Toward finding the gene for bovine platelet plasmin inhibitor.

Natalie Marie. Labbe
University of Windsor

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation

This online database contains the full-text of PhD dissertations and Masters' theses of University of Windsor students from 1954 forward. These documents are made available for personal study and research purposes only, in accordance with the Canadian Copyright Act and the Creative Commons license—CC BY-NC-ND (Attribution, Non-Commercial, No Derivative Works). Under this license, works must always be attributed to the copyright holder (original author), cannot be used for any commercial purposes, and may not be altered. Any other use would require the permission of the copyright holder. Students may inquire about withdrawing their dissertation and/or thesis from this database. For additional inquiries, please contact the repository administrator via email (scholarship@uwindsor.ca) or by telephone at 519-253-3000ext. 3208.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
TOWARD FINDING THE GENE FOR BOVINE PLATELET PLASMIN INHIBITOR

by

Natalie M. Labbé

A Thesis
Submitted to the Faculty of Graduate Studies and Research
Through the School of Physical Science
In partial Fulfillment of the Requirements for the
Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
1997
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-52466-3
ABSTRACT

TOWARD FINDING THE GENE FOR BOVINE PLATELET PLASMIN INHIBITOR

by

Natalie M. Labbé

Bovine platelet plasmin inhibitor (BPPI) is a novel Kunitz inhibitor, recently discovered in platelets (Li, 1992) for which an expression system is sought. Because the protein’s origin of synthesis was unknown, a search for a genomic clone was undertaken.

PCR was used in an attempt to amplify the BPPI gene from lymphocyte, bovine genomic DNA. The first set of primers used was designed according to the N and C termini of the amino acid sequence of BPPI (P1/P2). These were based on a degenerate nucleotide sequence derived from its amino acid sequence. Amplification with this set of primers was unsuccessful.

The second approach in amplifying the gene was to increase the specificity of the primers in the 3’ end (P3/P4) using a table of codon usage of the bovine genome. Two successive amplifications led to the formation of a 230 bp fragment. The sequence of this product was not comparable to the degenerate sequence of BPPI.

A third set of primers (P6/P7) was designed according to the gene sequence of SI (II). An inferred amino acid sequence from the SI (II) gene, outside of the region
encoding the mature protein shared almost identical homology with the amino acid sequence of BPPI, hence its use as a template in the design of primers.

The use of P6/P7 for amplification, resulted in the formation of two products. One was sized at 450 bp and the other at 172 bp. Sequencing of these genes indicated no similarities with the BPPI or SI (II) genes.

Amplification of the BPPI gene from genomic DNA was unsuccessful using these particular primers.

Future work could involve the construction of a cDNA library from a suitable source such as megakaryocytes.
Dedicated to my mother and father

Madeleine and Robert Labbé
ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to Dr. Lana Lee for her patience and guidance throughout the course of this study and for the opportunity to work on this project.

I would also like to thank the other members of my committee, Dr. Bulent Mutus and Dr. Alden Warner for taking the time to read and evaluate this thesis.

I am very grateful to Dr. T. Christopoulous and his group for their help in getting me started on this project. My gratitude is also extended to Ann-Marie Butler from the department of Biological Sciences who has given me tremendous insight in this project and who has also cloned and sequenced one of the PCR fragments in question.

I would like to acknowledge the Heart and Stroke foundation of Ontario for funding this research.

I thank my friends and fellow graduate students Domenic Perri, Mauro Acchione, Paul Malinowski, Mike Weller and Bahe Rajendran for their friendship and for whom I feel I am very fortunate to have met.

Thank you to my siblings Jérôme, Thomas, Denise, Grégoire, Andrée, Roselyne, Daniel, Antoine and Marc whom I love dearly and for whom their friendship and our tight familial bond means more to me than words can express. Finally, I thank my very dear friend Jessica Hamilton for her support and simply for being such a wonderful friend.
# TABLE OF CONTENTS

Abstract vi
Dedication vii
Acknowledgements viii
List of Tables xi
List of Figures xii
List of Abbreviations xiv

1. Introduction

1.1 Introduction 1
1.2 Objectives 12

2. Materials and Methods

2.1 Materials 15
2.2 Methods 19
   2.2.1 General Procedure for Casting and Electrophoresis of an Agarose Gel 19
   2.2.2 General Procedure for Gel Loading 20
   2.2.3 Isolation of Genomic DNA from Whole Calf Blood 21
   2.2.4 Design of Primers for PCR 23
   2.2.5 Basic Methodology of PCR 23
   2.2.6 PCR using P1 and P2 Primers 26
   2.2.7 PCR using P3 and P4 Primers 27
   2.2.8 Excision and Amplification of the 230 bp Region of the Agarose Gel 27
   2.2.9 Hemi Nested PCR using P3 and the Internal Primer P5 28
   2.2.10 PCR using P6 and P7 Primers 29
   2.2.11 Cloning of the 230, 172 and 450 bp PCR Products into the pCR®.2.1 Vector 29
      2.11.1 Ligation 29
      2.11.2 LB Medium and Plates 30
      2.11.3 Transformation 31
   2.2.12 Plasmid Isolation 32
   2.2.13 Estimation of the Quantity and Purity of Plasmids Isolated 33
   2.2.14 Restriction Analysis of Plasmids 34
   2.2.15 PCR on Insert in Plasmids 35
   2.2.16 Glycerol Stocks 35
   2.2.17 Sequencing of the Inserts in the pCR®.2.1 Vector 36
      2.2.17.1 Sequencing of the 230 bp Insert 36
      2.2.17.2 Sequencing of the 172 and 450 bp Inserts 37
   2.2.18 Analysis of the 230, 172 and 450 bp Sequences 40
   2.2.19 Design of Ideal Primers Using Primer3 Output 41
3. Results

3.1 Isolation of Genomic DNA from Whole Calf Blood
3.2 Design of Primers for PCR
3.3 PCR using P1 and P2 Primers
3.4 PCR using P3 and P4 Primers
3.5 Excision and Amplification of the 230 bp Region of the Agarose Gel
3.6 Hemi Nested PCR using P3 and the Internal Primer P5
3.7 PCR using P6 and P7 Primers
3.8 Cloning of the 230, 172 and 450 bp PCR Products into the pCR®2.1 Vector
   3.8.1 Ligation
   3.8.2 Transformation
3.9 Plasmid Isolation
3.10 Estimation of the Quantity and Purity of Plasmids Isolated
3.11 Restriction Analysis of Plasmids
3.12 PCR on Inserts in Plasmids
3.13 Glycerol Stocks
3.14 Sequencing of the Inserts in the pCR®2.1 Vector
   3.14.1 Sequencing of the 230 bp Insert
   3.14.2 Sequencing of the 172 and 450 bp Inserts
3.15 Analysis of the 230, 172 and 450 bp Sequences
3.16 Design of Ideal Primers using Primer3 Output

4. Discussion

4.1 Isolation of Genomic DNA from Whole Calf Blood
4.2 Design of Primers for PCR
4.3 Basic Methodology of PCR
4.4 PCR using P1 and P2 Primers
4.5 PCR using P3 and P4 Primers
4.6 Excision and Amplification of the 230 bp Region of the Agarose Gel
4.7 Hemi Nested PCR using P3 and the Internal Primer P5
4.8 PCR using P6 and P7 Primers
4.9 Cloning of the 230, 172 and 450 bp PCR Products into the pCR®2.1 Vector
   4.9.1 Ligation
   4.9.2 Transformation
4.10 Plasmid Isolation
4.11 Estimation of the Quantity and Purity of Plasmids Isolated
4.12 Restriction Analysis of Plasmids
4.13 PCR on Inserts in Plasmids
4.14 Glycerol Stocks
4.15 Sequencing of the Inserts in the pCR®2.1 Vector
4.16 Analysis of the 230, 172 and 450 bp Sequences
4.17 Design of Ideal Primers using Primer3 Output
5. Future work

6. Conclusion

Appendix

Appendix A: The pCR®2.1 Vector
Appendix B1: Nucleotide sequence producing High-scoring Segment
               Pairs for the 230 bp insert.
Appendix B2: Protein sequence producing High-scoring Segment
               Pairs for the 230 bp insert.
Appendix B3: Nucleotide sequence producing High-scoring Segment
               Pairs for the 172 bp insert.
Appendix B4: Protein sequence producing High-scoring Segment
               Pairs for the 172 bp insert.
Appendix B5: Nucleotide sequence producing High-scoring Segment
               Pairs for the 450 bp insert.
Appendix B6: Protein sequence producing High-scoring Segment
               Pairs for the 450 bp insert.

References

Vita Auctoris
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>TABLE</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Coagulation factors</td>
<td>4</td>
</tr>
<tr>
<td>3-1</td>
<td>A summary of the primers used for probing BPPI</td>
<td>44</td>
</tr>
<tr>
<td>3-2</td>
<td>A list of ideal primers indicated by the program Primer3 using the SI (II) gene as template</td>
<td>71</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>The coagulation pathway</td>
<td>3</td>
</tr>
<tr>
<td>1-2</td>
<td>Cα backbone of BPTI</td>
<td>7</td>
</tr>
<tr>
<td>1-3</td>
<td>Alignment of the amino acid sequences of BPPI with BPPI and SI (II)</td>
<td>10</td>
</tr>
<tr>
<td>1-4</td>
<td>Postulated structure of BPPI</td>
<td>13</td>
</tr>
<tr>
<td>2-1</td>
<td>Amino acid sequence of BPPI and primers</td>
<td>24</td>
</tr>
<tr>
<td>2-2</td>
<td>Alignment of primary sequence of BPPI and (SI) (II) with the SI (II) gene</td>
<td>25</td>
</tr>
<tr>
<td>3-1</td>
<td>0.5 % agarose gel of bovine genomic DNA</td>
<td>43</td>
</tr>
<tr>
<td>3-2</td>
<td>2.0 % agarose gel of PCR products generated with P3 and P4 primers</td>
<td>46</td>
</tr>
<tr>
<td>3-3</td>
<td>2.0 % agarose gel of amplification of the 230 bp region</td>
<td>48</td>
</tr>
<tr>
<td>3-4</td>
<td>2.0 % agarose gel of amplification using P3/P5 primers and agarose as template</td>
<td>50</td>
</tr>
<tr>
<td>3-5</td>
<td>2.0 % agarose gel of PCR products generated with P6 and P7 primers</td>
<td>51</td>
</tr>
<tr>
<td>3-6</td>
<td>2.0 % agarose gel of digestion of plasmid containing the 230 bp insert</td>
<td>53</td>
</tr>
<tr>
<td>3-7</td>
<td>1.5 % agarose gel of digestion and PCR of plasmid containing the 172 bp insert</td>
<td>54</td>
</tr>
<tr>
<td>3-8</td>
<td>1.3 % agarose gel of the digestion of plasmid containing the 450 bp insert</td>
<td>55</td>
</tr>
<tr>
<td>3-9</td>
<td>Spectrum of wavelength vs. absorbance of plasmid DNA</td>
<td>58</td>
</tr>
</tbody>
</table>
3-10  1.4 % agarose gel of digestion and PCR of plasmid containing 450 bp insert 60
3-11  Autoradiograph of the 230 bp sequence 63
3-12  Trace of the 172 bp nucleotide sequence generated in the 5’ to 3’ direction 64
3-13  Trace of the 172 bp nucleotide sequence generated in the 3’ to 5’ direction 65
3-14  Trace of the 450 bp nucleotide sequence generated in the 5’ to 3’ direction 66
3-15  Compilation of the 230, 172 bp sequences 67
3-16  The 450 bp sequence 69
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BPPI</td>
<td>bovine platelet plasmin inhibitor</td>
</tr>
<tr>
<td>BPTI</td>
<td>bovine pancreatic trypsin inhibitor</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>d/ddNTP</td>
<td>deoxynucleotide/dideoxynucleotide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
</tr>
<tr>
<td>FS</td>
<td>for sequencing</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>(^1)H-NMR</td>
<td>proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>P1</td>
<td>primer 1</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PIR</td>
<td>Protein Information Resource</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
</tbody>
</table>
SI  spleen inhibitor
SOC  media containing 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 1mM MgCl₂, 20 mM glucose

*Taq*  *Thermus aquaticus*

TBE  tris-HCl, borate, EDTA

TE  tris-HCl, EDTA

TKM  Tris-HCl, KCl, MgCl₂

*Tₘ*  melting temperature

UV-Vis  ultraviolet-visible

X-Gal  5-bromo-4-chloro-3-indolyl-β-D-galactoside
1.1 Introduction

Hemostasis is the term given to the process by which the circulatory system maintains the flow of blood and heals injuries to prevent excessive bleeding. Blood fluidity is critically maintained by inhibition of coagulation, inhibition of platelet aggregation and promotion of fibrinolysis (Colman et al., 1994).

When a vessel is ruptured, the shed blood becomes exposed to subendothelial components or adhesive proteins such as collagen, tissue factor and von Willebrand factor. This exposure in turn initiates blood coagulation. More specifically, the platelets floating in the blood become stimulated first to link themselves onto the damaged blood vessel through the von Willebrand factor and secondly to link themselves onto each other through fibrinogen. This adhesion and aggregation of platelets occurs via glycoprotein receptors on platelets that form or become exposed as a result of platelet activation. It is this process which initiates coagulation (Bachman, 1994).

Platelets are derived from megakaryocytes in the bone marrow. Their organelles are used for storage, the predominant one being the $\alpha$ - granule which contains many components relevant to clotting such as fibrinogen, factor X, factor V and $\alpha_1$-antitrypsin. The other two organelles are the dense granule and the lysosome. The former containing ATP, ADP, pyrophosphate and calcium and the latter, a variety of acid hydrolases. Agonists known to cause aggregation and secretion of platelets are thrombin, ADP and collagen. They interact with specific receptors on the platelet and cause the activation of phospholipase C. An important result of the activation of this enzyme is the mobilization of calcium. Many processes in platelet activation are calcium dependent. The increased
intracellular calcium concentration mediates reactions that cause fusion of the dense, alpha and lysosomal granular membranes with the membrane of the platelets resulting in the release of their contents (Colman et al., 1994).

In brief, clot formation can be initiated by the intrinsic or extrinsic pathway (Figure 1-1). The intrinsic pathway is named such because it is principally, but not exclusively activated by components contained within the vascular system. The extrinsic pathway, conversely, is exclusively activated by tissue factor, an intrinsic membrane protein. This pathway involves components from blood and vascular elements. As the diagram indicates, the end result of each pathway is the activation of factor X. The activated factor X proteolytically cleaves prothrombin, a zymogen, which after cleavage becomes thrombin, a serine protease homologous to trypsin. Thrombin then catalyzes the cleavage of specific bonds within fibrinogen. As a result, the soluble fibrinogen undergoes restructuring and becomes the insoluble fibrin (Colman et al., 1994). Table 1-1 is a list of some the factors of the coagulation pathway and their common names.

Coagulation is largely controlled by serine proteases. In fact, aside from thrombin, six other clotting factors are zymogens of serine proteases that are activated by other serine proteases earlier in the cascade. The remaining clotting factors or accessory factors are also activated by serine proteases in the cascade. These accessory factors in turn enhance the rate of activation of some of the zymogens (Voet and Voet, 1995).

All of the proteases involved with the coagulation or fibrinolytic pathways are serine proteases of the chymotrypsin superfamily. This class comprises many of the digestive enzymes such as trypsin, chymotrypsin and plasmin, all of the coagulation and fibrinolytic proteases and those involved with the complement system. Serine proteases
Figure 1-1: The coagulation pathway. Tissue factor, which becomes exposed to blood when tissue is injured forms a complex with factor VIIa and phospholipid (PL) then activates both factors IX and X. The intrinsic pathway includes contact activation of factor XI by the XIIa activated high molecular weight kininogen (HKa) complex. Then factor XIa activates factor IX to IXa that in turn activates factor X to Xa by complex formation with PL and VIIIa. Factor Xa forms a complex with Va and phospholipid and causes the cleavage of prothrombin to thrombin. Thrombin then cleaves fibrinogen into the monomers that then polymerize to form fibrin. Thrombin also converts factor XIII to XIIIa which crosslinks fibrin into a more solid structure. a represents active, s, soft clot and h, hard clot. (Colman et al., 1994)
**Table 1-1: Coagulation Factors** (Colman et al., 1994).

<table>
<thead>
<tr>
<th>Factor Number</th>
<th>Common Name</th>
<th>Subunit Mass (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fibrinogen</td>
<td>340</td>
</tr>
<tr>
<td>II</td>
<td>Prothrombin</td>
<td>72</td>
</tr>
<tr>
<td>III</td>
<td>Tissue Factor or thromboplastin</td>
<td>37</td>
</tr>
<tr>
<td>IV</td>
<td>Ca$^{2+}$</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>Proaccelerin</td>
<td>330</td>
</tr>
<tr>
<td>VII</td>
<td>Proconvertin</td>
<td>50</td>
</tr>
<tr>
<td>VIII</td>
<td>Antihemophilic factor</td>
<td>330</td>
</tr>
<tr>
<td>IX</td>
<td>Christmas factor</td>
<td>56</td>
</tr>
<tr>
<td>X</td>
<td>Stuart factor</td>
<td>56</td>
</tr>
<tr>
<td>XI</td>
<td>Plasma thromboplastin antecedent (PTA)</td>
<td>160</td>
</tr>
<tr>
<td>XII</td>
<td>Hageman factor</td>
<td>80</td>
</tr>
<tr>
<td>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>
</tbody>
</table>
are classified as such because they share a common mechanism of catalysis. This mechanism takes place through a reactive serine residue or serine-195 as seen in trypsin, the classical serine protease. It has been studied quite extensively in terms of its structure and mechanism (Salvesen and Salvatore, 1994).

Trypsin is a digestive protease not involved directly with coagulation or fibrinolysis. It is a monomeric, 240 residue enzyme which is folded into two domains each of which contains mostly antiparallel \( \beta \)-sheets in a barrel arrangement with a minor amount of helix. The active site consists of three essential residues, His 57, Ser 195 and Asp 102 which are referred to as the catalytic triad. This active site is nested between the two domains. The structure of the enzyme defines its specificity. For example, an aspartate residue that lies at the back of the binding pocket allows for the attraction and interaction of positively charged side chains such as arginine or lysine. Moreover, its specificity pocket is large, allowing it to bind such side chains as Arg and Lys (Moran et al., 1994).

Because the coagulation and fibrinolytic processes are controlled by proteases, by necessity there exist inhibitors to control the activities of these proteases. These inhibitors bind and inhibit the proteases responsible for clot formation. It is speculated that some, once bound to their target serine protease act as effectors for receptor mediated clearance (Salvesen and Salvatore, 1994). One extensively studied serine protease inhibitor of the Kunitz family, not directly involved in coagulation or fibrinolysis is bovine pancreatic trypsin inhibitor or BPTI. It is a very strong inhibitor of bovine trypsin with a \( K_d \) value of \( 6 \times 10^{-14} \) M (Vincent and Lazdunski, 1972) and trypsin-like enzymes. BPTI was the first inhibitor to be isolated in crystalline form. It was the first to be
sequenced, the first to have its 3-D structure determined crystallographically, and it was the first to be synthesized in the laboratory (Laskowski and Kato, 1980).

The strength of the attachment of BPTI to trypsin results from a very close fit of the reactive site loop of the inhibitor (Figure 1-2) and the active site of the enzyme. The reactive site loop consists of a sequence of six residues starting at Cys14 to Ile19. In binding, the Lys15 (P₁) side chain of the reactive site of BPTI sits in the specificity pocket of the trypsin active site. It and its adjacent residue Ala16 (P₁'), form the 'scissile bond' of the substrate that would normally be cleaved (Bachman, 1994).

Kunitz type inhibitors are a class of mostly low molecular weight, basic proteins that show activity toward serine proteases. They are not suicide substrates and do not form irreversible covalent bonds to their target enzyme (Cronlund and Walsh, 1992). Instead they form tight but reversible interactions. Kunitz inhibitors have at least one 'Kunitz' domain that consists of a well conserved, 60 amino acid sequence most frequently found in the NH₂ terminus of the inhibitor with only one domain. A notable feature of this inhibitor is the presence of six cysteine residues that form three disulphide bonds which appear to give the domain high thermal stability (Stallings-Mann et al., 1994). The locations of the cysteine residues in the primary sequence are critical for proper folding and inhibitory structure (Cronlund and Walsh, 1992). This inhibitory structure consists of two loops that bind the target protease. The first defines specificity and the second forms an interface with the protease. The P₁ residue located centrally within the first loop mimics the substrate that the enzyme recognizes. It defines the class of enzymes it will inhibit. Most frequently P₁ is a lysine but it is not unusual to find an arginine at this site, a likely substitution, since the premise of attachment is based on the
Figure 1-2: Cα backbone of BPTI. The reactive site loop residues are displayed by three-letter code. The full circles represent cysteinyI residues. The P1 residue in the reactive site is Lys 15 (Groeger et al., 1991).
positive charge. Differences within these two binding loops among other inhibitors of this class are said to contribute to more subtle specificities (Stallings-Mann et al., 1994).

Acting to oppose coagulation, is the fibrinolytic pathway. Plasmin, a key proponent, catalyzes the degradation of fibrin to soluble fragments and helps to limit the clot to the area of injury. It is also a member of the serine protease family. Plasmin, on the surface of the clot is shielded from α₂-antiplasmin, allowing for dissolution of the clot. In contrast, in general circulation, plasmin is rapidly inactivated by α₂-antiplasmin (Bachman, 1994).

Plasmin starts off as the zymogen plasminogen and is activated by serine proteases such as trypsin, urokinase, tissue-type plasminogen activator (t-PA) and even plasmin itself. Plasminogen is a 92 kDa zymogen referred to as Glu₁-plasminogen when in its native state. As the name implies, it has a glutamate residue as its first amino acid. Hydrolysis of the bond between Arg561-Val562 by activators such as urokinase and t-PA renders it a two-chained active molecule. Two cysteine bonds attach the resulting A and B chains. Further cleavage by plasmin at one of the following bonds Arg68-Met69, Lys77-Lys78 or Lys78-Val79 causes the loss of the N terminal portion of the A chain. The resulting fragment now beginning with a Lys residue is named Lys-plasminogen. This form of plasminogen displays an enhanced binding affinity for fibrinogen (Hajjar and Nachman, 1994).

Conversely, several inhibitors inhibit plasmin. These inhibitors tend to form equimolar complexes with it. Its principal inhibitor, α₂-antiplasmin is a 70 kDa glycoprotein that forms an irreversible attachment to plasmin. It actually acts in three ways to inhibit fibrinolysis, the first is in binding to its active site irreversibly, the second
is to inhibit plasminogen from binding to fibrin, and the last is by cross-linking to the α-chains of fibrinogen during clotting. Inactivation of plasmin occurs through formation of a stable complex with the serine in the active site (Francis and Marder, 1994) and occupation of the lysine binding site by Lys452 of α₂-antiplasmin (Bachman, 1994). An individual homozygous for a defective α₂-antiplasmin gene has a serious tendency to bleed.

Bovine platelet plasmin inhibitor (BPPI) is the name given to a recently discovered Kunitz inhibitor. Characterization indicated that it was quite homologous with BPTI, another member of the Kunitz inhibitor family. BPPI was discovered by accident when isolating other specific proteins from bovine platelets (Li, 1992). When starting with 40 - 45 L of fresh bovine blood, a typical yield was between 0.5 to 1.5 mg of this protein. Select kinetic studies indicated a general inhibition toward serine proteases. Its name was assigned because it displayed greater inhibitory and tighter binding capacity toward plasmin than trypsin. Weak inhibition of XIA, Xa and IXa was also observed (Walz et al., 1995). To date, its amino acid sequence (Li, 1992), select kinetic studies, and some preliminary structural studies (Baldwin, 1995) have been established. Its amino acid sequence shares homology with BPTI (Li, 1992) and bovine spleen inhibitor (SI) (II) (Creighton and Charles, 1987), two very well established serine protease Kunitz inhibitors. Alignment of the primary sequences of BPPI with BPTI and SI (II) (Figure 1-3) depicts the minor differences among their primary structures. The P₁ reactive site residue in BPPI, BPTI, and SI is Lys.

This figure also contains the primary sequences of SI (I) and (III), which appear to be intermediates in the processing of the 100 residue primary expression product of the
BPPI  \( \text{NH}_2-\text{TPGCDTSNQA KAQRPDFCLE PPYTGPCKAR MIRYFYNAKA} \)
BPTI  \( \text{NH}_2--------------\text{RPDFCLE PPYFGPCKAR IIRYFYNAKA} \)
SI(II) \( \text{NH}_2--------------\text{RPDFCLE PPYTGPCKAK MIRYFYNAKA} \)
SI(I)  \( \text{NH}_2--------------\text{AQRPDFCLE PPYTGPCKAK MIRYFYNAKA} \)
SI(III) \( \text{NH}_2--------------\text{RPDFCLE PPYTGPCKAK MIRYFYNAKA} \)

\[ \text{GFCETFVYGG CKAKSNF RS AECDMRTC GG AIGPRENL-COOH} \]
\[ \text{GLCQT FVYGG C RAKRN FKS AECDMRTC GG A----------COOH} \]
\[ \text{GFCETFVYGG CKAKSNF RS AECDMRTC GG A----------COOH} \]
\[ \text{GFCETFVYGG CKAKSNF RS AECDMRTC GG AIGPREN----------COOH} \]
\[ \text{GFCETFVYGG CKAKSNF RS AECDMRTC GG AIGPR----------COOH} \]

\text{Figure 1-3: Alignment of the amino acid sequence of BPPI with BPTI and SI (II). BPPI is extended at both the} \text{ amino and carboxy termini by 13 and 7 residues respectively, in comparison to BPTI and SI (II) (Li, 1992). SI} \text{ intermediates are listed for comparison. These intermediates are thought to follow the order of I, III then II in protein processing (Barra et al., 1991). The seven differences in sequence between BPTI and SI (II) are indicated by asterisks and P}_2^* \text{. The difference in primary sequence between BPPI and SI (II) lies at the P}_2^* \text{ residue. The P}_1^* \text{ residue of the reactive site is Lys in all three proteins (Li, 1992).} \]
SI (II) gene leading to the mature SI (II) inhibitor. The order of the intermediates in processing had a suggested pattern of I, III and II. These intermediates differ only in extent of proteolysis on their N and C termini (Barra et al., 1990). Within the protein, BPPI differs in amino acid sequence by only one residue at the P2 position (Figure 1-3) in comparison with SI (II).

Isoelectric focusing of BPPI demonstrated that it had a net charge of +3, (Baldwin, 1995) a value that was basic and typical of Kunitz type inhibitors. A secondary structure prediction of BPPI was made (Li, 1992) based on the rules of Chou-Fasman (not shown), and CD studies were performed to complement this prediction. The CD studies indicated that BPPI contained approximately 15 % α-helix, 27 % β-sheet, 43 % random coil and 15 % β-turn. These results were comparable with X-ray crystallographic data of BPTI, which indicated a content of 17 % α-helix, 33 % β-sheet, 36 % random coil and 14 % β-turn (Walter and Huber, 1983). The great deal of similarity in secondary structure indicated that the variations in amino acids among the two did not have a great deal of effect on the secondary structure (Baldwin, 1995).

Three dimensional structure prediction was performed using the Homology program and the X-ray coordinates of BPTI. This prediction supported the secondary structure results indicating that the N and C termini of BPPI contained more random coil than BPTI (Baldwin, 1995).

Gel filtration studies and kinetic studies performed suggested that BPPI was a dimer with two inhibitory sites. The gel filtration studies resulted in a calculated molecular weight of 19,000 Da (Baldwin, 1995) whereas the amino acid sequence indicated a molecular weight of 8,580 Da. Furthermore, kinetic studies showed an
inhibition of trypsin in a stoichiometric ratio of 1.8:1 (Baldwin, 1995). These results supported the postulated dimer structure of BPPI (Li, 1992) where an inter-subunit disulphide bond joined the monomers at their Cys4 position (Figure 1-4).

Interest is taken in this protein because of its possible role in coagulation and fibrinolysis. Though its role is not yet clearly defined in vivo, speculation suggests that its release from the platelet results in locally higher concentrations that may act to modulate platelet thrombus formation at the site of injury. Moreover, its low molecular weight may help in its ability to infuse and act quickly in limiting the clot to the area. This is nonetheless an over simplification of its role in the intricate and complex system of coagulation in which there remains much to be discovered.

The study of the inhibitors and activators of the proteases of blood clotting receive a large amount of attention principally because they are used in the treatment of strokes and other blood related abnormalities. For example, intravenous tissue plasminogen activator (t-PA) is one of the most commonly used agents for the treatment of strokes in the United States (Hennekens et al., 1995).

Another example is the use of Kunitz inhibitors in the in vitro inhibition of coagulation factors. Though very rudimentary, these studies involve the construction of a novel series of BPTI-derived analogues which are then tested against various factors to evaluate their effectiveness in antithrombic activity (Stassen et al., 1995).

1.2 Objectives

To further study BPPI in terms of its structure-function relationship, a project in which its gene was sought was undertaken. The isolation of this gene and insertion into
Figure 1-4: Postulated structure of BPPI. The intra-chain disulphide bonds in BPPI are Cys18/68, Cys27/51 and Cys43/64. The two monomers are joined by an inter-disulphide bond at the Cys4 positions of each subunit (Li, 1992).
an appropriate vector could lead to the construction of recombinant protein. This would enable investigations of the protein’s properties in vivo and perhaps elucidate its evolutionary relationship to other homologous proteins.

NMR is a powerful tool for structure determination. Bovine platelet plasmin inhibitor’s low molecular weight and preliminary NMR studies performed on it (Baldwin, 1995) indicate that it is a good candidate for further structural studies. However, the limited yields of protein do not permit complete structural analysis. A typical NMR sample would contain approximately 4.5 mg of BPPI. Thus a cloned gene would enable the production of recombinant protein at quantities necessary for NMR studies.

The immediate intention is to amplify the full gene of BPPI by PCR on bovine genomic DNA so that it can be incorporated directly into a suitable vector for expression.
2. Materials and Methods

2.1 Materials

Micropipetting was carried out using Gilson Pipetman, models P1000, P200, P20 and P10 variable pipettors (Mandel Scientific). Measurements of mass were made with either a Mettler P1200N or Mettler AE50 balance (Mettler Scientific). pH measurements were performed with an Accumet pH meter, model 810 (Allied Fisher Scientific). Agarose gels were electrophoresed with a Power pac 300 (Biorad). Photographs of gels were taken with an MP-4+ Camera System (Polaroid) on film type 667, 3\(\frac{1}{4}\) x 4\(\frac{1}{4}\)” (Polaroid). The UV transilluminator placed at the base of the camera was from Biorad. Centrifugations were performed with one of the following: Sorvall RTG000B refrigerated centrifuge (Du Pont), model 59A Micro-Centrifuge Fisher Scientific (Fisher) or J2-HS centrifuge equipped with the JA-20 rotor (Beckman). The temperature controlled water bath used was Model 1201 by VWR Scientific. All spectrophotometric measurements were performed with a Shimadzu UV-Vis recording spectrophotometer model UV-160 (Shimadzu). Readings were measured using 1 cm quartz (UV range) cuvettes from Hellma, Canada. The thermocycler used for PCR was the DNA Thermal Cycler from Perkin Elmer Cetus. The UV hood used to decontaminate all PCR equipment was a Biological Safety Cabinet, Class II, Type A/B3 hood (NUAIRÉ). The shaker used was an Orbit Lab-line Incubator-Shaker by VWR Scientific. The vortex used was by Fisher.

Tris base (Sigma), boric acid (Anachemia), EDTA (Sigma), ethidium bromide (Sigma), agarose (Sigma type I-B; low EEO), sucrose (BDH), SDS (Sigma), bromophenol blue (BDH).

Molecular weight standard used in agarose gel electrophoresis was one of the
following: λ DNA digested with EcoRI and HindIII (Sigma), pBR322 plasmid digested with BsuRI (HaeIII) (MBI Fermentas) or ΦX174 plasmid digested with BsuRI (HaeIII) (MBI Fermentas).

Genomic DNA was isolated from calf blood acquired from Gord’s Abattoir Leamington, Ont. Chemicals and minor equipment used in its isolation were trisodium citric acid (Sigma), glucose (Sigma), KCl (AnaLaR), MgCl₂ (Sigma), NaCl (BDH), Nonidet P-40 (United States Biochemical Corp.), 15 mL disposable, conical, centrifuge tubes (VWR), 1.7 mL microcentrifuge tubes (VWR) and anhydrous ethanol (BDH).

DNAAgency (Malvern, PA) synthesized primers for PCR. The first set of primers designed according to the BPPI amino acid sequence was designated P1 and P2 for the left and right primers, respectively. Their sequences were 5’-ACNCCNGGNTGYGAYAC-3’ and 5’-AGRTTTYTCNCNGNGNCC-3’. N represents a mixture of A, G, C and T. R represents A and G. Y represents T and C.

The second set of primers P3 and P4, left and right, respectively was also designed according to the amino acid sequence of BPPI (Li, 1992). Their sequences were: 5’-ACNCCNGGNTGYGACACC-3’ and 5’-NAGRTTTYTCNCGGGGGCC-3’. An internal probe designated P5, also part of this set had the following sequence 5’-NGCYTTNGCRTTRTARAARTA-3’.

The third set of primers P6 and P7, was designed according to the spleen inhibitor (SI) (II) nucleotide sequence (Creighton and Charles, 1987). Their sequences were: 5’-ACTCCAGGGTGTGACACC-3’ and 5’-CAGGTTCTCCCGGGGCC-3’.

PCR was performed using reagents from Eclipse BIO/CAN Scientific. MgCl₂ was provided as a 25 mM stock solution. The reaction buffer was 10 x, containing 20
mM (NH₄)₂SO₄, 75 mM Tris HCl and 0.01 % Tween 20. The polymerase used for amplification was Ultratherm Thermophilic DNA polymerase. The nucleotides used were provided as a 10 mM nucleotide mix by Sigma. The PCR tubes were thin walled 600 µL tubes from Biorad. The mineral oil used to overlay the reaction mixture and to coat the wells of the thermocycler was from Sigma.

Cloning was performed using the Original TA Cloning® Kit by Invitrogen. This kit contained 10 x ligation buffer (60 mM Tris-HCl pH 7.5, 60 mM MgCl₂, 50 mM NaCl, 1 mg/mL BSA, 70 mM β- mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol and 10 mM spermidine), pCR®2.1 linearized plasmid (25 ng/µL in 10 mM Tris-HCl, 1 mM EDTA pH 7.5) and T4 DNA ligase (4.0 Weiss units/µL). Methanol and acetic acid purchased from BDH.

Cultures were grown on 10 cm plates. Components used in or with medium were yeast extract of autolysed yeast cells (BBL Becton Dickinson), tryptase peptone (BBL Becton Dickinson), bacto agar (Difco), kanamycin monosulphate salt (Sigma), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma) and dimethylformamide (Sigma).

The host used for the ligated plasmids was E. coli INVαF' competent cells (Invitrogen). Chemicals and minor equipment used in transformation were β-mercaptoethanol (Invitrogen), SOC medium containing 2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂-6H₂O and 20 mM glucose (Invitrogen) and 50 mL sterile conical centrifuge tubes (VWR).

Chemicals and minor equipment used for plasmid isolation and manipulation were wooden toothpicks (Natural Touch®), potassium acetate (Sigma), glacial acetic acid (BDH), anhydrous ethanol (BDH), sodium acetate (Sigma), bovine pancreatic RNase A
(Sigma), EcoRI (10 units/µL) and digestion buffer containing 10 mM HCl (MBI Fermentas), glycerol (Sigma) and Pasteur pipettes (Maple Leaf).

Sequencing of the 230 bp insert was performed using the fmol sequencing kit by Promega. This kit contained fmol™ 5x Sequencing buffer (250 mM Tris-HCl, pH 9.0 and 10 mM MgCl₂), fmol™ Sequencing Stop Solution (10 mM NaOH, 95 % formamide, 0.05 % bromophenol blue and 0.05 % xylene cyanole), d/ddNTP mixes and Sequencing Grade Taq DNA Polymerase (5 U/µL). The d/ddGTP mix contained 30 µ dGTP, 20 µM 7-deaza-dGTP, 20 µM dATP, 20 µM dTTP and 20 µM dCTP. The d/ddATP mix contained 350 µM ddATP, 20 µM 7-deaza-dGTP, 20 µM dATP, 20 µM dTTP and 20 µM dCTP. The d/ddTTP mix contained 600 µM ddTTP, 20 µM 7-deaza-dGTP, 20 µM dATP, 20 µM dTTP and 20 µM dCTP. The d/ddCTP contained 200 µM ddCTP, 20 µM 7-deaza-dGTP, 20 µM dATP, 20 µM dTTP and 20 µM dCTP. RNA Polymerase T7 promoter primer (20mer) 5'-TAATACGACTCACTATAGGG-3' and pUC/M13 reverse primer (17mer) 5'-CAGGAAACAGCTATGAC-3' were also from Promega. XAR-2 X-ray film used for autoradiography by Kodak.

Sequencing of the 172 and 450 bp inserts was performed on an ABI 373A DNA Sequencer (PE-Applied Biosystems) with an ABI PRISM™ Dye Primer Cycle Sequencing Core Kit by Perkin Elmer. This kit contained 5x sequencing buffer (400 mM Tris-HCl, pH9.0 and 10 mM MgCl₂), mix A containing optimized quantities of ddATP, dATP, dCTP, 7-deaza-dGTP and dTTP, mix C containing optimized quantities of ddCTP, dATP, dCTP, 7-deaza-dGTP and dTTP, mix G containing optimized quantities of ddGTP, dATP, dCTP, 7-deaza-dGTP and dTTP and mix T containing optimized quantities of ddTTP, dATP, dCTP, 7-deaza-dGTP and dTTP. AmpliTaq® Polymerase,
FS with thermal stable pyrophosphatase, FAM, JOE, TAMRA and ROX Dye primers were also part of this kit. The concentrations of d/ddNTPs and AmpliTaq® Polymerase FS were not available to the consumer. Other chemicals used for sequencing were deionized formamide, Blue dextran, acrylamide and urea (all from Sigma).

2.2 Methods

2.2.1 General Procedure for Casting and Electrophoresis of an Agarose Gel

To simplify the procedure of gel making and running, a stock solution of 5 x TBE containing 0.45 M Tris base, 0.45 M boric acid and 0.01 M EDTA, pH 8.0, was made and stored at 4 °C. This solution was diluted to 1 x, and ethidium bromide was added to this working solution to a final concentration of 1.2 μg/mL. This solution was used for both the gel and as electrophoresis buffer. The appropriate quantity of agarose was dissolved in 50 mL of 1 x TBE (depending on the range of resolution needed) and melted in a microwave oven for 1 min. stirring every 15 s. A homemade horizontal 10 x 8 cm mini gel electrophoresis unit was used for casting and running gels. The comb and gel borders were set up before the gel was poured. The gel solution was allowed to cool to approximately 60 °C before being cast into the apparatus. Solidification of the agarose was usually achieved within 25 min. Once the agarose was solid, the apparatus was filled with enough 1 x TBE buffer to completely cover the gel, then the comb and borders were removed.

In order to minimize the contact of ethidium bromide with lab equipment, certain pieces of glassware were reserved for use with ethidium bromide only. Moreover, storage, dispensing and decontamination of ethidium bromide were restricted to the
fumehood.

All agarose gels and electrophoresis buffers containing ethidium bromide were disposed of accordingly. All solutions and gels to be decontaminated were placed in a plastic container. One volume of 0.5 M KMnO₄ and one volume of 2.5 N HCl were added to this container and mixed. The mixture was left out at room temperature overnight. One volume of 2.5 N NaOH was added to neutralize the solution before discarding. The gel was placed in the garbage and the solution poured in the sink with copious amounts of water.

2.2.2 General Procedure for Gel Loading

A stock solution of gel loading buffer consisting of 40 % sucrose, 0.5 % SDS and 0.001 % bromophenol blue (or just enough to give colour) was used for loading all samples. Standards were made with 0.5 µL of oligonucleotide marker, 7 µL of gel loading buffer and 6 µL of 1 x TBE. Samples were made up with 20 µL of PCR product, that was carefully removed from the PCR tube by placing the pipette tip right below the layer of mineral oil, 10 µL of gel loading buffer and 5 µL of 1 x TBE. These samples were then loaded into the wells using an appropriate pipettor.

One of the following three standards was used: lambda DNA digested with EcoRI and HindIII, 614 µg DNA at 0.4 mg/mL (size of fragments ranging from 21,226 to 564 bp): pBR322 DNA digested with BsuRI (HaeIII), 0.5 mg / mL (size of fragments ranging from 587 to 89 bp) or φX174 DNA digested with BsuRI (HaeIII), 0.5 mg /mL (size of fragments ranging from 1350 to 118 bp) depending on which range was needed. Once loaded, the gel was run at 70 V for 90 min., unless otherwise stated. To document
the gel, a photograph was taken using Polaroid film. This was done by placing the gel on a transilluminator situated at the base of the camera. After focussing the camera, a photograph was taken. The film was exposed for 45 s.

This was the general procedure followed for visualization and documentation of DNA. Agarose concentration, markers used and voltage applied to the gel were the parameters that were varied from experiment to experiment.

2.2.3 Isolation of Genomic DNA from Whole Calf Blood

Fresh bovine blood was collected upon the slaughtering of a calf. Generally, the blood was collected in a 1L bottle containing 100 mL of anticoagulant. The anticoagulant contained 0.122 M trisodium citric acid and 0.111 M glucose set at a pH of 7.8. Once collected, the mixture was very carefully mixed by slowly inverting the bottle to its side a couple of times, then placed on ice.

The isolation of DNA was done following a procedure from the University of Windsor, Department of Chemistry and Biochemistry, intermediary metabolism lab manual. Prior to starting the isolation, the following buffers were made: TKM1 low salt buffer (10 mM Tris HCl pH 7.6, 10 mM KCl, 10 mM MgCl₂ and 2 mM EDTA) and TKM2 high salt buffer (10 mM Tris HCl pH 7.6, 10 mM KCl, 10 mM MgCl₂, 0.4 M NaCl and 2 mM EDTA). Generally, 5 mL of blood, 15 mL of TKM1 buffer and 125 μL Nonidet P-40 were placed in a 15 mL disposable, conical centrifuge tube. The tube was mixed by inversion and centrifuged in a Sorvall RTG000B refrigerated centrifuge at 950 x g for 10 min. at room temperature. The supernatant was poured off and the pellet saved. This pellet was suspended/washed in another 5 mL of TKM1 and centrifuged
again. The washing and centrifugation procedure was repeated until the pellet was white. The pellet was then suspended in 800 µL of TKM2 and transferred to a 1.7 mL microcentrifuge tube containing 50 µL of 10 % SDS. The tube was capped and mixed by inversion. Following an incubation of 10 min. in a 55 °C water bath, 300 µL of 6 M NaCl were added and mixed well. The mixture was centrifuged in a Fisher, model 59A Micro-centrifuge at 12,600 x g for 5 min. The resulting supernatant and 2.2 mL of anhydrous ethanol were placed in a glass test tube, the test tube capped and inverted until the DNA precipitated. The DNA was removed with a glass pipette with the tip bent up and placed in a microcentrifuge tube containing 500 µL of 10 mM Tris HCl, 1 mM EDTA, pH 8.0. The mixture was then placed in a 65 °C water bath for 15 min. then placed at −20 °C for storage.

The concentration and purity of the sample were determined by measuring the absorbance of the sample at 260 and 280 nm using a UV-Vis spectrophotometer. A ratio of the absorbance of 260 nm to 280 nm was calculated to determine sample purity, where 1.8 was considered pure (Sambrook et al., 1989). The quantity of DNA was calculated using the estimate that one absorbance unit was approximately equal to 50 µg/mL of double stranded DNA (Sambrook et al., 1989).

Four, 7 and 15 µL of this DNA solution were loaded in separate lanes and electrophoresed in a 0.5 % agarose gel as outlined in ‘general methods’ for visualization. The gel was made by dissolving 0.25 g agarose in 50 mL of 1 x TBE. Lambda DNA digested with EcoRI/HindIII was used as a nucleotide marker. Once the gel was loaded, it was run at 50 Volts for 2.5 h.
2.2.4 Design of Primers for PCR

The first two sets of primers were designed according to the amino acid sequence of BPPI as determined by Li (Li, 1992). The first set of primers designated P1 and P2 were degenerate 17mers, and the second set, designated P3 and P4 were degenerate 18mers with increased specificity in the 3' end, as taken from a table of codon usage of the bovine genome (Wada et al., 1990). An internal probe, a 21mer, designated P5 was also designed according to a selected internal sequence of BPPI (Figure 2-1).

A third set of primers, 18mers, was designed according to the sequence of one of the isoinhibitors of BPTI, the spleen inhibitor (SI) (II) (Creighton and Charles, 1987). This set of primers was designated P6 and P7 for the left and right primers, respectively (Figure 2-2).

2.2.5 Basic Methodology of PCR

Distilled, deionized water was autoclaved at 15 psi for 20 min. on slow exhaust. All dilutions with water and the water used for the polymerase chain reaction was autoclaved. Upon receiving the primers, each was dissolved in enough water to give a final stock solution of 100 pmol/μL. From this stock solution, 10 pmol/μL aliquots of working solutions were made and stored at 4 °C. The reagents (MgCl₂ and 10 x buffer) were aliquoted into separate autoclaved microcentrifuge tubes as well. Pipette tips and 600 μL reaction tubes were autoclaved and stored for PCR use only. Prior to setting up a reaction, pipettors, the marker used for labeling tubes, tip boxes (lid open) and bottle containing water (lid open) were placed under UV light for 2 h. Other precautions taken
Figure 2-1: Amino acid sequence of BPPI and primers. Degenerate primers were designed according to the amino acid sequence of BPPI (Li, 1992). P₁ and P₂ designate the left and right primers of the first set designed. P₃ and P₄ designate the left and right primers of the second set. P₃ and P₄ are the same as P₁ and P₂ with the exception that they are specific in the 3’ end. The regions of specificity as underlined, were taken from a table of codon usage (Wada, 1990). P₅ is a 21 base, degenerate internal primer also designed according to this sequence. N represents a mixture of A, G, C and T. Y represents a mixture of C and T. R represents a mixture of A and G. Note that a product resulting from an amplification using P₃/P₅ spans 40 amino acids or 120 bp.
Figure 2-2: Alignment of primary sequence of BPPI and SI (II) with the SI (II) gene. Intron were excluded from the SI (II) gene. Arrowheads indicate the start and end of the middle exon encoding the mature SI (II) protein. Amino acids in italic are those inferred from the SI (II) nucleotide sequence. Underlined regions of the SI (II) gene correspond to the sequences in which primers P6 and P7 were designed. Note that the primers fall out of the region encoding the mature SI (II) protein (BPPI sequence from Li, 1992). (SI (II) gene from Creighton and Charles, 1987)
were setting up the reaction in a separate room from where the products were analyzed, frequent cleaning of the area used, keeping separate pipettors for PCR and frequent changes of gloves during set up.

A typical reaction mixture contained 64.5 μL of water, 10 μL of 10 x buffer, 10 μL of 25 mM MgCl₂, 4 μL of 1 mM dNTP mix, 5 μL of each 10 pmol/μL primer and 1 μL of 25 -30 ng/μL template. The mixture was overlaid with 70 μL of mineral oil to prevent evaporation. The wells of the thermal cycler were lined with 70 μL of mineral oil prior to inserting the tubes. The following program was used: a hot start of 95 °C for 5 min, denaturation of DNA at 95 °C for 1 min., annealing of primers at 60 °C for 30 s., and elongation at 72 °C for 1 min for 25 - 30 cycles. On the last cycle, elongation was extended for 10 min. The cycling step was then linked to a 4 °C soak file. One half of a microliter or 2.5 units of Ultratherm DNA polymerase was added within the last two minutes of the hot start. Generally, three other control PCR reactions were set up to confirm that the DNA generated were not artifacts of the amplification. One control contained all but the left primer, the second all but the right primer, and a negative with everything but the template. The reagents for the reaction were always added last to the negative. Once the cycling was finished, the samples were removed from thermocycler and 20 μL was loaded onto an agarose gel with the appropriate marker(s). Once finished, the gel was photographed for documentation.

2.2.6 PCR using P1 and P2 Primers

PCR was set up using primers P1 and P2 as described in ‘Basic Methodology of PCR’. This first reaction was performed using an annealing temperature of 58 °C. The
subsequent PCR reactions were variations of this first PCR. The second variation was to lower the annealing temperature from 58 °C to 50 °C, 45 °C and finally to 40 °C. The third was increasing the mass of template ten fold such that the final mass was 0.3 μg. The fourth set was to vary the amount of MgCl₂ starting at 2.5 mM and changing it to 1.0, 3.0 and finally 5.0 mM. The last variation was an amplification using 2 x the quantity of primer; 100 pmol as opposed to 50 with an annealing temperature of 45 °C. The results of these reactions were analyzed on 1.7 - 2.0 % agarose gels.

2.2.7 PCR using P3 and P4 Primers

P3 and P4, the second set designed, were primers analogous to P1 and P2 but with specificity in the 3’ end as taken from a codon usage table (Wada et al., 1990). To start, PCR was set up with an annealing temperature of 40 °C as opposed to 60 as outlined in ‘basic methodology of PCR’. Two subsequent reactions were set up keeping all conditions the same with the exception of the annealing temperature. One was performed using 55 °C and second using 60 °C. For each reaction performed, three controls were set up. The first contained all but the P3 primer, the second all but the P4 primer and the last one, all but the template. Twenty microliters of each of these reaction mixtures from the amplifications described were loaded onto 2.0 % agarose gels and electrophoresed at 70 Volts for 90 min.

2.2.8 Excision and Amplification of the 230 bp Region of the Agarose Gel

A piece of saran wrap was placed on a transilluminator. The gel containing the P3 and P4 primer amplification ‘products’ was placed on the saran wrap. A section of
the agarose in the range of approximately 230 base pairs was excised using a sterile scalpel blade. Excision of agarose was performed very cautiously, allowing the blade to touch only the area desired for amplification. A small piece of the agarose, the size of the tip of a pen was used in place of the microliter of bovine genomic DNA template usually used. This little fragment was placed in a 600 μL tube and the reagents added as outlined in ‘basic methodology of PCR’. Cycling was performed using an annealing temperature of 60 °C. Again, three controls were made up. One with all but the right primer, one with all but the left primer and a negative with all but the template. These samples were electrophoresed in a 2.0 % agarose gel for 2 h at 70 Volts.

2.2.9 **Hemi-Nested PCR using P3 and the Internal Primer P5**

The 230 bp band amplified in ‘Excision and amplification of the 230 bp region of the agarose gel’ was used in the same manner as template for a hemi-nested PCR. A nested PCR is a second PCR performed with a set of primers that are nested a few bases in from and overlapping a first set of primers. This nested PCR produces a slightly smaller piece of DNA within the target. A hemi nested PCR is one in which only one of the primers from the second set is nested, while the second one is the same as the one from the first set. This again will result in a slightly smaller fragment with only one of the termini being shorter than the other.

Again, a small piece of agarose containing the 230 bp band was cut out with a sterile scalpel blade and allocated into three PCR tubes, two of which would serve as controls. The hemi nested PCR was set up using the internal probe P5 (used as a right primer) and P3 as a primer set. This PCR would be used to determine whether the
fragment isolated was in fact the right one if a fragment proportional to the length of nucleotide segment encompassed by these two primers on the BPPI amino acid sequence was produced. This fragment in theory would be 120 bp in size (Figure 2-1).

PCR was set up as outlined in ‘basic methodology of PCR’ using an annealing temperature of 60 °C. Twenty microliters of the amplification reaction was electrophoresed in a 2.0 % agarose gel for 2h at 70 V.

2.2.10 PCR using P6 and P7 Primers

Primers P6 and P7 were those designed according to the nucleotide sequence of the SI (II) gene outside of the region encoding the mature protein (Figure 2-2). PCR was set up as described in ‘basic methodology of PCR’ with the exception of the annealing temperature. The first reaction set was performed with an annealing temperature of 55 °C, and the second was performed at 60 °C to reduce smearing. Once again, appropriate controls were also set up. The products of amplification were loaded and electrophoresed on a 2.0 % agarose gel at 70 V for 2 h.

2.2.11 Cloning of 230, 172 and 450 bp Products into pCR® 2.1 Vector

The ligation and transformation of the 230 bp insert was performed by Ann Marie Butler from the Department of Biological Sciences at the University of Windsor. The procedure followed for ligation and transformation was outlined in the Original TA Cloning Kit manual by Invitrogen.

2.2.11.1 Ligation

The same day that ligation was to be performed, a new PCR was set up with the
exception that the final elongation was extended for 30 min. The inserts to be ligated were the 230, 172 and the 450 bp products. Thus the respective conditions used to generate each product was repeated.

Ligation was set up by adding the following to a 600 μL thin walled tube: 1 μL of a 2 in 10 dilution of the newly generated PCR product, 1 μL of 10 x ligation buffer containing 60 mM Tris-HCl pH 7.5, 60 mM MgCl₂, 50 mM NaCl, 1 mg/mL BSA, 70 mM β- mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol and 10 mM spermidine, 2 μL of pCR®2.1 vector (25 ng/μL), 6 μL sterile water and 1 μL of T4 DNA ligase (4.0 Weiss units). The solution was mixed by tapping the bottom of the tube gently then placed in the thermocycler overnight using the soak mode programmed at 14 °C.

2.2.11.2 LB Medium and Plates

One liter of Luria Bertani medium containing 1.0 % tryptone, 0.5 % yeast extract and 1.0 % NaCl, pH 7.0 was made with deionized water. Five hundred milliliters of this solution was transferred to another 1 L Erlenmeyer flask designated for agar. Seven and a half grams of agar (15 g/L) was added to the flask and stirred. Both flasks were capped with aluminum foil and autoclaved on slow exhaust for 20 min. at 15 psi. Twenty-five milligrams or 50 μg/mL of kanamycin were added to each flask once the solutions attained 55 °C. After the addition of kanamycin, the solution containing the agar was aliquoted in 30 mL portions into approximately 14 sterile culture plates using aseptic technique. Once solid, the plates were placed in a 37 °C incubator overnight. Both the plates and the medium were stored at 4 °C until needed.

In order to distinguish colonies based on their ability to metabolize X-Gal 40 μL
of a 40 mg/mL X-Gal solution was spread over the plated agar with a bent glass rod prior to use. This procedure was performed using aseptic technique. Once the X-Gal solution was absorbed the plates were ready for use.

The 40 mg/mL X-Gal solution was made by dissolving 200 mg of X-Gal in 5 mL of dimethylformamide. The solution was stored in a dark bottle at −20 °C.

All media or plates containing any E. coli were disposed of accordingly. The media and containers used to hold it were soaked overnight in 10% bleach, while the plates were autoclaved at 15 psi for 20 min. then discarded.

2.2.11.3 Transformation

The procedure followed for transformation was outlined in the Original TA Cloning® Kit manual. Two microliters of 0.5 M β-mercaptoethanol and 2 μL of the ligation reaction were added to a 1.7 mL vial containing a 50 μL suspension of competent EcoRI INVαF' cells on ice. Heat shock was performed by placing the tube in a 42 °C water bath for exactly 30 s then on ice for 2 min. Two hundred and fifty microliters of SOC media containing 2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂-6H₂O and 20 mM glucose was added to the tube at room temperature. The tube was capped and placed horizontally at 37 °C in a rotary, shaking incubator for 1 h.

Two LB plates containing X-Gal were equilibrated in a 37 °C incubator for 30 min. prior to use. Fifty microliters of the transformation mixture was spread on one plate and 200 μL on the second plate using a bent glass rod. The glass rod was flamed with ethanol between use. Once the liquid was absorbed, the plates were placed upside down in a 37 °C incubator overnight. The plates were usually left in the incubator for 18 h or
until the colonies attained a sufficient size. Colour development was achieved by placing the plates at 4 °C for 1 h.

2.2.12 Plasmid Isolation

Colonies were purified and plasmids isolated by the alkali lysis method, using a scaled up version of a mini prep outlined in Sambrook et al., 1989. Typically, 8-12 colonies from each transformation were chosen for plasmid isolation. Colonies, which were round, white (slightly blue in the case of the colonies containing small inserts) and with a diameter of approximately 2 mm, were chosen for further investigation. Each colony was labeled on the plate for the purpose of reference. A small portion of the colony was transferred with a sterile toothpick to a labeled, sterile, 50 mL conical tube containing 10 mL of LB media with 50 µg/mL kanamycin. These tubes were loosely capped and placed in a 37 °C shaker for 8 h.

After incubation, the tubes were centrifuged at 5,930 x g in a Beckman J2-HS centrifuge with the JA-20 rotor for 15 min. at 4 °C. Without disrupting the pellet, as much supernatant as possible was removed using a glass pipette attached to a vacuum line. This pellet was suspended in 200 µL of ice cold solution I by vortexing. Solution I was made of 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8. The suspension was transferred to a microcentrifuge tube and placed on ice for 5 min. Four hundred microliters of freshly prepared solution II containing 0.2 N NaOH and 1 % SDS was added to the tube, the tube was mixed by inversion and placed on ice for 5 min. Three hundred microliters of ice cold solution III was added, vortexed for 2 s., then placed on ice for 5 min. Solution III was made of 60 mL of 5 M KCH₃COO, 11.5 mL of glacial
acetic acid and 28.5 mL of water. The resulting concentration of solution III was 3 M K⁺ and 5 M CH₃COO⁻. The tube was then spun in a Fisher, model 59A Micro-Centrifuge at 12,600 x g for 5 min at 4 °C and 600 µL of the resulting supernatant was transferred to a clean microcentrifuge tube, being careful to not transfer any precipitate. The DNA was precipitated by adding 600 µL of anhydrous ethanol and 60 µL (1/10 vol.) 0.3 M NaCH₃COO, pH 5.2. The solution was vortexed briefly, and incubated at room temperature for 2 min. The tube was centrifuged in a Fisher, model 59A Micro-Centrifuge at 12,600 x g for 5 min. and the supernatant discarded. The resulting pellet was rinsed by adding 1 mL cold 70 % ethanol with very gentle tapping of the bottom of the tube. The supernatant was aspirated and the pellet allowed to air dry for approximately 10 min. at room temperature. The pellet was then suspended in 100 µL of TE, pH 8.0 buffer containing DNase free RNase A and placed in a 37 °C water bath for 5 h. The DNase free RNase A solution was made by dissolving 1 mg/mL pancreatic RNase A in a pH 8.0, 10 mM Tris-HCl and 1 mM EDTA solution and boiling for 10 min. The DNA was once again recovered by precipitation with ethanol as described above. Once the resulting pellet was completely dry, it was suspended in 60 µL sterile water.

2.2.13 Estimation of Quantity and Purity of Plasmids Isolated

A UV-Vis spectrophotometer was set in photometric mode with 260 and 280 nm as the first and second wavelength respectively. Each plasmid sample was measured by placing 3 µL of the 60 µL plasmid in sterile water solution and 497 µL of water in an appropriate cuvette. The contents of the cuvette were mixed well with the pipette tip used to dispense the solution, then the absorbance was measured and recorded. The
quantity of plasmid was calculated using the assumption that one absorbance unit at 260 nm was approximately equivalent to 50 µg/mL of double stranded DNA (Sambrook et al., 1989). The purity was taken from the ratio of the absorbance at 260 nm to the absorbance at 280 nm (Sambrook et al., 1989). Cuvettes were cleaned between samples with one rinse of ethanol followed by five rinses with deionized water. These measurements were repeated for each of the plasmids isolated.

2.2.14 Restriction Analysis of Plasmids

An appropriate volume of plasmid sample, corresponding to approximately 0.1 to 1 µg DNA was digested by 1 to 3 units of EcoRI. Digestion was performed by placing 0.1 – 1 µg of the plasmid, 7 µL of sterile water, 1 µL of 10 x digestion buffer and 1 µL of the enzyme diluted previously in 1 x digestion buffer into a microcentrifuge tube. The 10 x digestion buffer consisted of 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 mM NaCl, 0.02 % Triton X-100. The tube was capped, gently tapped to mix, spun down briefly, and placed in a 37 °C water bath for 2 h.

In general, all the plasmids isolated were subjected to digestion with EcoRI in order to determine if an insert of the appropriate size was ligated into the vector. This includes the plasmids containing the 230 bp, 172 bp and 450 bp inserts. The 172 and 450 bp products, before insertion, were also subjected to digestion as described above. These were performed as controls to detect any possible EcoRI sites within them.

Both the digested plasmids and the digested PCR products (before insertion) were electrophoresed on 1.3 – 2.0 % agarose gels alongside their corresponding undigested plasmids. The digested samples were prepared for loading by adding 10 µL of gel
loading buffer and 5 μL of 1 x TBE directly to the tube. The undigested samples were prepared by adding 1 μL of the undigested sample, 10 μL of gel loading buffer and 5 μL of 1 x TBE.

2.2.15 PCR on Insert in Plasmids

The clones thought to contain the corresponding P6/P7 generated insert as determined by size through restriction analysis were subjected to PCR to confirm that the DNA inserted was recognized by the P6 and P7 primers. This was done using the same parameters to generate the DNA in the first place. In this case however, the template was 3 - 4 ng of plasmid.

The resulting amplification from PCR on the 450 bp insert containing plasmid was loaded and electrophoresed on a 1.4 % agarose gel and the products from PCR on the 172 bp insert containing plasmid on a 1.5 % agarose gel.

2.2.16 Glycerol Stocks

A solution of 80 % glycerol in sterile water was made and aliquoted into 20 mL scintillation vials. These vials were loosely capped and autoclaved at 15 psi for 20 min.

Fresh overnight cultures were grown to no more than 0.6 absorbance units at 600 nm. Using aseptic technique, 850 μL of the fresh culture and 150 μL of 80 % glycerol were transferred to an autoclaved 1.7 mL tube equipped with a sealing, threaded lid. The tube was sealed, frozen in liquid nitrogen and placed in a - 80 °C freezer for long term storage. Glycerol stocks were made using colonies containing plasmids with appropriately sized inserts as determined by such diagnostic tests as restriction digestion.
with EcoRI and amplification by PCR.

2.2.17 Sequencing of the Inserts in the pCR®2.1 Vector

Sequencing of the 230 bp insert was performed by Ann Marie Butler from the Department of Biological Sciences at the University of Windsor, Ontario. The 172 and 450 bp inserts were sequenced by Dr. Mike Hagen at the Biological Sciences Core Facility at Wayne State University, Detroit, Michigan.

2.2.17.1 Sequencing of the 230 bp Insert

This procedure was performed as outlined in the Promega fmol DNA Sequencing System manual. Four 600 μL tubes were labeled G, A, T and C. Two microliters of each of the d/ddNTP mixes were added to the appropriately labeled tubes. A primer/template/enzyme mix was made by adding the following to a 600 μL microcentrifuge tube: 1 μL or 100 ng of the plasmid template, 5 μL of fmol 5x sequencing buffer containing 250 mM Tris-HCl, pH 9.0 and 10 mM MgCl₂, 1.5 μL of ³²P labeled primer (1.5 pmol), 8.5 μL sterile water and 1 μL of Sequencing Grade Taq DNA Polymerase (5 U/μL). This enzyme/primer/template solution was mixed and aliquoted into 4 μL portions in each of the 600 μL tubes containing the d/ddNTP mix. Each reaction mixture was overlaid with one drop of mineral oil and spun briefly.

This procedure was performed in duplicate, one set of four tubes contained the ³²P labeled M13 reverse primer and another, the ³²P labeled T7 primer. Thus a reaction in which the sequence was to be determined in both directions, consisted of eight tubes in total.
The thermal cycler was programmed to cycle between denaturation, annealing and extension. It consisted of 95 °C for 2 min., to start, denaturation at 95 °C for 30 s, annealing at 42 °C for 30 s and extension at 70 °C for 1 min. The thermocycler was programmed for 30 cycles and to end with a final soak at 4 °C. The tubes were placed in the block during the first step after it attained a temperature of 95 °C.

After cycling, each reaction was stopped by adding 3 μL of fmol Sequencing Stop Solution containing 10 mM NaOH, 95 % formamide, 0.05 % bromophenol blue and 0.05 % xylene cyanole. These samples were heated to 70 °C for 2 min. before loading into the wells. Three microliters of each sample were loaded onto a 7 % acrylamide gel containing 40 % w/v urea in 1 x TBE buffer. The gel was electrophoresed at 1600 V.

Once electrophoresed, the gel was fixed for 5 min. in a 10 % methanol and 10 % acetic acid solution. The gel was then dried for 30 – 40 min. under a vacuum on a commercial gel dryer at 80 °C. An autoradiograph was made by exposing the gel to X-ray film for 16 hours at room temperature.

The 230 bp insert was sequenced in both directions but only the M13 reverse reaction was electrophoresed and autoradiographed.

2.2.17.2 Sequencing of the 172 and 450 bp Inserts

Sequencing of the 172 and 450 bp inserts was performed following a procedure outlined in the ABI PRISM™ Dye Primer Cycle Sequencing Core Kit manual. M13 Reverse and T7 dye-labeled primers were used to detect the sequences generated using AmpliTaq® cycle sequencing methodology. This involved four separate base-specific extension reactions, each containing the appropriate dye labeled primer.
Four 600 μL tubes were labeled G, A, T and C. The appropriate d/ddNTP mix was added to the appropriate 600 μL tube; 1 μL of the A and C mix and 2 μL of the G and T mixes. Mix A contained optimized quantities of ddATP, dATP, dCTP, 7-deaza-dGTP and dTTP. Mix C contained optimized quantities of ddCTP, dATP, dCTP, t-deaza-dGTP and dTTP. Mix G contained optimized quantities of ddGTP, dATP, dCTP, 7-deaza-dGTP and dTTP. Mix T contained optimized quantities of ddTTP, dATP, dCTP, 7-deaza-dGTP and dTTP. The concentrations of these mixtures were unavailable to the consumer.

The next reagents added to the tubes were the dye-labeled primers. One microliter of A (JOE) and C (FAM) dye labeled M13 reverse primer were added to the tubes labeled A and C. Two microliters of G (TAMRA) and T (ROX) dye labeled M13 reverse primer were added to the tubes labeled G and T.

One microliter of 5 x sequencing buffer was added to the A and C tubes and 2 to the G and T tubes. The sequencing buffer contained 400 mM Tris-HCl, pH 9.0 and 10 mM MgCl₂. One microliter of AmpliTaq® DNA polymerase, FS solution was added to each of the A and C tubes and 2 to the G and T tubes. The concentration of the DNA polymerase was not available to the purchaser.

The plasmids were prepared for sequencing by suspending them in sterile water at a concentration of 200 ng/μL. For sequencing, 1.2 μg of plasmid was required for each primer at this concentration. One microliter of the 200 ng/μL insert containing plasmid was added to the A and C tubes and 2 to the G and T tubes.

The same procedure was followed to sequence the inserts in the 3’ to 5’ direction. For this procedure dye labeled T7 primer was used in each of the tubes as opposed to the
dye labeled M13 reverse primer.

The 172 bp insert was sequenced with both the M13 reverse and the T7 primer. The 450 bp insert was sequenced with the M13 reverse primer only.

Each reaction mixture was overlaid with approximately 20 μL of mineral oil. Cycling involved a rapid thermal ramp to 95 °C for 30 seconds, a rapid thermal ramp to 55 °C for 15 seconds and a rapid thermal ramp to 70 °C for 60 s. Cycling was repeated for 25 cycles and ended with a thermal ramp to 4 °C.

Once the cycling was complete, the products generated were combined and purified using a simple ethanol precipitation. Two microliters of 3 M sodium acetate, pH 4.6 and 50 μL 95 % ethanol were placed in a 1.5 mL microcentrifuge tube. The entire 20 μL content of the reaction tube was transferred to the ethanol mixture, vortexed and placed on ice for 10 min. This mixture was centrifuged in a micro-centrifuge at 12,600 x g for 15-30 min. The resulting supernatant aspirated and the pellet rinsed with 250 μL of 70 % ethanol. The 70 % ethanol was again aspirated and the pellet allowed to air dry to completion before proceeding. The pellet was then suspended in loading buffer. The loading buffer was prepared by combining deionized formamide and 25 mM EDTA (pH 8.0) containing 50 mg/mL Blue dextran at a ratio of 5:1 formamide to EDTA/Blue dextran. Three to four microliters of this loading buffer were added to the sample. The sample was vortexed, spun down, heated to 90 °C for two minutes and stored on ice until ready to load.

The DNA fragments generated were loaded in a 6 % acrylamide and 8.3 M urea in 1 x TBE gel and electrophoresed at 1700 V. A typical gel had 36 wells and dimensions of 39 cm in length, 21.6 cm in width and 4 mm in thickness. Fragments were
detected by the ABI 373 DNA Sequencer.

2.2.18 Analysis of the 230, 172 and 450 bp Sequences

The sequence of the 230 bp product was read directly from the autoradiograph. Each lane in the gel corresponded to the termination products of one of the bases. Lane one corresponded to the fragments ending in G, lane 2, fragments ending in A, lane 3, fragments ending in T and lane 4, fragments ending in C. Starting from the top of the gel, the bases were read off sequentially until the bands were indiscernible. Within the sequence, the P3 primer was located. This sequence, starting at the P3 primer, was entered into the Basic Local Alignment Search Tool (BLAST), a freeware program linked to the National Center for Biotechnology Information (NCBI) at http://www3.ncbi.nlm.nih.gov/. The original BLAST algorithm is described in the Journal of Molecular Biology (Altschul et al., 1990) but credit for this particular program is given to Madden et al., 1997. Two searches on the sequence were made. The first was performed with the use of the program blastn, which compared the nucleotide sequence query against a nucleotide sequence database. The second was performed using the program blastx that compared the nucleotide sequence query translated in all six reading frames against a protein sequence database. The databases searched by the program were GenBank, EMBL, DDBJ, PDB, SwissProt and PIR. Statistics were also provided for the matches found.

The sequences of the 172 and 450 bp fragments were read directly off the traces provided by the ABI 373A Sequencer. The same procedure for analysis was followed for these sequences. In short, the P6/P7 primers were located in the sequences. The
sequences were then entered into both the blastn and blastx BLAST programs to perform database searches and statistics for judging the matches found.

2.2.19 Design of Ideal Primers using Primer3 Output

Primer3 Output is a freeware program found at http://www.genome.wi.mit.edu (Rozen and Skaltsky, 1997). The entire SI (II) sequence was entered in the space given. Product size and primer sizes were specified. The product size chosen was 230 - 900 bp and the primer size chosen was 18 bp. The query was submitted.
3. Results

3.1 Isolation of Genomic DNA from Whole Calf Blood

A typical preparation of 5 mL of blood containing anticoagulant yielded between 10 to 30 μg genomic DNA with a purity greater than 1.8 as calculated from the ratio of its absorbance at 260 and 280 nm (Sambrook et al., 1989).

In isolating the DNA, the first centrifugation resulted in a pellet that was usually washed and suspended in TKM1 three times before it became free of reddish brown colour. Care was taken to remove all the supernatant between washes. In the final step of the preparation, precipitation with ethanol, the DNA was visualized within approximately one minute after inversion of the test tube. After incubating the tube at 65 °C for 15 min., which was found to be sufficient time to remove traces of ethanol, the DNA was removed from the test tube and placed in a microcentrifuge tube containing 500 μL of TE, pH 8 storage buffer.

Four, 7 and 15 μL of this solution, corresponding to approximately 0.2, 0.4 and 0.9 μg DNA were loaded onto a 0.5 % gel (Figure 3-1). The resulting bands were broad. An estimate of the size of this band by regression analysis was 40 kb.

3.2 Design of Primers for PCR

A summary of all the PCR primers and internal probes are listed in Table 3-1. The first set P1 and P2 and second set P3 and P4 were designed according to the amino acid sequence of BPPI (Li, 1992). The first set were 17 bases in length, each having a degeneracy of 256 fold, GC content of 42–76 % and melting temperatures of 50 – 60 °C. The second set had less degeneracy: P3 had a value of 128 fold and P4 a value of 64 fold
Figure 3-1: 0.5 % agarose gel of bovine genomic DNA. The genomic DNA was sized at approximately 40 kb. Lanes 1, 2 and 3 contain 0.9 μg, 0.4 μg and 0.2 μg DNA respectively. Lane 4 contains the marker λ DNA/EcoRI + Hind III. Bands are at 21,226, 5,148, 4,973, 4,268, 3,530, 2,027, 1,904, 1,584, 1,375, 947, 831 and 564 base pairs.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Degeneracy</th>
<th>% GC</th>
<th>*Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>ACNCCNGGNTGYGAYAC</td>
<td>256</td>
<td>47 - 76</td>
<td>50 - 60</td>
</tr>
<tr>
<td>P2</td>
<td>AGRTTYTCNCGGNCC</td>
<td>256</td>
<td>42 - 72</td>
<td>50 - 60</td>
</tr>
<tr>
<td>P3</td>
<td>ACNCCNGGNTGYGACACC</td>
<td>128</td>
<td>56 - 78</td>
<td>56 - 64</td>
</tr>
<tr>
<td>P4</td>
<td>NAGRTTYTCNCGGGGGC</td>
<td>64</td>
<td>56 - 78</td>
<td>56 - 64</td>
</tr>
<tr>
<td>P5</td>
<td>NGCYTTNGCRRTRTRARAARTA</td>
<td>572</td>
<td>25 -50</td>
<td>50 - 64</td>
</tr>
<tr>
<td>P6</td>
<td>ACTCCAGGGTTGTGACACC</td>
<td>0</td>
<td>61</td>
<td>58</td>
</tr>
<tr>
<td>P7</td>
<td>CAGTTTCTCGGGGCCC</td>
<td>0</td>
<td>78</td>
<td>64</td>
</tr>
</tbody>
</table>

*melting temperature based on the simple equation 2(A+T) + 4(G+C)*

**Table 3-1: A summary of the primers used for probing BPPI.** This table lists the primer's sequence, degeneracy, % GC, and melting temperature. N, Y and R represent points of degeneracy. N represents a mixture of A, G, C and T, Y represents a mixture of C and T, R represents a mixture of A and G.
degeneracy. The increased specificity in the 3' end narrowed the ranges of GC content and melting temperatures (Table 3-1).

The third set was designed according to the DNA sequence of the spleen inhibitor (II) (Creighton and Charles, 1987). These primers contained no degenerate bases and had defined GC content and melting temperatures.

3.3 PCR using P1 and P2 Primers

All PCR reactions were performed as outlined in ‘basic methodology for PCR’ as outlined in section 2.2.5. The first PCR was performed using an annealing temperature of 58 °C to start. The second set was performed lowering the annealing temperature from 58 °C to 50, 45, and 40 °C. Proper controls were set up as described in ‘basic methodology for PCR’. The third was performed with an increase in the mass of the template to 0.3 μg. The fourth set was a variation of the concentration of MgCl₂ from 2.5 mM to 1.0, 3.0 and 5.0 mM. A final PCR was performed using 2 x the quantity of primer; 100 pmol as opposed to 50 with an annealing temperature of 45 °C. None of these reactions resulted in amplification.

3.4 PCR using P3 and P4 Primers

PCR was set up using the primers P3 and P4, and applying the conditions outlined in ‘basic methodology of PCR’ with the exception that an annealing temperature of 40 °C was used. This resulted in the formation of two bands at 1350 and 600 bp (Figure 3-2) and a very faint smear ranging from approximately 600 bp to 200 bp. The control PCR
Figure 3-2: 2.0 % agarose gel of PCR products generated with P3 and P4 primers. Lane 1 contains PCR products generated by P3 and P4. Lane 2 contains the pBR322 marker. Lane 3 (control) contains PCR products generated by the P4 primer only. Lane 4 (control) contains PCR products generated by the P3 primer only and lane 5 contains a negative control of PCR with P3 and P4 and no template. Note that in lane 1 the two bands at 1350 and 600 bp generated, are artifacts of the P4 primer (they are also present in lane 4). The pBR322 marker contains nucleotide fragments of the following sizes: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104 and 89 bp. Note that numbers without arrows indicated select nucleotide marker band sizes in lane 2 and those with arrows indicate PCR products in lane 1.
reaction, in which only the right primer P4 was used, demonstrated the presence of these two bands as well.

Two subsequent PCR reactions were performed using an increased stringency in the annealing temperature. One was performed at 55 °C and the next at 60 °C. The increase in annealing temperature resulted in the same banding pattern as the amplification at 40 °C but with crisper bands at 1350 and 600 bp and an almost invisible smear of products between 600 and 200 bp. Figure 3-2 is a 2.0 % agarose gel electrophoresed for 90 min. at 70 Volts containing the products from the amplification using an annealing temperature of 60 °C. It demonstrates the 1350 and 600 bp bands in both lanes one and three and a very faint smear ranging from approximately 600 to 200 bp. Note that lane one is a PCR performed with both primers and lane three is a control PCR performed with only the P4 primer.

3.5 Excision and Amplification of the 230 bp Region of the Agarose Gel

A small section of the agarose gel containing the ‘products’ of the P3/P4 amplification (Figure 3-2) was excised. The region taken was adjacent to the φX174 digested with Hae III, 234 bp marker. This region corresponded to a hypothetical size of the BPPI gene. This excised morsel was used as a template and further amplified with P3/P4 primers using the parameters outlined in ‘basic methodology of PCR’. Products were electrophoresed in a 2.0 % agarose gel in lanes 1, 2 and 3 in Figure 3-3. This band was sized at 230 bp by regression analysis.
Figure 3-3: 2.0 % agarose gel of amplification of the 230 bp region. Lanes 1, 2, and 3 all contain the same amplified product; sized at 230 bp. Lane 4 contains the φX174 marker. Marker sizes are 1353, 1078, 872, 603, 310, 281, 271, 234, 194 and 118 bp. Note that the numbers without arrows indicate select marker bands in lane 4.
3.6 Nested PCR using P3 and the Internal Primer P5

The amplified product, forming a discrete band at 230 bp (Figure 3-3) from the PCR with P3 and P4 primers was used as a template for a nested amplification using the internal probe P5 as a right primer and the original left primer P3. This amplification was performed using the same parameters to generate the 230 bp band outlined in ‘basic methodology of PCR’ using an annealing temperature of 60 °C. Visualization of the products on a 2.0 % agarose gel shows the generation of a product at approximately 130 bp in size, as determined by regression analysis (lane 2, Figure 3-4).

3.7 PCR using P6 and P7 Primers

PCR was set up as described in ‘basic methodology of PCR’ with proper controls using P6 and P7 primers and an annealing temperature of 55 °C. PCR using an annealing temperature of 55 °C produced too much smearing, thus, a follow up of this reaction was performed using an annealing temperature of 60 °C. Visualization of the products from the reaction performed with an annealing temperature of 60 °C on an agarose gel (Figure 3-5) shows two legitimate crispier bands and less smearing. These bands were sized at 450 and 172 bp using regression analysis. The band at 450 was more prominent than the one at 172.

3.8 Cloning of 230, 172 and 450 bp PCR Products into the pCR® 2.1 Vector

The ligation and transformation of the 230 bp insert was performed by Ann Marie Butler from the Department of Biological Sciences at the University of Windsor.
Figure 3-4: 2.0 % agarose gel of amplification using P3/P5 primers and agarose template. Amplification was performed using P3/P5 and a small morsel of agarose gel excised from 230 bp region of a primary amplification with P3/P5. Lane 1 contains the φX174 marker and lane 2 contains the amplification product at 130 bp (band barely visible). Numbers without arrows correspond to sizes of some of the molecular weight bands in lane 1. The sizes of the nucleotide markers are 1353, 1078, 872, 603, 310, 281, 271, 234, 194 and 118.
Figure 3-5: 2.0 % agarose gel of PCR products generated with P6 and P7 primers. Lane 1 contains the pBR322 marker. Lane 2 contains PCR products using only the right primer P7 (control). Lane 3 contains PCR products using the left primer P6 only (control). Lane 4 contains PCR products using both the left and right primers. Lane 5 contains the φX174 marker. Lane 6 is a negative control of a PCR with both primers and no template. The 450 and 172 bp products (lane 4, bands barely visible) are indicated by arrows. The pBR322 marker contains bands of the following sizes: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104 and 89 bp. The φX174 marker in lane 5 contains the following bands: 1353, 1078, 872, 603, 310, 281, 271, 234, 194 and 118 bp (select band sizes are indicated on the right side of the diagram without arrows).
3.8.1 Ligation

Ligation was performed while the PCR generated fragments were no more than 2 h old. Each of the 230, 172 and 450 bp products were generated using the appropriate conditions described in ‘Methods’. Lane 2 in Figure 3-6 contains the 230 bp band generated specifically for insertion. Lane 2 in Figure 3-7 shows the 172 bp fragment generated for insertion. This lane contains two products sized at 172 and 236 bp. The 236 bp band appeared on three separate occasions when the 172 bp band was amplified for ligation. The 172 bp band was more pronounced. This mixture of bands was used in the ligation reaction on the premise that statistically, the likelihood of incorporation would favour the more prominent 172 bp product. Lane 3 of Figure 3-8 contains the 450 bp generated product before insertion into the plasmid.

Ligation of the newly generated PCR fragments into the pCR®2.1 was complete within 14 to 18 h at 14 °C.

3.8.2 Transformation

Two microliters of the ligation mixture were used for transformation into the EcoRI INVαF’ competent cells. Following heat shock, a brief incubation, and plating of the newly transformed cells, the plates were incubated for 18 h. This was found to be sufficient time for the growth of appropriately sized colonies. The number of colonies that appeared after incubation varied greatly for each transformation. The smallest number of colonies, blue and white included, was seven and the largest was thirty for the plates containing 50 μL of cell suspension. Colonies which were well rounded, white and having a diameter of approximately 2 mm had a success rate of 70 – 90 % of
Figure 3-6: 2.0 % agarose gel of digestion of plasmid containing the 230 bp insert. Lane 1 contains a 1 kb ladder, lane 2 contains the 230 bp PCR product, lane 3 contains the pCR®2.1 plasmid before digestion, lane 4 the plasmid cut with EcoRI and lanes 5, 6 and 7 contain EcoRI cut, insert containing plasmids. Size of 230 bp product outlined on right side of diagram with an arrow. Nucleic acid markers are 10.0, 8.0, 6.0, 5.0, 4.0, 3.0, 2.5, 2.0, 1.5, 1.0 and 0.5 kb. Sizes of select marker bands are outlined on the left of the diagram. Photo courtesy of Ann-Marie Butler.
Figure 3-7: 1.5 % agarose gel of digestion and PCR of plasmid containing the 172 bp insert. Lane 1 contains the marker ϕX174, lane 2 PCR products to be inserted, lane 3 digestion of PCR product with EcoRI (control), lane 4 PCR on plasmid (clone 2) containing insert, lane 5 digestion of clone 2 with EcoRI, lane 6 clone 2 plasmid before digestion. Note that the PCR insert contains no EcoRI sites (lane 3), and that the insert generated by PCR on bovine genomic DNA (lane 2) is the same size as the band produced by PCR on the plasmid (lane 4) and digestion of the plasmid (lane 5). Lane 2 contains two bands, one at 236 bp and the other at 172 bp as indicated by the arrows on the left of the diagram. The ϕX174 nucleotide markers in lane 1 are 1353, 1078, 872, 603, 310, 281, 271, 234, 194 and 118 bp (select ϕX174 markers are indicated on the left side of the diagram without arrows). Note the banding pattern of the plasmid before and after digestion. Lane 6 contains two high molecular weight bands of plasmid origin at 3750 and 2400 bp. Lane 5 (after digestion) contains one very intense band at 2500 bp (indicated by arrows on the right side of the diagram).
Figure 3-8: 1.3 % agarose gel of digestion of plasmid containing the 450 bp insert. Lane 1 contains the φX174 marker. Lane 2 contains the 450 bp insert digested with EcoRI as a control. Lane 3 contains the 450 bp PCR product before insertion. Lane 4 contains clone 10 plasmid subjected to digestion with EcoRI. Lane 5 contains clone 10 plasmid before digestion. Lane 6 contains clone 9 plasmid subjected to digestion with EcoRI. Lane 7 contains clone 9 plasmid before digestion. Note that clone 9 contains the 450 bp insert (lane 6) and clone 10 does not (lane 4). Note also that the 450 bp insert contains no EcoRI sites (lane 2). The 450 bp fragment is outlined by the arrow on the left side of the diagram. The φX174 marker contains fragments of the following sizes: 1353, 1078, 872, 603, 310, 281, 271, 234, 194 and 118 bp (select φX174 markers are indicated on the left of the diagram without arrows).
containing plasmids with the appropriately sized inserts.

In the case of the *E. coli* with plasmids containing the 230 and 172 bp inserts, however, only a small fraction of the total colonies that grew on the plates were white. Colonies that were slightly blue in colour were also chosen for plasmid isolation and found to contain the appropriate inserts.

3.9 **Plasmid Isolation**

Colonies were purified and their plasmids isolated using the alkali lysis method (Sambrook *et al.*, 1989). Incubation of the colonies in media for 8 to 9 h. in a 37 °C rotatory incubator shaker was found to be the ideal time for optimal recovery of plasmids.

RNA was usually digested after a 5 h. incubation with RNase A. Digestion products were detected by the appearance of a smear of small nucleotide segments of approximately 50 – 100 bases in size when visualized on an agarose gel (not shown). Following digestion, the plasmids were once again recovered by ethanol precipitation then resuspended in 60 μL of sterile water. Samples to be sequenced were suspended in water as opposed to TE which would interfere with sequencing.

3.10 **Estimation of Quantity and Purity of Plasmids Isolated**

A UV-Vis spectrophotometer and the appropriate cuvettes were used to assess the quantity and purity of each sample processed. Absorbances at 260 and 280 nm were measured by placing 3 μL of the plasmid solution and 497 μL of water in a cuvette. A typical preparation yielded values between 0.1 – 0.2 absorption units at 260 nm and 0.05 – 0.1 absorption units at 280 nm. Purity of plasmids or the ratio of the absorbance at 260
nm to 280 nm usually resulted in a value equal to or slightly greater than 1.8. Sample quantities were usually between 1 to 4 µg of DNA per sample. The spectrum in Figure 3-9 obtained at a wavelength range of 240 to 310 nm was typical of all plasmids prepared.

3.11 *Restriction Analysis of Plasmids*

Digestion of the plasmids with *EcoRI* was complete after 2 h of incubation at 37 °C. Restriction analysis of plasmids containing the 230 bp insert is shown in the agarose gel in Figure 3-6. Lanes 5, 6 and 7 contain plasmids digested with *EcoRI*. Each lane contains a new band sized at 230 bp. This same procedure was followed for the two other plasmids containing the 172 and 450 bp products.

Typically a digestion was performed on the PCR product itself as a control to assure that there were no *EcoRI* digestion sites in the PCR product/insert. No digestion sites were found in any of the inserts.

Figure 3-7 is a 1.5 % agarose gel, which exhibits the digestion of clone 2, 172 bp insert containing plasmid with *EcoRI*. Note the appearance of a small sized band at 172 bp in lane 5. Lane 6 shows the plasmid before digestion for comparison. Recall that this particular ligation/transformation was performed using a pool of 172 and 236 bp products in which the 172 bp product was the more prominent product (Lane 2 in Figure 3-7). Of all the plasmids isolated and digested from this ligation/transformation, only the 172 bp product from the pool of the 172 and 236 bp fragments was incorporated into the plasmids.

Note also in Figure 3-7, the banding pattern of the plasmid before and after digestion. Lane 6 contains two large nucleotide bands at 3750 bp and 2400 bp of plasmid
Figure 3-9: Spectrum of wavelength vs. absorbance of plasmid DNA. This spectrum obtained at a wavelength range of 240 to 310 nm was typical of all plasmids prepared.
origin. Digestion of the plasmid (lane 5) results in one remaining high molecular weight band at 2500 bp and the 172 bp band below.

This figure also shows that no digestion sites existed in the 172 bp insert when comparing its banding pattern (lane 3) to its undigested counterpart in lane 2.

Figure 3-8 is a photograph of a 1.3 % agarose gel that shows the before and after digestion of clone 9, the 450 bp insert containing plasmid, with EcoRI in lanes 6 and 7, respectively. In lane 6, a 450 bp band appears after digestion of this plasmid. Conversely, clone 10, another of the plasmids isolated and digested did not contain the 450 bp insert. This was demonstrated by the lack of a 450 bp band in lane 4. The last point of interest from this digestion was that the 450 bp product did not contain any EcoRI digestion sites (lane 2).

3.12 PCR on Inserts in Plasmids

PCR was generally performed only on plasmids that through restriction analysis proved to contain an insert of the appropriate size. It was performed using the same conditions used to generate the inserts in the first place.

All of the plasmids subjected to PCR always resulted in an amplification of a product of the appropriate size as demonstrated when visualized on an agarose gel. Figure 3-7 is a 1.5 % agarose gel which shows that the insert in clone 2 produces amplification of a 172 bp insert when used as a template for PCR using primers P6/P7 (lane 4).

Figure 3-10 is a 1.4 % agarose gel which demonstrated that clone 2 plasmid used as a template does produce amplification of a 450 bp product when subjected to PCR.
Figure 3-10: 1.4 % agarose gel of the digestion and PCR on plasmids containing the 450 bp insert. Lane 1 contains the φX174 marker. Lane 2 contains digestion of clone 9 plasmid with EcoRI. Lane 3 contains digestion of clone 6 plasmid with EcoRI. Lane 4 contains digestion of clone 3 plasmid with EcoRI. Lane 5 contains the 450 bp insert generated using bovine genomic DNA as template. Lane 6 contains PCR on clone 2 plasmid. Lane 7 contains PCR on clone 2 plasmid using both primers P6 and 7 and no template. Lanes 2, 3 and 4 show that clones 9, 6 and 3 each contain the 450 bp insert, lanes 6 and 7 show that the 450 PCR bp band is a legitimate PCR product. The 450 bp fragment is indicated by the arrow on the right side of the diagram. The φX174 marker contains fragments of the following sizes: 1353, 1078, 872, 603, 310, 281, 271, 234, 194 and 118 bp (select φX174 markers are indicated on the left side of the diagram without arrows).
with the primers P6 and P7.

At this point it was conclusive that the 230, 172 and 450 bp inserts were incorporated in the plasmids.

3.13 Glycerol Stocks

Glycerol stocks of clones containing inserts were successfully made using overnight cultures exhibiting an absorbance of no more than 0.6 at 600 nm. Viability of glycerol stocks was demonstrated by growth and analysis upon transfer and incubation of 50 µL of the stock into 3 mL of LB broth. All clones that contained inserts of the appropriate sizes, as determined by digestion with EcoRI and amplification with the appropriate primers, were made up into glycerol stocks for long term storage in a -80 °C freezer.

3.14 Sequencing of the Inserts in the pCR\textsuperscript{2.1} Vector

The 230 bp insert was sequenced by Ann Marie Butler from the Biology Department at the University of Windsor, Ontario, and the 172 and 450 bp inserts were sequenced by Dr. Mike Hagen at the Biological Sciences Core Facility at Wayne State University, Detroit, Michigan.

3.14.1 Sequencing of the 230 bp Insert

The Promega fmol DNA Sequencing System was used for the 230 bp insert. The sequence was determined only in one direction, 5' to 3', using the M13 reverse \textsuperscript{32}P labeled primer. The sequence of this insert was read directly off the autoradiograph.
(Figure 3-11). The first lane in the autoradiograph corresponded to the base G, the second to A, the third to T and the fourth to C. Approximately 220 bases were comfortably read from it. Within the sequence, the primer P3 was located quite easily down stream from the M13 Reverse primer.

3.14.2 Sequencing of the 172 and 450 bp Inserts

These inserts were sequenced using the ABI PRISM™ Dye Primer Cycle Sequencing Core Kit by Perkin Elmer. The sequences of the 172 and 450 bp products were read off the traces. The traces were easily interpreted since each peak was colour coded. G was represented by the colour black, A by green and T and C by red and blue, respectively, on the output. Note that both outputs, the autoradiograph and traces, contained some vector sequence before the actual sequence started. For the 172 bp insert, two plasmids were sequenced in both directions 5' to 3' and 3' to 5' using M13 reverse and T7 dye-labeled primers, respectively. Figure 3-12 is a trace of the nucleotide sequence of the 172 bp insert using the M13 Reverse dye-labeled primer and Figure 3-13 is a trace of the nucleotide sequence generated using the T7 dye-labeled primer. Each of the 172 bp insert containing plasmids sequenced, gave the same results in both directions. The 450 bp insert was only sequenced using the M13 Reverse dye-labeled primer. The trace of the sequence using the M13 Reverse dye-labeled primers is shown in Figure 3-14.

3.15 Analysis of the 230, 172 and 450 bp Sequences

Figure 3-15 is a compilation of the sequences of the 230, 172 bp products and
Figure 3-11: Autoradiograph of the 230 bp sequence. Each lane of the sequencing gel corresponds to the termination products of one of the four bases. The lanes are G, A, T and C from left to right. The arrow indicates the first base (A) of the sequence (Autoradiograph courtesy of A. Butler).
Figure 3-12: Trace of the 172 bp nucleotide sequence generated in the 5' to 3' direction. The sequence was generated with the use of the M13 Reverse dye-labeled primer. Arrows encompass the sequence of the insert. Output from the ABI Applied Biosystems Model 373A.
Figure 3-13: Trace of the 172 bp nucleotide sequence generated in the 3' to 5' direction. This sequence was determined using the T7 primer. Arrows encompass the sequence of the insert. Output from the ABI Applied Biosystems Model 373A.
Figure 3-14: Trace of the 450 bp nucleotide sequence generated in the 5' to 3' direction. This sequence was generated using the M13 Reverse primer. Arrows encompass the sequence of the insert. Output from the ABI Applied Biosystems Model 377.
Figure 3-15: Compilation of the 230, 172 sequences. Sequence A is the sequence of the 230 bp product. B is a compilation of both the M13 Reverse and T7 primer sequences of the 172 bp fragment. The inferred primary sequence is indicated in one letter code above each of the nucleotide sequences. The primers used in generating the fragments are underlined. Primer labels are indicated in bold type. Asterisks denote stop codons.
Figure 3-16 is the sequence of the 450 bp product. Inferred amino acid sequences are listed above each sequence. The 230 bp sequence was taken from the autoradiograph (Figure 3-11). The 172 bp sequence was taken from the traces in Figures 3-12 and 3-13. The 450 bp sequence was taken from Figure 3-14. Vector, M13 Reverse and T7 sequences were not included in this diagram. The 230 bp sequence did not match up to the degenerate nucleotide sequence of BPPI. Nor did its inferred amino acid sequence share any likeness to the amino acid sequence of BPPI (Figure 2-1). Likewise, the nucleotide sequences and inferred amino acid sequences of the 172 and 450 bp products shared no homology with the nucleotide sequence or primary sequence of the spleen inhibitor (II) (Figure 2-2).

The sequences of the 230, 172 and 450 bp inserts were entered into the Basic Local Alignment Search Tool (BLAST) program. This program enabled a comparison of the nucleotide/protein sequence entered to nucleotide and protein databases of known sequences. Along with the matches found for the queries, statistics were provided to give an estimate of how likely the matches were.

All sequences were subjected to both nucleotide and protein searches. The blastn program performed searches of the query against nucleotide databases and the blastx program translated the entered nucleotide sequence into all reading frames then compared the resulting proteins to known proteins in the respective databases.

Nucleotide and amino acid sequences producing high-scoring segment pairs are summarized in Appendix B. For each match, the identification and the name of the nucleotide or protein sequence is given. A portion of the query sequence is aligned with this match. The reading frame or plus/minus strand for the query is specified. The span
Figure 3-16: The 450 bp sequence. The inferred primary sequence is indicated in one letter code above each of the nucleotide sequences. The primers used in generating the fragments are underlined. Primer labels are indicated in bold type. Asterisks denote stop codons.
of nucleotide/amino acid is given for both the query and the match. Within this span, identities and positive matches are summarized as a percentage of the total alignment. For example, the 230 bp nucleotide sequence entered shared a 67 % homology over a span of 58 bases with bovine mRNA for T-cell receptor beta chain (Appendix B1).

The results of each query showed that none of the sequences generated by PCR matched to any significant extent with any known nucleotide or protein sequence.

3.16 Design of Ideal Primers using Primer3 Output

The published amino acid sequence of the spleen inhibitor (II), an isoinhibitor of BPTI (Creighton and Charles, 1987) (Figure 2-2) had almost the same amino acid sequence as BPPI. The exception was that BPPI was longer by twenty residues and within the sequence, one residue differed. It had thirteen and seven more amino acids on its N and C termini, respectively. The full nucleotide sequence for SI (II), including introns has been established (Creighton and Charles, 1987). An inferred amino acid sequence based on the three exons of this sequence demonstrated an exact similarity to the amino acid sequence of BPPI in the 13 and 7 residues which flank the N and C termini. By entering the full nucleotide sequence (exons only) of SI (II) gene into the program titled ‘Primer3 Output’ at http://www.genome.wi.mit.edu, (Rozen and Skaletsky, 1997) a list of possible primers were generated, these were listed in Table 3-2. The table also lists the melting temperature, % GC of the primers and the size of the product that would theoretically be produced if used for amplification.
| Left primer       | Right primer       | Tm (°C) | % GC content | Size of product (bp)
|------------------|-------------------|---------|--------------|----------------------
| cccgctctcactcccct | gatgctccagtcatggg | 60.04, 60.00 | 66.67, 61.11 | 710                  |
| cggctctgcctccata  | gatgctccagtcatggg | 60.05, 60.00 | 61.11, 61.11 | 594                  |
| cggctctgcctccata  | ggaggggaggtgagaa  | 60.05, 59.98 | 61.11, 61.11 | 401                  |
| cggctctgcctccata  | tcagcatctcagggagc | 60.05, 60.03 | 61.11, 61.11 | 314                  |

Table 3-2: A list of ideal primers indicated by the program Primer3 using the SI (II) gene as template. Left and right primers are listed in the 5' to 3' direction. Melting temperature and % GC are listed under the appropriate column and separated by a comma for the left and right primers, respectively. The last column is the size of product (excluding introns) that would be amplified by the primers.
4. Discussion

4.1 Isolation of Genomic DNA from Whole Calf Blood

Typically, a 5 mL preparation of whole calf blood yielded between 10 and 30 μg of DNA. The quantity of DNA in a sample was calculated from its absorbance measurement at 260 nm. This estimate was calculated using the assumption that one absorbance unit at 260 nm was approximately equivalent to 50 μg/mL of double stranded DNA. Moreover, the purity of the sample or the ratio of the absorbance at 260 to 280 nm, Abs$_{260}$/Abs$_{280}$ (Sambrook et al., 1989) was generally found to be 1.8 or slightly greater. Electrophoresis of the genomic DNA on a 0.5 % agarose gel (Figure 3-1) revealed that the DNA was at approximately 40 kb, as sized by regression analysis. The size of this genomic DNA was as expected using this particular method of isolation. Moreover, these fragments were suitable for this application.

4.2 Design of Primers for PCR

Primers for PCR are oligonucleotides of 15 – 30 bases that hybridize to opposite strands flanking the region of interest in the target DNA. In general, when choosing primers, they should have 50 to 60 % G+C content and a balanced T$_m$ ranging from 55 °C to 80 °C. A simple calculation for the T$_m$ of primers is the sum of 2 °C for every A and T and 4 °C for each G and C in the primer (Thein and Wallace, 1986).

In designing degenerate primers, first one must choose amino acids that are coded by the minimum number of codons. For example, Lys and Arg can be encoded by 6 possible codons, whereas Cys and Asp are only encoded by two. While the length of these primers can contain as few as 15 – 20 nucleotides, the degeneracy must not exceed
516 fold (Innis et al., 1990). Another consideration in the prevention of mispriming is to avoid primers with a degenerate base in the 3’ end. For example, when designing the left primer, the last base or the degenerate base from the last codon should not be included (Sambrook et al., 1989).

The primers for amplification of the BPPI gene were chosen so that they would encompass the entire coding region of the nucleotide sequence. If this gene could be amplified in its entirety, then it could be cloned directly into an expression system.

The first set of primers designed according to the amino acid sequence of BPPI, sets one (P1/P2) and two (P3/P4), (Figure 2-1) were taken directly by transferring the six N and C termini amino acids into the corresponding degenerate codons, using a standard genetic code table (Voet and Voet, 1995). The left primers, P1 and P3, were precisely 17 and 18 bases of the 5’ end nascent strand so that they would anneal to the 3’ end of the 3’ to 5’ sense strand and extend in the 5’ to 3’ direction. The right primers, P2 and P4 were 17 and 18 bases of the 5’ end sense strand that would anneal to the 3’ end of the 5’ to 3’ nascent strand and extend in the 5’ to 3’ direction.

Because amplification did not occur with P1/P2, presumably due to the fact that the primers may have contained too much degeneracy, the second set, P3/P4 was designed with the added advantage that it lacked degeneracy in the 3’ end. For this, a codon usage table that listed the propensity of usage of codons for each amino acid in the bovine species was used (Wada et al., 1990). This table summarized a weighted average of how frequently a particular codon was used to specify a particular amino acid.

In addition to the set P3 and P4, an internal primer P5, was designed as a right primer so that it would bind within the sequence sought, on the 5’ to 3’ strand and
elongate in the 5' to 3' direction. This primer, originally designed to be a probe for Southern blotting was used as a right primer along with the primer P3 to produce a smaller nested product upon amplification. Performing an amplification using the primer pair P3/P5 would serve as a diagnostic tool to determine if the right gene was amplified. This smaller product would in theory be 120 bp in size when counting three bases for each of the amino acids that span and include the length encompassed by the primers P3 and P5 (Figure 2-1).

BPTI and SI (II) are protease inhibitors that share homology in amino acid sequence and function with BPPI (Li, 1992). BPTI and SI (II) are both 58 amino acids in length within which only seven residues differ (Figure 1-3). These differences are conservative and do not affect the tertiary structure and inhibitory activity too significantly (Fioretti et. al., 1988). In comparison, BPPI is 78 residues in length (Li, 1992), having 13 additional amino acids on the amino terminus and 7 on the carboxy terminus. Within these 78 residues, 58 of them line up with the 58 residues of the SI (II) primary sequence, within which only one residue differs (Figure 1-3). The nucleotide sequences for both BPTI (Anderson & Kingston, 1983) (Creighton & Charles, 1987) and SI (II) (Creighton & Charles, 1987) have been determined. The full genes of SI (II) and BPTI consisted of two introns and three exons, virtually identical in sequence. The middle exon, exon two, of both genes encoded the entire mature protein, and exons one and three encoded extrapeptides which were lost to posttranslational modifications (Creighton & Charles, 1987).

Due to the very high degree of amino acid homology and similarity in function between BPTI, SI (II) and BPPI, primer design was based on the assumption that the
BPPI gene was also encoded by one exon, uninterrupted by introns. The idea was to amplify the gene in its entirety by choosing primers in the extreme amino and carboxy termini of the protein that could then be cloned directly into a suitable vector for expression.

Primers P6 and P7 were designed according to the bovine spleen inhibitor (SI) (II) gene. An amino acid sequence was inferred from the SI (II) gene in the regions encoding extrapeptides lost to posttranslational modification (Figure 2-2). This inferred amino acid sequence matched the primary sequence of BPPI with the exception of one residue (Figure 1-3). The high degree in homology between these Kunitz inhibitors SI (II) and BPPI, prompted the use of the SI (II) gene as a model in the design of more suitable and defined primers for the amplification of BPPI (Figure 2-2).

Overall, the primers designed were correctly synthesized by DNAgency since they were fully recognized within the clones sequenced.

4.3 Basic Methodology of PCR

As described in ‘methods’, all precautions necessary to avoid contamination during PCR were taken. These included autoclaving any water used, placing equipment under UV light for 2 h prior to use, aliquotting reagents, and setting up reactions in a different room than that of which products were analyzed.

A typical reaction was performed in a 600 µL reaction tube and placed in a thermocycler well containing mineral oil for thermal conductivity. The reaction volume was 100 µL containing 25 – 30 ng genomic DNA or a small cube of agarose. In general, 0.05 – 1.0 µg DNA is used for amplifications of single-copy loci (Innis et al., 1990).
This gene however, was suspected to be part of a multigene family. Multigene families are commonly observed in eukaryotic genomes. Their evolution results from processes such as gene duplication, point mutation and gene conversion. Winged bean Kunitz chymotrypsin inhibitor (WCI) is an example of a protein encoded by a multigene family. WCI accumulates in an organ specific and temporally regulated manner (Habu et al., 1997). An organ specific accumulation has already been observed for BPTI and its iso-inhibitors. A study of the relative quantities of BPTI and its iso-inhibitors I, SI (II) and III in various bovine tissues indicated that BPTI always prevailed in the pituitary, lung, liver, and spleen. It was only in the spleen that SI (II) approached in quantity relative to BPTI. Aside from this iso-inhibitors I, SI (II) and III were found in approximately the same quantities in the various tissues examined, but their total quantities increased in the order of lung, pituitary gland, liver and spleen (Fioretti et al., 1988). On the basis that the gene for BPPI was a part of a multigene family, only 0.5 – 2 ng of genomic template should be used for amplification (Innis et al., 1990). Thus the quantity of template was a compromise based on the assumption that BPPI was part of the Kunitz inhibitor multigene family. Given that it was not, slightly more template than 0.5 – 2 ng would allow for its amplification. Hence the rationale of using 25 – 30 ng of genomic DNA template as opposed to 0.05 to 1.0 μg.

A recommended primer quantity was between 0.1 and 0.5 μM. A typical reaction contained 0.5 μM of each primer. A higher concentration would result in mispriming and accumulation of non-specific product (Innis et al., 1990). Moreover, primer-dimers would be encouraged to form (Sambrook et al., 1989).
For a 100 μL reaction, 20 - 200 μM of dNTPs are recommended for a balance between yield, specificity and fidelity (Innis et al., 1990). Moreover each should be present in the same amount to avoid misincorporation errors. In general the specificity and sensitivity are increased by the addition of a quantity of dNTPs tailored to the length and composition of the gene sought (Sardelli and Williams, 1997). For a typical amplification, 40 μM was used.

Magnesium ion concentration affects primer annealing, strand dissociation temperatures of both template and PCR product, specificity, formation of primer-dimer artifacts, enzyme activity, and fidelity. Template DNA, primers and dNTPs bind magnesium and Taq DNA Polymerase requires free magnesium for proper activity. Because dNTPS bind magnesium in a 1:1 ratio, it is recommended to have 0.5 – 2.5 mM MgCl₂ over the concentration of the quantity of dNTPs (Innis et al., 1990). For the present amplifications, 2.5 mM of MgCl₂ was used per reaction, an effective quantity of 2.46 mM.

In cycling, a hot start is recommended for two reasons. The first being to eliminate competing side reactions such as the amplification of non-target sequences and primer oligomerization and secondly to inactivate harmful proteases and nucleases. A manual hot start is performed by adding all but the DNA Polymerase to the tube and setting it in the thermocycler pre programmed for a five min. incubation at 95 °C. Within the last two minutes of the 95 °C incubation, the enzyme is added right below the mineral oil. Heating the reaction like this eliminates any reactions, which occur at room temperature, and it ensures the complete denaturation of complex starting templates (Sardelli and Williams, 1997).
Following this initial 95 °C incubation, cycling started with a denaturation at 95 °C for one minute, annealing of primers at 60 °C for 30 s. and elongation at 72 °C for 1 min. Cycling was repeated for 25 – 30 times and ended with a final elongation time of 10 min. The reaction was stopped with a 4 °C incubation.

Typically, the annealing temperature chosen is based on the true T_m of the primer and its length. For the most part, an applicable annealing temperature is 5 °C below the true T_m of the primers. Annealing temperatures between 55 and 72 °C generally yields the best results (Innis et al., 1990). At typical primer concentrations (0.2 μM) annealing only takes a few seconds.

Primer elongation depends upon the length and concentration of the target sequence and somewhat on temperature. Elongation is performed at 72 °C because this temperature is near optimal for extending primers on an M13-based model template. An extension time of one min. at 72 °C is considered sufficient for a 2 kb product to form (Innis et al., 1990). A final extension of 10 min. results in the incorporation of template independent A overhangs.

4.4 PCR using P1 and P2 Primers

Each of the attempted PCR reactions using primers P1 and P2 lead to no amplification. The only products produced in any of the reactions performed were primer dimers. Each lane in the agarose gels used to display these ‘products’ demonstrated that not even a faint smear of products was produced.

The first PCR performed was set up as described in ‘basic methodology of PCR’ using an annealing temperature of 58 °C to start. This temperature was on the upper end
of the range of melting temperatures of the primers P1/P2 having a range of 50 to 60 °C (Table 3-1). This range is broad due to the degeneracy of the primers. No amplification occurred thus the stringency of the reaction was lowered by decreasing the annealing temperature from 58 °C consecutively to 50 °C, 45 °C and finally down to 40 °C in an attempt to get any amplification of the template.

The next attempt was to increase the mass of the template from 30 ng to 300 ng in the case where the gene was not a part of a multigene family, an assumption made in deciding the quantity of template used. This once again resulted in no amplification.

Another attempt was in varying the quantity of MgCl₂ from 1.0 mM up to 5.0 mM using the same rationale of adjusting the stringency of the reaction. Magnesium concentration may affect primer annealing, formation of primer dimer artifacts and enzymatic activity and fidelity (Innis et al., 1990). This quantity must be optimized for each particular system. The variation of magnesium resulted in no amplification.

All of the reagents of the reaction were tested using a template and set of primers known to amplify. Since this reaction produced the product predicted, the system used for PCR was deemed acceptable and the template/primers questioned.

A final attempt was in increasing the mass of the primers from 50 pmol to 100 pmol since the primers were largely degenerate; containing a mixture of 256 different species each. The idea here was to increase the concentration of the primers such that the right sequence would be present in a high enough quantity among the mixture to bind and amplify.
Each of these reactions resulted in no amplification. At this point it was assumed that there simply existed far too much degeneracy in this first set of primers in order to carry on any successful amplification.

4.5 PCR using P3 and P4 Primers

Primers P3 and P4 were designed in order to try and eliminate some of the ambiguity of the first set P1/P2 that had 256 fold degeneracy in each primer. P3/P4 were analogous to P1/P2 with the exception that the 3' ends were not degenerate. Any mismatch in the 3' end of a primer can make unnecessary extension products that get carried through to successive amplifications (Innis et al., 1990). This degeneracy was eliminated using a table of codon usage for the bovine species (Wada et al., 1990). However, because the table of codon usage is a weighted average of the possible codons specifying a particular amino acid, there is still a small margin of error in choosing the most frequently used codon for that particular species.

The first PCR was set up so that the annealing temperature was 40 °C. This low stringency temperature was chosen as a starting point simply to get any amplification to occur. The resulting amplification products were two bands at 1350 and 600 bp that were also present in the P4 only, control lane, and a very faint smear of products ranging from approximately 600 bp down to 200 bp. In an attempt to obtain more defined products in the 200 – 600 bp region, or to increase the stringency so that fewer nonspecific products were formed this reaction was repeated using an annealing temperature of 55 °C then 60 °C. This heightened stringency caused a reduction in the smear of products formed and a sharpening of the bands at 1350 and 600 bp (Figure 3-2). The increase in stringency
however did not result in any discernible bands in the 200 – 600 bp region as anticipated. Because the bands resulting from the PCR with P3/P4 primers (lane 1) were also present in the control PCR (P4 primer only, lane 3) these were believed to result from two P4 primers acting as both the left and right primers, referred to as artifacts.

4.6 Excision and Amplification of the 230 bp Region of the Agarose Gel

No discernible bands were visualized upon amplification with the P3/P4 primers. This prompted an attempt to subject a region of a gel containing the ‘products’ of a primary amplification to a second amplification. The small fragment of agarose gel that was excised from the lane, adjacent to the 234 bp φX174 marker (Figure 3-3) which was used as a template for a further amplification with the P3/P4 primers, resulted in a broad band sized at 230 bp by regression analysis (Figure 3-3). An annealing temperature of 60 °C was used for this amplification, since it fell in the range of the melting temperatures of these primers which was 56 – 78 °C (Table 3-1). The melting temperature is not a defined value for these primers due to the fact that they are degenerate. The value of 56 °C is one that assumes that each of the wobble bases in the primer is either an A or a T. This is the lowest melting temperature that the primer can theoretically have. The highest melting temperature that the primer can have is 78 °C where every wobble base in the primer would be a G or C.

It was also noted that a piece of agarose, the size of the tip of a pen was sufficient as template for amplification.
4.7 Hemi-Nested PCR using P3 and the Internal Primer P5

A morsel of the 230 bp agarose band as generated by the P3 and P4 primers was used as a template for a nested PCR, using P3 and P5 as left and right primers. This nested PCR was used as a diagnostic test to determine if the 230 band was the gene sought. If it were the gene sought, then a corresponding smaller fragment would be produced, one of 120 bp in size. The amplification using these primers and template resulted in an amplification of a 130 bp fragment (Figure 3-4).

4.8 PCR using P6 and P7 Primers

PCR was performed using P6 and P7 primers as described in ‘basic methodology of PCR’ using an annealing temperature of 55 °C to start. This temperature was chosen as a starting point because the melting temperatures of the two primers are 58 and 64 °C for the left and right primers, respectively (Table 3-1). The rationale was to choose a suitable temperature that would allow both of the primers to anneal to the template solidly before extension occurred. This first reaction resulted in a prominent and legitimate band at 450 bp and a very faint smear of products running along the lane. In an attempt to reduce this smear, the same reaction was set up using an annealing temperature of 60 °C. This reaction produced two legitimate products sized at 450 and 172 bp. The band at 450 bp was still more prominent than the one at 172 bp. Using a higher annealing temperature produced sharper bands and less smearing.

4.9 Cloning of the 230, 172 and 450 bp Products in the pCR®2.1 Vector
Ligation and transformation of the 230 bp product was performed by Ann Marie Butler from the Department of Biological Sciences at the University of Windsor.

4.9.1 Ligation

Ligation involved making new PCR products on the same day that they were to be incorporated into the vectors and allowing the last elongation cycle of the polymerase chain reaction to continue for 30 min. so that A overhangs were produced. Moreover, the products were ligated within hours of the PCR products having been made so that they did not lose their A overhangs, which were necessary for ligation into the vector. Each PCR fragment to be incorporated was generated using the same conditions used to produce it in the first place. Lane 2 in Figure 3-6 shows the 230 bp band to be ligated into the vector, lane 2 of Figure 3-7 shows the 172 bp product to be ligated. Figure 3-8, lane 3, shows the amplification of the 450 bp product ready for ligation.

Note that in Figure 3-7, amplification of the 172 bp band resulted in the production of a 236 bp band as well. This amplification was performed three times and always resulted in the formation of this second, higher molecular weight band. This phenomena was typical of excessive cycling which resulted in the conversion of PCR products into random length higher molecular weight fragments (Bell, 1991). Nonetheless, the 172 bp band was always produced as a more prominent band. This mixture of products for ligation consistently resulted in the incorporation of the 172 bp fragment into the vector as demonstrated by digestion of the plasmids after transformation and growth of the *E. coli*.
The linearized pCR\textsuperscript{®}2.1 vector had the capability of incorporating PCR products in a site flanked by T overhangs. The T overhangs bind to the single A overhangs flanking the 3' ends of the PCR product. See Appendix A for site of incorporation into the vector.

In general, it was found that ligation was successful for each of the inserts incorporated into the competent *E. coli* INV\alpha F' cells as exhibited by the growth and analysis of the colonies and plasmids. The pCR\textsuperscript{®}2.1 vector carried a short segment of DNA containing the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (*lacZ*) (Appendix A). Within this coding region was a polycloning site, which upon ligation of a PCR product into this site, resulted in the addition of nucleotides and disruption of this gene. The addition of nucleotides resulted in the addition of amino acids to the amino terminal portion of the protein upon translation. When the *lacZ* gene was interrupted by a segment of DNA larger than 200 bp, the resulting N-terminal portion of the protein was disrupted to the point where it could not associate itself with its C-terminus counterpart to form the active enzyme. When the gene had a small segment incorporated (< 200 bp) the N and C portions could still be joined to form the active β-galactosidase enzyme. The joining of the N and C termini, termed α-complementation (Sambrook *et al.*, 1989) allowed for blue-white screening of colonies. α-Complementation signified the formation of the active β-galactosidase enzyme and hence the breakdown of the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate. The breakdown of X-Gal imparted a blue colour to the colony. Conversely, when the gene had been successfully interrupted, the colony
remained white. Thus the blue-white screening of colonies facilitated selection for further investigation.

4.9.2 Transformation

In general, it was found that transformation was successful for each of the inserts incorporated into the competent *E. coli* INVαF' cells as exhibited by the growth and analysis of the cells.

As predicted, the inserts of 172 and 230 bp only partially interrupted the gene, such that both white and slightly blue colonies contained inserts. Moreover, the quantity of blue colonies found on the plates exceeded the white.

Growth of the transformed cells containing the 450 bp insert resulted in a larger portion of white colonies than blue ones. In this case, only white colonies were chosen for further investigation. Ninety percent of the white colonies chosen for further investigation contained the 450 bp insert.

4.10 Plasmid Isolation

Plasmid isolation was performed on the colonies believed to contain inserts. Colonies were purified and isolated by the alkali lysis method (Sambrook *et al.*, 1989). Their isolation allowed for both digestion and amplification of plasmids; tools serving in the identification of clones containing the appropriate inserts.

In general, it was found that the length of time of growth of clones in 10 mL of media should not exceed 8 to 9 h of incubation. When overgrown, a preparation resulted
in high yield but poor quality DNA. Moreover, increased growth also resulted in larger quantities of RNA that would then require longer incubations with RNase A.

4.11 Estimation of Quantity and Purity of Plasmids Isolated

Measurements of quantity and purity of plasmids were calculated using the absorbances at 260 and 280 nm. These values were typically 1 – 4 μg of plasmid isolated per preparation with a purity of 1.8 or slightly greater.

The purity of the preparation was estimated by comparing the quantity of DNA to the quantity of protein in the sample, where the quantity of protein was estimated by the absorbance at 280 nm. Essentially, the purity of the sample was a measure of the ratio of DNA to protein.

It was found that whenever the overnight cultures were overgrown, or incubated for more than 9 h at 37 °C, the samples processed were high in yield of DNA but were lacking in purity.

4.12 Restriction Analysis of Plasmids

Analysis of the inserts in the pCR®2.1 vector by restriction digestion was facilitated by EcoRI sites located on either side of the cloning site (Appendix A). Digestion of this insert resulted in a product with ten and five extra bases flanking the 5’ and 3’ ends of the insert. Moreover, a product cut out by EcoRI always electrophoresed at a slightly higher molecular weight when comparing its distance traveled with the PCR product before insertion. For example, a comparison of the 450 bp product before insertion (lane 5 of Figure 3-10) to it after it has been cut out of the vector in lanes 2, 3
and 4 demonstrates that it is slightly larger due to the addition of 5' and 3' nucleotide segments contributed by the vector.

Restriction analysis was the first test performed on the plasmids to determine whether the inserts were worthy of being sequenced. When the size of the fragment incorporated matched the size of the PCR product inserted, the plasmid was considered for further investigation. An average of 70 – 90% of the plasmids isolated and subjected to digestion, contained inserts of the appropriate size.

Digestion performed on the insert itself before insertion into the plasmid was used as a control so that if any sites existed in the insert, the resulting banding pattern would be recognized upon digestion of the plasmid.

In each of the cases, the 230, 172 and 450 bp products were successfully incorporated into the vectors based on analysis by restriction digestion with EcoRI.

4.13 PCR on Insert in Plasmids

The plasmids containing inserts of the appropriate sizes as demonstrated by digestion with EcoRI, were subjected to PCR. PCR on the insert was performed using the plasmid as a template, and the appropriate primers. Amplification of a fragment of DNA that matched the size of the insert gave an indication that the appropriate fragments were incorporated. PCR was performed on the 172 and 450 bp containing plasmids. Each resulted in the respective amplification of 172 and 450 bp products.

4.14 Glycerol Stocks
Glycerol stocks were made up of the clones that contained inserts of the appropriate sizes and responded to amplification upon PCR with appropriate primers. They were made using fresh overnight cultures having an absorbance of no more than 0.6 at 600 nm. A small quantity of fresh culture was transferred to a glycerol solution and frozen and stored at –80 °C. Viability of the glycerol stocks was demonstrated when a small volume was transferred to fresh LB media and incubated at 37 °C. Within a few hours the media became cloudy.

4.15 Sequencing of the Inserts in the pCR®2.1 Vector

Of the plasmids isolated, all were subjected to digestion with EcoRI. Those indicating that they contained inserts of appropriate sizes were used as templates for PCR to determine if the segment inserted responded to amplification by the primers used to generate them in the first place. Clones which responded in a positive manner to both of these criteria were sent away to be sequenced.

Sequencing of inserts was performed by two applications of the Sanger method. One of the methods was the fmol™ DNA Sequencing System provided as a kit by Promega. The other was the ABI PRISM™ Dye Primer Cycle Sequencing Core Kit by Perkin Elmer. The premise of both of these applications relied on the extension of fragments using a DNA Polymerase and four separate base specific extension reactions. The fmol system made use of $^{32}$P end labeled primers and Taq DNA Polymerase and the second made use of fluorescent dye-labeled primers and the AmpliTaq® DNA Polymerase, FS.
Though the procedures were slightly different, the principle was the same. Four separate reactions were set up each containing a mixture of dNTPs and one of the ddNTPs. For example, in the case of the A termination reaction, a mixture of the four dNTPs and ddATP were mixed with the template, a labeled primer (\(^{32}\)P or Joe dye-primer) and DNA Polymerase. Cycling in the thermocycler through temperatures suitable to cause denaturation, annealing and extension of the template resulted in the formation of randomly sized fragments terminated at the 2'-3'-dideoxy-adenosine-triphosphate. These fragments were then separated by size on 6–7 % polyacrylamide gels and detected by autoradiography in the case of the \(^{32}\)P labeled primers or fluorescence in the case of the dye-labeled primers.

Sequencing was facilitated by the use of the pCR\(^{\circledR}\)2.1 vector which contained M13 Reverse and T7 primer sites (Appendix A). Sequencing with the M13 Reverse labeled primer, yielded determination of the 5' – 3' nascent strand whereas the T7 labeled primer yielded the sequence of the 5' – 3' sense strand but in the 3' to 5' direction. In other words, the T7 would give the complementary strand backward relative to the nascent strand.

4.16 Analysis of the 230, 172 and 450 bp Sequences

The sequence of the 230 bp product was read off the autoradiograph. Each lane of the autoradiographed gel corresponded to the termination fragments of each of the G, A, T and C reactions. The first lane contained the G termination fragments, the second, the A termination fragments and the third and fourth, the T and C termination fragments,
respectively. The sequence was read by starting at the top of the autoradiograph and reading sequentially down the gel until the bands were indiscernible.

The sequences of the 172 and 450 bp products were read right off the traces. The traces were easily interpreted since each peak was colour coded. All of the termination products were compiled before loading onto the gel. Thus the output was a result of the sequential emergence of the G, A, T and C termination fragments, based on size, as they came off the gel. These fragments were read fluorometrically based on the excitation and emission properties of each of the dyes. The fluorometric reading was then translated by the ABI PRISM Sequencer into an output that was colour coded to correspond to each of the labels. G was represented by the colour black, A was represented by green and T and C were represented by red and blue, respectively.

Note that both outputs, the autoradiograph and traces, contained some vector sequence before the actual sequence started. Figure 3-15 is a compilation of the sequences of the 230, 172 and 450 bp products.

The sequence of the 230 bp product did not correspond to the degenerate nucleotide sequence of BPPI. The translation product of this sequence did not match up to the amino acid sequence of BPPI either. Both the nucleotide sequence and translation products that were entered into the Basic Local Alignment Tool (BLAST) did not show any significant matches with any known gene or protein. The same was true for the 172 and 450 bp products.

The BLAST program was a link off the National Center for Biotechnology Information (NCBI) WebPages at http://www3.ncbi.nlm.nih.gov/. It performed database searching and statistics evaluating the matches that it found. This program was capable
of accommodating both nucleotide and amino acid sequences. It searched databases such as GenBank, EMBL, DDBJ, PDB and SwissProt.

4.17 Design of Ideal Primers using Primer3 Output

The design of ideal primers for PCR of the BPPI gene was based on the three exons of the SI (II) sequence. Though the mature SI (II) protein was entirely encoded within the second exon, an inferred amino acid sequence that spanned into the first and third exons of the SI (II) gene was identical to the amino acid sequence of BPPI. Thus, the full nucleotide sequence of SI (II) was used as a template for the design of primers for BPPI.

Primers for BPPI were chosen by entering the full encoding region of the SI (II) gene into the space provided in the Primer3 Output program. The program then proceeded to find primers within this sequence with optimal length, equivalent melting temperatures and G + C content. The resulting primers were listed in Table 3-2.

5. Future work

A different approach in isolating the gene of BPPI would involve the construction of a megakaryocyte cDNA library. Though it may be very presumptuous to assume that BPPI is expressed in the megakaryocyte as opposed to imported into the platelet after transcription, such a library has been successfully constructed (Gewirtz et al., 1995).

cDNA clones for both SI (II) and BPTI have been isolated from lung and liver (Kingston and Anderson, 1986; Creighton and Charles, 1987). A clone for BPTI was sought in order to gain more information about its physiological role, its biosynthesis and folding studies. To date, BPTI has been the subject of intense structural studies and is the
most thoroughly characterized protein in terms of its in vitro folding pattern. More recently, its in vivo folding has been studied (Creighton et al., 1993). One of the first expression system used for the fabrication of the BPTI protein consisted of BPTI cDNA incorporated into the pUC9 plasmid and transformed into E. coli (Creighton and Charles, 1987). More recently, its natural gene has been expressed by an in vitro transcription/translation system where its cDNA was cloned into phagemid Bluescript and translated in a wheat germ system. This system, coupled with the use of microsomes was used to trap intermediates in the production of mature BPTI (Creighton et al., 1993).

6. Conclusion

PCR was performed on bovine genomic DNA using primers based on the degenerate nucleotide sequence of BPPI as inferred from its amino acid sequence. The first set of primers P1/P2 had 256 fold degeneracy for each primer and consequently large ranges of Tm and % GC content. The use of these primers in PCR resulted in no amplification presumably due to the large degree of degeneracy.

A second set of primers, P3/P4 were also based on the inferred nucleotide sequence of BPPI, but with the added advantage that they were specific in the 3’ ends based on the propensity of usage of codons in the bovine species. Preliminary PCR with these primers resulted in no significant amplification. A secondary amplification was performed on a piece of agarose excised from a gel containing ‘products’ from a primary amplification. This resulted in the formation of a broad band at 230 bp. Sequencing of this fragment indicated that it had no similarity to the degenerate BPPI gene, nor did its inferred primary sequence match that of BPPI.
A third set of primers, P6/P7 designed according to the nucleotide sequence of SI (II) were used amplify the BPPI/SI (II) gene. PCR resulted in two products at 172 and 450 bp. Sequencing of these products demonstrated that neither was comparable to the SI (II) gene sequence, nor to the degenerate nucleotide sequence of BPPI.

The identification and isolation of the BPPI gene may require the use of a megakaryocyte cDNA library.
Appendix A  The pCR®2.1 vector. This figure demonstrates a PCR insert incorporated in the pCR®2.1 vector. The upper portion of the diagram is a segment of sequence within the lacZ alpha fragment containing the multiple cloning sites and restriction digestion sites that surround the region where the PCR fragment is incorporated. The lower portion is an overview of the closed circular vector. The lacZ alpha fragment is denoted the ‘start’ position of the sequence and ranges from 1 to 571 bases. Following the lacZ alpha fragment are the F1 ori site (530-944), the Kanamycin resistance gene (1278-2072), the Ampicillin resistance gene (2090-2950) and the CoIE1 origin (3095-3768). Primer sites available are M13 Reverse Primer (205-221), T7 Promoter (346-365), M13 Forward (-20) Primer (373-388) and the M13 Forward (-40) Primer (392-408). Note that the EcoRI sites are the first set of digestion sites that flank the PCR incorporation point. Reprinted with permission from Invitrogen.
Bovine mRNA for T-cell receptor beta chain, leader sequence, variable region, diversity region, and joining region
Length = 405

Plus Strand HSPs:

Identities = 39/58 (67%), Positives = 39/58 (67%), Strand = Plus / Plus

Query:  
```
97 ATTTCCCTAGCCAGACCTTGCTGCTAGAAATGCTGAAAGAAGGGGCTCTGGCTTG
```

Sbjct:  
```
4 ATTTCAACAACACAGACAGAGATTCCCTTGGAGCTGGGAGAAGACAGGCTCTGGCTTG
```

Appendix B1: Nucleotide sequence producing High-scoring Segment Pairs for 230 bp insert. Search performed by BLASTN (Altschul et al., 1990).
1

**emb|Z70289|HSP4G12** Human DNA sequence from fosmid 4G12 on chromosome 22q11.2-qter Contains STS and polymorphic CA repeat
Length = 43,770

**Minus Strand HSPs:**

Identities = 33/46 (71%) , Positives = 33/46 (71%) , Strand = Minus / Plus
Query: 162 CCGGGGCCCCAGCAGCCGCTCCCTGGGAGACTGAAGCAGAGGGAT 117
Sbjct: 4177 CCGGTGCTTGTAATCCCAGCTACTCGGGAGACTGAAGCAGAAAGAT 4222

Identities = 25/31 (80%) , Positives = 25/31 (80%) , Strand = Minus / Plus
Query: 60 GAGCTAGTGGGCTGGGAGGGAGGACTGGAA 30
Sbjct: 12759 GGGCTTGTGGGCTGGGAGGGAGGACTGGAA 12789

2

**emb|Z95326|HS344F17** Human DNA sequence from PAC 344F17 on chromosome 6q22.1-6q22.33 contains STS
Length = 85,832

**Plus Strand HSPs:**

Identities = 30/35 (85%) , Positives = 30/35 (85%) , Strand = Plus / Plus
Query: 113 ACCATCCCCCTGGCTCAGTCCTCAGGAAGACTGGG 147
Sbjct: 22061 AGCAATCCCTCCCCGCTAGTCCTCAGGAGAGCTGGG 22095

Appendix B3: Nucleotide sequence producing High-scoring Segment Pairs for 172 bp insert. Search performed by BLASTN (Altschul et al., 1990).
gi|1136800 (U28728) Efs [Mus musculus] gi|1373344 (U57686)
p130Cas-related protein Sin [Mus musculus] prf||2205344A embryonal
fyn-associated substrate [Mus musculus]
Length = 560

Plus Strand HSPs:

Identities = 12/15 (80%), Positives = 13/15 (86%), Frame = +2

Query: 32 PASPSQPTSSQPTPE 76
        PASP+QP SS PTPE
Sbjct: 78 PASPTQPGSSCTPTPE 92

Identities = 9/19 (47%), Positives = 12/19 (63%), Frame = +3

Query: 60 PSPHLNHLPWPSAPSPGP 116
        P+ ++ P PSPA SP P
Sbjct: 307 PALPVSEAPAPSPAPSPAP 325

Identities = 8/14 (57%), Positives = 9/14 (64%), Frame = +1

Query: 133 LPGSWLLGPGEPE 174
        LPG L P G+PE
Sbjct: 343 LPYGGGLKPEGDPE 356

2

sp|P41110|IF4G RABIT EUKARYOTIC TRANSLATON INITIATION FACTOR 4 GAMMA
(EIF-4-GAMMA) (EIF-4G) (EIF4G) (P220) pir||I46707 initiation factor
4-gamma - rabbit gi|404775 (L22090) initiation factor 4-gamma
[Oryctolagus cuniculus]
Length = 1402

Plus Strand HSPs:

Identities = 8/16 (50%), Positives = 10/16 (62%), Frame = +2

Query: 38 SPSQPTSSQPTPEPPP 85
        SPS+ S P+P P P
Sbjct: 57 SPSESQPSSPSPTPSP 72

Identities = 11/22 (50%), Positives = 13/22 (59%), Frame = +2

Query: 68 TPEPPPLAQPCLPRTNPSASV 133
        +PEP PLA+P LE S V
Sbjct: 120 SPEPTPLAEPFLEVEVTLSKPV 141

Identities = 14/24 (58%), Positives = 17/24 (70%), Frame = +2

Query: 38 SPSQPTSSQPTPEPPPLAQPCLEP 109
        S SQP+S PTP PPP+ +P EP
Sbjct: 59 SESQPSSPSPTPSPPPILEPGSEP 82

Appendix B4: Protein sequence producing High-scoring Segment Pairs for 172 bp
insert. Search performed by BLASTX (Altschul et al., 1990; Gish and States, 1993).
gb|L02870|HUMCOL7A1 Human alpha-1 type VII collagen (COL7A1) mRNA, complete cds.
Length = 9287

Minus Strand HSPs:

Identities = 32/39 (82%), Positives = 32/39 (82%), Strand = Minus / Plus

Query: 428 CCTCCTTCACCTCCAGGTTGAAAGGAAAACCTGAGGCTC 390

Sbjct: 7254 CCTCCTGCCCTCCAGGTTGAAAGGAGATCTGGGCCCTC 7292

gb|L81819|HSL81819 Homo sapiens (subclone 1_h3 from P1 H36) DNA sequence
Length = 3937

Plus Strand HSPs:

Identities = 39/56 (69%), Positives = 39/56 (69%), Strand = Plus / Plus

Query: 109 GCACCACCTGTGCTCCCACTAACATTACAGTCAAGTCATTCTGCTCGCTCCAAAAGCAA 164

Sbjct: 3206 GCACCACCTGTGCTCCACCGAGGCTGATGGGATGATTTGAAGACACTGCTCCAACAAACAA 3261

Appendix B5: Nucleotide sequence producing High-scoring Segment Pairs for 450 bp insert. Search performed by BLASTN (Altschul et al., 1990).
1

**gml**|**PID**|**d1020288** (D84239) IgG Fc binding protein [Homo sapiens]  
**Length** = 5405

**Minus Strand HSPs:**

**Identities** = 9/24 (37%), **Positives** = 12/24 (50%), **Frame** = -1

**Query:**  411  CEGKPEALGSTDGSFSQETMGVCV  340  
            C G  +L + +G S GCVC  
**Sbjct:**  2350  CPGSCPISAPAEGCESACREGCVC  2373  

**Identities** = 6/18 (33%), **Positives** = 11/18 (61%), **Frame** = -2

**Query:**  251  LLATRALMNCVCSCMA  198  
            +L+  C+ ++  C CWA  
**Sbjct:**  3195  VLSADRCVPLNGGCGCA  3212  

**Identities** = 6/18 (33%), **Positives** = 11/18 (61%), **Frame** = -2

**Query:**  251  LLATRALMNCVCSCMA  198  
            +L+  C+ ++  C CWA  
**Sbjct:**  4396  VLSADRCVPLNGGCGCA  4413

---

**Appendix B6:** Protein sequence producing High-scoring Segment Pairs for 450 bp insert. Search performed by BLASTX (Altschul et al., 1990; Gish and States, 1993).
References


Dept. of Chemistry and Biochemistry, University of Windsor (1994) Intermediary Metabolism I lab manual, 2nd edition.


Vita Auctoris

NATALIE MARIE LABBE

PLACE OF BIRTH: Windsor, Ontario

DATE OF BIRTH: February 21, 1971

EDUCATION: St. Anne's High School, Windsor, Ontario 1985-1990

University of Windsor, Windsor, Ontario 1990-1995 Honours B.Sc. Biochemistry


PUBLICATION