinogen treated micRoplate wells. To stimulate the cells ADP (final concentration 20 uM) was added to the wells prior to the addition of the platelets. The cells were then treated as outlined in the Methods and Materials section. Each point is representative of a minimum three replicates and the standard error is indicated (1 standard deviation unit).

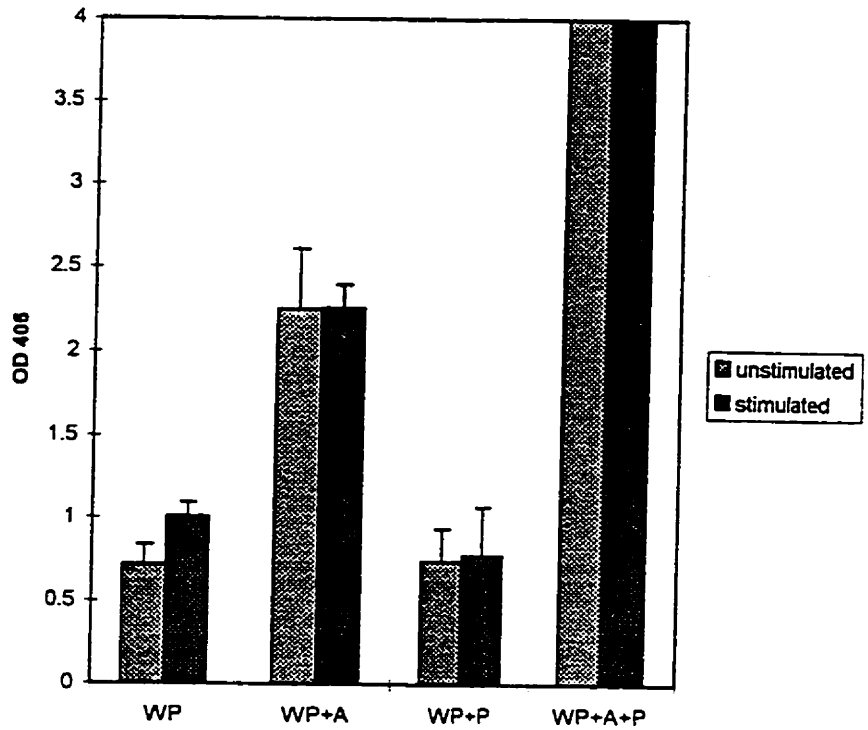

4
3.5
3
2.5
2
1.5
1
0.5
0
OD 406
WP
WP+A
WP+P
WP+A+P
unstimulated
stimulated

Figure 26. Platelet Adhesion - Controls for Substrates.

Presented is the adhesion of unstimulated and stimulated platelets to 4 different substrates using the established methods for the platelet adhesion assay. Platelets were prepared as outlined in the Methods and Materials section. 1.5 X 10^{7} platelets were aliquoted onto one of the following: BSA, fibrinogen, fibronectin, or vitronectin treated microplate wells. To stimulate the cells ADP (final concentration 20 uM) was added to the wells prior to the platelets. The cells were then treated as outlined in the Methods and Materials section. Each point is representative of a minimum three replicates and the standard error is indicated (1 standard deviation unit).

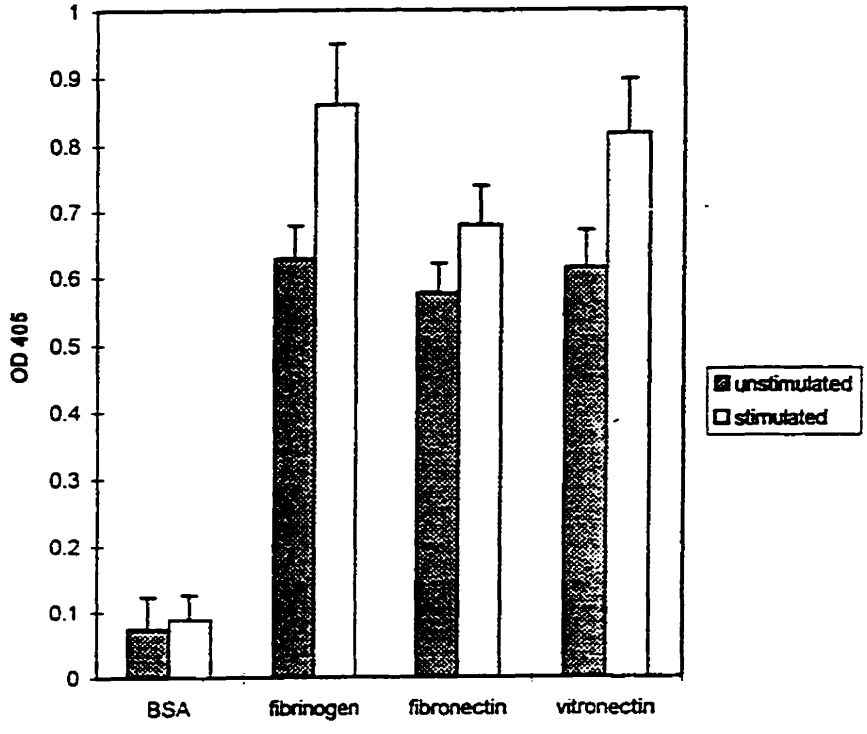

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 405
BSA
fibrinogen
fibronectin
vitronectin
unstimulated
stimulated

Figure 27. Platelet Adhesion - Effect of EDTA.

Presented is the adhesion of unstimulated and stimulated platelets to fibrinogen to compare the effect of EDTA on the four different substrates for platelet adhesion. Platelets were prepared as outlined in the Methods and Materials section. Where indicated, EDTA (final concentration 5 mM) was incubated with the platelets for 10 minutes at room temperature prior to addition to the microplate well. 1.5×10^7 platelets were aliquoted onto one of the following: BSA, fibrinogen, fibronectin, or vitronectin treated microplate wells. To stimulate the cells ADP (final concentration 20 uM) was added to the wells prior to the platelets. The cells were then treated as outlined in the Methods and Materials section. Each point is representative of a minimum three replicates and the standard error is indicated (1 standard deviation unit).

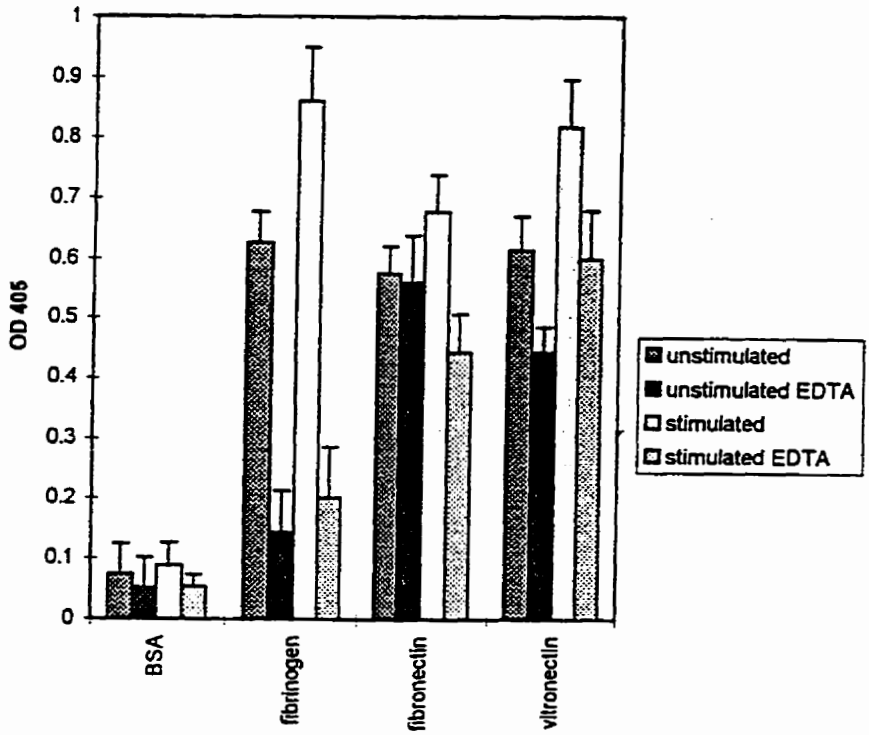

OD 405
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
BSA
fibrinogen
fibronectin
vitronectin
unstimulated
unstimulated EDTA
stimulated
stimulated EDTA

Figure 28. HUVEC Adhesion - Testing Stain and Plates.

The data presented compares two staining and plating techniques for HUVEC. HUVECs were diluted to 3 X 10^6 cells/ml. For the 96 well culture plate, 100 ul of cells were plated onto fibronectin treated wells and incubated at 37 °C for 24 hours, to ensure that the cells were confluent. For the 48 well culture plate, 300 ul of cells were plated and incubated in the same manner. The cells were then washed with PBS and fixed with 10% formalin using 100 ul for 96 well plate or 300 ul for 48 well plate. The cells were either stained with toluidine blue (TB, 1% in 10% formalin) or crystal violet (CV, 0.5% in ddH_2O) (50 ul for 96 well plate, 150 ul for 48 well plate) for 60 minutes at room temperature. The wells were washed extensively and air dried overnight. The toluidine blue stained cells were solubilized with 10% SDS and the crystal violet stained cells were solubilized with 50% ethylene glycol. The absorbance readings for the 96 well plates were performed on a microplate reader at 620 nm (Bio-Tech Instruments), as this wavelength setting was the closest to 595 nm, which is the maximum absorbance wavelength for the stains. For the 48 well plates, the solution was removed to a new cuvette and an absorbance reading taken in a spectrophotometer at 595 nm. Each data point is representative of at least three replicates and the standard error is presented (1 standard deviation unit).

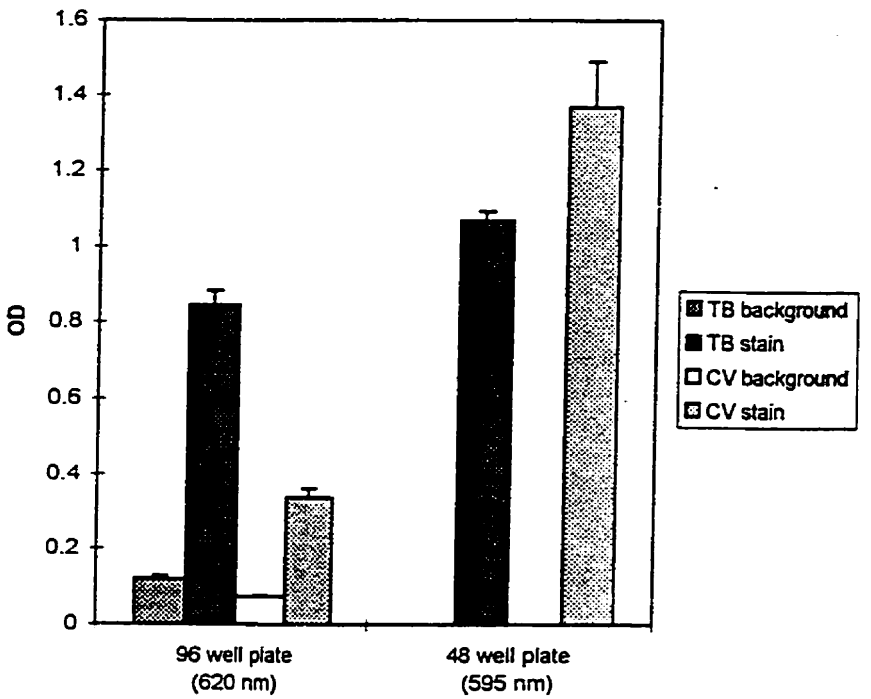

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD
96 well plate
(620 nm)
48 well plate
(595 nm)
TB background
TB stain
CV background
CV stain

Figure 29. HUVEC Adhesion - Dose Response oF Number of Cells.

This dose response curve shows the trend of the number of HUVECs plated and the detection method of toluidine blue staining. Three curves are presented to indicate the trend of HUVEC adhesion to each of fibrinogen (□), fibronectin (Δ), and vitronectin (O) as substrates. HUVEC were plated in the following concentrations: 0.6 X 10^3, 1.2 X 10^3, 1.8 X 10^3, and 2.4 X 10^3 on the various substrates and incubated at 37°C for 2 hours. The cells were then washed with PBS, fixed, and stained as outlined in the Materials and Methods section. Each data point is representative of at least three replicates and the standard error is presented (1 standard deviation unit).

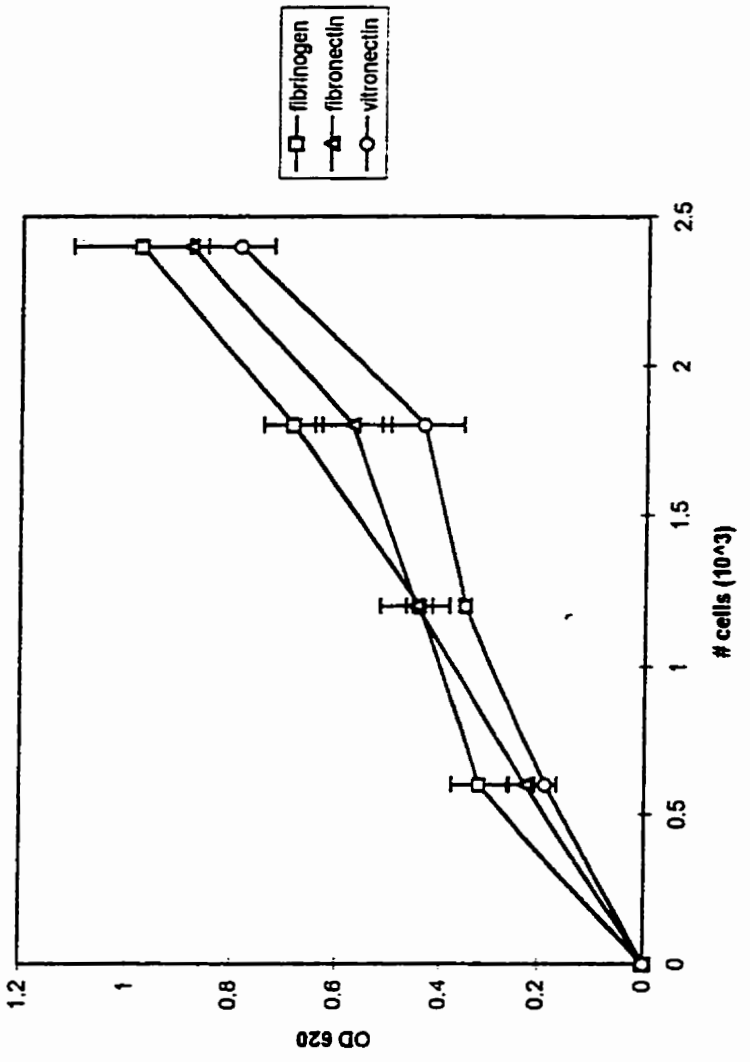

fibrinogen
fibronectin
vitronectin
OD 620
cells (10^3)
0
0.2
0.4
0.6
0.8
1
1.2
0
0.5
1
1.5
2
2.5

Figure 30. HUVEC Adhesion - Time Course of Cell Adhesion.

This time course shows the adhesion of HUVEC to the three ligands over 4 hours. Three curves are presented to indicate the trend for HUVEC adhesion to each of fibrinogen (□), fibronectin (Δ), and vitronectin (O) as ligands. 1.5 X 10^3 HUVEC were plated on the various substrates and incubated at 37°C for the time indicated. The cells were washed with PBS, fixed, and stained as outlined in the Materials and Methods section. Each data point is representative of three replicates and the standard error is presented (1 standard deviation unit).

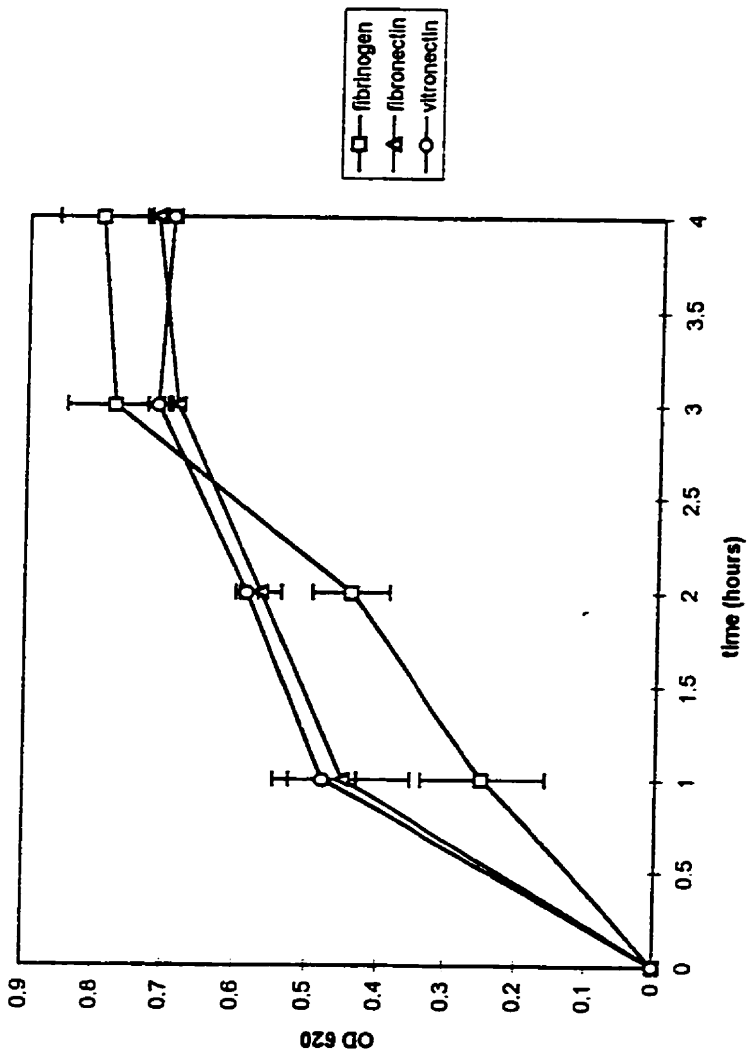

fibrinogen
fibronectin
vitronectin
OD 620
time (hours)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
0.5
1
1.5
2
2.5
3
3.5
4

Figure 31. HUVEC Adhesion - Controls for Substrates.

Presented is the adhesion of HUVEC to three ligands using the standardized methods established for the HUVEC adhesion assay. 1.5 X 10^3 HUVEC were allowed to adhere to fibrinogen, fibonectin, or vitronectin coated wells for two hours. The cells were then washed with PBS, fixed, and stained as outlined in the Materials and Methods section. Each point is representative of three replicates and the standard error is indicated (one standard deviation unit).

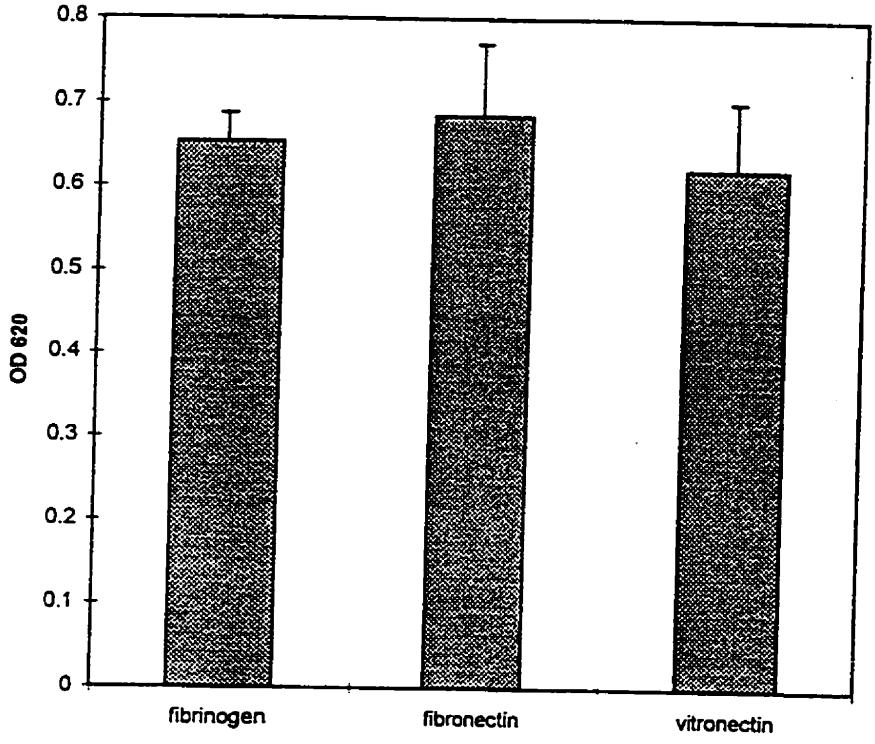

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 620
fibrinogen
fibronectin
vitronectin

Figure 32. Disintegrin Inhibition of Platelet Aggregation.

The dose response curves for Mambin and Barbourin inhibition of platelet aggregation are shown. Disintegrins were incubated with the platelets for 10 minutes at room temperature prior to addition of the agonist. Platelet aggregations were performed as outlined in the Materials and Methods section. The inhibition by Mambin is indicated by "◆" and Barbourin is indicated by "□". Each point is representative of three trials and the standard error is presented (one standard deviation unit).

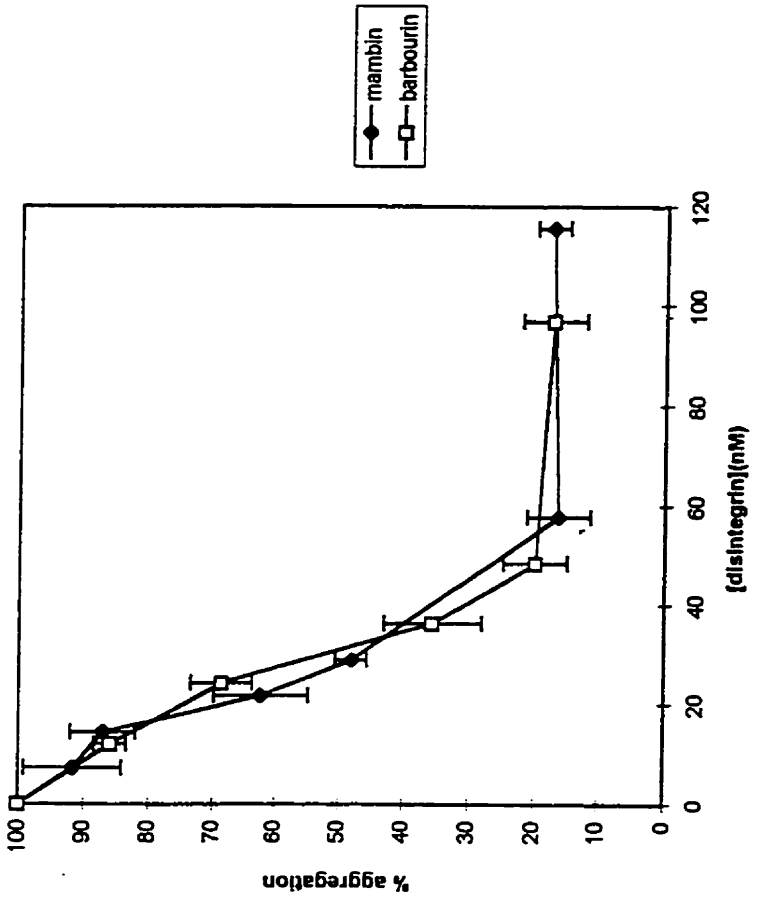

mambin
barbourin
% aggregation
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
[disintegrin](nM)

Figure 33. Disintegrin Inhibition of Unstimulated Platelet Adhesion to Fibrinogen.

The dose response curves for Mambin and Barourin inhibition of unstimulated platelet adhesion to fibrinogen are shown. Disintegrins were incubated with the platelets for 10 minutes at room temperature prior to addition to the fibrinogen treated wells. Platelet adhesions were performed as outlined in the Materials and Methods section. The inhibition by Mambin is indicated by "◆" and Barbourin is indicated by "□". Each point is representative of three trials and the standard error is presented (one standard deviation unit).

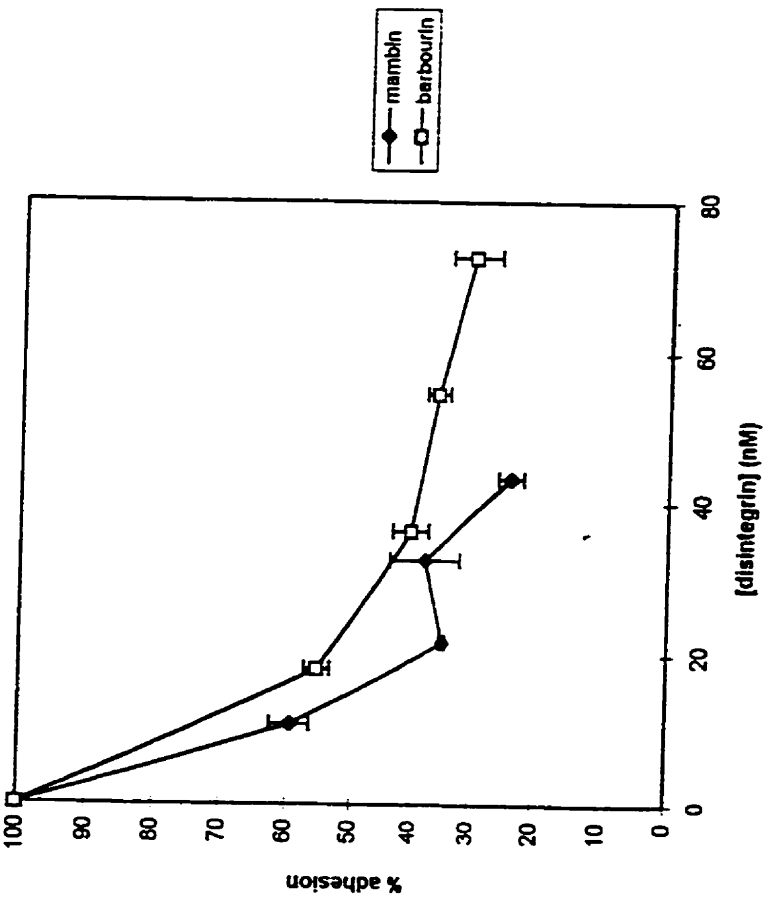

mambin
barbourin
% adhesion
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
[disintegrin] (nM)

Figure 34. Disintegrin Inhibition of Stimulated Platelet Adhesion to Fibrinogen.

The dose response curves for Mambin and Barbourin inhibition of ADP stimulated platelet adhesion to fibrinogen are shown. Disintegrins were incubated with the platelets for 10 minutes at room temperature prior to addition to the fibrinogen treated wells. Platelet adhesions were performed as outlined in the Materials and Methods section. The inhibition by Mambin is indicated by "◆" and Barbourin is indicated by "□". Each point is representative of three trials and the standard error is presented (one standard deviation unit).

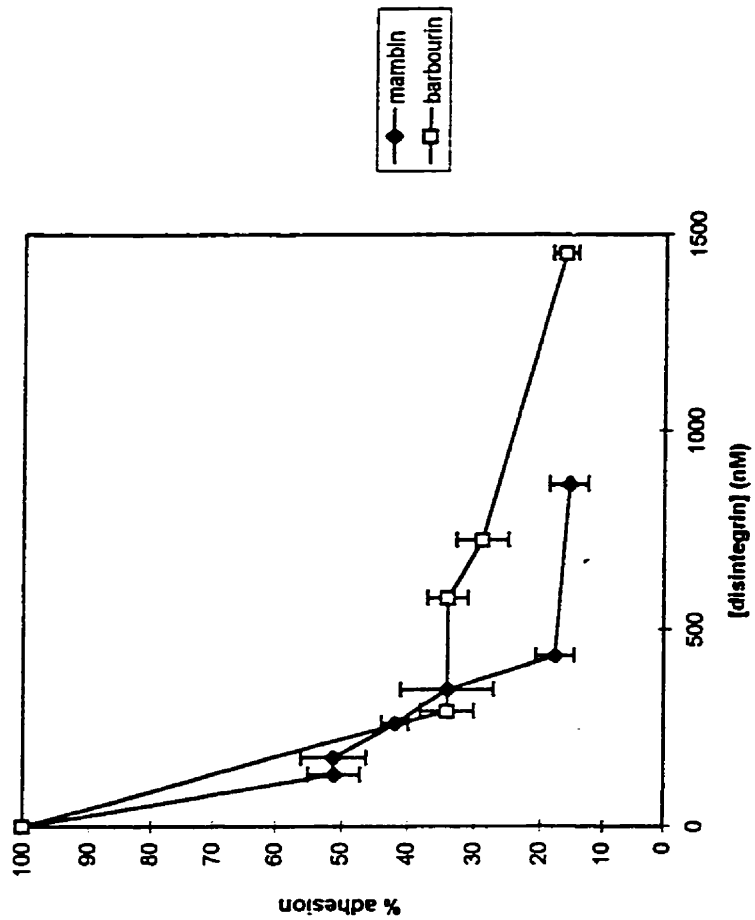

mambin
barbourin
% adhesion
100
90
80
70
60
50
40
30
20
10
0
0
500
1000
1500
[disintegrin] (nM)

Figure 35. Disintegrin Inhibition of Stimulated Platelet Adhesion to Fibronectin.

The dose response curves for Mambin and Barbourin inhibition of ADP stimulated platelet adhesion to fibronectin are shown. Disintegrins were incubated with the platelets for 10 minutes at room temperature prior to addition to the fibronectin treated wells. Platelet adhesions were performed as outlined in the Materials and Methods section. The inhibition by Mambin is indicated by "◆" and Barbourin is indicated by "□". Each point is representative of three trials and the standard error is presented (one standard deviation unit).

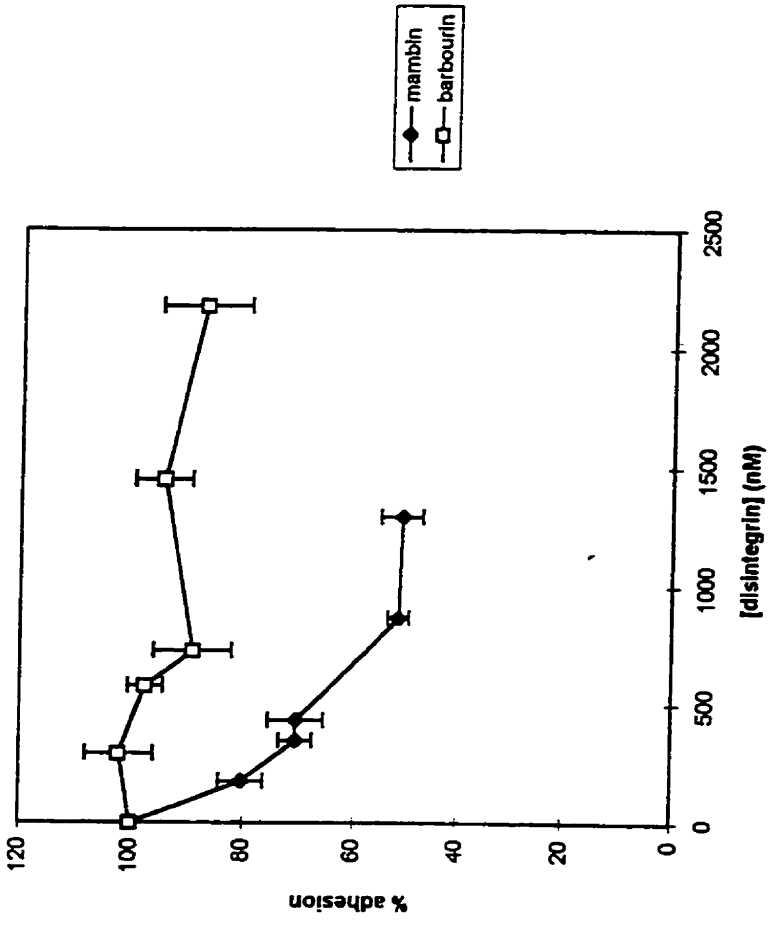

mambin
barbourin
% adhesion
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
[disintegrin] (nM)

Figure 36. Disintegrin Inhibition of Stimulated Platelet Adhesion to Vitronectin.

The dose response curves for Mambin and Barbourin inhibition of ADP stimulated platelet adhesion to vitronectin are shown. Disintegrins were incubated with the platelets for 10 minutes at room temperature prior to addition to the vitronectin treated wells. Platelet adhesions were performed as outlined in the Materials and Methods section. The inhibition by Mambin is indicated by "◆" and Barbourin is indicated by "□". Each point is representative of three trials and the standard error is presented (one standard deviation unit).

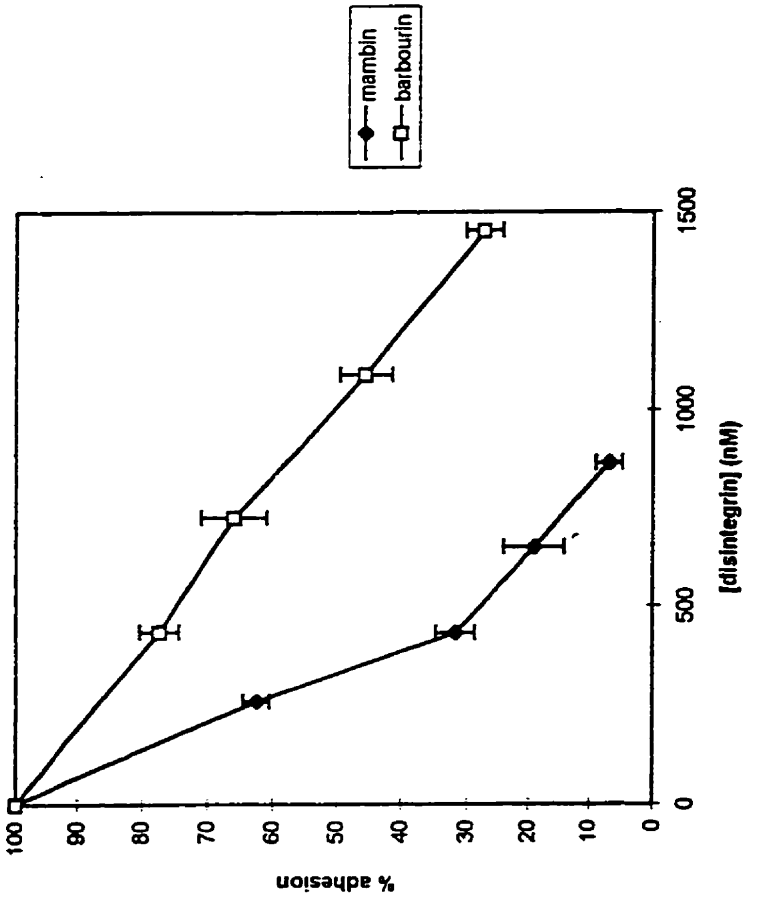

mambin
barbourin
% adhesion
100
90
80
70
60
50
40
30
20
10
0
0
500
1000
1500
[disintegrin] (nM)

Figure 37. Disintegrin Inhibition of HUVEC Adhesion to Fibrinogen.

The dose response curves for Mambin and Barbourin inhibition of HUVEC adhesion to fibrinogen are shown. Disintegrins were incubated with the HUVEC for 10 minutes at room temperature prior to addition to the fibrinogen treated wells. HUVEC adhesions were performed as outlined in the Materials and Methods section. The inhibition by Mambin is indicated by "◆" and Barbourin is indicated by "□". Each point is representative of three trials and the standard error is presented (one standard deviation unit).

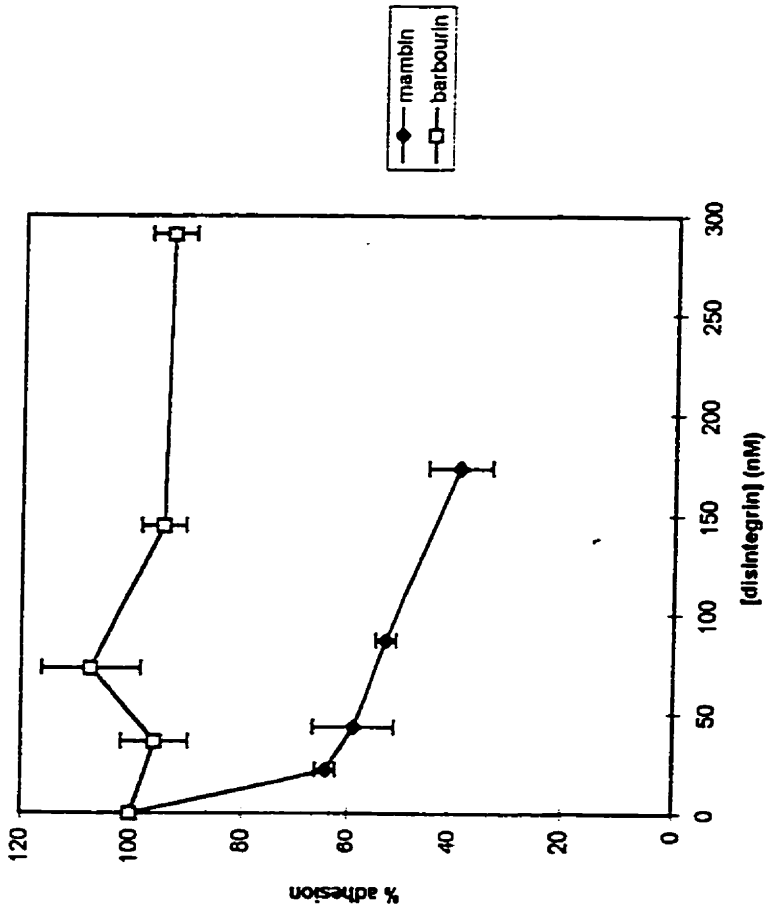

mambin
barbourin
% adhesion
120
100
80
60
40
20
0
0
50
100
150
200
250
300
[disintegrin] (nM)

Figure 38. Disintegrin Inhibition of HUVEC Adhesion to Fibronectin.

The dose response curves for Mambin and Barbourin inhibition of HUVEC adhesion to fibronectin are shown. Disintegrins were incubated with the HUVEC for 10 minutes at room temperature prior to addition to the fibronectin treated wells. HUVEC adhesions were performed as outlined in the Materials and Methods section. The inhibition by Mambin is indicated by "◆" and Barbourin is indicated by "□". Each point is representative of three trials and the standard error is presented (one standard deviation unit).

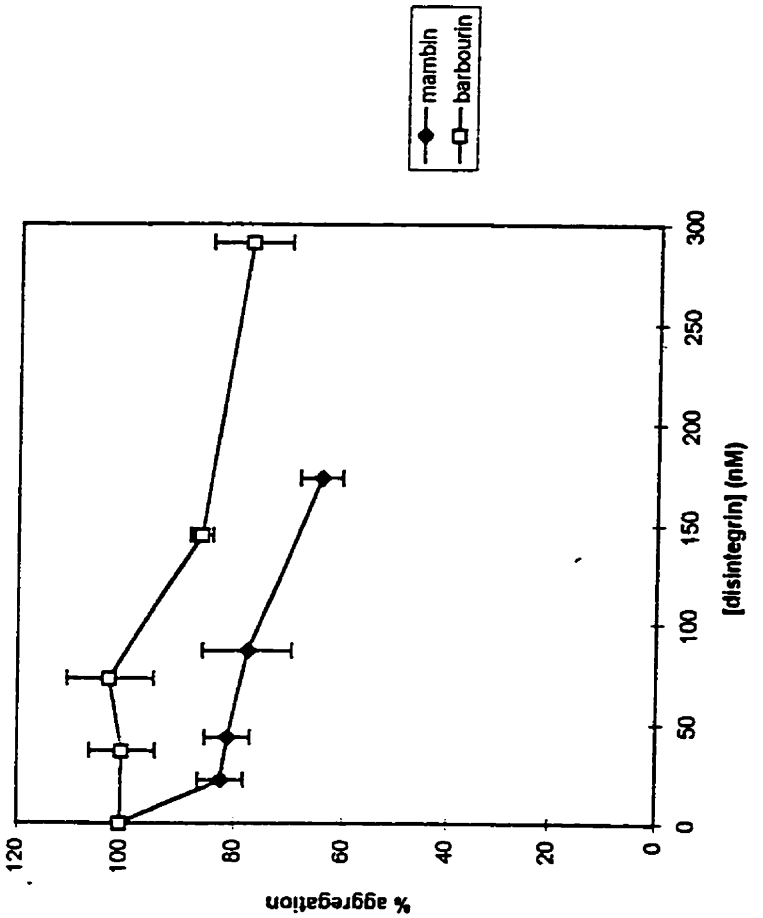
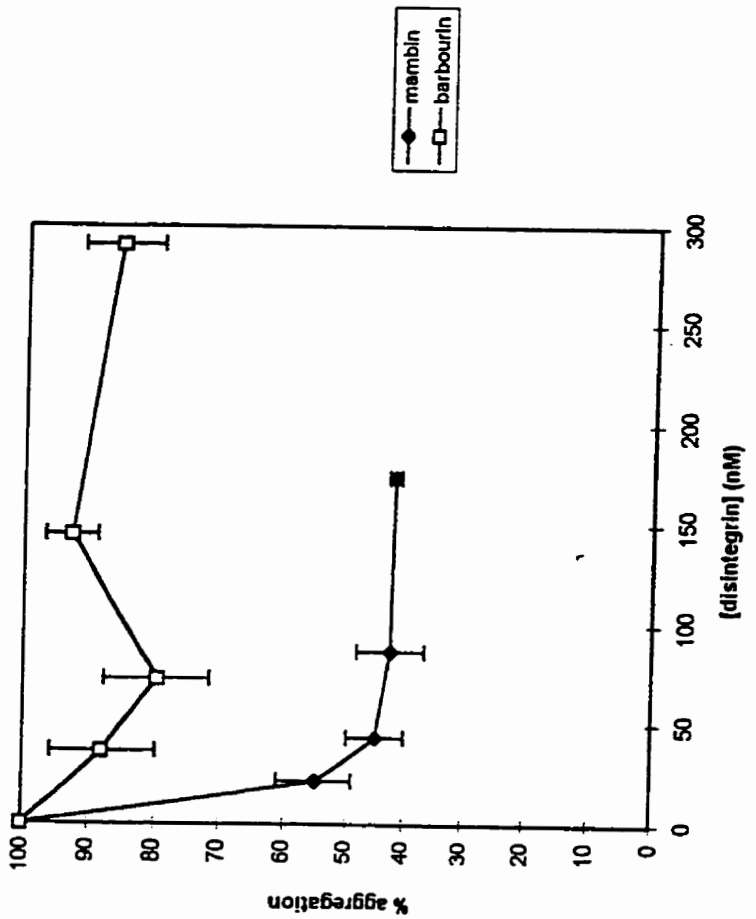

mambin
barbourin
% aggregation
[disintegrin] (nM)
0
20
40
60
80
100
120
0
50
100
150
200
250
300

Figure 39. Disintegrin Inhibition of HUVEC Adhesion to Vitronectin.

The dose response curves for Mambin and Barbourin inhibition of HUVEC adhesion to vitronectin are shown. Disintegrins were incubated with the HUVEC for 10 minutes at room temperature prior to addition to the vitronectin treated wells. HUVEC adhesions were performed as outlined in the Materials and Methods section. The inhibition by Mambin is indicated by "◆" and Barbourin is indicated by "□". Each point is representative of three trials and the standard error is presented (one standard deviation unit).


mambin
barbourin
% aggregation
[disintegrin] (nM)
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
250
300

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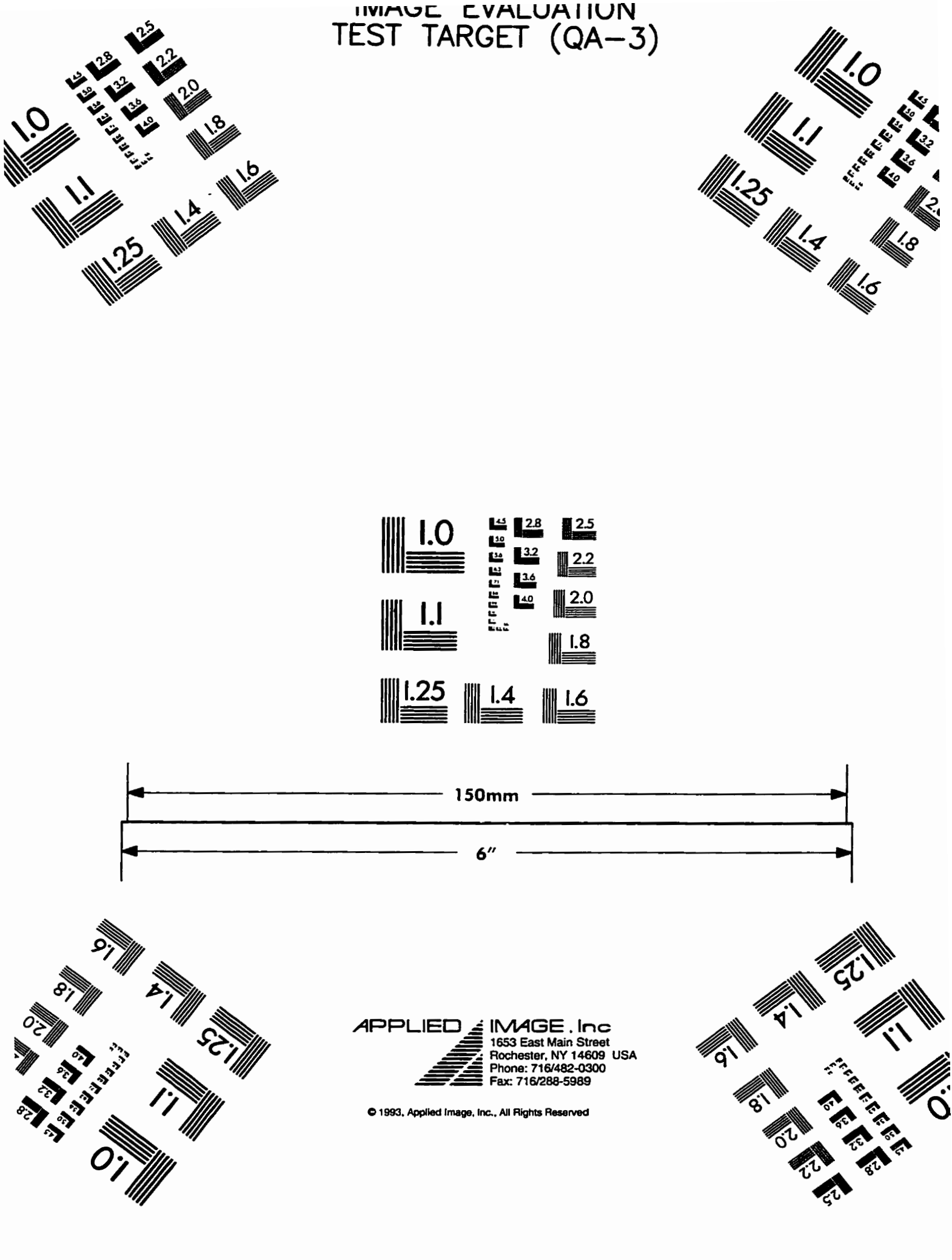
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