Purification, characterization, and functional investigations of novel platelet proteins.

Jeffrey Stephen. Baldwin  
*University of Windsor*
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PURIFICATION, CHARACTERIZATION, AND FUNCTIONAL INVESTIGATIONS OF NOVEL PLATELET PROTEINS

by

Jeffrey S. Baldwin

A Thesis
Submitted to the Faculty of Graduate Studies and Research through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

1995
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ABSTRACT

PURIFICATION, CHARACTERIZATION, AND FUNCTIONAL INVESTIGATIONS OF NOVEL PLATELET PROTEINS

by

Jeffrey S. Baldwin

Blood platelets play a central role in maintaining the hemostatic system. Contained within the α-granules of blood platelets are a variety of proteins involved in the coagulation process. Recently, a novel Kunitz inhibitor of trypsin-like enzymes has been discovered in bovine platelets that has a higher degree of association for plasmin than trypsin, and thereby has been termed bovine platelet plasmin inhibitor (BPPI). The amino acid sequence (Li, 1992) of this new protein has been obtained and extensive kinetic analysis of BPPI with many of the serine proteases involved in blood coagulation has been investigated (Walz et al., manuscript in preparation). However, basic problems in the purification, resulting in protein that was essentially insoluble, has hampered structural determinations by x-ray or NMR analysis. BPPI has the added property of tenaciously binding to cellulose-based membranes which narrows the possibilities to desalt efficiently and concentrate the sample. An investigation into the cause of the protein insolubility has revealed that an excessively high concentration of acetonitrile, used to elute the protein from a C18 reversed-phase cartridge, was most probably the cause of the insolubility.
Concentrated BPPI solutions permitted the preparation of a suitable sample for structural determination by NMR, and preliminary $^1$H-NOESY and $^1$H-TOCSY spectra have been obtained. However, given the high degree of sequence homology with a bovine pancreatic trypsin inhibitor (BPTI), a protein whose three-dimensional structure has been extensively characterized, a structural determination of BPPI has been performed using molecular modelling. Circular dichroism studies have indicated that the secondary structural elements in BPPI are similar to those of BPPI and are accurately predicted in the model. A search for a similar protein in human platelets has resulted in the discovery of a 12,000 Da (by SDS-PAGE) protein that also inhibits trypsin and plasmin and has preliminarily been termed human platelet plasmin inhibitor (HPPI).

An interesting peptide has also been prepared by enzymatically digesting bovine platelet factor four, a heparin-neutralizing protein, with Staphylococcus aureus V8 protease. The carboxy-terminal peptide, termed V2, was found to possess significant heparin-binding ability, relative to other proteins with heparin affinity, indicating the presence of stable secondary structural elements. Circular dichroism spectroscopy of V2 revealed an $\alpha$-helical content similar to b-PF4. The oligomeric structure of the peptide in solution is believed to be that of a dimer based on molecular weight estimations by gel permeation chromatography.
DEDICATION

To my family

with love and gratitude.
ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my supervisor Dr. Lana Lee, for her guidance and support throughout this study. I am honoured to have had the opportunity to be a graduate student in her lab.

I am also grateful to Dr. Timothy Veenstra and Dr. Christopher Talpas for their assistance in the laboratory and for their friendship.

I thank Dr. Gary Shaw, at the University of Western Ontario, for acquiring the nuclear magnetic resonance spectra and Dr. Serge Vinogradov, at Wayne State University, for the use of his CD spectrometer.

I would also like to acknowledge Dr. Bulent Mutus and Dr. David Cotter for serving as members of my thesis committee and to Dr. A.G. Szabo for chairing my defense. My gratitude is extended to the Heart and Stroke Foundation of Ontario for funding this research and to the University of Windsor for additional support through travel grants.

Of course, I would like to thank my friends and fellow graduate students Cynthia Balion, Stephanie White, Domenic Perri, Susan Bortolin, Barbara Galvan, Christopher Howard, Marie Tannous, Zayna Khayat, Joseph Macri, Norman Chiu, Nancy Foley, Nikki Fransen, Anne Charlton, and Daniel Trepanier (and I apologize if I forgot anyone), for making my time spent at the University of Windsor so memorable. Finally, my love and thanks to my best friend Sandy Batten.
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<tr>
<td>ACN</td>
<td>acetonitrile</td>
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<td>N-α-benzoyl-DL-arginine-p-nitroanilide</td>
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<td>bis-acrylamide</td>
<td>N,N'-methylene-bis-acrylamide</td>
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<td>b-PF4</td>
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<td>BPPI</td>
<td>bovine platelet plasmin inhibitor</td>
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<tr>
<td>BPTI</td>
<td>bovine pancreatic trypsin inhibitor</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>2-hydroxy-1-1(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphththioic acid</td>
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<td>carboxymethyl-Sephadex</td>
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<td>DIFP</td>
<td>diisopropylfluorophosphate</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<tr>
<td>DQF-COSY</td>
<td>double quantum filtered correlation spectroscopy</td>
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<td>DSS</td>
<td>sodium 2,2-dimethyl-2-silapentane-5-sulphonate</td>
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<td>D₂O</td>
<td>deuterium oxidé</td>
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<td>EACA</td>
<td>ε-aminocaproic acid</td>
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<td>N,N'-ethylenediamine tetra-acetic acid</td>
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<td>(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])</td>
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<td>MRE</td>
<td>mean residue ellipticity</td>
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<td>polymethylpentene</td>
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<td>TFA</td>
<td>trifluoroacetic acid</td>
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<td>(tris[hydroxymethyl]aminomethane)</td>
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<td>$t_1$</td>
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<tr>
<td>$t_2$</td>
<td>number of increments in the second dimension</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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Introduction

Hemostasis and Platelets

The process of hemostasis maintains blood in the fluid state under physiological conditions, but also allows for the rapid and efficient response to vascular injury to prevent blood loss. Figure 1.1 presents a general schematic diagram that depicts the coagulation cascade, and some of the factors and inhibitors involved in hemostasis are summarized in Table 1.1. The major blood constituent involved in hemostasis are platelets, which are derived from megakaryocytes found in bone marrow. Platelets have a diameter of approximately 3 μm and contain α-granules, that are the prevalent organelle. Secretion of proteins from α-granules can be triggered by a variety of stimuli ranging from weak agonists, such as ADP, to the relatively strong agonistic properties of thrombin. Among the proteins secreted from the α-granules is factor V which stimulates factor X and thereby accelerates prothrombin activation. The result is the production of additional thrombin, thus enhancing the platelet secretory response. The other organelles contained within platelets are dense granules and lysosomes (Zucker, 1980). The dense granules contain ATP, ADP, pyrophosphate, serotonin, and divalent metals, whereas the lysosomes contain a large number of acid glycosidases (Holmsen, 1980). Of course, the processes resulting in platelet secretion must also be modulated by specific regulatory mechanisms. These include polysaccharides, such as heparin, and several plasma protease inhibitors of which antithrombin-III and α1-antitrypsin appear to be most important. Thrombin converts fibrinogen to fibrin which then polymerizes to form the fibrin clot.
Figure 1.1: The clotting cascade. The central event involved in clotting is considered to involve tissue factor (TF), which is normally not exposed to blood. However, with vascular or endothelial cell injury, TF, together with Factor VIIa and phospholipid (PL), act to convert Factor IX to IXa and Factor X to Xa. The "intrinsic pathway" includes contact activation of Factor XI by the XIIa/activated high molecular weight kininogen (HKa) complex. Factor XIIa also converts Factor IX to IXa in turn converts Factor X to Xa, in concert with Factors VIIIa and PL. Factor Xa then converts, with Factor Va and PL, prothrombin to thrombin. Thrombin cleaves fibrinopeptides (FPA, FPB) from fibrinogen, allowing the resultant fibrin monomers to polymerize, and converts Factor XIII to XIIIa, which crosslinks (XL) the fibrin clot. Natural plasma inhibitors retard clotting as follows: C1-inhibitor (C1 INH) neutralizes Factor XIIa, tissue factor pathway inhibitor (TFPI) blocks Factor VIIa/TF, and antithrombin III (ATIII) blocks Factors IXa, Xa and thrombin. Rectangles represent sites of inhibitor action (Colman et al., 1994).
### Table 1.1

**Blood Coagulation Factors and Inhibitors**

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<td>Prothrombin</td>
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<td>Factor III</td>
<td>Tissue factor or thromboplastin</td>
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<td>Factor IV</td>
<td>Ca^{2+}</td>
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<td>Factor VII</td>
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<td>Christmas factor</td>
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<td>Stuart factor</td>
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</tr>
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<td>Factor XI</td>
<td>Plasma thromboplastin antecedent (PTA)</td>
<td>160</td>
</tr>
<tr>
<td>Factor XII</td>
<td>Hageman factor</td>
<td>80</td>
</tr>
<tr>
<td>Factor XIII</td>
<td>Fibrin-stabilizing factor (FSF)</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>Prekallikrein</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>High molecular weight kininogen (HMK)</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Antithrombin III (ATIII)</td>
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</tr>
<tr>
<td></td>
<td>C1 Inhibitor (C1 INH)</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Tissue Factor Pathway Inhibitor (TFPI)</td>
<td>40</td>
</tr>
</tbody>
</table>
Platelets do not adhere to the normal vascular endothelium, but will adhere to an endothelial disruption. The cut end of a blood vessel, for example, provides sites for the adhesive protein von Willebrand factor (vWF) to bind, and in turn the platelets adhere through the platelet glycoprotein (GP) Ib/IX complex, located on the platelet surface, that recognizes vWF. Aggregation is mediated by glycoproteins IIb/IIIa (GPIIb/IIIa) bridged to GPIIb/IIIa on another platelet by fibrinogen. These adhesive proteins participate in the formation of a bridge from platelets to the subendothelial wall. The various agonists such as ADP interact with specific receptors and activate phospholipase C. This enzyme in turn catalyzes the cleavage of phosphatidylinositol bisphosphate to inositol triphosphate, which immobilizes divalent calcium to activate myosin light chain kinase, which in turn phosphorylates myosin light chain (MLC-P\textsubscript{PO\textsubscript{4}}). Calcium also activates phospholipase A\textsubscript{2} to release arachidonic acid from phospholipids, which is then converted to thromboxane A\textsubscript{2}. The other product of the cleavage of inositol bisphosphate is diacylglycerol, which stimulates protein kinase C to phosphorylate a protein called P47-P\textsubscript{PO\textsubscript{4}}, which along with thromboxane A\textsubscript{2} and MLC-P\textsubscript{PO\textsubscript{4}}, stimulate secretion of products of the dense, alpha, and lysosomal granules (Colman et al., 1994).

Plasmin is a member of the serine protease family of enzymes, formed through the proteolytic cleavage of an 86 kDa zymogen called plasminogen. The active zymogen consists of a heavy (A) polypeptide chain (MW = 55,000 Da) attached by a disulfide bond to a light (B) chain (MW = 25,000 Da). Activation of plasminogen can be achieved with enzymes such as urokinase, trypsin, tissue plasminogen activator, and even plasmin
itself. Native plasminogen has an amino-terminal glutamic acid (referred to as Glu-
plasminogen), but is converted by limited plasmin digestion at the Arg68-Met69, Lys77-
Lys78, or Lys78-Val79 bond, to Lys-plasminogen. Final conversion to active Lys-
plasmin results from the cleavage of the Arg561-Val562 bond. The B-chain of the newly
formed two-chain molecule remains attached to the A-chain by two disulfide bonds. The
B-chain contains the active site, which is composed of the three amino acids His603,
Asp646, and Ser741, which is similar to that of trypsin (Bachmann, 1994).

The major role of plasmin is the degradation of fibrin (fibrinolysis) to soluble
products. As implied above, plasmin also functions as an endoproteinase, which cleaves
on the carboxy-terminal side of lysine and arginine, with a greater specificity for lysine.
Plasmin on the surface of the fibrin clot, however, is protected from α2-antiplasmin, and
clot dissolution occurs with the formation of fibrin degradation products. This reaction
serves to limit fibrinolysis to the region of the clot.

Tissue plasminogen activator (t-PA) is a 68 kDa serine protease that is quite an
inefficient activator of plasminogen in the absence of fibrin. However, in the presence
of fibrin, t-PA greatly increases plasminogen activation that is a result of the assembly
formation of plasminogen and t-PA on the surface of fibrin. Fibrin acts to increase the
local plasminogen concentration by creating an additional interaction between t-PA and
its substrate. The high affinity of t-PA for plasminogen in the presence of fibrin thereby
allows efficient activation of the fibrin clot, while no efficient plasminogen activation by
t-PA occurs in plasma (Hoylaerts et al., 1982).

Plasmin inactivators are also important physiologically. Individuals with an α2-
antiplasmin deficiency have a serious tendency to bleed. There are numerous inactivators of plasmin that include, but are not limited to, EACA, organophosphates, and \( \alpha_2 \)-antiplasmin. The latter molecule, often called plasmin inhibitor, is the primary inhibitor of plasmin. It is a single-chain polypeptide with a molecular weight of 70,000 Da and contains approximately 13% carbohydrate. The three functional properties of \( \alpha_2 \)-antiplasmin are to: 1, inhibit plasmin very rapidly; 2, interfere with adsorption of plasminogen to fibrin, and 3, undergo crosslinking with the \( \alpha \)-chains of fibrin during clotting. The carboxy-terminal Lys452 of \( \alpha_2 \)-antiplasmin interacts with the lysine binding site (LBS) of plasmin, while the amino-terminal glutamine residue is covalently crosslinked to a carboxy-terminal lysine in the \( \alpha \)-chain of fibrin by Factor XIIIa (Bachmann, 1994).

**Kunitz-type Inhibitors**

The standard mechanism of all Kunitz-type inhibitors is that on each molecule lies at least one peptide bond called the reactive site, that specifically interacts with the active site of the enzyme, and that the inhibitor itself is a very good substrate. The reactive site peptide bond is encompassed in at least one disulfide loop, which ensures that the two peptide chains cannot dissociate during the conversion of the virgin to the modified inhibitor. The reactive site residue, P1, generally corresponds to the specificity of the enzyme to be inhibited. Aprotinin or its many isoforms (Siekmann *et al.*, 1988), which have a P1 lysyl or arginyl residue tend to inhibit trypsin or trypsin-like enzymes. In contrast, when the P1 residue is replaced by an aromatic residue, such as tyrosine or
phenylalanine, chymotrypsin and chymotrypsin-like enzymes are inhibited. Interestingly, the exchange of a lysine for an arginine residue at the P1 position leaves the inhibitor specificity and strength approximately the same. This is a remarkable feature that is unique to proteinase inhibitors. Biological activity is most often lost in proteins in which the active site residue has been replaced, even by closely related residues (Laskowski and Kato, 1980).

**Bovine Pancreatic Trypsin Inhibitor (BPTI)**

BPTI is a serine protease inhibitor that belongs to the Kunitz family of inhibitors and is known by a variety of names including aprotinin, spleen isoform II, and Trasylol®, which is a trademark of Bayer. This molecule has surpassed serum albumin and ribonuclease as a favourite for investigation by physical protein chemists because it is small (only 58 residues), soluble, very stable, and both its sequence and three-dimensional structure (both x-ray and NMR determinations) are known. Several isoforms have been found in various bovine organs including lung, liver, and spleen (Fioretti et al., 1983), sheep and ox lung (Fioretti et al., 1988), as well as from porcine uterus (Stallings-Mann, 1994). The purified inhibitor is a very strong inhibitor of trypsin, with a $K_d$ value of $6 \times 10^{-14}$ M (Vincent and Lazdunski, 1972), and of trypsin-like enzymes. Enzymatic semisynthetic methods have demonstrated that aprotinin homologues mutated in the P1 position (Figure 1.2) maintain their inhibitory function to trypsin and kallikrein and have $K_d$ values $< 10^{-10}$ (Groeger et al., 1991). Similarly, a recombinant [Arg$_{15}$Glu$_{52}$] aprotinin expression system was found to have excellent inhibitory activity toward plasmin (Kd
Figure 1.2: Cα backbone of BPTI illustrating the reactive site amino acid residues. The filled circles represent cysteiny1 residues and the open circles all other residues. The amino acid residues around the reactive site are displayed by the three-letter code (from Groeger et al., 1991).
Figure 1.3: Alignment of the primary sequences of BPPI and BPTI. A representation of the amino acid sequence alignment of BPPI (Li, 1992) and BPTI (Kassell, 1970) to give maximum consensus.
= 1.3 x 10^{-10}, kallikrein (Kd = 3.2 x 10^{-10}), and trypsin (Kd < 10^{-11}) (Auerswald et al., 1987; Auerswald et al., 1988).

**Bovine Platelet Plasmin Inhibitor**

A new aprotinin homologue has been found in bovine platelets and has been termed bovine platelet plasmin inhibitor (BPPI) based on initial kinetic measurements that the inhibitor has slightly higher association for plasmin than trypsin (Walz et al., manuscript in preparation). In addition, BPPI is only a weak inhibitor of factors XIa, Xa, and IXa. BPPI is secreted from the α-granules of blood platelets using a strong agonist such as calcium ionophore A-23187. It is a low molecular weight protein that has no affinity for heparin. The amino acid sequence was determined by overlapping amino-terminal sequence data using cyanogen bromide and trypsin digested peptides (Li, 1992). BPPI has the highest homology with BPTI according to amino acid alignment. However, BPPI has 20 more amino acids, 13 at the N-terminus and 7 at the C-terminus, than BPTI, as shown in figure 1.3. BPPI is postulated to be a dimer whose structure mimicks that of BPTI, having intra-chain disulfide bonds between Cys18/68, Cys27/51, and Cys43/64, in addition to a inter-subunit disulfide bond between Cys4 of two monomers. There is also approximately 30% homology with other Kunitz-type proteinases, including lipoprotein associated coagulation inhibitor, and human inter-α-trypsin inhibitor (Li, 1992).

**Platelet Factor 4-Related Proteins**

Activated platelets release a large amount of two proteins, platelet factor 4 (PF4) and
β-thromboglobulin (β-TG), which mediate several inflammatory processes. The PF4-related proteins are small proteins ranging in molecular weights, in the monomeric form, from 6,000 Da to 25,000 Da, and most are less than 10,000 Da. Several proteins such as PF4 have sites for N- or O-linked glycosylation. The PF4 family of proteins, all share four conserved cysteine residues. These residues form intramolecular disulfide bonds that link cysteines 1 and 3 and cysteines 2 and 4, in PF4 and β-thromboglobulin (Begg et al., 1978; St. Charles et al., 1989).

β-thromboglobulin and PF4 are best characterized by their heparin-neutralizing activity (Rucinski et al., 1979). However, they also exhibit several inflammatory activities. Both are chemoattractants for fibroblasts and monocytes and PF4 is a chemoattractant for neutrophils (Deuel et al., 1981). Connective tissue activating peptide (CTAP-III) which may be the proteolytic product of β-TG has been shown to stimulate DNA synthesis and multiple aspects of glycosaminoglycan and proteoglycan metabolism (Castor et al., 1983). A novel cleavage product of β-TG was recently found to induce neutrophil degranulation (Walz and Baggiohini, 1989).

Experimentally-induced immunosuppression in mice has ben shown to be blocked by PF4 (Barone et al., 1988), however the mechanism of action has not been elucidated. Another recent finding is the inhibition of angiogenesis by recombinant human platelet factor 4 and related peptides, apparently by preventing endothelial cell proliferation in response to growth factors (Maione et al., 1990).

Peptides related to the carboxy terminus of human platelet factor 4 have also been shown to possess antibacterial activity. A peptide (C13) that corresponds to the last 13
amino acids of the carboxyl terminus of PF4 had antibacterial activity against *E. Coli* in normal human serum. Amino acid substitutions that disrupted the α-helical nature of the C13 peptide rendered it inactive (Darveau, 1992).

Interleukin-8 (IL-8), another PF4 related protein, is a potent neutrophil chemoattractant that is rapidly induced in monocytes (Walz *et al.*, 1987) and it appears to amplify the early neutrophilic response to various inflammatory stimuli. IL-8 is an attractive target for novel anti-inflammatory strategies. A possible therapy could involve the administration of an anti-IL-8 antibody to block the destructive neutrophil response in rheumatoid arthritis (Stoeckle and Barker, 1990).

**Bovine Platelet Factor 4**

Bovine platelet factor four (b-PF4) is a relatively low molecular weight protein found in the α-granules of blood platelets. The activation of platelets during tissue injury, results in excretion of b-PF4 by exocytosis. PF-4 was found to have heparin neutralizing ability which functions as an anti-coagulant (Deutsch *et al.*, 1955). Heparin binds to antithrombin III, which enhances its ability to bind thrombin. The amount of free thrombin in the plasma is therefore reduced which prevents activation of the blood coagulation cascade. It has been shown, however, that PF4 can displace heparin from the antithrombin III-thrombin complex resulting in an increase in free thrombin to initiate clotting (Loscalzo *et al.*, 1985).

Bovine PF4 is a tetramer in solution, consisting of four identical subunits each having a molecular weight of 9,505 Da, by amino acid sequencing. Each subunit is composed
of 88 amino acids (Ciaglowski et al., 1986) and contains two disulfide bonds between Cys25/Cys61 and Cys27/Cys67. The x-ray crystallographic structure of b-PF4 has been determined and consists of three antiparallel β-sheets arranged in a Greek key, one α-helix, and an extended loop (St. Charles et al., 1989). There are seven lysine residues in each monomer, six of which are located near the carboxyl terminus.

The enzymatic digestion of b-PF4 by Staphylococcus aureus V8 protease, produces the V1 (residues 1 - 43) and V2 (residues 44 - 88) fragments. The V2 fragment contains 6 of the 7 lysine residues found in the parent protein. These positively charged lysyl residues are implicated in the ability of the parent molecule (b-PF4) to bind to the negatively charged groups on heparin molecules (Stuckey et al., 1992). It has been shown that heparin molecules wrap around a belt of positive charges formed by the alignment of lysine residues in b-PF4 (Talpas et al., 1991).

Objectives of this Study

Basic biochemical characterizations of BPPI were performed to determine whether this molecule behaved similarly to BPTI, which has been extensively characterized. In addition, modifications in the purification of the inhibitor were necessary to obtain a soluble purified product. A three dimensional model of BPPI was proposed based on its high degree of sequence homology with BPTI.

Similarly, characterization of V2, with respect to its heparin binding ability, oligomeric structure, and presence of stable secondary structural elements, were examined.
Materials and Methods

General

All solutions were prepared with distilled-deionized water. Liquid handling of volumes of less than 1 mL was facilitated by the appropriate Gilson Pipetman (models P20, P200, P1000) from Mandel Scientific Co. Ltd., Guelph, Ontario. Mass determinations were made with either a Mettler P1200N or Mettler AE50 balance from Fisher Scientific Inc., Ottawa, Ontario. All electrophoresis experiments were performed with a model EC452 power supply from E-C Apparatus Corporation, St. Petersburg, Florida. Trypsin, from bovine pancreas, was used in experiments requiring this enzyme. All pH measurements were determined with a Fisher Accumet Model 810 pH meter from Allied Fisher Scientific (Pittsburgh, PA) or a Corning Model 240 pH meter from Corning Science Products (Corning, NY). All NMR experiments were performed at the University of Western Ontario, London, Ontario, with a 500 MHz Unity-500 spectrometer (Varian Associates Inc., Palo Alto, CA), equipped with a 5 mm triple resonance probe, at 298 K.

Isolation of Bovine Platelet Plasmin Inhibitor

Chemicals required for the isolation of bovine platelet plasmin inhibitor (BPPI) were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. This procedure is a modification of Ciaglowski et al., 1986 and Li, 1992. One day prior to sample collection, four litres of anticoagulant were prepared consisting of 3.6% (w/v) sodium citrate and 2.0% (w/v) dextrose, pH 7.8 were prepared. Four hundred millilitres
anticoagulant were transferred to twelve four-litre polypropylene collection jugs and refrigerated. Approximately 45 litres of fresh bovine blood (Gord's Abattoir, Leamington, Ontario) was collected into a basin and then quickly transferred to the jugs containing anticoagulant equipped with a coarse strainer to remove any large debris. The jugs were then gently inverted to ensure adequate mixing and kept at room temperature until processed.

The blood was transferred to a 2 L polymethylpentene (PMP) beaker to facilitate dispensing into 1 L polycarbonate centrifuge bottles. The samples were then centrifuged for 15 minutes at 3,300 x g (4,000 rpm) in a Beckman model J-6B centrifuge equipped with a JS-4.2 rotor at 14° C. The plasma layer was then aspirated off until the plasma level was just above the platelet layer. A turkey baster was then used to transfer the platelets from the centrifuge bottle to a 1 L PMP beaker containing an equal volume of wash buffer containing 3.9 mM potassium phosphate, 25.9 mM sodium phosphate, 5.5 mM dextrose at pH 7.35. This solution was placed on ice until all the whole blood fractions had been processed. The platelet fractions in the wash buffer were then centrifuged as described above. The supernatant was aspirated and discarded and the wash step repeated until the supernatant was clear (about 3 - 5 times). The platelet layer (approximately 75 mL) was then transferred to a 250 mL polycarbonate centrifuge bottle containing 100 mL of resuspension buffer consisting of 15 mM Tris, 0.14 M sodium chloride, 5 mM dextrose, 2 mM calcium chloride at pH 7.6.

Platelet activation was accomplished by addition of 1 mL of 0.5 M EACA as a plasmin inhibitor, 2 mL of 0.1 M calcium chloride, and 200 µL of calcium ionophore
A23187 (2 mg/mL in ethanol) with gently swirling. Within one minute, the platelets were activated as indicated by visible clumping, at which time 40 μL DIFP (1.0 g/mL) was added as a general serine protease inhibitor. The activated platelets were centrifuged under conditions described above, in a Sorvall model RC2-B centrifuge, equipped with a GSA rotor. The supernatant, containing the platelet releasate, was aspirated and transferred to a 250 mL polypropylene bottle. This material was then applied to an Affi-Gel (Bio-Rad Laboratories, Richmond, CA) Heparin Affinity Chromatography Gel column (15 cm x 1.5 cm) equilibrated with 15 mM Tris, 0.15 M sodium chloride, 2 mM calcium chloride, 0.02% (w/v) sodium azide at pH 7.6 (buffer A) at ambient temperature. The flow rate was controlled with a Pharmacia P-1 peristaltic pump (Pharmacia Fine Chemicals, Uppsala, Sweden) at 1 mL/min and the eluent was monitored with a Gilson 111B UV detector at 280 nm (Gilson Medical Electronics Inc., Middleton, WI) and recorded on a Kipp & Zonen model BD41 chart recorder (Kipp & Zonen, Delft, Netherlands) with an absorbance scale of 0.1 AUFS. The unbound fraction was collected in a 250 mL polypropylene bottle. The column was then washed with buffer A, until a baseline was established. The heparin-binding proteins were then eluted with 15 mM Tris, 1.0 M sodium chloride, at pH 7.6 and collected in a 125 mL polypropylene bottle and frozen at -20°C.

The heparin-affinity unbound fraction was then batch processed with CM-Sephadex weak cation exchange media prepared as follows. Five grams of CM-Sephadex were swollen in 200 mL of buffer A in a 500 mL Erlenmeyer flask, covered with an aluminum foil cap containing several pin-holes, and boiled for 1 hour. The media was then allowed
to cool and settle, and the buffer solution decanted, followed by two more buffer changes (no boiling required) to remove the fines. The unbound fraction was then added to the media, after the final decanting, and shaken on an orbital shaker (Lab-Line Instruments, VWR Scientific, Mississauga, Ontario) at 140 rpm overnight at room temperature. The media was then removed from the shaker and filtered through a coarse-porosity fritted disc Buchner funnel and then washed with 400 mL of buffer A. The media was then transferred to a 100 mL beaker, resuspended in 75 mL of buffer A and poured into a chromatography column (25 cm x 1.5 cm). The column was then washed with buffer A at a flow rate of 1 mL/min, until a baseline was established by monitoring at 280 nm with a scale of 0.2 AUFS. Elution was carried out with 15 mM Tris, 0.55 M sodium chloride, 2 mM calcium chloride, 0.02% (w/v) sodium azide, at pH 7.6. The eluted proteins were collected in 6 mL fractions in a Pharmacia Frac-100 fraction collector (Pharmacia Fine Chemicals, Uppsala, Sweden). The protein-containing fractions were then pooled, transferred to a 300 mL lyophilization flask, frozen in a methanol/dry ice mixture, and lyophilized overnight.

The lyophilized product was then resuspended in 15 mL of 0.1% (v/v) TFA and applied to a Sep-Pak Plus C18 solid phase extraction cartridge (Waters Chromatography Division, Milford, MA), connected to a 10 mL syringe, equilibrated with 5 mL of methanol and rinsed with 10 mL of water. The column was then washed with 10 mL of 0.1% (v/v) TFA and then bound material was eluted with 60% (v/v) acetonitrile into a 20 mL glass scintillation vial. The liquid sample was then frozen in methanol/dry ice and lyophilized overnight. Typically, 0.5 - 1.0 mg of protein was obtained, depending on the
volume of viable platelets isolated, and was stored at 4°C.

SDS-PAGE of BPPI

Discontinuous polyacrylamide electrophoresis was performed according to Laemmli, 1970. A 100 mL stock acrylamide solution containing ratio of 30:0.8 (w/w) acrylamide:bis-acrylamide was prepared and filtered through Whatman # 1 filter paper (Whatman International, VWR Scientific, Mississauga, Ontario) and stored at 4°C. Seventeen per cent polyacrylamide slab gels were cast in pairs using the Mighty Small™ minigel unit SE275 (Hoefer Scientific Instruments, San Francisco, CA) by combining 14.2 mL stock acrylamide, 9.4 mL 1.0 M Tris (pH 8.8), 0.25 mL 10% (w/v) SDS, and 1.15 mL water. Polymerization was initiated with the addition of 71 μL of 10% (w/v) ammonium persulfate and 7.1 μL TEMED. The solution was pipetted into the casting apparatus and a thin layer of isopropanol was layered on top. Polymerization was typically achieved within 30 minutes and the alcohol layer decanted. The stacking gel was formed by preparing a solution of 2.5 mL stock acrylamide, 1.5 mL 1.25 M Tris (pH 6.8), 0.15 mL 10% (w/v) SDS, 10.85 mL water, and 45 μL of 0.1% (w/v) bromophenol blue and polymerized with the addition of 150 μL 10% (w/v) ammonium persulfate and 15 μL TEMED. The stacking gel was layered on top of the resolving gel, and combs were inserted to form the sample wells. Polymerization was generally complete after approximately 10 minutes, at which time the gels were carefully removed from the casting chamber, rinsed with water, and stored in an plastic bag at 4°C, until required.

A small quantity (approximately 0.1 mg) of lyophilized BPPI was dissolved in 50 μL
of water in a microcentrifuge tube. An equal volume of 2X sample buffer consisting of 1.0 mL 10% (w/v) SDS, 0.25 mL 1.25 M Tris (pH 6.8), 0.25 mL 2-mercaptoethanol, 0.20 mL glycerol, 0.1 mL 0.1% (w/v) bromophenol blue, and 0.70 mL of water was then added to each tube. The sample, as well as a mixture of Sigma protein standards (MW-SDS-70L), was boiled for 3 minutes. A slab gel was attached to the electrophoresis unit and filled with electrophoresis buffer composed of 194 mM glycine, 0.1% (w/v) SDS, and 25 mM Tris. The BPPI sample and protein standards were loaded into separate wells with a 25 µL Hamilton syringe. Electrophoresis was performed at 200 V and was complete in approximately 50 minutes. The gel was then removed from the running unit and placed in staining solution consisting of 0.05% (w/v) Coomassie blue R, 50% (v/v) methanol, and 10% (v/v) glacial acetic acid for 1 hour. The gel was then transferred to a 50% (v/v) methanol/10% (v/v) glacial acetic acid solution and allowed to destain overnight. Destained gels were preserved by sandwiching the gel between two sheets of BioGelWrap™ G101 (BioDesign Inc. of New York, Carmel, New York) and dried for 30 minutes under a 60 Watt light bulb placed at a distance of 20 cm.

Isoelectric Focusing of Purified BPPI

Isoelectric focusing was performed using the Bio-Rad Model 111 Mini IEF Cell and capillary gel casting tray (Bio-Rad Laboratories, Richmond, CA). Two types of IEF experiments were employed. The first, used Bio-Lyte 3/10 ampholytes to give a broad pH gradient. Secondly, Bio-Lyte 8/10 ampholytes provided a narrow pH gradient to verify the isoelectric point determined in the former experiment. The chemicals required
to cast one 125 x 65 x 0.4 mm gel are summarized below:

<table>
<thead>
<tr>
<th>Component</th>
<th>3/10</th>
<th>8/10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer-ampholyte solution</td>
<td>3/10</td>
<td>8/10</td>
</tr>
<tr>
<td>Water</td>
<td>2.75 mL</td>
<td>1.5 mL</td>
</tr>
<tr>
<td>Acrylamide (25:1)</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>25% (w/v) glycerol</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ampholyte</td>
<td>0.25</td>
<td>0.5</td>
</tr>
</tbody>
</table>

At this point, the solution was filtered through a 0.45 μm HATF filter and then degassed for 5 minutes in a 25 mL stoppered Erlenmeyer flask. Catalysis was then initiated by adding 7.5 μL of 10% (w/v) ammonium persulfate, 25 μL 0.1% (w/v) riboflavin-5'-phosphate, and 3 μL TEMED. The Bio-Rad Mini IEF system employed a polyacrylamide gel support film, since the gels are extremely thin and were cast according to the manufacturer's instructions. A 0.5 mg/mL BPPI solution was prepared in a 2% (v/v) ampholyte solution. A 2 μL sample was applied to the gel surface using the gel template and allowed to diffuse into the gel for 5 minutes. The template was then carefully removed from the gel. Electrophoresis conditions for the first experiment (3/10 ampholytes) required focusing beginning at 100 V for 15 minutes, increased to 200 V for 15 minutes, and finally increased to 450 V for an additional 60 minutes. The conditions for the second experiment (8/10 ampholytes) required focusing beginning at 100 V for 15 minutes, followed by an increase to 200 V for 10 minutes. The gel support film was removed from the glass plate upon completion of electrophoresis and incubated in staining solution consisting of 27% (v/v) ethanol, 10% (v/v) acetic acid, 0.04% (w/v) Coomassie blue-R, 0.05% (w/v) crocein scarlet (Bio-Rad Laboratories, Richmond, CA) and 0.5%
(w/v) copper sulphate for 1 hour with gently agitation. Initially, the gel was destained by incubating the gel in a 12% (v/v) ethanol, 7% (v/v) acetic acid, 0.5% (w/v) copper sulphate solution for 2 - 3 changes, followed by immersion in a 25% (v/v) ethanol/7% (v/v) acetic acid solution for approximately 1 hour. The gel was allowed to dry overnight in the fume hood.

**Gel Filtration of BPPI**

A molecular weight estimation was performed with a Pharmacia Superose™ 12 HR 10/30 FPLC column, on a Waters 600 multisolvent delivery system, equipped with a Waters 441 absorbance detector and a Waters 745 data module. The column was equilibrated with buffer A, prepared with Milli-Q™ quality water and filtered through a 0.45 μm HATF membrane (Waters Chromatography Division, Milford, MA). A 25 μL injection of a combination of BSA/cytochrome c (0.5 mg/mL each) and a 25 μL injection of a combination of BPTI/carbonic anhydrase (0.5 mg/mL each) were used as standards, after allowing the column time to re-equilibrate between each set of standards. The flow rate was maintained at 0.75 mL/min for the duration of the 45 minute run time. The eluted proteins were detected at 254 nm, and the data module chart speed set at 0.25 cm/min. A 0.5 mg/mL BPPI solution was prepared in buffer A and a 20 μL sample was injected.

**Solubilization Studies of BPPI**

Approximately 0.2 mg of lyophilized BPPI was placed in a series of 1.5 mL
microcentrifuge tubes that contained 100 µL of 50 mM sodium acetate buffer, having pH values of 4.0, 4.5, 5.0, and 5.5. Another series of tubes was prepared to which 100 µL of 50 mM sodium phosphate buffer, having pH values of 6.5, 7.0, and 7.5 was added. Concentration measurements were determined by Coomassie blue protein dye-binding assay (Bradford, 1976) performed as follows. Coomassie blue G (0.06 g) was dissolved in 80 mL of water and stirred for 1 hour, at which time 3.2 mL of 60% perchloric acid was added and the volume brought up to 100 mL with water. The dye solution was mixed thoroughly and filtered through Whatman #1 filter paper. A 1 mg/mL solution of BSA was prepared as a standard, given that a 1 mg/mL solution has an absorbance of 0.667 at 280 nm (Van Kley & Stahmann, 1959). The standard series was prepared by transferring 2 - 14 µL of BSA, in 2 µL increments, to separate test tubes. In addition, two tubes without BSA were prepared as blanks. Water was added to each test tube to bring the volume up to 200 µL. Finally, 800 µL of dye was added to each test tube which were then vortexed. The samples were measured at 620 nm after a 5 minute incubation period.

**Reversed-phase HPLC of BPPI**

This chromatographic process was performed on a Waters 600 HPLC and model 441 UV detector with a Delta-Pak™ C18 (3.9 mm x 300 mm) column equilibrated with 0.05% (v/v) TFA. A small amount of lyophilized BPPI (approximately 100 µg) was dissolved in 300 µL equilibration buffer, of which 200 µL was actually injected. The protein was eluted with increasing acetonitrile (ACN) concentration in 0.05% (v/v) TFA as follows:
0% ACN for 5 minutes, 0 - 25% ACN for 5 minutes, 25 - 50% ACN for 40 minutes, 50 - 70% ACN for 5 minutes, 70% ACN for 5 minutes, and 70 - 0% ACN for 5 minutes. The absorbance of the eluent was monitored at 214 nm and recorded on the data module set at a chart speed of 0.25 cm/min.

**Supplementary Solubility Studies**

BPPI samples previously not soluble above 0.5 mg/mL were dissolved in (10:1) 0.1% (v/v) TFA:protein solution and concentrated with a Sep-Pak cartridge as before. However, the bound protein was eluted with 38% (v/v) ACN. The lyophilized product was resuspended in 100 - 150 μL of buffer A and stored at 4°C in a microcentrifuge tube. Any insoluble material was resuspended in 0.1% (v/v) TFA and lyophilized again. The lyophilized protein was then dissolved in the resuspended solution. The solution was then adjusted to pH 8.5 by addition of approximately 1 μL of 0.5 M sodium hydroxide. Protein concentrations were determined by the Bradford assay as described above.

**Amino Acid Analysis of BPPI (Bidlingmeyer et al., 1984)**

A 50 μL aliquot of a 0.5 mg/mL solution of BPPI in water was transferred to a culture tube. The sample was dried to 65 mTorr using the Water Pico-Tag™ Workstation. The reaction vial was removed and 200 μL of constant boiling grade 6N HCl (Pierce, Rockford, IL) containing 1% (v/v) phenol, was added to the bottom of the reaction vial. Hydrolysis was carried out at 110°C for 20 hours, at which time the sample was transferred to a fresh reaction vial and dried along with 5 μL of a mixture of amino acid
standards (Pierce, Rockford, IL), which were introduced at this step. The dried samples were then redried with 20 μL of a 2:2:1 mixture of methanol:water:TEA, followed by derivitization with 20 μL of a 7:1:1:1 mixture of methanol:water:TEA:PITC for 20 minutes at room temperature. The samples were then dried to 65 mTorr and allowed to continue drying for 30 minutes at this pressure to ensure all excess reagent was removed. The derivatized samples were dissolved in sample diluent containing 5% (v/v) ACN, 4.75 mM disodium hydrogen phosphate, at pH 7.4. Effective separation of 17 amino acids was achieved within the first 13 minutes under the conditions in Table 2.1.

Typically, the derivatized samples were resuspended in 200 μL of sample diluent and 4 μL was injected into a Waters Pico-Tag column installed in the Waters 600 Multisolvent delivery system, equipped with a column heating unit that maintained the column temperature at 38°C. The model 441 detector was set at 254 nm and the chart speed of the integrator was set at 1 cm/min for 13 minutes with an attenuation of 64. Quantification of amino acid residues was based on elution times and peak areas of the amino acid standards.

**Trypsin Inhibition Assay of BPPI**

This assay is a modification of Fioretti et al., 1983. A dilute BPPI solution (approximately 0.1 mg/mL) was prepared in 50 mM Hepes, pH 8.0. A 400 μL aliquot of this solution was incubated with 200 μL of 0.046 M Tris buffer at pH 8.1, and 40 μL of trypsin (0.1344 mg/mL), from bovine pancreas, for 5 minutes at room temperature.
### Table 2.1

**Amino Acid Analysis Elution Gradient**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL/min)</th>
<th>A&lt;sup&gt;1&lt;/sup&gt;%</th>
<th>B&lt;sup&gt;2&lt;/sup&gt;%</th>
<th>C&lt;sup&gt;3&lt;/sup&gt;%</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>10.0</td>
<td>1.0</td>
<td>56</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>10.3</td>
<td>1.0</td>
<td>0</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>12.0</td>
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<td>40</td>
<td>60</td>
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</tr>
<tr>
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<tr>
<td>30.5</td>
<td>1.0</td>
<td>94</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

<sup>1</sup> 0.14 M sodium acetate trihydrate/0.05% (v/v) TEA, adjusted to pH 6.4 with HPLC grade glacial acetic acid and filtered through a 0.45 μm HATF filter.

<sup>2</sup> Milli-Q quality water filtered through a 0.45 μm HATF filter.

<sup>3</sup> HPLC grade acetonitrile filtered through a 0.45 μm HVHP filter.
After the incubation was complete, 300 μL of BAPNA (0.008 M) was added and quickly mixed. A control experiment was also performed by replacing the BPPI solution with Hepes buffer. Absorbances were recorded in continual scan mode at 405 nm for 180 seconds.

Stability Studies of the BPPI/Trypsin Complex in SDS

This procedure is a variation of Arakawa et al., 1992. Six microcentrifuge tubes each containing a 5 μL aliquot of a 5 μg/μL BPPI solution and 30 μL of a 2 mg/mL trypsin, from bovine pancreas, solution were prepared, gently mixed and incubated for 10 minutes at room temperature. Two control tubes were also prepared separately containing only trypsin or BPTI. A 35 μL aliquot of sample buffer containing 250 μL glycerol, 250 μL 10% SDS stock solution, 50 μL 1.25 M Tris at pH 6.8, and 1.95 mL of water was then added to each tube. The samples were then incubated at temperatures ranging from 4° to 95°C for 10 minutes and then electrophoresed on a 17% resolving/5% stacking SDS-PAGE gel at a constant current of 18 mA. The gels were stained, destained, and preserved as described previously.

One and Two-dimensional NMR Spectroscopy of BPPI

A 1 mM solution of BPPI was prepared in 450 μL of water at an alkaline pH of approximately 8.5 in a microcentrifuge tube. The solution was then adjusted to pH 7.1 with dilute HCl. A 1 μL aliquot of 10 mM DSS (MSD Isotopes, Montreal, PQ) was added as an internal standard. The volume of the solution was then made up to 500 μL.
with D$_2$O and transferred to a high quality model PP535 NMR tube (Wilmad, Buena, NJ) and centrifuged briefly.

A one dimensional experiment was first performed to determine the viability of the sample for more detailed spectral acquisition. The experiment was completed with the collection of 512 transients and the presaturation method of solvent suppression was employed. A MLEV-17 spin lock of 50 ms was used in the collection of the TOCSY spectra. The TOCSY and NOESY spectra were collected with 256 time increments in the $t_1$ domain and zero-filled to 4096 points. A total of 64 transients were averaged for each $t_1$ increment. The presaturation method of solvent suppression was employed during the TOCSY experiment, whereas the solvent peak was saturated by adding an inversion recovery sequence to the standard NOESY pulse sequence. Mixing times of 100 and 200 milliseconds were used in the two NOESY experiments. A 6000 Hz spectral width was collected in both dimensions.

**Three-dimensional Structural Determination of BPPI using Homology**

The amino acid sequence of BPPI was entered, by one letter code, into a standard text file created with vi editor within UNIX on a Silicon Graphics R4000 Indigo Workstation in the following format: filename.seq. This sequence file was then read by the Homology module operating within InsightII (Biosym Technologies, San Diego, CA). The x-ray crystallographic structure of bovine pancreatic trypsin inhibitor (Walter and Huber, 1983) was obtained from Brookhaven National Laboratory Protein Databank (Bernstein et al, 1977) file 4PTI and displayed using InsightII. The primary sequence of BPTI was
derived from the crystal structure using the *Homology* program. The sequences of BPPI and BPTI were aligned according to sequence homology, and the coordinates for the reference protein (BPTI) were copied to the model protein (BPPI). The coordinates for the N- and C-termini of BPPI were added by employing the *EndRepair* command within *Homology*.

The generated BPPI structure was then subjected to energy minimization using the *Discover* module. The minimization was carried out for 1000 iterations using the conjugate gradient. The minimized structure was then copied and the BPPI dimer was generated, within the *BioPolymer* module, by forming a disulfide bond between the Cys-4 residue from both monomers. Again, the BPPI dimer was subjected to energy minimization for 3500 iterations using the conjugate gradient.

**Circular Dichroism Spectroscopy of BPPI**

The circular dichroism spectrum of BPPI was recorded at Wayne State University, Department of Chemistry, with a Jasco-600 Spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan) with a 1 mm path length cell. A 117 μM BPPI sample was prepared in 1.5 mL of 0.1 mM K₂HPO₄ at pH 7.7, using an extinction coefficient of 5960 M⁻¹cm⁻¹ calculated by the method of Gill and von Hippel, 1989. Similarly, a 142 μM BPTI sample was prepared in 1.5 mL of 0.1 mM K₂HPO₄ at pH 7.7, as a reference standard, using an extinction coefficient of 5395 M⁻¹cm⁻¹ (Makhatadze *et al.*, 1993). The CD spectrum was recorded from 250 to 185 nm using 65 data points at a scan rate of 2 nm/min. The phosphate buffer CD spectrum was subtracted from the protein spectrum.
to give the final corrected spectrum of BPPI. The secondary structural elements of BPPI were calculated based on the approximation that a protein CD spectrum in the amide region can be represented as a linear combination of the four different contributing elements to the secondary structure (Chang et al., 1978).

Isolation of Human Platelets

This procedure employs the same chemicals as described for the purification of bovine platelets, however there are many modifications in the procedure outlined here. Platelet enriched fractions, obtained from the Red Cross, were emptied into 1 L Nalgene PMP beakers designated for platelet work only. The material was transferred to 500 mL polycarbonate centrifuge bottles, balances, and centrifuged for 20 minutes at 4,000 x g in a J2-HS centrifuge equipped with a JA-10 rotor at room temperature. The plasma fraction was aspirated, leaving the platelets and residual red cells not removed in earlier processing. The platelets, from each 500 mL centrifuge bottle, were removed with a turkey baster and transferred to a 250 mL polycarbonate centrifuge bottle. A 50 mL volume of wash buffer was added to the platelets with brief and gentle mixing. The suspension was then centrifuged at room temperature for 20 minutes in a J2-HS centrifuge equipped with a JA-14 rotor at 4,000 x g. The supernatant was decanted and the washing procedure repeated. After the second wash, the platelets were resuspended in 50 mL of resuspension buffer. Two millilitres of 0.1 M calcium chloride and 1 mL 0.5 M EACA were added by pipetting down the side of the bottle and gently swirled for about 30 seconds. Two hundred microlitres of 2 mg/mL calcium ionophore A23187 (in DMSO)
were added and gently swirled for about 15 seconds. Platelet activation was noted by the formation of white clumps usually within 1 minute. PMSF was added after the activation process to a final concentration of 0.1 mM from a 200 mM stock solution (in ethanol). The mixture was then centrifuged as above for 30 minutes. The resulting supernatant, containing the platelet proteins, was a straw-yellow colour and was transferred to a 200 mL polypropylene bottle. Any fibrin that may have formed was removed with a plastic pipette. The sample was applied to an Affi-gel heparin column (15 cm x 1.5 cm) equilibrated with buffer A to establish a baseline. The unbound fraction and the washings (approximately 200 mL) were collected in a plastic bottle and frozen.

Isolation of a Human Platelet Plasmin Inhibitor (HPPI)

The heparin-agarose unbound fraction was applied to a trypsin-agarose affinity column prepared as follows. Three millilitres of Affi-Gel 10 beads (Bio-Rad Laboratories, Richmond, CA) were washed with 100 mL of cold water on a 50 mL coarse-porosity fritted disc Buchner funnel. The beads were transferred in cold water to a 50 mL conical tube (Falcon Labware, VWR Scientific, Mississauga, ON), centrifuged briefly, and the supernatant decanted. Trypsin (25 mg) in 6 mL of 0.046 M Hepes, 0.0115 M calcium chloride buffer, at pH 8.0 was added to the beads and incubated at 4°C on a rotary wheel for 5 hours. After incubation, 300 µL of TEA was added, and incubated for 1 hour on a rotary wheel at 4°C. The sample was then briefly centrifuged and the supernatant aspirated and saved to determine the amount of trypsin bound to the column. The slurry was then poured into a Bio-Rad Econo column (90 mm x 10) and
washed with 50 mL of buffer A at 4°C.

After the heparin-agarose unbound fraction had been applied, the column was washed with buffer A to establish a baseline. The bound proteins were eluted with water, followed by 10 mM HCl, and the absorbance was monitored at 280 nm and recorded with chart recorder settings of 0.5 mm/min with an offset of 0.1 AUFS. The bound protein solution (approximately 30 - 50 mL) was made up to 200 mL with buffer A, and batch processed with 3 g of CM-Sephadex as described previously. Proteins bound to the ion-exchange media were eluted with 15 mM Tris, 2 mM calcium chloride, 1.0 M sodium chloride, 0.02% (w/v) sodium azide, at pH 7.6. The detection wavelength was 280 nm with an absorbance scale of 0.05 AUFS, and 6 mL fractions were collected. Final purification of the protein was performed as described for the bovine species.

The purity and molecular weight of both the CM-Sephadex bound and unbound fractions were examined on 17% polyacrylamide gels using SDS-PAGE, as described previously with the exception of the staining method. The gel was stained in a solution consisting of 0.05% (w/v) Cal-Red® (Hong et al., 1993), 50% (v/v) methanol, and 10% (v/v) acetic acid for 6 hours. The gel was allowed to destain overnight and was preserved with BioGelWrap™.

Trypsin Inhibition Assay of HPPI

The lyophilized HPPI sample was resuspended in 75 μL of 50 mM Hepes/20 mM calcium chloride, at pH 8.0. A 25 μL aliquot of this solution was transferred to a microcentrifuge tube and incubated with 100 ng of trypsin, from bovine pancreas, and 200
μL of 0.046 M Tris, at pH 8.1 for 10 minutes at room temperature. When the incubation was complete, 300 μL of 0.008 M BAPNA was added and quickly mixed. The absorbance was monitored in continual scan mode at 405 nm for 180 seconds.

Plasmin Inhibition Assay of HPPI

Human plasmin (Sigma, P-4895) was prepared by dissolving the solid protein/buffer mixture (5.4 mg = 1.296 U) in 1 mL of buffer consisting of 0.1 M Hepes, 0.1 M sodium chloride, 0.1% (w/v) PEG 8000, at pH 7.4. Two hundred microlitre aliquots were transferred to microcentrifuge tubes and frozen at -20°C. The assay was performed by incubating 300 μL Hepes, pH 7.4, 400 μL inhibitor, and 50 μL of plasmin solution for 30 minutes at 25°C. Following the incubation, 10 μL of 40 mM BAPNA was added, quickly mixed, and the absorbance was monitored at 405 nm for 600 seconds. A control experiment was also performed as above, replacing the inhibitor solution with Hepes buffer.

Purification of Bovine Platelet Factor Four

Bovine PF4 (b-PF4) was purified similarly to a method as described by Ciaglowski et al., 1986. Platelet isolation, activation, and heparin-agarose chromatography are described above. Bovine PF4 was eluted from the heparin-agarose column with 50 mM Tris/1.5 M NaCl. This fraction was concentrated with an Amicon model 8050 stirred ultrafiltration (Amicon Inc., Beverly, MA) cell containing a YM5 membrane (MW cutoff of 5,000 Daltons) to approximately 7 mL. The concentrated solution was then applied
to a calibrated Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) gel filtration column (3 cm x 90 cm) equilibrated with 50 mM Tris/0.75 M NaCl at pH 7.4. The flow rate was maintained at 0.2 mL/min, 6 mL fraction were collected over 30 minutes, and monitored at 280 nm. The fraction eluted with a molecular weight similar to that of tetrameric b-PF4 (approximately 45,000 Da) was pooled and lyophilized. The lyophilized product was resuspended in 1 - 2 mL 0.03 M ammonium bicarbonate buffer and the sample desalted over a Sephadex G-15 column (3 cm x 10 cm) and then lyophilized. The purity of the product was verified using a 17% polyacrylamide gel (Laemmli, 1970) calibrated with Dalton Mark VII-L electrophoresis standards (Sigma).

Reduction and Alkylation of b-PF4

This procedure is similar to that of Ciaglowski et al., 1986. A 1 mg/mL solution of b-PF4 in 1.0 M Tris/0.3 M EDTA at pH 8.0 was incubated with a 20-fold molar excess of dithiothreitol over the concentration of cysteine. The solution was then flushed with nitrogen for 3 minutes, sealed with a rubber septum, and incubated at 37°C for 18 hours. A 5-fold molar excess of iodoacetamide was then added with a syringe, and then incubated at room temperature for 4 hours in the dark. The reaction was then terminated by adding 1.0 mL of 2-mercaptoethanol, frozen, and lyophilized in the dark.

Enzymatic Digestion of Reduced and Alkylated b-PF4

The reduced and alkylated protein was dissolved in 50 mM ammonium acetate buffer at pH 4.0 to give a final concentration of 1 mg/mL. *Staphylococcus aureus* V8 protease
(Worthington) was dissolved in Milli-Q quality water to a concentration of 0.27 mg/mL (Drapeau et al., 1972). An aliquot of the enzyme solution was added to the protein solution to give 1:50 (w:w) ratio between the enzyme and the protein. The solution was then incubated for 48 - 72 hours at 37°C. The digestion was terminated by freezing the sample to - 20°C and lyophilizing.

Peptide Mapping of Digestion Products

Separation of the generated peptides was performed with a Delta-Pak reversed-phase HPLC column (Waters) with eluted peptides monitored at 214 nm. The lyophilized product was dissolved in a minimal quantity (approximately 750 µL) of 0.05% (v/v) TFA and passed through a 0.45 µm filter to remove any insoluble products. The peptides were eluted at a flow rate of 1 mL/min, using a linear gradient of increasing ACN concentration as follows (Talpas et al., 1991): 0 - 20 min (0 - 20% ACN), 20 - 60 (20 - 40% ACN), and 60 - 90 min (40 - 70% ACN).

Affinity Chromatography of V2

The ability of the V2 fragment to bind heparin was examined with a Bio-Rad Econo Pac Heparin Cartridge (Bio-Rad Laboratories, Richmond, CA) connected to an HPLC. The experiment was performed at room temperature using a linear gradient from 0.15 M to 1.5 M sodium chloride over a period of 55 minutes, in 10 mM potassium phosphate buffer at pH 7.5 and monitored at 254 nm at a flow rate of 0.4 mL/min.
Oligomeric Determination of V2

A Superose-12 gel filtration column (Pharmacia), designed for use with HPLC, was calibrated with a series of protein standards consisting of bovine serum albumin, cytochrome c, carbonic anhydrase, and BPTI at a concentration of 0.5 mg/mL. The column buffer was 10 mM potassium phosphate/0.2 M sodium chloride at pH 7.5. The flow rate was 0.5 mL/min and the eluted proteins were detected at 254 nm.

Circular Dichroism (CD) Spectroscopy of V2

The circular dichroism spectrum of V2 was recorded, at Wayne State University, Department of Biochemistry (Faculty of Medicine) with an On-Line Instrument Systems (OLIS) UV/VIS/CD spectrometer (Olis Inc., Bogar, GA) with a 1 mm path length cell. A 32.5 μM solution of V2 was prepared in a 1 mM sodium phosphate/0.15 M NaCl buffer at pH 6.5. The CD spectrum was recorded from 250 to 196 nm using 54 data points with a time integral of 3 seconds per data point. The final spectrum of V2 was an average of 3 scans and was baseline subtracted. The exact concentration of the sample, determined by amino acid analysis, was required to convert the spectral readings in millidegrees (mdeg) to mean residue ellipticity (MRE). The CD spectrometer was calibrated with a 0.1 mg/mL aqueous sample of (+)-10-camphorsulfonic acid.

The α-helical content of V2 was determined by comparing the mean residue ellipticity at 222 nm (MRE_{222nm}) for the V2 spectra to the MRE_{222nm} of a complete α-helix (Toniolo et al., 1979) and of a complete random coil (Greenfield and Fasman, 1969).
NMR Spectroscopy of V2

A 1 mM solution of V2 was prepared in 20 mM sodium phosphate/0.15 M NaCl at pH 6.5 to give a final volume of 0.450 mL in a microcentrifuge tube. A 1 μL aliquot of 10 mM DSS (MSD Isotopes, Montreal, PQ) was added as an internal standard. The volume was made to 0.5 mL with the addition of D₂O (Cambridge Isotope Laboratories, Woburn, MA) and briefly centrifuged. The sample was stored at 4°C until required.

A DQF-COSY experiment was performed and a total of 2048 data points were collected in the t₂ domain and zero-filled to 4096 data points. There were 32 time increments collected in the t₁ domain each consisting of 32 transients. A spectral width of 6000 Hz was used in both dimensions and data acquisition was performed with solvent suppression by presaturation.
Results

Isolation of BPPI

An average preparation involved processing between 40 - 45 L of fresh bovine blood through various centrifugation and column chromatography steps (Ciaglowski et al., 1986 and Li, 1992). The unbound fraction from the heparin-agarose chromatography was then batch processed with CM-Sephadex. Figure 3.1 illustrates a typical elution profile for the fraction bound to the CM-Sephadex media. The protein was eluted with approximately 150 mL of buffer B consisting of 15 mM Tris, 0.55 M NaCl, 2 mM CaCl₂, and 0.02% (w/v) NaN₃, at pH 7.6. The overall peak shape was quite asymmetrical. Typically, 0.5 - 1.5 mg of protein is obtained for each preparation.

SDS-PAGE of BPPI

The purity of the protein from the CM-Sephadex chromatography was verified using SDS-PAGE (Laemmli, 1970). The samples were prepared for electrophoresis by concentrating the samples with a Sep-Pak and then lyophilizing them. Almost always, a single band with a molecular weight corresponding to approximately 12,000 Daltons was present after visualization with Coomassie Brilliant Blue stain as shown in lane 3 of figure 3.2. The band was normally quite broad relative to the protein standards (lane 4) regardless of the mass of protein applied. The molecular weight markers contained: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate, 36 kDa; carbonic anhydrase, 29 kDa, trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa;
Figure 3.1: CM-Sephadex ion exchange profile of the unbound heparin-agarose fraction. The eluted proteins were monitored at 280 nm. The unbound fraction from heparin-agarose chromatography was batch processed overnight with 5 g of CM-Sephadex in buffer A consisting of 15 mM Tris, 0.15 M NaCl, 2 mM CaCl₂, and 0.02% (w/v) NaN₃, at pH 7.6. The media was washed with buffer A to establish a baseline at a flow rate of 1.0 mL/min. The bound proteins were eluted with approximately 150 mL of buffer B consisting of 15 mM Tris, 0.55 M NaCl, 2 mM CaCl₂, and 0.02% (w/v) NaN₃, at pH 7.6.
Figure 3.2: Gel electrophoresis of BPPI. Lane 4, protein standards consisting of: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.2 kDa. Lane 1 and 2 contain samples of the proteins present in the unbound heparin-agarose fraction. Lane 3, 30 μg sample of the desalted and lyophilized solution from CM-Sephadex ion exchange chromatography.
α-lactalbumin, 14.2 kDa. Lanes 1 and 2 contain samples of the proteins present in the heparin-agarose unbound fraction, which contain two additional bands with molecular weights of 66,000 Da and 16,000 Da.

**Isoelectric Focusing of BPPI**

The isoelectric focusing experiments were performed with two pH gradients. Figures 3.3 and 3.4 show the experiments performed with the 3 to 10 and the 8 to 10 range ampholytes, respectively. Lanes 1 and 6 of figure 3.3 contain protein standards consisting of: cytochrome c, pI = 9.6; ribonuclease A, pI = 7.8; myoglobin, pI = 7.0; and β-lactoglobulin. Lanes 2 - 5 contain a 2 μL sample of a 0.5 mg/mL BPPI solution. The isoelectric point of BPPI was interpolated from the linear regression line generated from the relative migrations of the four protein standards for the 3 to 10 experiment. Lanes 1 and 2 of figure 3.4, contain 2 μL of cytochrome c, pI = 9.6 and 2 μL of aprotinin, pI = 10.5, respectively. The pI was calculated to be 10.4 for the pH 3/10 experiment, whereas the pH 8/10 experiment indicated a slightly lower pI value of 10.2.

**Gel Filtration of BPPI**

The molecular weight of BPPI in solution was estimated by gel permeation chromatography using a Superose-12 column calibrated with BSA, 66,000 Da; carbonic anhydrase, 29,000 Da; cytochrome c, 12,400 Da; and aprotinin, 6,500 Da. The column was equilibrated with buffer A consisting of 15 mM Tris, 0.15 M NaCl, 2 mM CaCl$_2$, and 0.02% (w/v) NaN$_3$, at pH 7.6. The absorbance was monitored at 254 nm and the flow
Figure 3.3: Broad range isoelectric focusing of BPPI. A 12 µL BPPI solution (0.5 mg/mL) was prepared in a 2% (v/v) 3/10 ampholyte solution. The experiment was conducted at a pH range from 3 to 10 using a voltage step gradient. Lanes 1 and 6 contain protein standards consisting of: cytochrome c, pI = 9.6; ribonuclease A, pI = 7.8; myoglobin, pI = 7.0; and β-lactoglobulin, pI = 5.1. Lanes 2-5 contain a 2 µL sample of 0.5 mg/mL BPPI solution. The isoelectric point of the BPPI solution was calculated to be 10.4, using the regression line of $y = 0.12011 x + 4.9579$. 
Figure 3.4: Narrow range isoelectric focusing of BPPI. The narrow range experiment was conducted from pH 8 to 10 using a voltage step gradient. A 5 μL BPPI solution (0.5 mg/mL) was prepared in a 2% (v/v) 8/10 ampholyte solution. The protein standards used were cytochrome c, pH = 9.6 (lane 1) and aprotinin, pH = 10.5 (lane 2). A 2 μL BPPI sample was applied to lane 3 of the gel. The pH was calculated to be 10.2 using the regression line of y = 0.05882 x + 7.235 that was generated from the protein standards.
Figure 3.5: Molecular weight estimation of BPPI. A linear regression plot of the elution times from gel filtration chromatography using a Superose-12 column calibrated with BSA, 66,000 Da; carbonic anhydrase, 29,000 Da; cytchrome c, 12,400 Da; and aprotinin, 6,500 Da. The column was equilibrated with buffer A consisting of 15 mM Tris, 0.15 M NaCl, 2 mM CaCl₂, and 0.02% (w/v) NaN₃, at pH 7.6. The flow rate was maintained at 0.75 mL/min and the eluted proteins were detected at 254 nm. A purified BPPI sample was applied and found to elute at 20.38 minutes (B), corresponding to a molecular weight of approximately 19,000 Da, using the regression line of \( y = 0.14529 \, x + 7.249 \).
rate maintained at 0.75 mL/min. Calculation of the molecular weight of 19,000 Da for BPPI, which eluted at approximately 20.4 minutes (B), was based on the linear regression line generated from the elution times of the four protein standards as presented in figure 3.5.

**Solubilization Studies of BPPI**

A variety of solubility studies were performed with BPPI including, but not limited to, a pH range study at constant buffer concentrations. The histogram shown in figure 3.6 typifies the concentration determinations of less than 0.5 mg/mL as calculated in most experiments using the Bradford protein assay. The value at which the solubility of BPPI was greatest in sodium acetate at pH of 4.0, was at a concentration of 0.48 mg/mL. Alternatively, the solubility was lowest in sodium acetate at pH 5.5. The solubility of BPPI nearest to physiological pH was approximately 0.35 mg/mL.

**Reversed-phase HPLC of BPPI**

A typical elution profile for this chromatographic procedure is illustrated in figure 3.7. BPPI samples purified by CM-Sephadex chromatography were applied to a Delta-Pak C18 reversed-phase HPLC column, without any further purification. The samples appeared to be quite pure, that was evident from the single peak that eluted at approximately 18 minutes, corresponding to an acetonitrile concentration of 38% (v/v) in 0.05% (v/v) TFA. The identity of this peak was verified using SDS-PAGE (not shown). The large peak eluting at approximately 3 minutes is an optical artifact, resulting from impurities and
Figure 3.6: Solubilization studies of BPPI. Protein samples were dissolved in 50 mM sodium acetate at pH 4 - 5.5 and 50 mM sodium phosphate at pH 6.5 - 7.5. Concentration measurements were determined by the Bradford protein assay using a UV-vis spectrometer at a wavelength of 620 nm.
Figure 3.7: Reversed-phase HPLC of BPPI. A Delta-Pak C18 reversed-phase HPLC chromatogram of a BPPI sample purified by CM-Sephadex chromatography. Approximately 70 μg of BPPI was applied to the column equilibrated with 0.05% (v/v) TFA. The protein was eluted with a linear gradient of increasing acetonitrile, in 0.05% (v/v) TFA, at a time of approximately 18 minutes (38% acetonitrile). The absorbance was monitored at 214 nm and the flow rate maintained at 1 mL/min.
was present in blank injections containing only buffer.

**Supplementary Solubility Studies**

A re-examination of the solubility of BPPI was performed after altering the method used to concentrate the sample, which involved eluting the bound proteins from a Sep-Pak C18 cartridge with 60% (v/v) acetonitrile. In this procedure, the samples were desalted and concentrated with a Sep-Pak cartridge and eluted with 38% (v/v) acetonitrile and lyophilized. The lyophilized product was resuspended in buffer A consisting of 15 mM Tris, 0.15 M NaCl, 2 mM CaCl₂, and 0.02% (w/v) NaN₃, at pH 7.6. The concentration was determined with the Bradford assay as before. However, figure 3.8 illustrates a dramatic increase in the solubility of BPPI. The standard curve, which covered a range from 0 to 14 µg of protein, was used to calculate a protein concentration of approximately 12 mg/mL for the BPPI solution.

**Amino Acid Analysis of BPPI**

The hydrolyzed and derivatized BPPI sample was chromatographed on a Pico-Tag™ reversed-phase HPLC column, which can resolve 17 standard amino acids (Bidlingmeier et al., 1984) as shown in figure 3.9. The individual amino acids were quantified using response factors calculated from the amino acid standards. The number of calculated residues for each amino acid was determined by dividing the picomoles for each individual amino acid by the total picomoles per residue as shown in table 3.1. An excellent indicator for the presence of a Kunitz-type inhibitor is the noted absence of
Figure 3.8: Supplementary solubility studies. Standard curve generated with BSA using 0 - 14 µg of protein. BPPI samples were concentrated with a Sep-Pak, lyophilized, and dissolved in 100 µL of buffer A consisting of 15 mM Tris, 0.15 M NaCl, 2 mM CaCl₂, and 0.02% (w/v) NaN₃, at pH 7.6. The concentration measurements were determined by the Bradford protein assay using a UV-vis spectrometer at a wavelength of 620 nm. The absorbance of the BPPI solution was 0.399, corresponding to a concentration of approximately 12 mg/mL using the regression line of \( y = 0.01645 x + 0.00992 \).
Figure 3.9: Amino acid analysis of BPPI. HPLC chromatogram of a hydrolyzed and PITC-derivatized BPPI sample applied to a Pico-Tag™ column. The amino acids were eluted with increasing acetonitrile concentration and detected at 254 nm.
Table 3.1
Amino Acid Analysis of BPPI*

<table>
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<tr>
<th>Amino Acid</th>
<th>Picomoles</th>
<th>Number of residues (calculated)</th>
<th>Number of residues (theoretical)</th>
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<td>2</td>
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<tr>
<td>Lys</td>
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</table>

*Quantification of the amino acid residues in BPPI. The molar amount of each amino acid was determined using the response factors for the 17 standards. The theoretical values are based on the amino acid sequence of BPPI (Li, 1992).
histidine as was the case for the BPPI sample analyzed. The theoretical number of residues was determined from the primary sequence of BPPI (Li, 1992).

Trypsin Inhibition Assay of BPPI

The ability of BPPI to inhibit the serine protease trypsin was examined using BAPNA as a substrate (Fioretti et al., 1983) following a five minute incubation of the protease and inhibitor at a ratio of 1:6.5 (w/w). Figure 3.10 illustrates the activity of trypsin without BPPI present (filled squares) and in the presence of BPPI (open squares). The conditions of excess inhibitor, used in this experiment, caused complete inhibition of trypsin hydrolysis of the chromogenic substrate.

Stability Studies of the BPPI/Trypsin Complex

The trypsin/BPPI solutions were incubated over a wide temperature range in the presence of 0.5% (w/v) SDS. The solutions were electrophoresed at room temperature as shown in figures 3.11 and 3.12. Lanes 9 and 10 on both figures 3.11 and 3.12 contain molecular weight markers consisting of: BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.2 kDa. Figure 3.11 represents the study conducted from 4°C to 45°C as follows: Lane 1, 4°C; lane 2, 25°C; lane 3, 30°C; lane 4, 40°C, and lane 5, 45°C. The appearance of a broad band having a molecular weight of approximately 45,000 Daltons corresponding to the molecular weight of the BPPI/trypsin complex is evident in each of these lanes. The
Figure 3.10: Trypsin inhibition assay of BPPI. Trypsin, from bovine pancreas, hydrolysis of BAPNA was measured in the absence of BPPI (filled squares) and in the presence of BPPI (open squares). The protease/inhibitor solution was incubated for 5 minutes at room temperature. The absorbance was recorded for 180 seconds at 405 nm.
Figure 3.11: Stability study of the BPPI/trypsin complex. The stability of the BPPI/Trypsin complex was examined, in the presence of 0.5% (w/v) SDS, over a temperature range of 4°C to 45°C. Six samples containing 60 μg of trypsin, from bovine pancreas, was incubated with 25 μg of BPPI for ten minutes. Subsequently, an SDS solution was added to give a final concentration of 0.5% (w/v) SDS. The solutions were then incubated for 10 minutes at 4°C, 25°C, 30°C, 40°C, or 45°C and then electrophoresed. Lane 1, 4°C; lane 2, 25°C; lane 3, 30°C; lane 4, 40°C; and lane 5, 45°C. Lanes 9 and 10 contains molecular weight markers consisting of: BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.2 kDa. The formation of a new band at approximately 45 kDa indicated the stability of the complex in the presence of SDS over the entire temperature range. Lane 7 contained only trypsin, and lane 8 contained only BPPI. Lane 6 contained only sample buffer.
Figure 3.12: Stability study of the BPPI/trypsin complex. The stability of the BPPI/trypsin complex was examined, in the presence of 0.5% (w/v) SDS, over a temperature range of 45°C to 95°C. Six samples containing 60 μg of trypsin, from bovine pancreas, was incubated with 25 μg of BPPI for ten minutes. Subsequently, an SDS solution was added to give a final concentration of 0.5% (w/v) SDS. The solutions were then incubated for 10 minutes at 45°C, 50°C, 60°C, 70°C, or 95°C and then electrophoresed. Lane 1, 45°C; lane 2, 50°C; lane 3, 60°C; lane 4, 70°C; and lane 5, 95°C. Lanes 9 and 10 contain molecular weight markers consisting of: BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.2 kDa. The formation of a new band at approximately 45 kDa indicated the BPPI/trypsin complex was stable up to a temperature of 60°C in the presence of SDS. Lane 7 contained only trypsin, and lane 8 contained only BPPI. Lane 6 contained only sample buffer.
study conducted between 45° and 95°C is shown in figure 3.12. The incubation temperature of the complex was as follows: Lane 1, 45°C; lane 2, 50°C; lane 3, 60°C; lane 4, 70°C; and lane 5, 95°C. The appearance of this 45 kDa band seems to diminish as the temperature is increased above 50 degrees Celsius. Figure 3.13 illustrates the densitometric analysis of the BPPI/trypsin complex from 4° to 95°C in the presence of 2% (w/v) SDS. The inhibitor/protease complex appeared stable to denaturation up to approximately 50°C when incubated with 2% (w/v) SDS.

One and Two-Dimensional 1H-NMR Spectroscopy of BPPI

A 1 mM BPPI sample was prepared in a 90:10 (v:v) ratio of H₂O:D₂O at pH 7.1, with DSS added to a final concentration of 20 μM. Figure 3.14 shows the 1-dimensional experiment, collected with 512 transients using the presaturation method of solvent suppression. The spectrum appears to have good line shape and resonance intensity, indicating the sample was suitable for additional NMR studies. The same sample was then used for the subsequent two-dimensional experiments.

The TOCSY (Braunschweiger and Ernst, 1983) and NOESY (Anil-Kumar et al., 1980) spectra are shown in figures 3.15 and 3.16, respectively. In both experiments, the spectra were collected with 256 time increments and a total of 64 transients were averaged for each t₁ time increment. The presaturation method of solvent suppression was employed for acquisition of the TOCSY spectrum, whereas saturation was accomplished by adding an inversion-recovery block before the NOESY pulse sequence. The mixing time for the NOESY experiment was 200 milliseconds. The fingerprint region for both spectra are
Figure 3.13: Stability study of the BPPI/trypsin complex. A densitometric analysis of the 45 kDa band of BPPI/trypsin solutions in the presence of 2% (w/v) SDS. Solutions containing 60 μg trypsin, from bovine pancreas, and 25 μg of BPPI were incubated for 10 minutes. Subsequently, an SDS solution was added to give a final concentration of 2% (w/v) SDS. The solutions were then incubated for 10 minutes at 20°, 40°, 60°, 80°, or 95° C. The relative intensities of the 45 kDa band were determined after correction for any background staining. A noted decrease is the intensity of the 45 kDa band occurs under incubation conditions above 60° C.
Figure 3.14: $^1$H-NMR spectrum of BPPI. A one-dimensional spectrum of BPPI was obtained to determine the quality of the sample. The sample (1 mM) was prepared in a 90:10 (v:v) ratio of $\text{H}_2\text{O}$-$\text{D}_2\text{O}$ at pH 7.1 with 20$\mu$M DSS added as an internal standard. A total of 512 transients were collected using the presaturation method of solvent suppression. The resonance intensities were strong and the peak shapes were sharp indicating the sample was suitable for further experiments.
Figure 3.15: 2-dimensional $^1$H-TOCSY spectrum of BPPI. The fingerprint region of a TOCSY spectrum of a 1 mM BPPI solution in a 90:10 (v:v) ratio of H$_2$O:D$_2$O. The spectrum was collected with 256 time increments and a total of 64 transients were averaged for each $t_1$ increment, using the presaturation method of solvent suppression. The presence of cross peaks in this region represent the spin patterns for the NH-CαH coupling for the individual amino acids.
Figure 3.16: 2-dimensional $^1$H-NOESY spectrum of BPPI. The fingerprint region of a NOESY spectrum of a 1 mM BPPI solution in 90:10 (v:v) ratio of H$_2$O:D$_2$O. The spectrum was collected with 256 time increments and a total of 64 transients were averaged for each $t_1$ increment with mixing time of 200 milliseconds. The presence of cross-peaks in this region indicate sequence-specific coupling between the $\alpha$H-NH resonances of adjacent residues.
shown. The TOCSY spectrum reveals the spin-systems for the individual amino acids and the NOESY spectrum gives potential sequence-specific information.

**Three-dimensional Structural Determination of BPPI using Homology**

The primary sequence of BPPI which contains 78 amino acids (Li, 1992) was derived from the sequence file generated with vi editor. This sequence was aligned with the primary sequence of the 58 residue BPTI molecule, which was extracted from the x-ray structure file 4PTI (Walter and Huber, 1983), obtained from the Protein Data Bank at Brookhaven National Laboratory (Bernstein *et al.*, 1977) to give maximum consensus, using the Homology program. The atomic coordinates for BPPI were generated from the atomic coordinates of BPTI. Figure 3.17 illustrates the homodimeric structure of BPPI which was created within InsightII and subjected to energy minimization.

**Circular Dichroism Spectroscopy of BPPI**

The CD spectrum (figure 3.18) was of a 117 μM BPPI sample, prepared in 0.1 mM potassium phosphate at pH 7.7, and was recorded from 250 to 185 nm using 65 data points at a scan rate of 2 nm/min. A similar spectrum was obtained of a 142 μM BPTI solution to use as a reference. All protein spectra were baseline corrected against the buffer. The relative amounts of secondary structural elements were calculated using the method of Chang *et al.*, 1978, and indicated that BPPI contains approximately: 15% α-helix, 27% β-sheet, 43% random coil, and 15% β-turn.
Figure 3.17: A three-dimensional model of a BPPI dimer. A BPPI homodimer was created within *InsightII* by forming a disulfide bond between the Cys-4 residues of two monomeric BPPI molecules. The dimeric structure was energy minimized with the *Discover* program.
Figure 3.18: CD Spectroscopy of BPPI. The CD spectrum of a 117 μM BPPI sample, prepared in 0.1 mM K₂HPO₄ at pH 7.7, was recorded from 250 - 185 nm using 65 data points at a scan rate of 2 nm/min. The CD spectrum of a 142 μM BPTI sample was also obtained under identical conditions. All protein spectra were baseline corrected. The relative percentages of secondary structural elements were determined by the method of Chang et al., 1978, indicating BPPI contains 15% α-helix, 27% β-sheet, 43% random coil, and 15% β-turn.
Isolation of Human Platelet Plasmin Inhibitor

Trypsin-agarose chromatography (Kassell et al., 1970) was used to bind any trypsin inhibitors that were present in the unbound fraction from heparin-agarose chromatography. The unbound heparin-agarose fraction was applied to the column at 2.5 mL/min in buffer and rinsed with buffer A consisting of 15 mM Tris, 0.15 M NaCl, 2 mM CaCl₂, and 0.02% (w/v) NaN₃, at pH 7.6 to establish a baseline. The bound proteins were eluted with water (fractions 1 - 14) followed by 10 mM HCl (fractions 16 - 26) as shown in figure 3.19. The protein solution was then batch processed with CM-Sephadex overnight. The proteins were eluted with 15 mM Tris/1.0 M NaCl at pH 7.6 and a typical chromatogram is illustrated in figure 3.20.

The purity of the product obtained from the above chromatographic procedures was examined by SDS-PAGE. Lane 2 in figure 3.21 illustrates that the isolated fraction contains a single protein band with a molecular weight of approximately 12,000 Daltons using the seven standards (BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.2 kDa) in lane 1 as reference. Lane 2 contains the proteins contained within the unbound fraction from CM-Sephadex chromatography which had a molecular weight of approximately 21,000 Da. Typically, 50 µg of protein is obtained from approximately 2 L of outdated platelets.

Trypsin Inhibition Assay of HPPI

The ability of HPPI to inhibit the serine protease trypsin was examined using BAPNA
Figure 3.19: Trypsin-agarose chromatography of the unbound heparin-agarose fraction from human platelets. The unbound heparin-agarose fraction was applied to the trypsin-affinity column at 2.5 mL/min. The column was rinsed with buffer A consisting of 15 mM Tris, 0.15 M NaCl, 2 mM CaCl$_2$, and 0.02% (w/v) NaN$_3$, at pH 7.6 to establish a baseline. The proteins were eluted with water, followed by 10 mM HCl and the absorbance was monitored at 280 nm.
Figure 3.20: CM-Sephadex chromatography of trypsin-agarose bound fraction. The bound fractions from the trypsin-affinity column was batch processed overnight with 3 g of CM-Sephadex. The column was washed with buffer A consisting of 15 mM Tris, 0.15 M NaCl, 2 mM CaCl₂, and 0.02% (w/v) NaN₃, at pH 7.6 to establish a baseline and the bound proteins eluted with 15 mM Tris/1.0 M NaCl, at pH 7.6. The eluted proteins were detected at 280 nm.
Figure 3.21: 17% (w/v) SDS-PAGE gel of HPPI. The molecular weight and purity of the fraction isolated from CM-Sephadex chromatography was verified by SDS-PAGE. Lane 2 contains a single protein band with a molecular weight of approximately 12,000 Da. Lane 1 contains the molecular weight standards consisting of: BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.2 kDa. Lane 3 contains the unbound CM-Sephadex fraction which appears to contain a single protein with a molecular weight of approximately 20,000 Da.
as a substrate (Fioretti *et al.*, 1983) following a ten minute incubation of the protease and inhibitor at a ratio of 4:3 (w/w). Figure 3.22 illustrates the activity of trypsin without HPPI present (filled squares) and in the presence of HPPI (open squares). The overall ability of HPPI to inhibit trypsin hydrolysis of BAPNA was approximately 77%, which was calculated as the ratio of the final absorbances, after ten minutes, for the inhibition and the control experiment.

**Plasmin Inhibition Assay of HPPI**

The ability of HPPI to inhibit human plasmin was examined using BAPNA as a substrate following a thirty minute incubation of the protease and inhibitor at a ratio of 3:2 (w/w). Figure 3.23 illustrates the activity of plasmin without HPPI present (open squares) and in the presence of HPPI (filled squares). The HPPI solution was able to completely inhibit plasmin hydrolysis of BAPNA.

**Purity of b-PF4**

The protein solution eluted from the heparin-agarose column with buffer consisting of 15 mM Tris, 1.5 M NaCl, 2 mM CaCl₂, and 0.02% (w/v) NaN₃, at pH 7.6, was concentrated by ultrafiltration and applied to a Sephadex G-75 column. The fraction eluting with a molecular weight of 45,000 Da was desalted and lyophilized. The purity of the solid product was verified by SDS-PAGE. As indicated in figure 3.24, there is a single band with a molecular weight of approximately 14,000 Da.
Figure 3.22: Trypsin inhibition assay of HPPI. Trypsin, from bovine pancreas, hydrolysis of BAPNA was measured in the absence of HPPI (filled squares) and in the presence of HPPI (open squares). The protease/inhibitor solution was incubated for 10 minutes at room temperature. The absorbance was recorded for 180 seconds at 405 nm.
Figure 3.23: Plasmin inhibition assay of HPPI. Plasmin hydrolysis of BAPNA was measured in the absence of HPPI (open squares) and in the presence of HPPI (closed squares). The protease/inhibitor solution was allowed to react for 30 minutes at room temperature. The absorbance was recorded for 300 seconds at 405 nm.
Figure 3.24: 17% (w/v) SDS-PAGE gel of b-PF4. The purity of the fraction eluted from the Sephadex G-75 column with a molecular weight of approximately 45,000 Da was determined with SDS-PAGE. The sample was prepared for electrophoresis by dialysis and lyophilization. Lane 2 contains the b-PF4 sample which had a molecular weight of approximately 14,000 Da under the reducing conditions of the experiment. The molecular weight markers, shown in lane 1, consisted of: BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.2 kDa.
Peptide Mapping of Digestion Products

Peptide mapping of the enzymatically digested b-PF4 was accomplished by reversed-phase HPLC with a Delta-Pak C18 column and is shown in figure 3.25. Two predominant peaks on the chromatogram correspond to V1, which eluted at 30 minutes, and to V2, which eluted at 50 minutes. The eluted peptides were detected at a wavelength of 214 nm and the flow rate was maintained at 1.0 mL/min.

Affinity Chromatography of V2

A comparison of the heparin-binding ability of V2 and the parent protein (b-PF4) is illustrated in figure 3.26. The protein solutions were applied to a Bio-Rad Econo Pac Heparin Cartridge equilibrated with 10 mM potassium phosphate, at pH 7.5. The flow rate was maintained at 0.4 mL/min and the absorbance detector set at 254 nm. Bovine PF4 eluted at a salt concentration of 1.26 M sodium chloride, whereas V2 was eluted at 0.76 M sodium chloride.

Oligomeric Determination of V2

Gel filtration chromatography was employed with a Superose-12 column equilibrated with 10 mM potassium phosphate/0.2 M NaCl, at pH 7.5, to determine the molecular weight of the V2 fragment in solution. The flow rate of the column was maintained at 0.75 mL/min and monitored at 254 nm. Figure 3.27 shows the elution time of V2 relative to the molecular weight standards used to calibrate the column. The V2 fragment had an elution time of 35.4 minutes, which corresponds to a molecular weight of approximately
Figure 3.25: Peptide mapping of digestion products. Bovine PF4 was enzymatically digested with *Staphylococcus aureus* V8 protease in 50 mM sodium acetate at pH 4.0 for 24 hours at 37° C. The peptides were lyophilized and resuspended in 0.05% (v/v) TFA and chromatographed on a Delta-Pak C18 column. The peptides were eluted with increasing acetonitrile concentration and the absorbance monitored at 214 nm. The two major fractions at 30 minutes and 50 minutes correspond to V1 and V2, respectively.
Figure 3.26: Affinity chromatography of V2. The heparin affinity of both V2 and b-PF4 was examined with a Bio-Rad Econo Pac Heparin Affinity Cartridge attached to an HPLC. Elutions were carried out with a linear gradient from 0.15 M to 1.5 M NaCl in 10 mM potassium phosphate at pH 7.5. The V2 peptide eluted at a salt concentration of 0.76 M (A), and b-PF4 eluted at a concentration of 1.26 M NaCl (B).
Figure 3.27: Oligomeric determination of V2. The molecular weight of the V2 fragment was determined by gel filtration using a Superose-12 column calibrated with BSA (I), 66,000 Da; carbonic anhydrase (II), 29,000 Da; cytochrome c (III), 12,400 Da; and aprotinin (IV), 6,500 Da. The elution time of V2, at 35.4 minutes, is denoted by the symbol X.
9,200 Da.

**CD Spectroscopy of V2**

The CD spectrum is shown in figure 3.28. A 32.5 μM V2 sample was prepared in 1 mM phosphate at pH 6.5. The mean residue ellipticity of the V2 fragment at 222 nm was -5,798 deg.cm²/dmol and the α-helical content was calculated to be 23%, based on the observed mean residue ellipticity of 3,900 deg.cm²/dmol for a random coil (Greenfield and Fasman, 1969) and -38,000 deg.cm²/dmol for an α-helix (Toniolo et al., 1979). The final spectrum of V2 was an average of 3 scans and recorded from 250 to 196 nm using a time integral of 3 seconds per data point.

**NMR Spectroscopy of V2**

A DQF-COSY experiment was performed on a 1 mM V2 solution prepared at a 90:10 ratio (v:v) of H₂O:D₂O in 20 mM sodium phosphate/0.15 M NaCl at pH 6.5, containing 20 μM DSS as an internal standard. The experiment was performed with 32 time increments in the t₁ domain each consisting of 32 transients and the presaturation method of solvent suppression was employed. Figure 3.29 illustrates the upfield region of a COSY spectrum showing the cross-peaks for valine, leucine, and isoleucine residues.
Figure 3.28: CD spectroscopy of V2. The circular dichroism spectrum of the V2 fragment was obtained from 250 - 196 nm with a time integral of 3 seconds per point. The peptide was prepared at a concentration of 32.5 μM in 1 mM sodium phosphate/0.15 M NaCl at pH 6.5. The mean residue ellipticity at 222 nm was determined to be -5,798 deg.cm²/dmol.
Figure 3.29: DQF-COSY Spectrum of V2. The $^1$H-NMR spectrum of a 1 mM V2 sample, prepared in 20 mM sodium phosphate/0.15 M NaCl, at pH 6.5, with DSS added at a final concentration of 20 µM. There were 32 time increments collected in the $t_1$ domain each consisting of 32 transients.
Discussion

Isolation of BPPI

The proper handling of the bovine blood during collection and processing was an important factor in obtaining an adequate yield and a pure product. Several key steps were closely monitored to maintain the integrity of blood. One requirement involved the quick transfer of the blood from the collection pans to the jugs containing anticoagulant. Any indication of clotting before transfer required the sample to be discarded. Secondly, care was taken to avoid contamination of the pooled platelets with erythrocytes. Finally, chemical activation with calcium ionophore A-23187 was preferred, over the freeze/thaw technique, as the method of platelet activation. The ionophore requires a very short period of time to cause platelet aggregation, in comparison to the three cycles of freezing the sample in a methanol/dry ice bath and then heating in a 38°C water bath. The extent to which the platelets were activated was also increased with the ionophore and generally resulted in a highly clarified platelet releasate solution following centrifugation.

A preliminary affinity chromatography technique was used to separate the heparin-binding proteins from the proteins without heparin affinity, such as BPPI, followed by cation exchange chromatography. The ion exchange elution profile, shown in figure 3.1 appears quite asymmetrical. The very large shoulder the chromatogram possesses is not an indication of an impure sample. The asymmetry is caused by the compression of the ion exchange media upon the step elution with a high salt concentration. Other groups have reported the efficiency of isolating aprotinin-like serine protease inhibitors can be
improved by using a Mono S strong cation exchange column designed for FPLC systems, which have large capacities and allow rapid purification (Stallings-Mann et al., 1994).

**SDS-PAGE of BPPI**

Gel electrophoresis on a 17% polyacrylamide gel provided a rapid and efficient method to obtain a molecular weight estimation of the fraction obtained from the CM-Sephadex column and to verify the purity of the isolated fraction. Lane 3 of figure 3.2 shows a single band with a molecular weight corresponding to approximately 12,000 Da. This value appears inflated when compared to the molecular weight of 8,600 Da as determined from the primary sequence. However, control experiments with BPTI, which has a molecular weight of only 6,500 Da, also exhibited a molecular weight that was larger than expected, but anomalous migrations often occur with proteins less than 14,000 Da. The appearance of the band, believed to be BPPI was also quite broad, which may be a consequence of a possible glycosylation site. It has been reported that similar inhibitors isolated from bovine spleen contain a carbohydrate moiety (Fioretti et al., 1983). No consensus sequence exists for the presence of N-linked glycosylation. However, there are three serine and five threonine residues to which O-linked carbohydrates may attach. Lanes 1 and 2 in figure 3.2 contain protein samples from the unbound heparin agarose fraction.

**Isoelectric Focusing of BPPI**

Isoelectric point (pI) determinations were performed on a horizontal capillary gel
system. Comparisons of the primary sequences of BPPI and BPTI would indicate that BPPI and BPTI would have very similar isoelectric points. The experimentally determined isoelectric point of BPPI shown in figure 3.3, using ampholytes in the range of pH 3 - pH 10, was 10.4, using the regression line calculated from the protein standards. This value corresponds very closely to the value for BPTI, which has a pI of 10.5 (Chauvet et al., 1964). An experiment using ampholytes in the range of pH 8 - pH 10, was performed to distinguish better the isoelectric points of the BPPI and BPTI. Cytochrome c and BPTI were used as the standards which have pI values of 9.6 and 10.5, respectively. The experiment more definitively indicated that BPPI does have a lower pI than BPTI. The pI determined in this experiment was approximately 10.2, as shown in figure 3.4. Comparisons of the amino acid sequences of the two proteins would support this experimental observation. Overall, the net charge on BPPI is +3, whereas the net charge of BPTI is +5 which would account for its decreased isoelectric point relative to BPTI.

Gel Filtration of BPPI

The molecular weight of BPPI is 8,580 Da by amino acid sequencing (Li, 1992). The quaternary structure of BPPI was investigated by gel permeation chromatography. Figure 3.5 illustrates that BPPI had an elution time that was greater than the times observed for both aprotinin (6,500 Da) and cytochrome c (12,400 Da). The calculated molecular weight of 19,000 Da, determined from the regression line, indicates that, under the conditions employed in the experiment, BPPI is most likely a dimer in solution. Kinetic
experiments performed on BPPI would support this observation. It was found that BPPI could inhibit plasmin or trypsin at a stoichiometry of 1.8:1 on a molar basis (Walz et al., manuscript in preparation). This ratio would indicate that each molecule of BPPI has two inhibitory sites, implying the quaternary structure of the molecule is that of a dimer.

**Solubilization Studies of BPPI**

The solubility of BPPI was examined under many different conditions. Attempts to increase the solubility involved varying the pH from 4 to 9, altering the buffer strength and the buffers themselves, increasing the ionic strength, and decreasing the polarity of the solutions used to resuspend the solid BPPI samples. The results from the pH range study are shown in figure 3.6 and were typical of all solubility studies. In all cases, the protein concentration was near or below 0.5 mg/mL, but was least soluble in sodium acetate at pH 5.5. The relative insolubility of BPPI in solution was quite unexpected, since BPTI itself is soluble (Green and Work, 1953) in water, 70% methanol, 70% ethanol, and 50% acetone. In addition, stable solutions of BPTI have been prepared, at concentrations as high as 20 mM in unbuffered solutions, that were used in pioneering two-dimensional NMR experiments (Wagner et al., 1987).

**Reversed-phase HPLC of BPPI**

The lack of solubility of BPPI prompted an examination of the protocol used to purify the protein. The initial purification steps of centrifugation, heparin-agarose chromatography and CM-Sephadex chromatography were performed under near
physiological pH and ionic strength conditions. If, in fact, BPPI did have similar solubility characteristics to BPTI, it seemed probable that the difficulty in obtaining concentrated samples was a result of the final step in the purification involving desalting and concentrating the protein sample. Initially, the standard protein techniques of dialysis and ultrafiltration were used to desalt the samples, which were then concentrated by lyophilizing them. However, the amount of protein recovered after this procedure was less than 0.1 mg. Alternatively, the protein could be concentrated using a reversed-phase Sep-Pak C18 cartridge with sample elution under conditions of 60% (v/v) acetonitrile (Walz, personal communication). This technique worked very well to desalt, however resuspended solutions were quite insoluble as reported above. Under the pretense that these elution conditions were too harsh, a reversed-phase HPLC experiment was performed to determine the exact concentration of acetonitrile required to elute BPPI from a C18 column. The procedure used a Delta-Pak C18 (Waters Associates) which contains the same chromatographic media contained within the Sep-Pak cartridge.

The BPPI solutions applied to the column were eluted under conditions of increasing acetonitrile concentration using a linear gradient. Interestingly, it was found that BPPI eluted from the reversed-phase media at an acetonitrile concentration of 38% (v/v), as shown in figure 3.7. This technique gave further evidence relating to the purity of the sample. The protein eluted as a single peak at approximately 18 minutes. The initial peak at 3 minutes was due to impurities in the buffer and also appeared in the blank injections.
Supplementary Solubility Studies

Subsequent purifications were performed exactly as described previously, with the exception of the final step in the protocol. The protein was desalted and concentrated on a Sep-Pak C18 cartridge, however only the bound protein was eluted with 38% (v/v) acetonitrile, and then lyophilized. The lyophilized product was then resuspended in a small volume of buffer consisting of 15 mM Tris, 0.15 M NaCl, 2 mM CaCl₂, and 0.02% (w/v) NaN₃, at pH 7.6. The initial appearance of the solution was a uniform cloudy mixture. The pH of the solution was increased to pH 8.5 with the addition of dilute sodium hydroxide which successfully clarified the solution. The protein concentration was determined by the Bradford protein dye-binding assay as before. The standard curve, based on increasing concentrations of BSA as shown in figure 3.8, was used to calculate the protein concentration. The absorbance of the BPPI solution (2μg/μL) was 0.399 at 620 nm. The regression line was used to calculate a protein concentration of approximately 12 mg/mL for the BPPI solution. This measurement is an estimate because the concentration of the solution did not fall within the linear region of the standard curve and no dilution was performed. The concentration determination of 12 mg/mL is, in all probability, a conservative one given the diminished absorbance at higher protein concentrations.

Amino Acid Analysis of BPPI

Gel electrophoresis is a rapid and efficient method to determine initially the purity of a protein sample. However, a more definitive determination of the identity of the
protein was performed with amino acid analysis. Recent methods have allowed the
determination of 17 of the 20 amino acids, in a relatively short period, using pre-column
derivatization of the hydrolyzed amino acids. Figure 3.9 is a chromatogram of a
hydrolyzed and derivatized BPPI sample. The area of each peak was integrated and the
amount of each amino acid was quantified using the response factors for the amino acid
standards. Reversed-phase HPLC of the derivatized amino acids is extremely sensitive,
and requires as little as 250 pmol of protein (Bidlingmeyer et al., 1984). Table 3.1
compares the calculated number of each amino acid to the theoretical value based on the
amino acid sequence (Li, 1992). The correlation is, in general, quite good, however,
some values do appear higher or lower than expected. Errors in the molar quantification
of some residues, such as Asx and Glx, often occur because of the difficulty in baseline
resolving these peaks. Free cysteine residues are usually degraded by acid hydrolysis and
therefore cannot be accurately quantified. Interestingly, the intramolecular disulfide bonds
(cystine residues) present within the BPPI molecule are stable to acid hydrolysis and are
quantified quite accurately. The amino acid analysis of BPTI, performed as a control,
also demonstrated the stability of the disulfides, indicating the presence of six cysteine
residues.

Trypsin Inhibition Assay of BPPI

Evidence from previous experiments indicated that the protein isolated from bovine
platelets was, in fact, BPPI. However, the protein may have lost its bioactivity during the
purification. Trypsin hydrolysis, in the presence of BPPI, of the substrate BAPNA was
used as assay for the activity of the inhibitor (Fioretti et al., 1983). A solution of trypsin, from bovine pancreas, and BPPI at a ratio of 1:6.5 (w:w) was incubated for five minutes and then the solution was assayed for trypsin activity. Figure 3.10 compares the trypsin hydrolysis of BAPNA in the presence of BPPI (open squares) and without BPPI (filled squares). It was quite clear that BPPI possessed inhibitory activity toward trypsin, and by inference to plasmin as well. BPPI (bovine platelet plasmin inhibitor) was named based on kinetic evidence indicating that, although it exhibited inhibitory activity toward trypsin, kallikrein, and factor XIII, it had the greatest specificity toward the enzyme plasmin (Walz et al., in preparation). Similar studies with recombinant aprotinin have demonstrated potent inhibitory activity toward a number of serine proteases including α-chymotrypsin, plasmin, kallikrein, and trypsin (Auerswald et al., 1988). For practical reasons, such as economy and overall enzyme stability, trypsin was the enzyme of choice to determine the inhibitory activity of BPPI.

**Stability Studies of the BPPI/Trypsin Complex**

Solutions of BPPI (MW = 18,000 Da) and trypsin, from bovine pancreas, (MW = 24,000 Da) were pre-incubated for 10 minutes at room temperature. A stock solution of SDS was then added to give a final concentration of 0.5% (w/v) SDS. These solutions were then incubated from 4°C to 95°C for 10 minutes and then electrophoresed on a 17% non-reducing acrylamide gel. Figure 3.11 illustrates that in the temperature range from 4°C to 45°C (lanes 1 to 5), a 45 kDa band was formed, which approximately corresponds to the molecular weight of the BPPI/trypsin complex. Lane 7 contained only
trypsin and the presence of several bands is probably a result of the autodigestive properties of trypsin. Lane 8 contained only BPPI, which was not completely purified. An advantage of the SDS-PAGE technique to study complex formation is that the samples do not have to be in highly purified forms. The formation of the 45 kDa band appears quite uniform from 25° to 45° C (lanes 2 - 5). However, this band does not occur as intensely when incubated at 4° C, which is probably a result of the decreased activity of trypsin at this temperature. A continuation of the stability study is illustrated in figure 3.12, which shows the temperature range of 45° to 95° C (lanes 1 - 5). The 45 kDa band began to disappear as the incubation temperature was increased above 50° C. However, a very faint band at 45 kDa is present even when incubated at temperatures as high as 95° C. As in the previous figure, lane 7 contained only trypsin and lane 8 contained only BPPI. It is evident from these control lanes that separately, the band at 45 kDa does not appear, however, upon incubation of both proteins together, the 45 kDa band appears.

Similarly, solutions of trypsin and BPPI were pre-incubated for 10 minutes, followed by the addition of an SDS stock solution to a final concentration of 2% (w/v) SDS. The solutions were then heated over a temperature range from 20° to 95° C for 10 minutes. Figure 3.13 depicts the intensities of the bands corresponding to a molecular weight of approximately 45 kDa based on the regression line of the standards as a function of temperature. The term relative volume represents the volume corrected for any background staining. Other investigators studied the complex formation of the trypsin/soybean trypsin inhibitor complex by SDS-PAGE, which indicated that trypsin was able to form a stable complex with the inhibitor even in the presence of SDS if it is mixed
with Bowman-Birk trypsin inhibitor or soybean trypsin inhibitor before the addition of SDS (Arakawa et al., 1992). They noticed when the mixture was heated that the complex band disappeared in a temperature-dependent manner. These experiments enabled the determination of the melting temperatures for the complex, which is a indicator of its stability. The melting temperature of the trypsin/soybean trypsin inhibitor complex was approximately 35°C, whereas the melting temperature of the trypsin/Bowman-Birk inhibitor complex had a higher melting temperature of approximately 41°C. It appears that the melting temperature of the trypsin/BPPI complex is about 50°C which is higher than that of the trypsin/inhibitor complexes studied previously. Stability in the presence of SDS is indicative of complex stability, since this detergent is routinely used in electrophoresis to bind to proteins, to disrupt their structure and shape, and to dissociate them into polypeptide chains. Resistance to SDS denaturation is therefore an indication of a very stable enzyme/inhibitor complex. The original conformations of the proteins are typically disrupted by SDS, however the nature of the conformation they adopt in SDS is not known (Tanford et al., 1974).

**One and Two-Dimensional ³H-NMR Spectroscopy of BPPI**

A 1 mM BPPI solution was prepared in 90:10 (v:v) ratio of water:deuterium oxide with DSS added to a final concentration of 2 μM, as an internal standard. A preliminary one-dimensional proton spectrum was obtained to determine the quality of the sample as shown in figure 3.14. The spectral line shapes and intensities were characteristic of a protein spectrum possessing secondary structural elements. The line widths can be used
as an indication of the aggregation state of a protein sample. The generally good line
widths, obtained in the spectrum of BPPI, would suggest the protein was not aggregating
under the experimental conditions used to collect the spectrum. (Wüthrich, 1986). The
solvent resonance, which is the large peak at approximately 5 ppm, was suppressed using
the method of presaturation. This resonance may seem uncharacteristically large,
however, given that the spectrum was recorded in 90% water the intensity does not seem
unreasonable and is not an indication of poor solvent suppression.

Two-dimensional TOSCY and NOESY experiments were performed to give further
insight into the secondary structural elements of the BPPI in solution. The finger-print
region of the TOSCY spectrum, shown in figure 3.15, represents the through-bond
connectivities of the NH and the αH protons. Similarly, the finger-print region of the
NOESY spectrum is shown in figure 3.16, that gives, predominantly, the through-space
connectivities of the αH-NH protons between sequentially neighbouring residues.

The two-dimensional experiments provide the basis for the determination of the
solution structure of BPPI. The spectra are more complicated than the spectra obtained
of BPTI (Wagner et al., 1987) however, these experiments indicate that full analysis is
possible leading to a complete structure determination. Spectral differences between the
two isoforms are predominanly due to changes in the conditions of spectral acquisition
and the dimeric nature of BPPI. Two dimensional data sets may typically require one to
two years to complete the resonance assignments and three-dimensional structure
determination.
Three-dimensional Structural Determination of BPPI using Homology

Protein structure prediction of BPPI, using the amino acid sequence of the protein (Li, 1992), was based on homology modelling. This approach uses a known protein structure, determined either by x-ray crystallography or NMR spectroscopy, as the template to which the unknown protein is modelled. There are only six amino acid substitutions in the sequence aligned primary sequences of BPPI and BPTI. The excellent sequence homology between BPTI, the structure of which has been determined, and BPPI makes modelling by homology an accurate method of structure prediction. Figure 3.17 illustrates the homodimeric structure of BPPI generated by comparative modelling.

The homology modelling was based on certain structural information obtained from biochemical analysis that was related to the oligomeric structure of BPPI. Gel filtration studies and kinetic analysis indicated that BPPI was a dimer in solution and that the dimerization occurred by the formation of a disulfide bond between the fourth cysteiny1 residue of each monomer (Li, 1992). In addition, reversed-polarity native gel electrophoresis (Reisfeld et al., 1962) was performed on BPPI samples incubated in iodoacetate (Creighton, 1980) showing no change in migration between treated and untreated samples (data not shown). The model predicts that the N- and C-terminus extensions of BPPI have a random coil structure. The α-helical and two-stranded antiparallel β-sheet appear to be maintained in the BPPI structure. The structure was extensively energy minimized using the Discover program (Biosym Technologies) to ensure any unfavourable side-chain conformations were removed.
Circular Dichroism Spectroscopy of BPPI

The secondary structural elements of BPPI were estimated using circular dichroism (CD) spectroscopy in the region from 250 to 185 nm. A reference protein spectrum of BPTI was also obtained under similar conditions. The x-ray crystallographic data (Walter and Huber, 1983) indicated that BPTI contained approximately 17% α-helix, 33% β-sheet, 36% random coil, and 14% β-turn, and these values were comparable to the measurements obtained by CD. The calculations based on the BPPI spectrum, which was similar to the spectrum obtained for BPTI, indicated secondary structural elements consisting of 15% α-helix, 27% β-sheet, 43% random coil, and 15% β-turn.

Molecular modelling studies of BPPI indicate that the amino terminus and carboxyl terminus extension do not possess any stable structural elements. This is supported by the CD data which indicated that the content of α-helix and β-sheet present in BPPI is similar to that of BPTI. The various amino acid substitutions in BPPI, are generally all conserved, and seem to suggest that these alterations in amino acids have not changed to the secondary structure of the protein to any significance, with reference to BPTI.

Isolation of Human Platelet Plasmin Inhibitor

The experimental procedure used to isolate human platelets was adapted from that used to purify bovine platelets (Ciaglowski et al., 1986). In contrast to the procedure used to purify BPPI, the unbound heparin-agarose fraction was then applied to a trypsin-agarose column to isolate inhibitory proteins within the solution, as shown in figure 3.19. The proteins bound to the trypsin-agarose column were eluted and then batch processed.
with CM-Sephadex (figure 3.20) which facilitated the isolation of a low molecular weight band (MW = 12,000 Da) visible by SDS-PAGE stained with Cal-Red as illustrated in lane 2 of figure 3.21. The approximate yield for this protein purification is about 50 μg. The age of the platelets, generally 10 - 12 day out-dated platelets that are no longer suitable for transfusion, may cause a significant loss in the amount of protein isolated in the purification.

Trypsin Inhibition Assay of HPPI

The low molecular weight fraction isolated by trypsin-agarose and CM-Sephadex chromatography was assayed for its ability to inhibit the the serine protease trypsin, from bovine pancreas, using BAPNA as a substrate. The enzyme and potential inhibitor were incubated at a ratio of 4:3 (w:w) for 10 minutes. Figure 3.22 shows that trypsin hydrolysis of the substrate was definitely inhibited by the bound CM-Sephadex fraction. The inhibition was calculated at approximately 77%, which represents the quotient of the absorbance of the chromogenic substrate following the 10 minute reaction with that of control experiment containing no inhibitor (filled squares). The determination of kinetic parameters was not performed given the low yield of protein.

Plasmin Inhibition Assay of HPPI

The observation that the protein present in the bound CM-Sephadex fraction could inhibit trypsin prompted a similar inhibition assay with plasmin. The ability of HPPI to inhibit this enzyme was also examined using BAPNA as a substrate. However, the
enzyme and inhibitor were incubated for 30 minutes at a ratio of 3:2 (w:w). The inhibition is represented in figure 3.23 (filled squares), and it appears to be complete. The control experiment performed without the inhibitor present is shown as the open squares. Interestingly, despite the slight excess enzyme present in solution, total inhibition was observed, that is in contrast to the assay performed with trypsin. This may indicate that the inhibitor found in the human species has a greater affinity for plasmin.

**Purification of b-PF4**

The purity of b-PF4 was determined by 17% SDS-PAGE as shown in lane 2 of figure 3.24. The gel indicated a single low molecular weight protein (MW = 14,000 Da) was present in this fraction. This molecular weight was larger than the expected 9,500 Da, which is the molecular weight per subunit based on the amino acid sequence. However, anomalous migrations are often observed with low molecular weight proteins. In addition, PF-4 has a glycosylation site located at the N-terminus of the protein (St. Charles et al., 1989) which would increase the apparent molecular weight observed by SDS-PAGE and does not indicate that a protein other than b-PF4 was erroneously purified in this case.

**Peptide Mapping of Digestion Products**

The amino acid sequence of b-PF4 has been determined by Ciaglowski et al., 1986. The lack of glutamic acid residues near the carboxyl terminus makes enzymatic digestion with *Staphylococcus aureus* V8 protase an efficient method to clip the protein into the two equally sized peptides, V1 and V2, through the predominant cleavage at the

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peptide bond between Glu-43 and Val-44 at pH 4.0 (Talpas et al., 1991).

The separation and identification of each of the observed peaks, termed V1 and V2, were performed by peptide mapping and amino acid analysis, respectively. Peptide mapping by reversed-phase HPLC of the enzymatic digest of b-PF4 by Staphylococcus aureus V8 protease is shown in figure 3.25. The two predominant peaks on the chromatogram correspond to V1, eluted at 30 minutes, and V2, eluted at 50 minutes. The results of the amino acid analysis (data not shown) of the V2 fragment indicated an excellent correlation between the expected number of amino acids in the V2 peptide, based on the amino acids found in residues 44 to 88, with those amino acids observed in peak V2.

**Affinity Chromatography of V2**

A comparison of the heparin binding ability of both the V2 fragment and b-PF4 is illustrated in figure 3.26. Bovine PF4 eluted at a salt concentration of 1.26 M, whereas the V2 fragment eluted at 0.76 M NaCl. Bovine PF4 binds heparin with a high degree of affinity ($K_d = 10^{7.5}$) (Loscalzo et al., 1985) and is generally eluted from heparin-agarose media with a salt concentration ranging from 1.2 M to 1.5 M NaCl (Holt et al., 1986), which is in agreement with the elution concentration of 1.26 M NaCl found in this study. Although the salt concentration required to elute the V2 fragment from the heparin affinity column is less than that required by the parent protein, V2 still possesses significant heparin binding capability that is comparable to that observed of the homologous proteins. Connective tissue activating peptide III (CTAP-III) was found to
be displaced from heparin-agarose media by 0.3 M NaCl (Castor et al., 1989), whereas
the platelet-specific proteins β-thromboglobulin (β-TG) and low affinity platelet factor
four (LA-PF4) eluted from heparin-agarose at 0.5 M NaCl (Moore et al., 1979; Rucinski
et al., 1979). The observed retention of V2 on heparin affinity columns gives strong
support to the proposed lysine binding model that indicates the heparin wraps around a
belt of positive charges formed by the lysine residues located near the carboxy-terminus
of the protein (Stuckey et al., 1992). The possibility of conformationally independent
non-specific electrostatic interactions with heparin was tested with the basic protein horse
heart cytochrome c (pI = 9.6), which did not bind at the 0.15 M NaCl concentration used
in the equilibrium buffer.

Oligomeric Determination of V2

The possible oligomeric structure of the V2 peptide in solution was determined by
a molecular weight determination by gel filtration chromatography. Figure 3.27 shows
the elution time of V2 relative to the molecular weight standards. The linear regression
line for the protein standards, bovine serum albumin, bovine erythrocyte carbonic
anhydrase, horse heart cytochrome c, and bovine lung aprotinin, for the gel filtration
experiment, was used to calculate a molecular weight of 9,200 Da for the V2 fragment,
based on its elution time of 35.4 minutes. The molecular weight of V2 based on its
amino acid sequence is 4,620 Da, and the molecular weight determined experimentally
would suggest that V2 forms a dimeric structure in solution. Biochemical and
crystallographic studies have demonstrated that platelet factor 4-related proteins form

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non-covalently linked multimers, which is a common feature of these proteins increasing the likelihood for oligomeric formation of V2 (Watanable et al., 1989; St. Charles et al., 1989).

The x-ray crystallographic structure of b-PF4 revealed each monomer was stabilized by extensive intramolecular hydrophobic interactions at the helix/sheet interface between Leu-42, Val-44 and Leu-56 in the β-sheet and residues Tyr-75, Leu-79, and Leu-83 in the α-helix. All of these contacts may be present in the V2 fragment to give a stable structure, with the exception of residue Leu-42. The dimerization may be rationalized on the basis of hydrophobic interactions at the helix/sheet interface of the subunit A monomer, that extends to include residues Val-144, Leu-156, Tyr-175, Leu-79, and Leu-83 of the adjacent B subunit monomer. Gel permeation chromatography indicated a possible dimer, and absolutely no indication of tetrameric formation, as seen with b-PF4. The x-ray crystallographic data support this observation as well. The formation of A/B and C/D dimers does not seem likely because of the interface which requires a salt bridge between positions Glu-43, which is absent in the V2 fragment, and residue Lys-65. This salt bridge is conserved in the sequences of all homotetrameric proteins homologous to b-PF4 (St. Charles et al., 1989).

**CD Spectroscopy of V2**

The presence of secondary structural elements was confirmed by circular dichroism spectroscopy of V2 as shown in figure 3.28. The mean residue ellipticity of the V2 fragment at 222 nm was used to calculate an α-helical content of 23%, based on the
observed mean residue ellipticities for both a random coil and \( \alpha \)-helix.

The observed 23\% \( \alpha \)-helical content is in excellent agreement with the 24\% \( \alpha \)-helical content of the corresponding residues of b-PF4 as determined by x-ray crystallographic methods (St. Charles et al., 1989). The \( \alpha \)-helical conformation occurs in the amino series of tyrosine-75 to glycine-85. Assuming these residues adopt the same conformation in V2, the content of \( \alpha \)-helix present is 23.8\%, which supports the CD data calculations.

The presence of \( \alpha \)-helical structure is of great importance to the functional properties of the protein. It has been mentioned previously, that this belt of positive charge is the region responsible for heparin neutralization. In addition, a synthetic peptide composed the 13 residues at the C-terminal end of PF4, which is the \( \alpha \)-helical region, has been found to be an effective antimicrobial agent (Darveau et al., 1992). Other investigators have found that the C-terminal region of platelet factor 4-related proteins have an active role as mediators in the inflammatory response (Stoeckle and Barker, 1990).

**NMR Spectroscopy of V2**

A double quantum filtered COSY spectrum was performed on a 1 mM V2 solution prepared at a 90:10 (v:v) ratio of water:deuterium oxide. Figure 3.29 illustrates the upfield region of the COSY spectrum. This region illustrates the methyl cross-peaks for lysine, leucine, and isoleucine, which contains a large amount of overlap. There are three isoleucine, seven leucine, and seven lysine residues in the primary sequence of the V2 fragment. The large number and similar resonance frequencies of the protons associated with these residues resulted in a great deal of degeneracy which would make
unambiguous assignments difficult. The process may be facilitated using three-
dimensional techniques that have proved invaluable at reducing degeneracy and allowing
the determination of much larger structures (Clore and Gronenborn, 1994). However, the
complete analysis of the NMR data was not necessary to gain considerable insight into
the structure of V2. The combination of biochemical techniques, in addition to circular
dichroism spectroscopy and molecular modelling, has resulted in a proposed structure that
can be rationalized on the basis of existing structural information.

Conclusions

Modifications in the BPPI purification protocol resulted in protein solubility greater
than 12 mg/mL. The estimated molecular weight of BPPI in solution was approximately
19,000 Da. Secondary structural elements analyzed by CD spectroscopy indicated 15% α-helix, 27% β-sheet, 43% random coil, and 15% turn for BPPI. It was also found that
the BPPI/trypsin complex was stable in solutions of SDS. A three dimensional model of
BPPI was generated based on its primary sequence homology with BPTI. A low
molecular weight protein that inhibits both plasmin and trypsin was found in human
platelets and has been termed HPPI.

It was found that the V2 fragment was able to bind heparin with a relatively high
degree of affinity and exists as a dimer in solution. CD spectroscopy indicated that V2
contained 15% α-helical structure which was in excellent agreement with the x-ray
coordinates of b-PF4.
References


**Vita Auctoris**

**JEFFREY STEPHEN BALDWIN**

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<td><strong>EDUCATION</strong></td>
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**SCHOLARSHIPS AND AWARDS**

1995  
Ontario Graduate Scholarship (declined)

1995  
University of Windsor Summer Research Scholarship (declined)

1995  
University of Windsor Postgraduate Tuition Scholarship (declined)

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PUBLICATIONS AND PRESENTATIONS

Refereed Journals


Abstracts and Presentations