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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

PURIFICATION, CHARACTERIZATION AND cDNA CLONING OF A TRYPSIN INHIBITOR FROM *PENTACLETHRA MACROLOBA*

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

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Norman, Oklahoma
1998

PURIFICATION, CHARACTERIZATION AND cDNA CLONING OF A TRYPSIN INHIBITOR FROM *PENTACLETHRA MACROLOBA*

A Dissertation APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

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List of Abbreviation

ATAC	Anhydrotrypsin affinity chromatography
ATI	α-Amylase / trypsin inhibitor
BANA	N-α-Benzoyl-DL-arginine-4-nitroanilide
BBI	Bowman-Birk protease inhibitor
BTI	*Brassica* trypsin inhibitor
CaMV	Cauliflower mosaic virus
CBB	Coomassie Brilliant Blue
Da	Dalton
DMSO	Dimethyl sulfoxide
DTT	Dithiotheitol
FMN	Flavin mononucleotide
GCG	Genetic computer group
I_{50}	50% inhibition
IEF	Isoelectric focusing electrophoresis
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
IU	Inhibition unit
KDa	Kilodalton
KTI	Kunitz trypsin inhibitor
MWCO	Molecular weight cutoff
MS	Murashige and Skoog medium
PMSF	Phenylmethylsulfonyl fluoride
PmLTI	*P. macroloba* low molecular weight trypsin inhibitor
PmSTI	*P. macroloba* high molecular weight trypsin inhibitor
PTI I/II	Potato trypsin inhibitor I/II
PVDF	Durapore polyvinylidene difluoride
PVP	Polyvinylpyrrolidone
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophorsis
SEC	Size exclusion chromatography
SI	Squash inhibitor
SPI	Serine protease inhibitor
TEMED	N,n,n',n'-tetramethylethylenediamine
TAC	Trypsin affinity chromatography
TI	Trypsin inhibitor
X-gal	5-Bromo-4-chloro-3-indolyl β-d-galactopyranoside

ABSTRACT

Two types of trypsin inhibitors, designated PmLTI and PmSTI, were isolated and purified from seeds of *Pentaclethra macroloba.* The two predominant forms of PmLTI have estimated molecular weights of 43 and 39 kDa, whereas the major forms of PmSTI have molecular weights around 7 to 8 kDa. SDS-PAGE analysis of samples indicated that PmLTI exists as a dimer, and PmSTI appears to be a monomer. Inhibitory activity of PmLTIs was about 2.78 and 3.93 *IU/mgTI* against bovine trypsin and *Heliocoverpa zea* midgut trypsin, respectively. Inhibitory activity of PmSTI was 50.94 and 14.23 *IU/mgTI* against bovine and *H. zea* midgut trypsin, respectively. PmSTI was still active after boiling for 30 minutes but lost activity immediately when boiled with reducing agent. PmSTI is the first low molecular weight TI isolated from seeds of the legume subfamily *Mimosaceae.*

Amino acid sequencing revealed that two PmSTI variants, one 60 and the other 61 amino acid residues in length, were present in seeds. Analysis of PmSTI sequence indicates that it belongs to the Bowman-Birk inhibitor family. Although PmSTI has an estimated pI of 8.15 based on experimental data, PmSTI did not bind to either an anion exchange column at pH 9.0 or a cation exchange column at pH 5.0. PmSTI is a tight binding inhibitor of trypsin with an inhibition constant of 0.1 nM against bovine trypsin, but did not inhibit chymotrypsin. A full-length 558-bp cDNA sequence encoding the precursor of PmSTI has been isolated and

characterized. The cDNA sequence possesses typical sequence motifs observed in plants. These include a Kozak ribosome binding site (GAAGATGG), a *cis*-acting signal for the 3'-end mRNA formation which consists of a TATGTA domain upstream of the transcription stop signal AATAAA and a GT or T rich downstream region. The deduced PmSTI precursor has 120 aa residues which comprises a 52 residue N-terminal peptide, a mature PmSTI sequence and an 8-residue carboxyl-terminal extension peptide.

The larval growth of the European corn borer, *Ostrinia nubilalis*, was reduced when grown on artificial diet containing either purified PmTI or PmSTI fusion protein expressed in *E. coli*. Likewise, high mortalities were observed with the free-living nematode *Caenohabditis elegans* cultured on *E. coli* expressing PmSTI.

A PmSTI cDNA clone was successfully expressed in tobacco plants after *Agrobacterium*-mediated plant transformation. Transformation and expression of the PmSTI coding sequence were confirmed by Southern blot analysis, trypsin inhibitor assay and Western blot. When the effects of the expression of the PmSTI transgene in tobacco plants was evaluated, results indicated that the transgenic plants exhibited some degree of protection to the tobacco hornworm (*Manduca sexta*).

CHAPTER I

INTRODUCTION TO PLANT SERINE PROTEASE INHIBITORS AND THEIR APPLICATION AND FUTURE

Biologically active compounds in plants have been widely investigated and tested for their broad application in agriculture, medicine and industry. Often these compounds are naturally present in various plant tissues, and some of them are systemically inducible in response to environmental factors (Ryan, 1990; Dixon and Lamb, 1990; Ryals *et al.*, 1992). Active compounds include polypeptides, such as enzyme inhibitors (Ryan, 1990), enzymes, lectins (Chrispeel and Raikhel, 1991) and other toxic proteins (Korth and Levings, 1993); and secondary metabolites such as alkaloids and terpenes (Janzen *et al.*, 1977; Baldwin, 1989). Recently, the study of plant serine protease inhibitors has attracted a lot of attention because of their potential use in agriculture as well as in medical sciences.

1.1 Distribution and biodiversity of plant serine protease inhibitors

The occurrence of serine protease inhibitors in plants was first reported in 1938 (Bowman, 1938 cited by Garcia-Olmedo *et al.*, 1987). These inhibitors are widely distributed throughout the plant kingdom

(Ryan, 1973). Most of the research on plant serine protease inhibitors has focused on inhibitors of trypsin. Some of these trypsin inhibitors were later found to inhibit other serine proteases, such as chymotrypsin, elastase, subtilisin and plasmin, and might be more meaningfully referred to as 'serine protease inhibitors (SPI)'. Many of these inhibitors are localized in storage organs such as seeds (Janzen *et al.*, 1986), fruits (Pham *et al.*, 1985), tubers (Krizaj *et al.*, 1993; Mitsumori *et al.*, 1994) and edible roots (Scott and Symes, 1996; Wang and Yeh, 1996). Plant seeds, especially those of legumes, are rich in serine protease inhibitors (Ryan, 1973). The presence and/or accumulation of inhibitors in other tissues and organs may be induced in response to biological and environmental factors (Ryan *et al.*, 1990; Ryals *et al.*, 1992). Since the presence of serine protease inhibitors affects the nutritional value of seeds and fruits, research on these inhibitors has frequently focused on the economically important species in the *Leguminosae* (legume), *Solanaceae*, *Gramineae*, *Araceae* (arrowhead), *Cucurbitaceae* (squash), *Cruciferae (Brassica)*, *Convolvulaceae*, and *Caricaceae* (papaya) (Garcia-Olmedo *et al.*, 1987).

Generally, protein inhibitors of serine proteases have been grouped into 16 different classes based on sequence similarity, topological

similarity and binding mechanism (Laskowski and Kato, 1980; Bode and Huber, 1992). More specifically, plant serine protease inhibitors can be divided into the following 7 groups, based on their origins as well as their structural similarity.

1). Kunitz serine protease inhibitor group (KSPI)

Most KSPIs are from the *Leguminosae* (including the subfamilies *Papiliondeae, Cesalpinoideae, and Mimosaceae*), *Araceae, Gramineae* and *Convolvulaceae*. The Kunitz serine protease inhibitors are approximately 20 KDa in molecular weight and contain two disulfide bonds (Laskowski and Kato, 1980). Often there are isoforms present in the same plant, which differ in activity and specificity (Garcia-Olmedo *et al.*, 1987). Some of them inhibit trypsin and other serine proteases but not chymotrypsin, whereas others are inhibitors of chymotrypsin but not of trypsin (Odani *et al.*, 1979; Kortt and Jermyn, 1981). KSPIs from the *Leguminosae* are generally composed of single subunits while inhibitors from other families like the *Araceae* exist as dimers (Hammer *et al.*, 1989). This group possesses only one reactive site in the protein sequence which binds 1:1 with the cognate protease.

2). Bowman-Birk serine protease inhibitor group (BBI)

Bowman-Birk serine protease inhibitors were first isolated from soybean and characterized by Bowman and Birk (cited by Ryan, 1973). Most BBIs have been isolated from seeds of the legume subfamily *Papiloinaceae*, while a few have been found in other legume subfamilies and cereal species (Garcia-Olmedo *et al.*, 1987). Most of them are developmentally expressed in the seeds. To date, there are only two known cases (alfalfa and maize) in which this type of inhibitor has been shown to be inducibly expressed (Brown and Ryan, 1984; Rohrmeier and Lehle, 1993). The BBI inhibitors are generally small polypeptides with MWs around 7 KDa (Garcia-Olmedo *et al.*, 1987). Bowman-Birk inhibitors have extremely stable tertiary structures linked by seven highly conserved disulfide bridges (Figure 1-1) (Laskowski and Kato, 1980). BBIs have two similar domains crosslinked together intramolecularly with a reactive site in each domain (Bode and Huber, 1992). For this reason BBIs have been called double-headed inhibitors. Members of this group inhibit most serine proteases (Table 1-1), but are most effective inhibitors of trypsin and chymotrypsin.

Table 1-1. Specificity and inhibitory constants (K_i) for several plant serine protease inhibitors.

Inhibitors	Trypsin (K_i)	Chymotrypsin (K_i)	Elastase (K_i)	Subtilisin (K_i)	Plasmin (K_i)	Other Proteases
ASTI (Potato PI I)	+ (0.34nM)	+ (0.41nM)	-	+ (0.37nM)	+ (38 nM)	cathesin
RTI (Brassica)	+ (0.3nM)	+ (410nM)	na	na	na	na
BSZx (α-amylase/ trypsin)	+	+	-	na	+	thrombin

ASTI, amaranth seed trypsin inhibitor (Hejgaard *et al.*, 1994); RTI, oil-rape trypsin inhibitor (Ceciliani *et al.*, 1994; BSZx, barley serpin Zx (Dahl *et al.*, 1996).

3). Squash inhibitor group (SI)

This group of inhibitors is only found in the *Cucurbitaceae*. SIs are small, monomeric, thermal stable proteins with molecular weights of about 5 KDa (Hamato *et al.*, 1995). SIs have three disulfide bridges (Figure 1-1) and exhibit very strong inhibitory activity against trypsin and other serine proteases such as elastase (Hamato *et al.*, 1995; Huang *et al.*, 1992). The reactive site of the SI's is the Arg-Ile, Leu-Ile or Lys-Ile residues located in the large loop between the first and the second cysteine residue. There are many isoinhibitors in this group (Pham *et al.*, 1985; Lee and Lin, 1995; Hamato *et al.*, 1995).

4). α-Amylase / trypsin inhibitor group (ATI)

The amylase/serine protease inhibitors which have been found only in cereals are approximately 120 residues in length (~ 12 KDa) (Strobl *et al.*, 1995). ATIs may be monomers, dimers or tetramers, each exhibiting different inhibitory specificity (Poerio *et al.*, 1991). The ATIs are usually glycosylated with a single sugar molecule bound per subunit (Strobl *et al.*, 1995). This group of inhibitors has four or five intramolecular disulfide bridges. All members of this group are bifunctional, with separate reactive sites inhibiting α-amylase or trypsin with a 1:1 binding ratio of inhibitor to enzyme (Garcia-Olmedo *et al.*, 1987).

5). Potato trypsin inhibitor group I (PTI I)

PTI I was first discovered in potato tuber, where it accumulated throughout tuber development (Ryan and Balls, 1962). Garcia-Olmedo *et al.* (1987) later showed that PTI I accumulated in leaves of potato and tomato in response to wounding. PTI I is approximately 41 KDa, and contains one disulfide bond (Figure 1-1) and one reactive site (Garcia-Olmedo *et al.*, 1987). PTI I inhibits chymotrypsin and usually has Leu,

Met or Asp at the P_1 position which is the carboxy-terminal residue of the reactive site (Wingate *et al.*, 1989).

6). Potato trypsin inhibitor group II (PTI II)

Like PTI I, PTI IIs are found in the *Solanaceae* family (Garcia-Olmedo *et al.*, 1987). PTI II-like inhibitors are dimeric with a subunit molecular weight of 23 KDa and 5 intramolecular disulfide bonds (Figure 1-1) (Strobl *et al.*, 1995). PTI IIs inhibit both trypsin and chymotrypsin independently and have two inhibitory sites per monomer, one for chymotrypsin and the other for trypsin (Pearce *et al.*, 1988).

7). Brassica trypsin inhibitor group (BTI)

Brassica trypsin inhibitors are a group of small inhibitory proteins with molecular weights of about 7 KDa (Menegatti *et al.*,1992). BTIs have only been found in the family *Cruciferae.* Sequences of several BTIs have been reported (Menegatti *et al.*, 1992; Ceciliani *et al.*, 1994). BTIs inhibit the catalytic activity of trypsin or α-chymotrypsin with an enzyme-inhibitor ratio of 1:1. The BTIs possess four disulfide bridges (Figure 1-1).

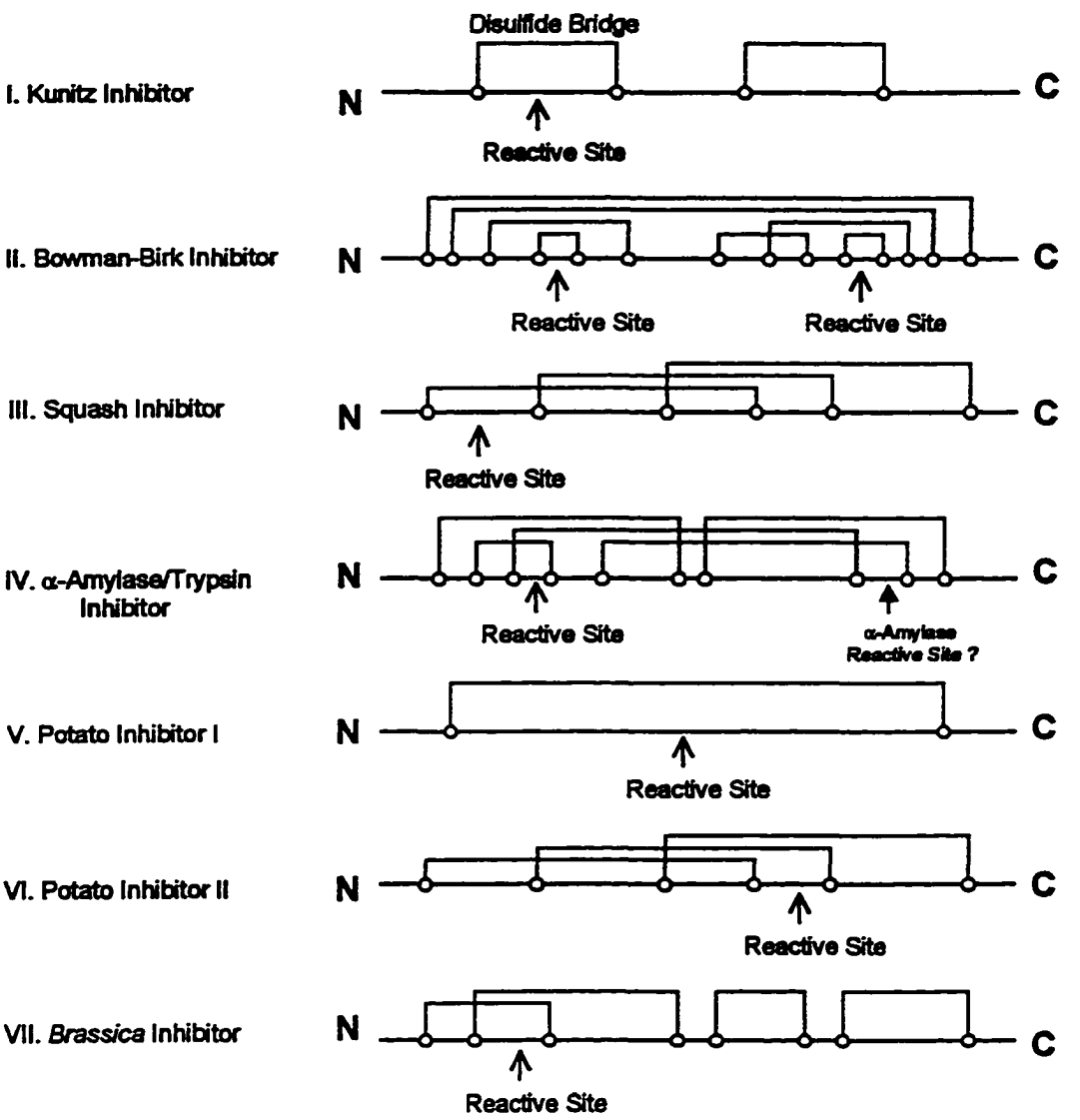

Figure 1-1. Structural features of plant serine protease inhibitors (Laskowski and Kato, 1980; Huang *et al.*, 1992; Menegatti *et al.*, 1992; Ceciliani *et al.*, 1994; Rozycki *et al.*, 1994; Strobl *et al.*, 1995). The position of a half cystine residue (o) and reactive sites (↑) against serine proteases are noted.

1.2 Important aspects of plant serine protease inhibitor structure

Bode and Huber (1992) summarized the relationships between the secondary structure of plant serine protease inhibitors and their interaction with protease. All plant serine protease inhibitors characterized to date have an exposed binding loop, which exhibits a characteristic canonical conformation unrelated to the structure (Figure 1-2) (Bode and Huber, 1992). However, the remaining structures outside the binding loop exhibit quite different folding motifs. For instance, the α-amylase/trypsin inhibitor binding loop for trypsin inhibition is stabilized by two alpha helices. This novel motif is not found in the secondary structures of other known serine protease inhibitors (Strobl *et al.*, 1995). The structure of squash inhibitors outside the loop is a very short central two-stranded β-ladder. No other secondary structure is formed (Holak *et al.*, 1989). For the BBI family, the molecule is double-headed. The folding shows internal symmetry in the central β-sheet, which is divided into two similar halves (Holak *et al.*, 1989).

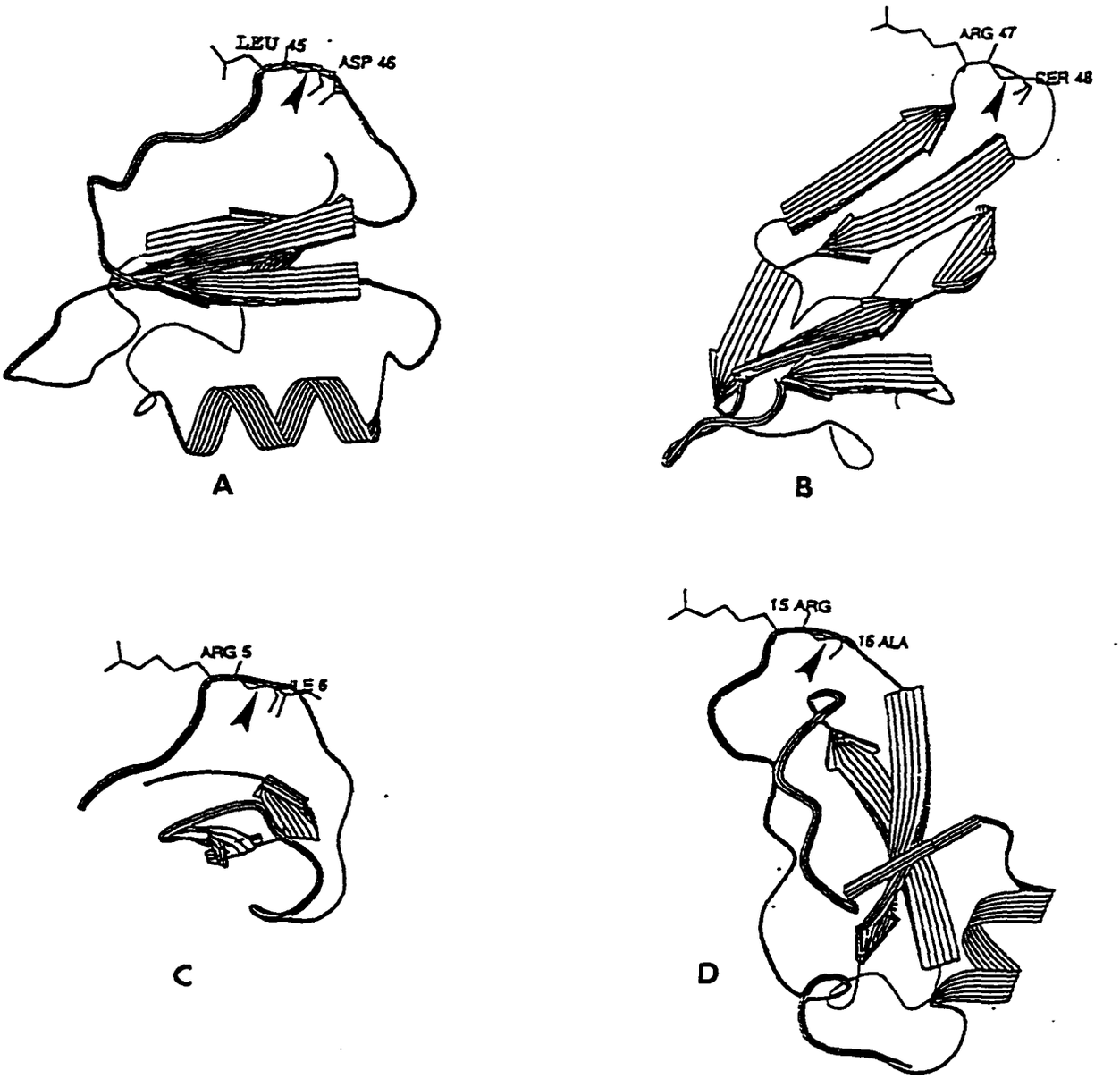

Figure 1-2. Secondary and tertiary structures of some serine protease inhibitors (Bode and Huber, 1992). The secondary and tertiary structures of some different inhibitors as presented by Bode and Huber (1992) are shown as ribbon structures. The reactive site of the inhibitors is indicated with an arrow and the structures of the two amino acids forming the reactive site are indicated. The wide arrow in the structure represents β-structure and the helix represents a α-helix. A, eglin c (potato inhibitor I group). B, mung bean trypsin inhibitor (Bowman-Birk inhibitor group). C, squash seed inhibitor I (squash inhibitor group). D, basic pancreatic trypsin inhibitor (Kunitz inhibitor group).

In spite of the differences in the secondary structure outside the binding loop, serine protease inhibitors have a compact shape and contain a hydrophobic core which may include one or more disulfide crosslinks (Bode and Huber, 1992; Strobl *et al.*, 1995).

The stability of the inhibitor structure depends on the secondary folding motif (α helix and β sheet), the number of disulfide bridges and the hydrophobicity of the inhibitor. These features lead to rigidity and stability in the protein structure. Some serine protease inhibitors such as BBIs contain few secondary motifs, but usually have high disulfide content (Werner and Wemmer, 1991; Strobl *et al.*, 1995). These inhibitors are stabilized by the crosslinking provided by the intramolecular disulfide bridges. All the inhibitors characterized form an apolar core that appears to be involved in thermal stability (Strobl *et al.*, 1995). The individual segments of this core domain interact cooperatively as a unit, which forms the supporting scaffold for the exposed binding loop. Even cleavage of the binding loop by the cognate protease may not alter protein folding and inhibitory activity (Strobl *et al.*, 1995).

1.3 Molecular biology of plant serine protease inhibitors

1). Multigene family of plant serine protease inhibitors.

Many genes and cDNA sequences of plant serine protease inhibitors have been isolated and characterized. Frequently, these inhibitors are encoded by a gene family containing at least two members (Hammond *et al.*, 1984; Bradshaw *et al.*, 1989; Adachi *et al.*, 1993; Ishikawa *et al.*, 1994). Jofuku and Goldberg (1989) demonstrated that in soybean the Kunitz trypsin inhibitor gene family contained at least 10 members. They also found that three distant genes from different chromosomes encoding Kunitz trypsin inhibitor had different expression patterns in soybean. Four genes encoding the winged-bean Kunitz-type inhibitor (WCI-3a, 3b, 2 and x) have been cloned and sequenced. Comparison of these genes indicated that they exhibited a high degree of homology at putative transcribed regions (Umemoto *et al.*, 1992).

The inhibitors of other plant SPI groups have been shown to be encoded by more than one gene. Soybean Bowman-Birk inhibitor is encoded by several genes. These genes may locate at different positions on the chromosome and the expression of these genes is controlled by different *cis*-elements (Hammond *et al.*, 1984). The wheat and barley α-amylase/trypsin inhibitor (ATI group) genes are dispersed over several

chromosomes (3, 4, 6 and 7 for wheat and 1, 2, 3 and 4 for barley) (Gautier *et al.*, 1990; Mena *et al.*, 1992). For potato trypsin inhibitor type I (PTI I), several genes were found in the tomato genome. At least one of them was induced in the leaves in response to wounding and oligosaccharide elicitors (Wingate *et al.*, 1989). The genes of tomato PTI I also include those expressed in the developing fruits (Wingate and Ryan, 1991). Likewise, potato trypsin inhibitor type II (PTI II) genes were analyzed and the expression of these different genes was under either developmental or environmental control (Sanchez-Serrano *et al.*, 1986; Keil *et al.*, 1986).

2). Common features of serine protease inhibitor genes.

Genes of plant serine trypsin inhibitors display typical features of eukaryotic genes. They include a TATA box and a CAAAT motif at the 5'-end. On the 3'-end, an AATAAA motif and a polyadenylation signal sequence are included (Keil *et al.*, 1986; Ishikawa *et al.*, 1994). However, the 5'-end of the inhibitor genes showed significant divergence in sequence homology and length among individual members in a multigene family. The variation in these 5'-end sequences (*cis*-control elements) presumably controls differences in gene expression (Ishikawa *et al.*, 1994).

3). Regulation of gene expression.

The differences in the expression patterns of serine protease inhibitor genes is related to differences in inhibitor gene function. Expression of these genes is subject to control by different regulatory elements (Ishikawa *et al.*, 1994). For example, both potato PTI I and II are expressed developmentally in flowers, and in response to mechanical wounding or microbial infection in leaves (McGurl *et al.*, 1995). Soybean Bowman-Birk inhibitor mRNA accumulated early during the mid-maturation stage of seed development and reached a steady state level later in development (Hammond *et al.*, 1984). BBIs are typically found in seeds, where their expression is developmentally regulated. The exception is alfalfa BBI, which is wound-induced (Brown and Ryan, 1984). Potato PTI I/II genes are expressed early during tuberization (Mitsumori *et al.*, 1994), while α-amylase/trypsin inhibitor genes are expressed only during the early stages of endosperm development (de la Hoz *et al.*, 1994).

1.4 Inhibitory mechanism and biological functions of plant serine protease inhibitors

1). Inhibitor specificity

The specificity and potency of inhibition depend on the amino acid sequence and structure of the inhibitor's reactive site and the active site of the target proteases (Birk, 1996). The carboxy-terminal residue of the inhibitory site, P_1, generally corresponds to the specificity of the cognate protease. Thus, inhibitors with Lys or Arg in the P_1 site inhibit trypsin and trypsin-like enzymes, while those with Tyr, Phe, Trp, Leu or Met in the P_1 site inhibit chymotrypsin and chymotrypsin-like enzymes. Inhibitors with Ala or Ser in the P_1 site inhibit elastase-like enzymes (Laskowski and Kato, 1980). However, this is not an absolute rule. Most of the serine protease inhibitors exhibit multiple specificities. For example, Table 1-1 lists some inhibitors which inhibit trypsin, chymotrypsin and other serine proteases.

Trypsin inhibitors from different plant species vary considerably in their ability to inhibit trypsin or other serine proteases from different sources (Belitz *et al.*, 1982; Christeller and Shaw, 1989; Hejgaard *et al.*, 1994; Dahl *et al.*, 1996). Likewise, the specific activity of trypsin inhibitors can be altered by *in vitro* mutagenesis. Wen *et al.* (1995) changed the cowpea trypsin inhibitor (CpTI) inhibitory specificity by *in vitro* mutagenesis. *Acacia confusa* trypsin inhibitor lost its inhibitory

activity due to the change of an amino acid residue in the reactive site (Hung *et al.*, 1994).

2). Mechanism of inhibition

Laskowski and Kato (1980) proposed a model for the mechanism of inhibition of serine protease inhibitors. On the surface of each inhibitor molecule lies at least one peptide bond called the reactive site, which specifically interacts with the active site of the cognate enzyme. The value of K_{cat}/K_m for the hydrolysis of this peptide bond at neutral pH is much higher than that of regular substrate, but the values of K_{cat} and K_m are both many orders of magnitude lower than the values for normal substrates. Thus, the protease inhibitor is more tightly bound to the protease than the substrate and the proteolysis of inhibitor is extremely slow because of the high K_{cat}/K_m and low K_{cat} and K_m values. Therefore, the serine protease inhibitors are slow and tight-binding inhibitors. The overall mechanism of enzyme inhibitor interaction is written below:

$$E + I \Leftrightarrow L \Leftrightarrow C \Leftrightarrow X \Leftrightarrow L^* \Leftrightarrow E + I^* \qquad (1)$$

where E is the protease; I and I* are the unmodified and modified inhibitors, respectively; L and L* refer to the loose, noncovalent (rapid dissociable) complex of E with I and I*, respectively; X is the relatively

long-lived intermediate in E + I* reaction, and C is the stable EI complex (Laskowski and Kato, 1980).

Results of studies of the secondary structure of the protease inhibitors and X-ray crystallography and NMR of the inhibitor-protease complex supported the model proposed by Laskowski and Kato (1980). Li *et al.* (1994) demonstrated that the binding domain of mung bean trypsin inhibitor was very rigid and resisted conformational changes even after binding to the trypsin active site. This structural rigidity of the binding domain decreased the rate or limited hydrolysis of the peptide bond of the inhibitor.

3). Proposed biological functions

a). Plant defense, sulfur storage, and endoprotease regulation.

Proposed physiological functions of plant protease inhibitors include regulation of endogenous proteases, sulfur storage and plant protection (Ryan, 1973; Richardson, 1977). Ryan *et al.* (1973) summarized evidence suggesting the role of trypsin inhibitors found in seeds in the regulation of endogenous protease activity. For example, a soybean protease was inhibited by both Kunitz and Bowman-Birk inhibitors (Morita *et al.*, 1996). Also, indirect evidence suggested that

serine protease inhibitors might serve as a site of sulfur storage (Richardson, 1977; Ryan, 1973).

Plant protease inhibitors are a group of naturally-occurring defense-related proteins which may be involved in protecting plants against insect predation (Ryan, 1990). The potential roles of protease inhibitors in plant protection have been summarized (Garcia-Olmedo, 1987; Laskowski and Kato, 1980; Ryan, 1990). Protease inhibitors have been reported to have effects on pests and microbial pathogens (Ryan, 1990; Birk, 1996; Wang and Yeh, 1996). These include effects on insects (Hilder *et al.*, 1987; Johnson *et al.*, 1989; Zhao *et al.*, 1995), nematodes (Hepher *et al.*, 1992) and pathogenic microorganisms (MacGibbon and Mann, 1986; McGurl *et al.*, 1995). Protease inhibitors were also induced by microbial invasion, or simply by wounding (Peng and Black, 1976; Ryals *et al.*, 1992). A trypsin inhibitor from crude pumpkin phloem exudate was effective against trypsin-like protease from fungal rot organisms (MacGibbon and Mann, 1986).

Effects of SPI on insect and nematode growth and development. Protease inhibitors as insect toxic agents may interfere with insect protein digestion, thereby limiting insect growth and development (Ryan, 1990).

Serine protease inhibitors target the major protease, a trypsin-like protease, in the insect digestive system (Hagenmaier, 1971; Purcell *et al.*, 1992). Trypsin, which is the control protease in digestion of insect dietary foods, regulates the activity of other proteases (Purcell *et al.*, 1992). Furthermore, serine protease inhibitor was assumed by Liener *et al.* (1980) to have an important secondary effect on insect growth and development. That is, when SPI enters the insect intestinal lumen, it binds to the proteases, inducing secretion of large amounts of protease and preventing from re-absorption of the SPI-bound proteases. The newly induced proteases could overcome any inhibition by SPI and could digest the dietary proteins. The overproduction of proteases, however, could use up the already-low essential amino acids, such as sulfur containing amino acids, leading to insufficient amino acids for normal protein synthesis (Broadway and Duffy, 1986).

b). Effects on malignant cells

It has been shown that protease inhibitors possess certain physiological activities that prevent, or at least greatly reduce, the radiologically or chemically induced malignant transformation of mammalian cells in culture and in experimental animals (Kennedy, 1993).

For instance, soybean Bowman-Birk inhibitor exhibited inhibitory activity against malignant transformed cells under certain conditions and had effects on various forms of cancer. Yavelow *et al.* (1985) reported that a crude soybean extract, if defatted with acetone, effectively blocked cell transformation *in vitro*. An active component of this crude extract was BBI. These observations, with epidemiological data, suggested that BBI may be a dietary anticarcinogen, particularly with respect to colon cancer. Weed *et al.* (1985) and Messadi *et al.* (1986) reported that BBI suppressed the growth of tumors, such as DMH-induced colon tumor and DMBA-induced tumor in hamster cheek pouch (human oral cancer).

Proposed mechanism for cancer prevention. The current hypothesis concerning the putative anticancer activity of protease inhibitors suggests that these proteins inhibit specific proteases, which are thought to be involved in the conversion of a cell to a malignant state (Kaneko *et al.*, 1986). Several researcher have suggested that protease inhibitor might suppress carcinogenesis by preventing the production of free radicals induced in cells (Coburn *et al.*, 1981, Troll *et al.*, 1982). Quigley (1979) proposed that cancer cells are capable of invading normal tissues through the action of proteases. On the other hand, structural similarities were

found between trypsin inhibitors such as BBI and tumor growth factor (Flecker, 1993). Thus, the anticarcinogenic activity of trypsin inhibitors may include the inhibition of proteolytic action as well as interference with tumor growth factor.

1.5 Application and future of plant serine inhibitors

1). Direct application of serine protease inhibitors for pest control.

Many higher plant genes encoding insecticidal and pathogenesis-related proteins exist in nature. These genes can be expressed in transgenic plants in a tissue or developmentally specific manner, or in response to environmental stimuli in order to protect the transgenic plant from attack. The genes can either be expressed singly or in combination so as to enhance the level of resistance to insect pests and pathogens. Plant trypsin inhibitor is one type of defense-related protein. Hilder *et al.* (1987) first introduced the Bowman-Birk trypsin inhibitor (TI) gene from soybean into tobacco and showed that transgenic plants were able to resist damage from selected insects. Transformation and expression of other trypsin inhibitor genes such as potato TI I and II also provided some protection against insects (Johnson *et al.*, 1989). Potato protease inhibitor II

expressed in rice provided resistance to pink stem borer (*Sesamia inferens*) (Duan *et al.*, 1996). Transgenic plants containing a TI gene that was not expressed, however, were susceptible to insect attack (Johnson *et al.*, 1989).

2). Engineering wide-spectrum protease inhibitors to avoid pest adaptation.

Pests can overcome the action of defense-related proteins by proteolysis of these proteins. Therefore, proteolysis is a factor which may limit the long-term use and benefit of the transgene (Jongsma *et al.*, 1995). Blocking the activity of pest proteases on a transgene by pyramiding the transgene with a gene for a wide spectrum protease inhibitor may enhance the protective effects of the transgene used to genetically engineer insect resistance (Michaud, 1997). Indeed, using the combination of a protease inhibitor gene with another insecticidal protein gene has been quite successful in protecting plants from insect attack (Lin *et al.*, 1989; Zhao *et al*, 1995).

3). Serine protease inhibitors for preventing tumor formation.

Investigations of the potential use of protease inhibitors, especially Bowman-Birk inhibitor, to prevent tumor formation suggest a possible value for the presence of these inhibitors in foods for humans and animals.

For example, Kennedy (1993) disclosed a method of using BBI as a treatment for cancer. The potential application of protease inhibitors in tumor prevention is evidenced by the number of patents issued in this area.

4). Serine protease inhibitor as a laboratory tool for enzyme purification.

Protease inhibitors are valuable laboratory tools for the purification of proteolytic enzymes. Immobilized SPI has already been used as an affinity ligand to purify specific proteases (Richardson, 1977).

CHAPTER II

ISOLATION, PURIFICATION OF TRYPSIN INHIBITORS FROM THE TROPICAL TREE *PENTACLETHRA MACROLOBA*

The tropical rain forest is a rich source of plant species from which to isolate trypsin inhibitors. Janzen *et al.* (1986) investigated seeds from 59 legumes from the tropical dry forest and showed that all of them were able to inhibit bovine trypsin, although to different extents depending on the species. Seeds of *Pentaclethra macroloba,* a legume from the tropical rainforest, were reported to be toxic to animals and insects (Hartshorn, 1983). Schubert and Ruzicka (unpublished) demonstrated the presence of trypsin inhibitory activity in extract of these seeds. Subsequently, seed extracts of *P. macroloba* were shown to reduce insect growth, and it was suggested that this might be due in part to the presence of trypsin inhibitor and/or lectin in these seeds (Chun *et al.*, 1994). Previously, we have isolated trypsin inhibitors from the seeds of *P. macroloba* (PmTIs) using affinity chromatography (Rathburn *et al.*, 1998). The affinity-purified PmTI sample reduced insect growth and increased larval mortality when incorporated into an artificial diet used to rear the insects. Inhibitory

activity in the affinity purified preparation of PmTI using in gel detection after SDS-PAGE showed that at least two types of trypsin inhibitors were present in the extracts. Our objective was to isolate, purify and clone the cDNA of trypsin inhibitors, which represent one type of the seed toxic proteins. The strategy used in this study is outlined in Figure 2-1. This chapter describes the isolation and purification of two types of PmTI using various purification methods.

MATERIALS AND METHODS

Materials. *Pentaclethra macroloba* seeds were collected at La Selva Biological Station, Costa Rica. The seeds were sliced, lyophilized, and stored at -20 °C after being transported to the United States. Seed extracts were prepared with or without heating as noted. Seeds (~20 g, collected on 7-13-93, lyophilized) were sliced as small as possible and mixed with 5% PVP and 50 ml ice cold 0.1 M Tris-Cl, pH 8.5 with 5 mM $MgCl_2$ and 0.045% (w/v) DTT. Seed slices were then homogenized using Brinkmann PT3000 homogenizer and filtered through a double-layer of cheesecloth and Miracloth. The filtrate was centrifuged at 20,000 x g for 10 minutes.

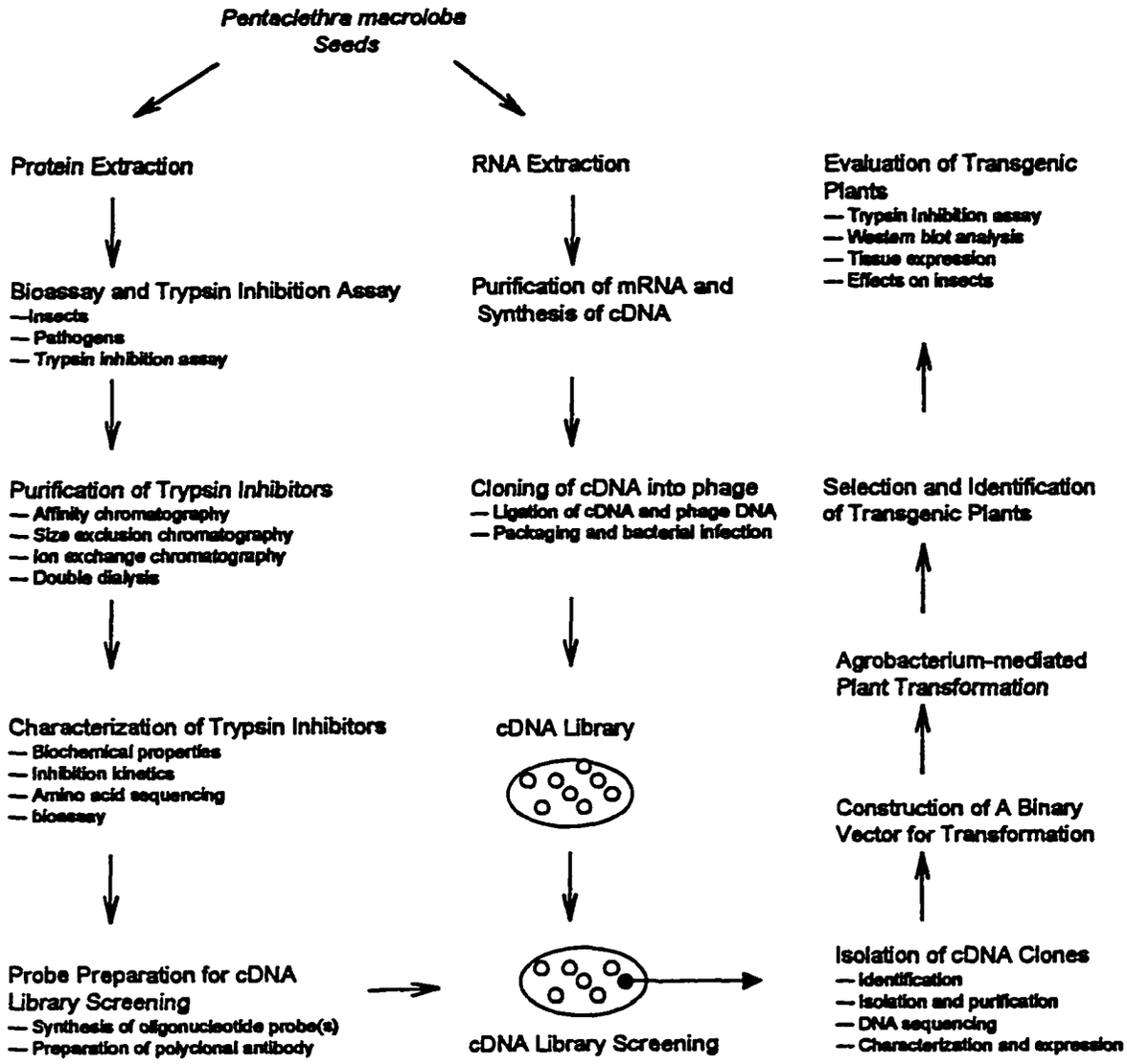

Figure 2-1. Strategy for the purification and cloning of cDNA encoding trypsin inhibitors from seeds of the tropical tree *P. macroloba.*

The supernatant (*ca* 80 ml) was pooled and heated at 70 °C for 15 minutes. The heat-treated supernatant was centrifuged again at 16,000 x g for 10 minutes. The resulting supernatant fluid was collected and subjected to dialysis (3,500 Da MWCO) for 24 to 48 hours. The dialyzed heat-treated extract was transferred to 50 ml sterile tubes for storage at -20°C. In some instances, the sliced seeds were boiled in 0.1 M Tris-Cl, pH 8.5, 5.0 mM $MgCl_2$ for 15 minutes before homogenization.

Affinity chromatography. Both trypsin and anhydrotrypsin affinity chromatography (called TAC and ATAC, respectively) were used to isolate trypsin inhibitors from crude seed extracts. TAC was performed according to the methods described in Appendix I. Anhydrotrypsin was prepared by the method of Pusztai *et al.* (1988) by treatment with PMSF until the residual trypsin activity was less than 0.5%. Anhydrotrypsin was then coupled to cyanogen bromide activated Sepharose-4B following the manufacturer's instructions (Appendix I).

The ATAC column was equilibrated with 0.1 M Tris-Cl, pH 8.5, 5.0 mM $MgCl_2$, 3.0 mM PMSF. Only fresh PMSF solution (in DMSO) was used since PMSF is unstable in water (Sambrook *et al.*, 1989).

Proteins bound to the column were eluted with 0.5 M Na-acetate buffer containing 0.5 M NaCl by using a decreasing pH step gradient from pH 7.0 to 2.0. Absorbance at 280 nm was used to monitor the elution of bound proteins and fractions that inhibited bovine trypsin were pooled, dialyzed (3,500 Da MWCO) against ultrapure water and lyophilized.

Size exclusion chromatography. Size exclusion chromatography (SEC) was performed at room temperature. A 1.5 cm x 100 cm Sephadex G-50 column was equilibrated with 50 mM Tris-Cl, pH 8.0, 0.25 M NaCl. Two to five mg of affinity-purified PmTI was applied to the column and protein was eluted at a flow rate of 15 ml/hour. Fractions of 2.5 ml were collected (Appendix I). Alternatively, proteins were separated by HPLC on a 1.5 x 60 cm Pharmacia Superose 12 SEC column. In some cases, samples were heated at 100 °C for 5 min in the presence of 2.0 mM ß-mercaptoethanol prior to being applied to the column. The column was equilibrated with deaerated 50 mM sodium phosphate buffer, pH 7.0, 0.15 M NaCl. The flow rate was maintained at 0.5 ml/min. Fractions (0.5 ml) were collected from 20 to 60 min after sample injection. Active fractions were pooled and dialyzed (3,500 Da MWCO) against water. In addition, SEC was used to determine the native molecular weight of PmTI's.

Ion exchange chromatography. Affinity purified PmTI was further purified by anion exchange chromatography using Pharmacia Q-Spharose, Resource Q and BioRad Q_5 columns. The columns were equilibrated with 100 mM Tris-Cl, pH 9.0, and fractions were eluted with a linear NaCl gradient from 0 to 0.5 M. For separation of isoforms of the large molecular weight TI (PmLTI), PmLTI purified by SEC on Sephadex G-50 was applied to a 2 x 9 cm Q-Sepharose column equilibrated with 100 mM Tris-HCl, pH 9.5. Proteins were eluted from the column using a linear NaCl gradient (0 to 0.15 M) followed by a 0.5 M NaCl wash. Fractions (2.0 ml) were collected and tested for inhibition of bovine trypsin.

Double dialysis. Samples after anion-exchange chromatography were dialyzed extensively against deionized water at 4 °C for several days using a technique combining two molecular weight cutoff (MWCO) membranes (3,500/10,000 or 3,500/12-14,000). The higher MWCO membrane containing the sample was placed inside the 3,500 MWCO membrane and the deionized water was changed at 12-hour intervals. The fluid between the two membranes was collected and subjected to affinity chromatography. In some instances, the same membrane combinations

were used to separate trypsin inhibitors purified by SEC or affinity chromatography.

SDS-PAGE. SDS-PAGE gels of 12-15% acrylamide were prepared and run according to the method of Laemmli *et al.* (1970) with modification as noted. Protein samples were denatured by boiling for three minutes in SDS sample buffer (BioRad) with and without 2 mM ß-mercaptoethanol. Some samples, referred to as 'native TI', were not boiled prior to electrophoresis. The estimated subunit molecular weight of the trypsin inhibitors was determined by electrophoresis on a 10-20% gradient SDS-PAGE gels under native and denatured conditions (Hammer *et al.*, 1989). Proteins were detected by staining with Coomassie Brilliant Blue R-250 (CBB) or silver stained.

In-gel activity assay. After SDS-PAGE electrophoresis, the gel was washed briefly with deionized water and then with 0.1 M K-phosphate buffer (equal volume of 0.2 M H_2PO_4 and K_2HPO_4). The gel was then immersed in a solution containing 15 mg bovine trypsin (Sigma, MO) dissolved in 150 ml 0.1 M K-phosphate buffer and incubated for about 20 minutes. The trypsin solution was poured out and the gel was

incubated in a container covered with Saran Wrap at 25° C for 30 minutes. The substrate solution (20 mg acetyl-*dl*-phenylalanine-β-napthyl ester, 10 ml of *N'N*-dimethylformamide, 40 mg tetrazotized *orth*-dianiside and 70 ml 0.05 M potassium phosphate buffer, pH 7.0) was finally added to the gel. The gel was incubated at room temperature until the bands could be detected. The reaction was stopped by transferring the gel to 2% (v/v) acetic acid.

Isoelectric Focusing Electrophoresis. IEF was carried out using a BioRad mini-IEF apparatus. The IEF gel solution was prepared by mixing 1.0 ml acrylamide solution (25% acrylamide [BioRad], 3% bisacrylamide), 1 ml glycerol, 0.5 ml Bio-lyte 8/10 (BioRad) and deionized H_2O to a final volume of 5.0 ml. The solution was degassed and then 50 µl of freshly prepared 10% ammonium persulfate, 50 µl of 0.1% FMN and 5 µl TEMED were added. The gel solution was poured into the chamber and allowed to polymerize for at least 30 minutes. After polymerization, protein samples were directly loaded onto the gel, the gel was pre-run at 100 and 200 volts for 15 minutes each and then IEF was performed at 400 volts for an hour. Following electrophoresis, proteins were detected by

staining with CBB-R250 while protease inhibitor was revealed using the in-gel trypsin activity detection.

Trypsin inhibition assay and data analysis. Assays were carried out in 0.1 M Tris-Cl buffer with different pH values. The reaction mixture was prepared by adding a solution of 100 µl trypsin (50 ng/µl in 0.1 M Tris-Cl) with different volumes of test samples. Assay buffer was added to a final volume of 973 µ. The reaction was initiated with the addition of 27 µl of substrate (40 mg/ml *p*-BANA in DMSO) to a final concentration of 2.5 mM. All assays were performed at 25°C. Data was automatically recorded t 410 nm in 30-second interval afor a total of 5 minutes with a Beckman DU7500 spectrophotometer.

Trypsin inhibition was expressed in terms of the amount of protein required to reduce the reaction rate to 50% of the rate of the control (50% inhibition, I_{50}) and inhibition units (*IU*). The values for I_{50} were obtained from a linear plot of percent inhibition versus the amount of protein (ng) in the assay. Each reaction was performed in triplicate. The relationship among the value for *50%* inhibition, the Y intercept and the slope is expressed as:

$$50\%\,Inhibition(I_{50}, ng) = \frac{1-2\bar{Y}}{2\bar{A}}$$

where $\bar{Y}$ is the y intercept, $\bar{A}$ is the slope of the linear relationship between percent inhibition and the protein amount (ng).

Inhibition unit (IU) is defined as the amount of trypsin inhibitor required to reduce hydrolysis by 1.0 μmole substrate (BANA) per minute. The relationship between IU and the rate of hydrolysis (ΔE /minute) is:

$$Inhibition\ Unit(IU) = \frac{\Delta E_0 - \Delta E_i}{8.8\ \mu\ \text{mole}^{-1}}$$

where ΔE_0 is the difference in absorbance in one minute in the absence of the inhibitor, ΔE_i is the change of absorbance per minute in the presence of the inhibitor. The light extinction coefficient for product at 410 nm is 8.8 $cm^2 \cdot \mu mole^{-1}$. When the relationship between 50% inhibition (I_{50}) and trypsin concentration is known, the equation of specific inhibitory activity can be simplified to:

$$Specific\ Inhibitory\ Actvity\ (IU / mg\ protein) = \frac{\frac{1}{2}\Delta E_0}{I_{50}(ng) \times 8.8 \times 10^{-6}}$$

where specific activity is expressed as the *inhibition unit* per mg protein, ΔE_0 is the difference in absorbance in one minute in the absence of

inhibitor, I_{50} is the 50% inhibition. The specific inhibitory activity is used to determine trypsin inhibition in samples during purification and to compare values for PmTI with those of other trypsin inhibitors for which the values are available.

Extraction of Heliocoverpa zea trypsin-like protease for inhibition assay. Larvae of *H. zea* were reared on a diet modified from Berger (1963) (see appendix II) and prepared as described by Waldbauer *et al.* (1984). The fifth instar larvae were briefly chilled on ice and then fixed to the cold dissection tray by pinning the dorsal side down. The ventral side of the larvae was carefully cut with scissors, leaving the gut exposed. Tris-Cl buffer (0.1 M, pH 9.5) was applied to rinse away the hemolymph. The contents of the lumen of the entire gut was collected, quickly chilled on ice, filtered through one layer of miracloth and centrifuged for 15 minutes at 25,000 x g at 4 °C. The protein concentration and the tryptic activity of the resulting supernatant fluid were determined. The crude gut extract was stored at –20 °C.

RESULTS

A. Affinity chromatography of trypsin inhibitors.

Approximately 95 mg of PmTI was purified from 20 g of seeds by trypsin affinity chromatography. By comparing the amount of protein necessary to inhibit trypsin activity by 50% with the spectrophotometric assay (I_{50}) for the crude extract and the I_{50} for purified PmTI, we estimated that PmTI accounted for about 3 % of the soluble seed protein.

Two major peaks were eluted from the trypsin affinity column (TAC) with pH 3.0 and pH 2.0 Na-acetate buffers (Figure 2-2). The pH 2.0 fraction corresponded to denatured proteins and did not inhibit trypsin as shown on the SDS-PAGE activity-stained gels. The pH 3.0 fraction contained two types of active trypsin inhibitors, designated PmLTI (large trypsin inhibitor) and PmSTI (small trypsin inhibitor) as shown in Figure 2-3, as well as several other polypeptides. Although PmSTI and the small polypeptides could have been derived from the PmLTI fragments cleaved by trypsin during purification, further study using the in-gel trypsin activity assay revealed two activity bands in the crude extracts of the same mobility as that of the affinity purified pH 3.0 fraction (Figure 2-2). This result suggests that two different trypsin inhibitors, i.e. PmLTI and PmSTI, originally existed in *P. macroloba* seeds, and that the small polypeptides

were fragments of PmLTI's. However, the possibility of proteolysis in the seed extracts during extraction can not be ruled out.

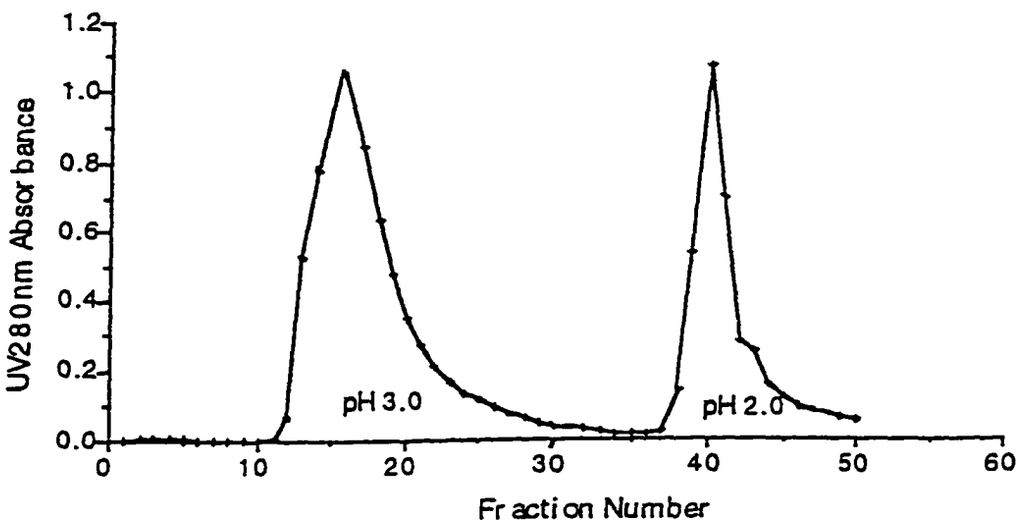

Figure 2-2. Affinity chromatography of *P. macroloba* seed extract on Sephadex 4B coupled with bovine trypsin. Twenty ml of seed extract in 0.1 M Tris-Cl, pH 8.5 with 5 mM $MgCl_2$ was loaded onto the column and the column was thoroughly washed with the same buffer. Proteins were eluted from the column using a pH 7.0 to 2.0 step gradient of 0.5 M Na acetate buffer containing 0.5 M NaCl.

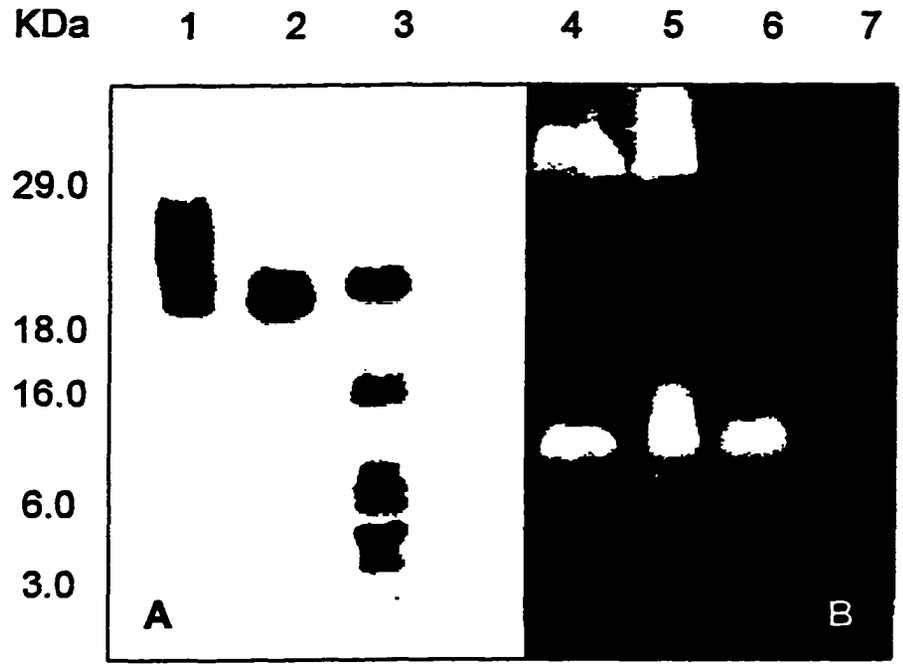

Figure 2-3. SDS-PAGE (15% gel) of the affinity-purified PmTI fractions eluted at pH 3.0. Lyophilized samples were dissolved in the SDS sample buffer as indicated. A, gel was stained with Coomassie Brilliant Blue; B, in-gel activity assay as described in 'Material and Methods'. An Equal amount (5 μg) of affinity-purified PmTI was loaded onto each lane. For crude extract, about 20 μg sample was loaded. Lanes 1 and 5, PmTI in SDS sample buffer without β-mercaptoethanol, unheated; lanes 2 and 6, PmTI in SDS sample buffer without β-mercaptoethanol, boiled; lanes 3 and 7, PmTI boiled in SDS sample buffer with β-mercaptoethanol; lane 4, crude extract in SDS sample buffer without β-mercaptoethanol, unheated.

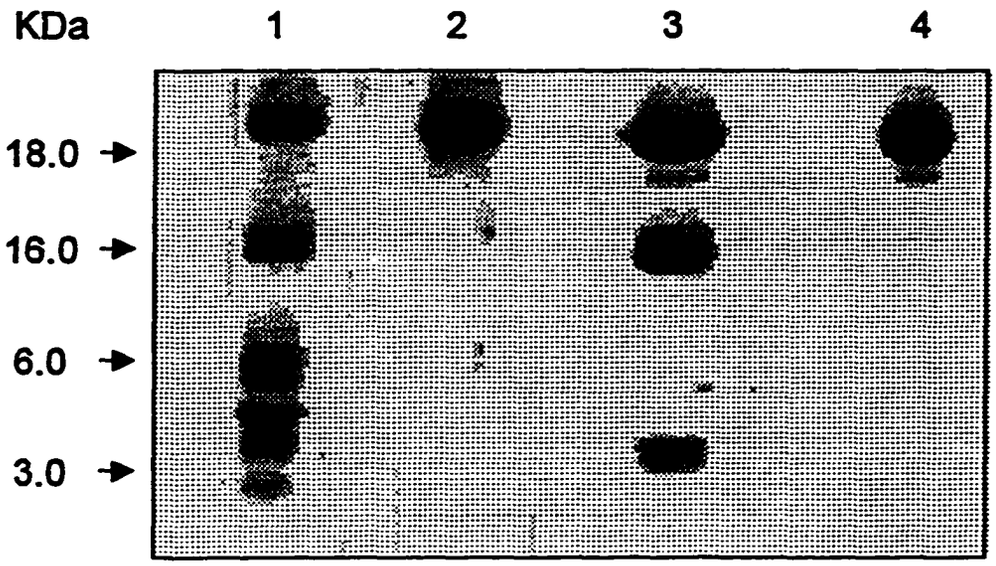

Figure 2-4. SDS-PAGE (15% gel) of TAC and ATAC purified PmTIs. Lyophilized PmTIs (about 10 μg each sample) were dissolved in SDS-PAGE sample buffer and boiled as indicated. Samples were subjected to electrophoresis and the gel was stained with Coomassie Brilliant Blue R-250. Lanes 1 and 2, TAC-purified PmTI; lanes 3 and 4, ATAC-purified PmTI. Lanes 1 and 3, samples in SDS sample buffer with β-mercaptoethanol; lanes 2 and 4, samples in SDS sample buffer without β-mercaptoethanol.

Anhydrotrypsin affinity chromatography (ATAC) was used in an attempt to determine whether the polypeptides observed on SDS-PAGE gels of heated and reduced PmTI were the result of cleavage of PmLTI by active trypsin during affinity purification and to purify unmodified PmTIs so that the property of the intact inhibitor could be examined. In this approach, the active site of trypsin is inactivated. However, inactivation does not affect the binding of trypsin-inhibitor. In these studies, trypsin activity was reduced by 98% after the treatment with 3 mM PMSF according to Pusztai *et al.*, (1988). Trypsin inhibitors isolated by ATAC were considered to be without modification.

Differences in the SDS-PAGE gel pattern were observed after using anhydrotrypsin affinity column (ATAC) when compared to TI isolated by TAC. A comparison of purified PmTIs by TAC and ATAC on SDS-PAGE revealed that the gel pattern of ATAC column had only four major bands (21, 15, 14, 4 KDa) under reducing conditions and one band (21 KDa) under non-reducing conditions (Figure 2-4). In contrast, the TAC purified inhibitor had more than seven bands under reducing conditions and also only one band (21 KDa) under non-reducing conditions. With in-gel activity staining, ATAC-purified PmTI showed stronger inhibitory activity

(bigger spot) than that of the TAC purified TI. These results confirm that PmTI is cleaved by the trypsin during purification

B. Size exclusion chromatography of P. macroloba trypsin inhibitors

Since two active trypsin inhibitors of apparently different molecular weight were detected in affinity-purified samples, size-exclusion chromatography was used to separate these two trypsin inhibitors. Affinity-purified trypsin inhibitors from both heated (70 °C) and unheated crude extracts were applied to a Sephadex G-50 column. Two well-separated peaks of trypsin inhibitor were obtained (Figure 2-5). Comparison of the inhibitory activity profiles of heated and unheated samples in Figure 2-6 demonstrated that the activity of heated PmLTI was slightly weaker than the activity of the unheated sample.

The amount of protein from the G-50 column required to inhibit 5 μg trypsin activity by 50% in the standard assay (I_{50} values) was determined using both bovine trypsin and *H. zea* trypsin-like protease. When the inhibitory activities of PmSTI and PmSTI were compared, it was clear that PmSTI was a much more effective inhibitor of both bovine trypsin and *H. zea* trypsin (Figure 2-6). Six times more PmSTI was required to inhibit *H. zea* trypsin than was required to inhibit bovine

trypsin by 50%. One possible reason for the low activity is that non-purified *H. zea* trypsin is mixed with other proteases.

Although the profiles of absorbance at 280 nm and the inhibitory activity suggested very good resolution of the two trypsin inhibitors, SDS-PAGE showed that the second peak in Figure 2-5 contained PmSTI mixed with higher MW polypeptides and that the peak I contained relatively pure PmLTI (Figure 2-7).

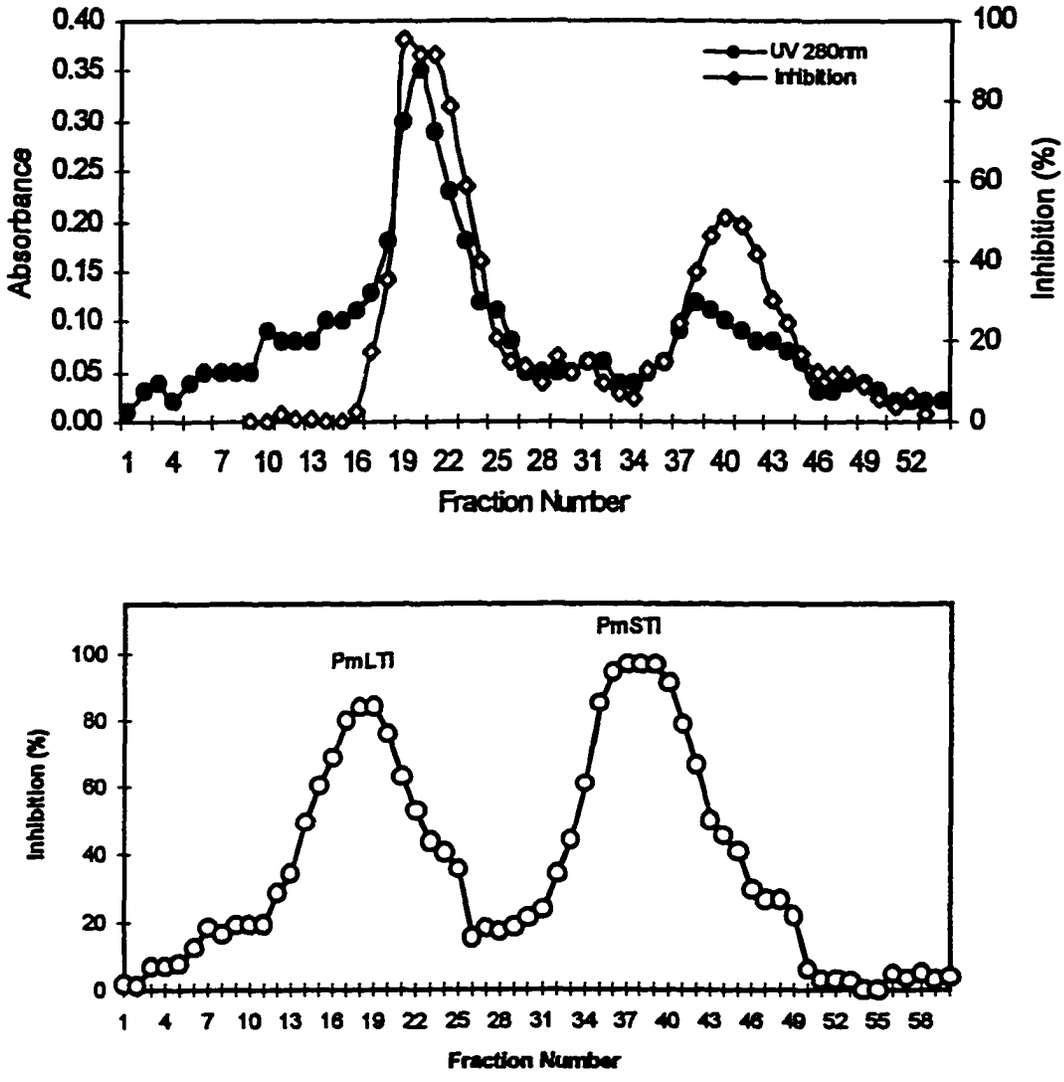

Figure 2-5. Size exclusion chromatography of affinity-purified PmTI on Sephadex G-50. Desalted and lyophilized PmTI (about 5 mg) was dissolved in 0.5 ml 50 mM Tris-Cl, pH 8.0 containing 0.25 M NaCl, and fractionated on a column (1.5 x 100 cm) equilibrated with the same buffer. The flow rate was 15 ml per hour; fraction size was 2.5 ml. The absorbance of each fraction at 280 nm was measured to monitor protein elution. Trypsin inhibitory activity was determined using the standard assay with 50µl of each fraction added per assay. A, affinity-purified sample from unheated crude extracts; B, sample from heated (70 °C) crude extracts.

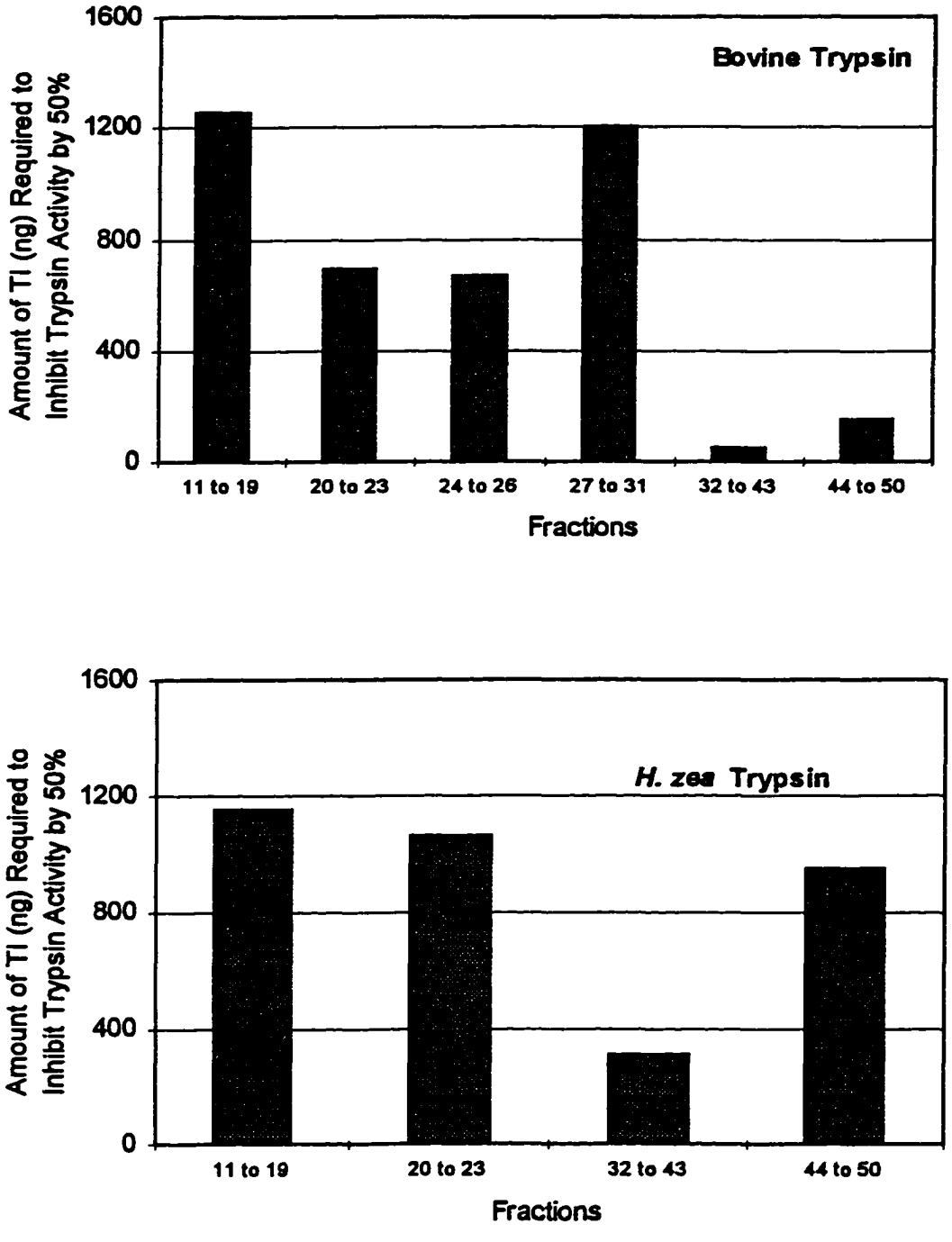

Figure 2-6. The amount of G-50 fractions required to inhibit the activity of 5 μg bovine trypsin and *H. zea* trypsin by 50%. Based on in-gel analysis, fraction 11 to 19 (Peak 1 referred to Figure 2-5) contained purified PmLTI; 20 to 23 (right side of peak 1) contained mixed PmTI; 32 to 43 (Peak 2) contained PmSTI; and 44 to 50 (right side of Peak 2) contained PmSTI and few other small peptides.

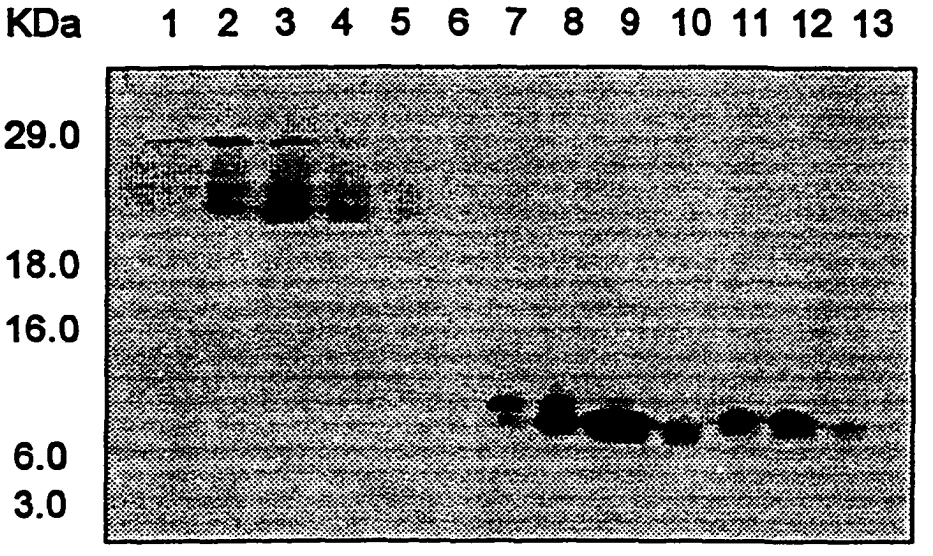

Figure 2-7. SDS-PAGE (15% gel) of fractions after gel filtration chromatography on a Sephadex G-50 column. Affinity-purified PmTI was fractionated by gel filtration as described in 'Materials and Methods'. Each individual fraction in the two major peaks was dried on a Speed-Vac and then dissolved in SDS-PAGE sample buffer without mercaptoethanol. Samples were boiled for 3 minutes before being loaded onto the gel. The gel was silver stained. Lanes 1-13 are from the same numbers of fractions after SEC.

C. Purification of PmSTI by double dialysis

Based on SDS-PAGE analysis, PmSTI is a small polypeptide with a molecular mass of about 7 KDa. As shown above, attempting to purify PmSTI away from PmLTI by SEC resulted in fractions of PmSTI contaminated with higher molecular weight proteins. This high molecular weight protein was detected when used high amount of PmSTI fraction. For this reason, other techniques were developed to obtain pure PmSTI free of contaminating proteins. PmSTI can be purified by the exclusion of PmLTI using techniques which discriminate against the high molecular weight PmLTI. Initially, centrifugation using Centricon® filters C-30 (30 KDa MWCO) and C-10 (10 KDa MWCO) was tested. Unexpectedly, PmSTI was retained by the C-10 filter, even though its estimated MW based on SDS-PAGE was less than 8,000 Da, and PmSTI was not retained by the C-30 filter (data not shown).

A double dialysis procedure using two combined membranes with different molecular weight cutoffs (MWCO) was used successfully to resolve PmLTI and PmSTI. This method was combined with affinity chromatography and gel filtration chromatography and used to purify large amounts of PmSTI. Crude extract was placed in a dialysis membrane with

a 12-14 KDa MWCO (or 10 KDa MWCO). This bag was then placed inside a second dialysis membrane with a 3,500 Da MWCO. PmSTI, along with other low MW proteins, were dialyzed out of the inner membrane and were retained by the outer dialysis membrane. The low molecular weight fraction between the two membranes was enriched for PmSTI and devoid of significant amounts of PmLTI. PmSTI was isolated by affinity chromatography from this enriched fraction. Gel filtration chromatography was used as the final step in the purification of PmSTI.

Alternatively, PmSTI fractions obtained by affinity chromatography were further purified by gel filtration followed by double dialysis. PmSTI-containing fractions after SEC were pooled and dialyzed against water using the double dialysis method. Protein retained within the 3,500 Da MWCO membrane exhibited strong inhibition of trypsin activity. Analysis of this fraction by SDS-PAGE revealed that this material was actually free of PmLTI (Figure 2-9). Unfortunately, a significant amount of PmSTI was still retained within the 12-14,000 Da MWCO membrane. The distribution of activity between the two fractions is presented in (Figure 2-8). Double dialysis with membranes of MWCO of 6-8,000 and 3,500 Da was also

used, but this method did not increase resolution and protein recovery (data not shown).

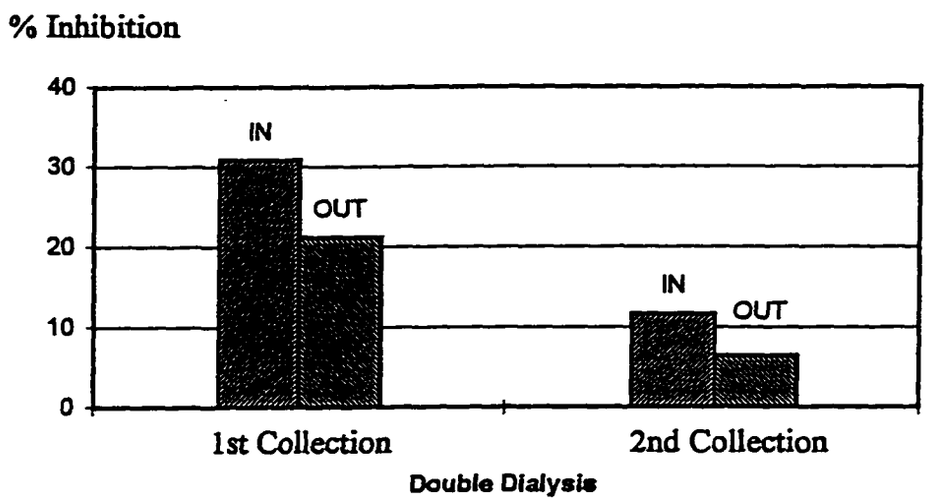

Figure 2-8. Trypsin inhibition of fractions after double dialysis of affinity-purified PmTI. 'IN' corresponds to fraction inside the 12-14,000 Da MWCO membrane; 'OUT' corresponds to fraction outside the 12-14,000 but retained in 3,500 Da MWCO membrane.

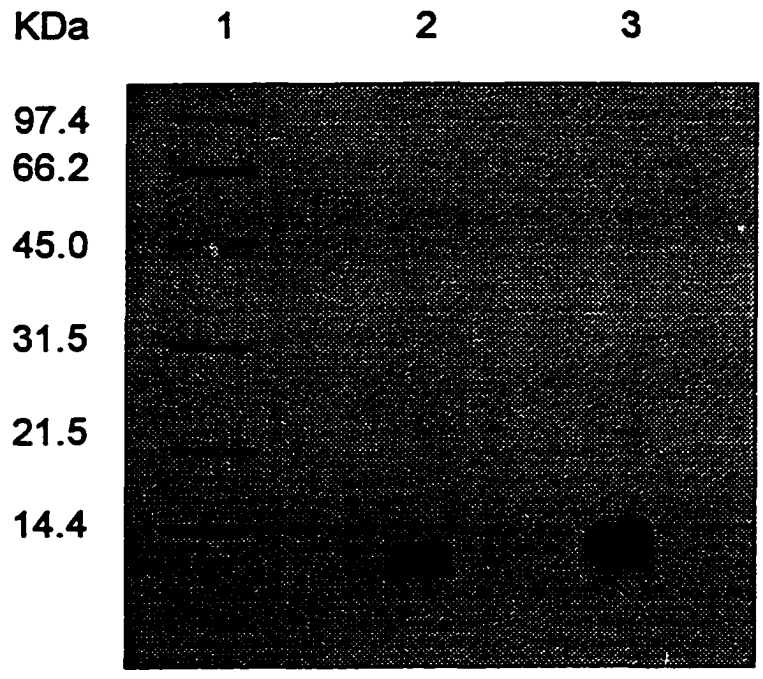

Figure 2-9. SDS-PAGE (15% gel) of PmSTI obtained after double dialysis. A sample (100 µl) of the fraction between the 3,500 Da MWCO and 12,000 Da MWCO membranes was dried in the Speed-Vac and dissolved in SDS-PAGE sample buffer without mercaptoethanol. Samples were boiled for 3 minutes before being loaded onto the gel. The gel was silver stained. Lane 1, molecular markers; lane 2, first collection; lane 3, second collection.

Purification of PmSTI using the double dialysis method as described was only partially successful, and the recovery of PmSTI in the volume between the two membranes was low. Multiple collection enabled us to elute more PmSTI out of the inner membrane from the original crude PmTI.

D. Identification and separation of PmTI isoforms

Only one Coomassie Brilliant Blue (CBB) stained band was observed after SDS-PAGE gel analysis of purified PmLTI. A single band was also observed with purified PmSTI. To determine whether isoforms of the two types of TI existed, these purified fractions were subjected to IEF. The protein banding pattern, as revealed by silver staining, and the activity profile of the purified inhibitors separated on an IEF gel using pH 8 to 10 ampholytes is presented in Figure 2-10. Two activity bands with pI's approximately 8.5 and 8.8 were revealed in the PmLTI sample. This result suggests that two isoforms of PmLTI may be present in *P. macroloba* seeds. For the PmSTI sample, two activity bands were also seen on the gel. One of these had an estimated pI of 8.15, while the other one had a pI below pH 8.0. This result indicates that there may be more than one form of PmSTI.

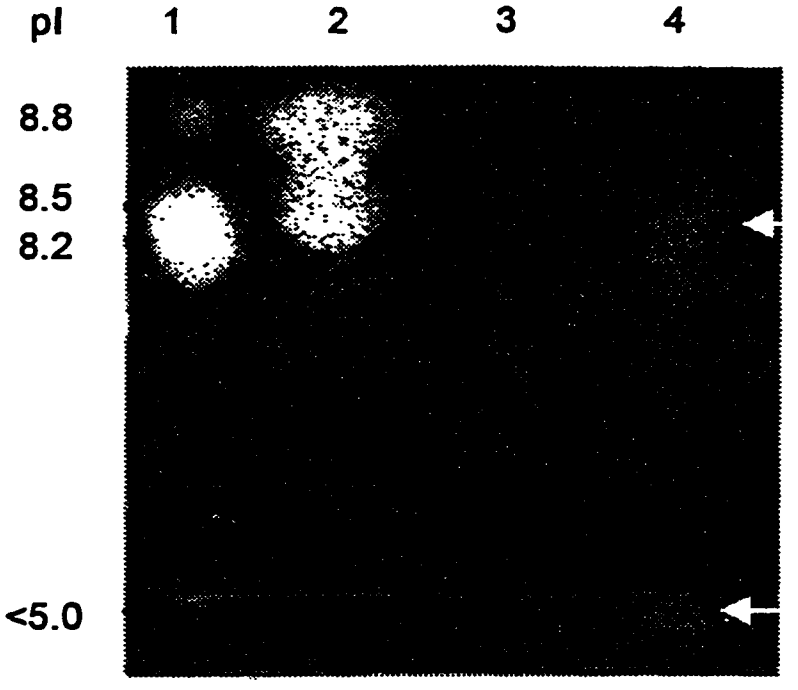

Figure 2-10. Isoelectric focusing electrophoresis of PmLTI and PmSTI. Purified PmLTI and PmSTI were lyophilized and dissolved in Nanopure water. Four µl (~ 1 µg) of each sample was loaded onto each lane. IEF was performed with an ampholyte range of 8 to 10. Pharmacia standard pI markers were used to estimate the pI values of PmLTI and PmSTI. The gel was stained using the in-gel activity assay. Lane 1, TAC-purified PmTI from heated (70 °C) extract. Lane 2, SEC-purified PmLTI. Lane 3, boiled PmLTI. Lane 4, PmSTI purified by double dialysis. PmSTI was indicated as noted (arrow).

Analysis of PmTI by native PAGE. Native PAGE was used to further test for the existence of multiple forms of PmLTI. When PmLTI was subjected to electrophoreses on a pH 10.0 native polyacrylamide gel, a protein band pattern similar to that observed after IEF was obtained using the in-gel activity stain (Figure 2-11). This result confirms the existence of at least two isoforms of PmLTI in seeds.

Separation of isoforms of PmLTI. Affinity purified PmLTI either consists of isoforms, isoforms modified during purification, or both. Separation of PmLTI isoforms was performed on a 2 x 9 cm Q-Sepharose column using a gradient of NaCl (0 to 0.15 M). Figure 2-12 showed the chromatography profile of purified PmLTI from affinity purified samples. Three major peaks were obtained while some minor peaks were also shown. SDS-PAGE of the fractions showed that the three major peaks had different protein banding patterns. Peak I had one protein band, Peak II had three bands and Peak III had seven bands. The patterns indicate that peak I and II may be the two isoforms of PmLTI while the peak III may be a modified PmLTI.

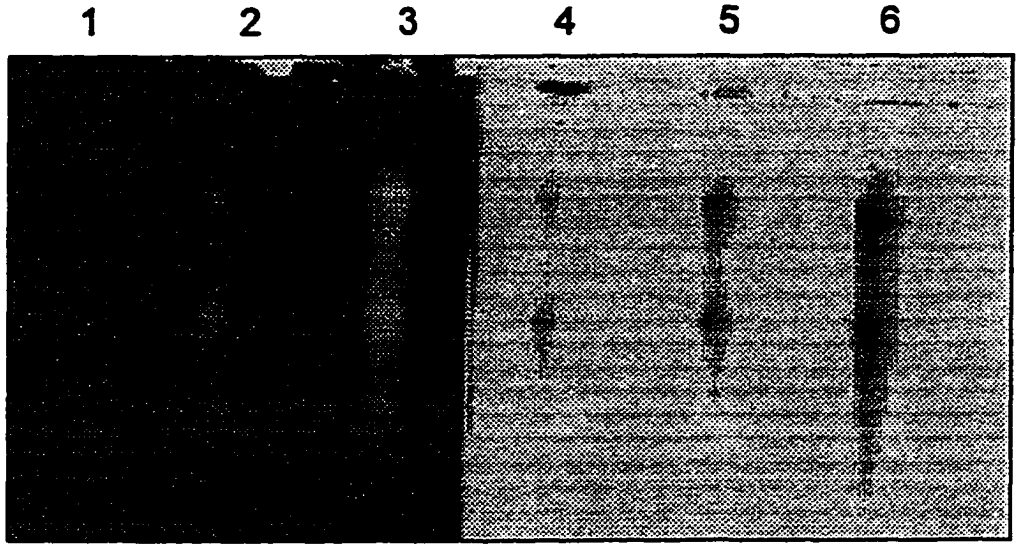

Figure 2-11. Native PAGE (12.5 %) of purified PmLTI at pH 10 using continuous electrophoresis system. The gel was prepared with pH 10 ethanolalmine-HCl buffer. The gel was cut into two parts. One (lane 1-3) was stained with CBB and the other (lane 4-6) was analyzed using the in-gel trypsin assay. Lanes 1,4: TAC-purified PmLTI from heated (70 °C) extract. Lanes 2,5: TAC-purified PmLTI from unheated extract. Lanes 3,6: ATAC-purified PmLTI from unheated extract.

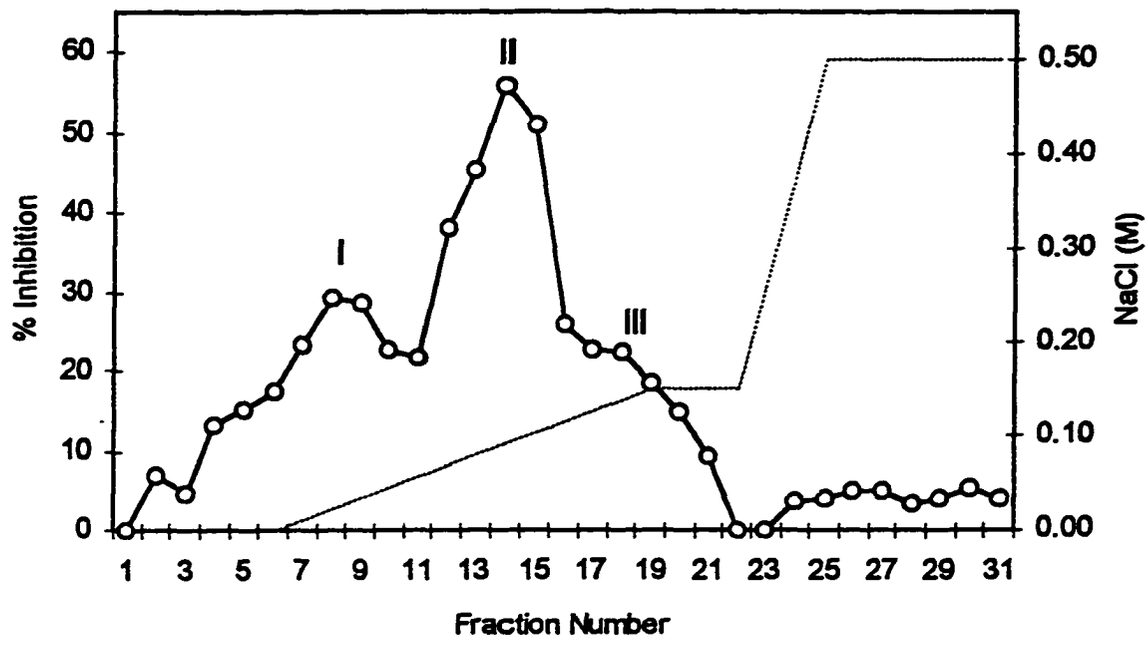

Figure 2-12. Anion exchange chromatography of PmLTI from affinity purified sample at pH 9.5 on a 2 x 9 cm Q-Sepharose column. Purified PmLTI was dissolved in 0.1 M Tris-Cl, pH 9.5 and loaded onto the column equilibrated with the same solution. Fractionation was conducted with a NaCl gradient from 0 to 0.15 M and then with 0.5 M NaCl. Inhibitory activity of the fractions was assayed against 5 μg bovine trypsin in 1 ml assay solution using 50 μl each fraction.

DISCUSSION

Pentaclethra macroloba seeds contain two serine protease inhibitors, named PmLTI and PmSTI corresponding to their sizes of molecular weight. Although PmSTI exists in very low amounts in seed extracts, the in-gel inhibition assay showed that inhibition was stronger for PmSTI than for PmLTI which is present at much higher amounts in the seed. Furthermore, SDS-PAGE analysis of affinity purified PmTI showed no PmSTI band on the gel by CBB staining despite clear evidence of its presence by the in-gel assay.

The results suggest that routine purification methods are not an effective approach to purify PmSTI. Taking advantage of its small size, enrichment of PmSTI was accomplished by double dialysis between two membranes of the 3,500 and 12-14,000 Da MWCO. PmSTI, which was retained by the 3,500 Da MWCO membrane, was then purified by affinity chromatography followed by HPLC. SDS-PAGE analysis indicated that a native PmSTI was purified.

Plant serine protease inhibitors usually have more than one isoinhibitor or isoform in the plant (Kortt and Jermyn, 1981). The seeds of most legume species contain more than one type of trypsin inhibitor.

Soybean harbors the well-known Kunitz TI and Bowman-Birk TI (Laskowski and Kato, 1980). *Mimosaceae* species have been found to contain trypsin inhibitors with molecular weights about 20 KDa, similar to the soybean Kunitz TI (Joubert, 1983; Kortt and Jermyn, 1981; Odani *et al.*, 1979; Richardson *et al.*, 1986). However, the occurrence of low molecular weight trypsin inhibitors in these *Mimosaceae* species has not been reported (Kortt and Jermyn, 1981; Garcia-Olmedo *et al.*, 1987). Our results indicate that PmLTI has at least two isoforms, while PmSTI could have more than one form in *P. macroloba* seeds.

The *Mimosaceae* is the most primitive sub-family of the *Leguminosae*. Inhibitors (Kunitz type) from this subfamily were considered to be a primitive type in legumes (Odani *et al.*, 1979). Members of the Kunitz family were also found in species other than the *Leguminosae*, such as in the *Araceae* and the *Alismataceae* (Chi *et al.*, 1985). PmLTI exhibits structural similarity (two chains linked by a disulfide bond) to other TI's from the *Mimosaceae*, but unlike other trypsin inhibitors of this subfamily, PmLTI exists as dimers (two single units associated together). PmSTI is the first low molecular weight trypsin inhibitor found in the legume subfamily *Mimosaceae*.

It has been proposed that an ancestral, unique trypsin inhibitor could generate multiple functions or multiple reactive sites by internal gene duplication and mutation (Odani and Ikenaka, 1976). One inhibitor with only one reactive site may evolve into an inhibitor with several independent reactive sites, capable of inhibiting various proteases or more than one trypsin molecule. PmSTI is the first small (less than 8 KDa) trypsin inhibitor found in the primitive *Leguminosae* subfamily *Mimosaceae* which only inhibits trypsin. These results suggest that PmSTI may be an evolutionary intermediate between inhibitors affecting trypsin alone and those with ability to inhibit both trypsin and chymotrypsin found in the subfamilies *Mimosaceae* and *Papilionaceae*.

One of our research goals is to enhance crop resistance to insect pests which cost billions of dollars in term of crop losses and control costs. Genes for trypsin inhibitors, such as BBTI, have been successfully transformed and expressed in tobacco species, conferring resistance to the insect pest *Heliothis virescens* (Hilder *et al.*, 1987). Both PmLTI and PmSTI are potential candidates to engineer into plant protection from insects.

CHAPTER III

CHARACTERIZATION, AMINO ACID SEQUENCE OF *P. MACROLOBA* TRYPSIN INHIBITORS

We have isolated and purified two trypsin inhibitors, PmLTI and PmSTI from *P. macroloba* seeds (Chapter II). Although PmLTI may be a Kunitz type trypsin inhibitor based on the SDS-PAGE gel patterns, PmSTI has not been classified into any inhibitor family since it is the first small inhibitor reported in the subfamily *Mimosaceae*. PmSTI has strong inhibitory activity against trypsin but not against chymotrypsin. PmSTI is a small polypeptide of about 7,000 to 10,000 Da estimated molecular weight based on SDS-PAGE. It is present in very low concentrations in affinity purified fractions. Since *P. macroloba* trypsin inhibitors were found to be partially modified during affinity purification, it was necessary to establish whether PmSTI was a proteolytic product derived from PmLTI or a different TI.

MATERIALS AND METHODS

Preparation of extract containing small proteins. Approximately 100 g of sliced and freeze-dried *P. macroloba* seeds were homogenized with a Polytron PT3000 homogenizer (Brinkmann) and extracted in 500

ml of ice cold 0.1 M Tris-Cl, 5 mM $MgCl_2$, pH 8.5. The suspension was stirred continuously at 4 °C up to 4 hours and then filtered through a double-layer of each cheesecloth and Miracloth. The filtrate was centrifuged at 17,500 rpm (SS34 rotor) at 4° C for 20 minutes to remove insoluble debris. The supernatant fluid was dialyzed against water with double dialysis membranes with a MW cutoff of 12,000 (inside) and 3,500 Da (outside). The fluid (small protein extract) between the two membranes was collected every 24 hours and lyophilized.

Purification of P. macroloba PmSTI. Lyophilized low molecular mass protein extracts were redissolved in 100 mM Tris-Cl, pH 9.0 and centrifuged briefly to remove debris. Samples were loaded onto a Pharmacia Resource Q column using a Pharmacia FPLC system. The column was equilibrated with 100 mM Tris-Cl, pH 9.0 buffer and the proteins were fractionated by gradient elution from 0 to 1.0 M NaCl in Tris buffer (50 ml) over 45 minutes. The flow rate was controlled at 1.0 ml/minute. Trypsin inhibition activity of protein fractions was analyzed using the standard assay. Activity peaks were pooled, dialyzed overnight against distilled water using dialysis tubing with a molecular mass cut-off of 3,500 Da. Active fractions were further purified by gel filtration

chromatography on a 1.0 x 100 cm Sephadex G-50 column or a 1 x 60 cm Superose 12 (Pharmacia) column. The fractions with strong antitrypsin activity were pooled. In order to fully understand the chromatographic behavior of PmSTI, a Mono S cation exchange column equilibrated with pH 5.0 buffer was used to fractionate active fractions after chromatography on the Resource Q column. Alternatively, affinity chromatography was used to purify PmSTI from the active fractions from the Resource Q column as previously described (Chapter II).

Isoelectricfocusing electrophoresis (IEF). A mini-IEF Chamber (BioRad) was utilized for isoelectricfocusing electrophoresis. The IEF gel was prepared according to the BioRad manual. Ampholytes with pH ranges of 3/10 and 8/10 were used. Trypsin inhibitory activity and protein bands were detected on IEF gels by the in-gel activity method and staining with Coomassie Brilliant Blue, respectively. Standard pI markers were separated on the same IEF gel and detected by protein staining. A plot of protein migration from the anode versus pI values for standard proteins was used to estimate the pI of TI's.

SDS-PAGE and immunodetection of the protein. Either reduced or nonreduced samples were analyzed by SDS-PAGE (15% separating gel and 3% stacking gel). All the samples were boiled for 3 minutes prior to electrophoresis. Two replicated gels were run, one for protein staining and the other for electroblotting onto nitrocellulose or PVDF membranes according to standard transfer procedures described in the manufacturer's manual. Immunoblots of PmSTI were processed as described by Promega using alkaline phosphatase conjugated secondary (goat anti-mouse) antibody (see protocol in Appendix I). Color detection was performed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (*Promega, Madison, WI*). Antibodies to PmSTI were obtained by immunizing a mouse with affinity purified PmSTI.

Inhibitory activity measurement and in-gel activity assay. The procedure for measuring trypsin inhibitory activity was described in Chapter II. Activity was measured in 0.1 M Tris buffer, pH 9.5 containing 5 mM $MgCl_2$. Trypsin (100 µl of a 50 ng/µl solution) was mixed with different amounts of PmSTI samples in the presence of 27 µl of 40 mg/ml *p*-BANA in DMSO (2.5 mM) in a final reaction volume of 1.0 ml. All

assays were performed at 25°C. Absorbance (410 nm) was automatically recorded at 30-second intervals for a total of 5 minutes with a Beckman DU 7500 spectrophotometer.

The in-gel activity assay was performed as described in Chapter II. After electrophoresis, the gel was washed briefly with deionized water and then with 0.1 M K-phosphate buffer (approximate pH 7.0). The gel was then immersed into the trypsin solution (15 mg trypsin dissolved in 150 ml 0.1 M K-phosphate buffer) and incubated for about 20 minutes. The trypsin solution was poured off and the gel was incubated at 25°C for 30 minutes. The substrate solution (20 mg acetyl-*dl*-phenylalanine-β-napthyl ester, 10 ml *N'N*-dimethyl formamide, 40 mg tetrazotized *orth*-dianiside and 70 ml 0.05 M potassium phosphate buffer, pH 7.0) was added to the gel. The gel was incubated at room temperature until the bands were visible. The reaction was stopped by transferring the gel to 2% acetic acid.

Protein modification and amino acid sequencing Purified PmSTI was isolated by micro HPLC on a column PLKP-S (1.0 x 50 mm), at a flow rate of 50 µl/min. HPLC was performed by the staff in the Molecular Biology Resource Center at the OU Health Sciences Center. The peak

fractions were collected for carboxy-methylation. The protein was dissolved in a 5-ml solution of 0.36 M Tris-HCl buffer, pH 8.6, 0.2 % ethylenediaminetetraacetic acid, and 5 M guanidine hydrochloride. The protein solution contained a β-mercaptoethanol to cysteine residue ratio of 150:1. The sample vial was purged with nitrogen, sealed and left at room temperature for up to 12 hours. The concentration of cysteine residues in the PmSTI was estimated using the analysis of amino acid composition. An equal molar of cysteine to monoiodoacetic acid ratio was gradually added to the reaction mixture. The reaction mixture was adjusted to pH 8.0 with 5 M NaOH and kept in the dark for 15 minutes. Finally, the mixture was dialyzed against water to remove salts. Chymotrypsin and lys-C specific protease were utilized for digestion of the carboxymethylated PmSTI. The protein digestion was performed by incubating the protein sample for 6 hours with the protease in the reaction buffer supplied by the manufacturer (Sigma, St. Louis MO).

Chymotrypsin and Lys-C protease digested PmSTIs were fractionated by micro HPLC on a 1.0x150 mm C_{18} column. Fractions were collected according to their absorbance at 216 nm. The amino acid composition was analyzed after acid hydrolysis of the carboxymethylated

ptotein. Fragments separated and purified by reverse phase chromatography (C_{18} column) were then sequenced by Auto Edman degradation methods. The analysis of amino acid composition and protein sequencing were performed by staff in OU Health Sciences Center Molecular Biology Resource Center.

N-terminal sequence analysis. Purified PmLTI and PmSTI were separated by SDS-PAGE (Laemmli, 1980) and electrotransferred onto PVDF membrane following the manufacturer's instructions (BioRad). The membrane was stained with CBB. The bands corresponding to PmLTI and PmSTI were cut out and sent for sequencing. The Edman degradation method used for amino acid sequencing was performed on a 477A Protein Sequencer/120A Analyzer from Applied Biosystems, Inc. by the University of Oklahoma Health Science Center Molecular Biology Resource Center.

RESULTS

A. Characterization of Inhibitory activity of PmTIs

1). Inhibitory activity of PmLTI and PmSTI against bovine trypsin. The amount of protein required to inhibit trypsin activity by 50% (I_{50}) was determined for both PmLTI and PmSTI in the presence of 2.5 mM

substrate, *p*-BANA. The values of I_{50} for PmSTI and PmLTI were 53 and 690 ng, respectively, at pH 9.5 in the presence of 5 mM $MgCl_2$. Under these conditions, PmSTI is a much more effective inhibitor of bovine trypsin than PmLTI.

When equimolar of trypsin and PmSTI were mixed and a molar excess of the substrate *p*-BANA was added to the reaction mixture, no hydrolysis was observed over twenty-four hours of incubation. This observation suggests that PmSTI is probably either a tight-binding or an irreversible trypsin inhibitor.

2). Specific inhibitory activity of PmTIs. The crude extracts, ATC-purified PmTI, and purified PmLTI and PmSTI from ATC-purified sample were tested for their specific inhibition against both *H. zea* trypsin and bovine trypsin. Results of inhibition assays against bovine and *H. zea* trypsins are compiled in Table 3-1. Both PmLTI and PmSTI inhibited bovine and *H. zea* trypsin differently. PmLTI exhibited higher specific activity against trypsin from *H. zea* than bovine trypsin. The specific activity was 2.8 and 3.9 IU/mg TI against bovine trypsin and *H. zea* trypsin, respectively. The specific activity of PmSTI was determined to be

50.94 IU/mg TI towards bovine trypsin and 14.23 IU/mg TI towards *H. zea* midgut trypsin.

Table 3-1. Comparison of the inhibitory activity of *Pentaclethra* trypsin inhibitors during purification with the activity of a commercial preparation of soybean trypsin inhibitors.

Sample	Specific Activity against bovine trypsin (IU/mg Protein)	Specific Activity against *H. zea* trypsin (IU/mg Protein)
Crude Extract	0.04	0.08
TAC purified TI	2.5	3.7
PmLTI [a)]	2.8	3.9
PmSTI [a)]	50.9	14.2
Kunitz TI [b)]	1.0	0.9
Bowman-Birk TI [b)]	5.0	2.1

a). Both PmLTI and PmSTI were purified from a TAC purified PmTI sample.
b). Kunitz and Bowman-Birk TIs were purchased from Sigma, St Louis MO.

The binding of inhibitor to trypsin was rather slow. Maximum inhibitory activity was obtained after preincubation of inhibitor fraction with trypsin for 10 min. (Figure 3-1). When TAC-purified PmTI, trypsin, and substrate were mixed together at the same time, the inhibition was lower than when PmTI and trypsin were incubated up to 10 minutes prior

to the addition of substrate. This result indicates that PmTI binds slowly before reaching its maximum inhibition.

3). Effect of temperature on PmTI inhibitory activity. Inhibitory activity of PmTI purified from an unheated crude extract was 3.7 *IU*/mg protein, whereas activity of a crude extract that was heated at 70°C for 5 minutes was only 2.2 IU/mg protein. Thus, heating resulted in the loss of approximately 40 % of the inhibitory activity.

SDS-PAGE analysis of heated and unheated samples of TAC-purified PmTI showed that the apparent MW of PmTI (both PmLTI and PmSTI) decreased upon heating. Table 3-2 summarizes the effects of heating on the inhibitory activity of both PmLTI and PmSTI, based on in-gel activity staining. Although treatment at 70°C caused a partial loss in activity of the large PmTI, heating at 70 °C had no apparent effect on PmSTI. Table 3-2 shows that the activity of PmLTI was completely lost when heated at 100 °C for only 5 seconds. In contrast, PmSTI only partially lost its activity after boiling for five minutes. PmSTI still retained inhibitory activity after boiling for 30 minutes. The activity, however, was lost completely when the sample was boiled for 5 minutes under reducing conditions.

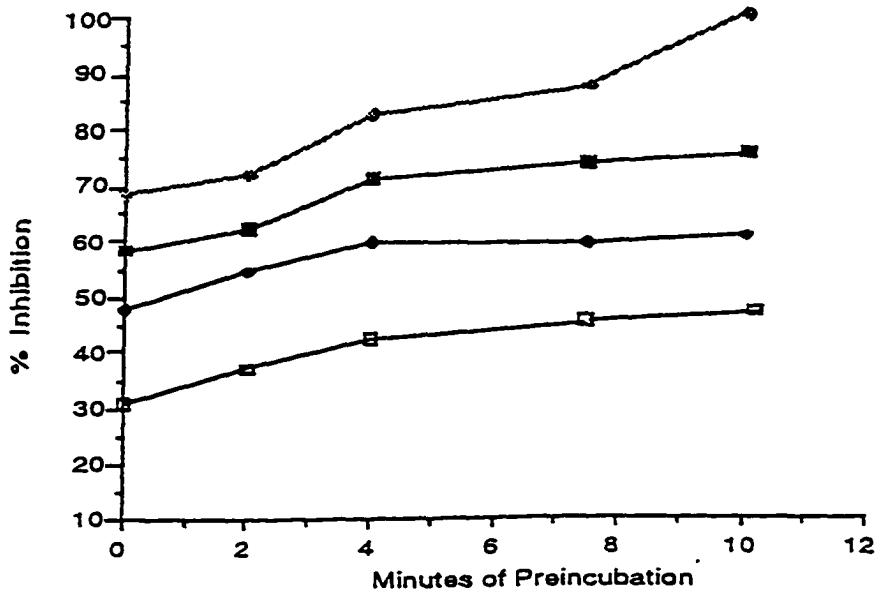

Figure 3-1. Inhibition of bovine trypsin by TAC-purified PmTI after preincubation with trypsin and PmTI. Both trypsin and PmTI were mixed and incubated for specified times. Substrate *p*-BANA was then added to the mixture and the tryptic activity was monitored constantly for 5 minutes at 25°C. Four different concentrations of PmTI were used.

Table 3-2. Effect of heating on the inhibitory activity of PmLTI and PmSTI.

Treatment	Inhibitory Activity	
	PmLTI	PmSTI
Buffer	+++	++++
Buffer + β-ME	+	++++
Buffer + Heat 70°C	++	++++
Buffer + Heat 70°C + β-ME	-	++
Buffer + Heat 100°C, 5 seconds	-	++++
Buffer + Heat 100°C + β-ME, 5 seconds	-	+
Buffer + Heat 100°C, 30 minutes	-	+++
Buffer + Heat 100°C + β-ME, 30 minutes	-	-

1). Inhibitory activity was estimated using the in-gel assay.
2). Buffer = SDS-PAGE sample buffer, β-ME = 2.5% (v/v) 2-mercaptoethanol.
3). ++++: very strong activity, +++: strong, ++: fair, +: low, -: no activity.

4). Determination of inhibitory constant (K_i) of PmSTI.

A plot of $1/V_i$ (V_i = rate of *p*-BANA hydrolysis in the presence of inhibitor) against total PmSTI concentration $[I]_t$ was nonlinear, hyperbolic and upward curving (Figure 3-2). This result suggests that PmSTI is an unusual type of protease inhibitor that does not obey the well-established kinetics of classical reversible inhibitors for which the plot of $1/V_i$ against $[I]_t$ is linear (Szedlacsek and Duggleby, 1995). Based on the plot of $1/V_i$ against $[I]_t$, PmSTI appears to be a tight-binding inhibitor (Szedlacsek and Duggleby, 1995).

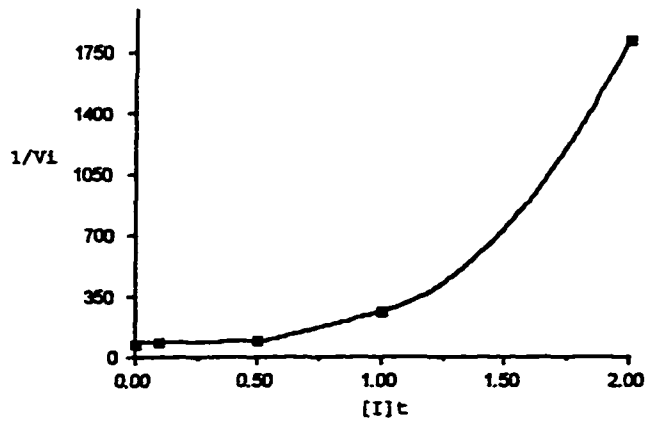

Figure 3-2: Plot of $[I]_t$ against $1/v_i$. Reaction mixture: [Trypsin], 1.0 μg/ml; *p*-BANA, 2.5 mM at 0.1 M Tris-Cl, 5 mM $MgCl_2$, pH 9.5. The reaction rate was measured at 410 nm continuously for five minutes.

PmSTI inhibiton kinetics were determined on the basis of the following equations derived by Morrison (1969) for a tight-binding inhibitor. Plots of $I_t/(1-v_i/v_o)$ as a function of v_o/v_i were linear with an intercept equal to E_t and a slope equal to a so-called apparent inhibition constant K_{iapp} (equation 1). K_{iapp} is a substrate-dependent inhibition constant expressed as equation (2) (Szedlacsek and Duggleby, 1995). K_{iapp} can be expressed as either equation (3) or (4) depending on the competitive or uncompetitive nature of the inhibitor.

$$\frac{I_t}{1-v_i/v_o} = K_{iapp} \times \frac{v_o}{v_i} + E_t \qquad (1)$$

where $[I]_t$ is the total concentration of PmSTI, V_i = initial rate in the presence of PmSTI, V_0 = initial rate in the absence of PmSTI, K_{iapp} = apparent inhibition constant, E_t = total concentration of trypsin.

For a noncompetitive inhibitor,

$$K_{iapp} = K_3 \times (1+\frac{A}{K_m})(1+K_3\frac{A}{K_mK_4}) \qquad (2)$$

where K_3 = the dissociation constant of the trypsin and PmSTI complex, K_4 = the dissociation constant of trypsin, substrate BANA and the PmSTI complex, A = substrate concentration, Km = Michaelis-Menten constant.

If $K_4 = \infty$ (i.e., competitive inhibitor), then

$$K_{iapp} = K_3 \times (1+\frac{A}{K_m}), \quad K_i = K_3 \qquad (3)$$

If $K_3 = \infty$ (i.e., uncompetitive inhibitor), then

$$K_{iapp} = K_4 \times (1+\frac{K_m}{A}), \quad K_i = K_4 \qquad (4)$$

Reaction rates were determined using different amounts of the inhibitor in the presence of different concentrations of substrate. The experimental data were expressed in the form of $\frac{I_t}{1-v_i/v_o}$ and v_i/v_o. K_{Iapp} values for different substrate concentrations were obtained from the plot of

$\frac{I_i}{1-v_i/v_o}$ vs. v_i/v_o (Table 3-3). K_{iapp} was then plotted against different substrate concentrations. This plot was approximately linear, and, therefore, fit equation (3) (Figure 3-3). This suggests that PmSTI is a competitive inhibitor. The inhibition constant (K_i) of PmSTI was 1.0×10^{-10} M. This value does not depend on the enzyme or substrate concentrations, while K_{Iapp} is affected by the enzyme or substrate concentrations.

Table 3-3. Effect of substrate concentration on the apparent inhibition constant (K_{iapp}) of PmSTI.

Substrate concentration (mM)	K_{iapp} (M)	$[E]_{total}$ ($\times 10^{-7}$ M)
1.25	3.69×10^{-9}	0.90
2.5	5.03×10^{-9}	1.14
5.0	1.55×10^{-8}	0.76
7.5	1.75×10^{-8}	1.10
10.0	2.63×10^{-8}	1.06

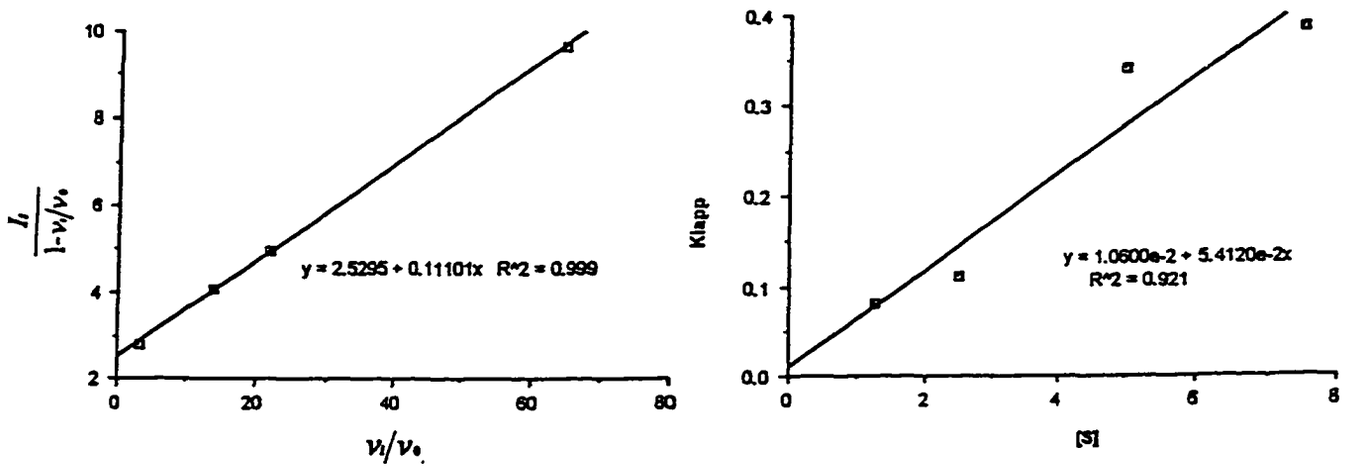

Figure 3-3. Determination of inhibition constant K_i. **A.** Plot of $\frac{I_i}{1-v_i/v_o}$ against v_i/v_o. The slope of the plot was the apparent inhibition constant (K_{iapp}) according the equation (1) in the text. **B.** Plot of apparent inhibition constant ($K_{I\ app}$) vs. substrate concentration (A). The Y-intercept, which was 1.01×10^{-10} (0.10 nM), was the inhibition constant, K_i, for PmSTI according to the equation (3) in the text.

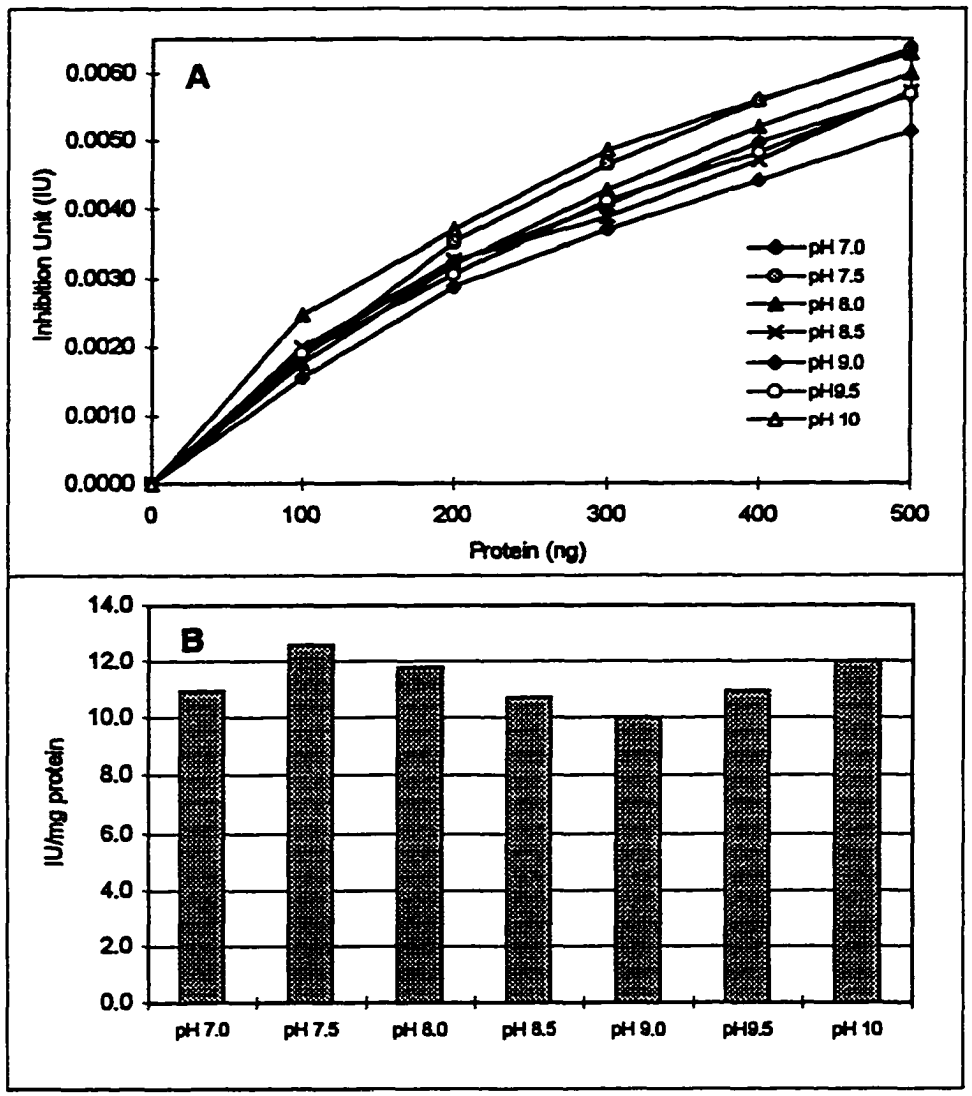

Figure 3-4: Effect of pH on PmSTI inhibitory activity against bovine trypsin. Enzyme and PmSTI were mixed and incubated for five minutes before the substrate *p*-BANA was added. Inhibition was determined by continuously monitoring the absorbance at 410 nm for five minutes at 25°C. Tris-Cl buffers with different pH values were used in the reaction. A: Inhibitory activity of PmSTI at different concentrations and pH values. Experimental data from three replicate assays were used. B: Specific activity (IU/mg protein) of PmSTI at different pH's.

5). Effect of pH on PmSTI inhibitory activity. The inhibitory activity of purified PmSTI was determined at different pH values ranging from 7.0 to 10.0. There was no significant difference in specific inhibitory activities at any of the tested pH's (Figure 3-4). The specific inhibitory activity of PmSTI was the lowest at pH 9.0 and the highest at pH 7.5. This result suggests that PmST activity is relatively unaffected by pH over the range from 7 to 10.

B. Biochemical properties.

1). Determination of molecular weight. The molecular mass of PmLTIs was estimated by size exclusion chromatography to be about 40,000 Da. To examine the molecular structure of PmLTI further, samples of PmLTI were subjected to denaturing electrophoresis on a gradient SDS-PAGE gel from 10-20% acrylamide in the absence of reductant with or without boiling the samples. Two bands with estimated molecular weights of 43,000 and 39,000 Da were detected in the unheated sample of PmLTI. However, one major band with an estimated MW of 21.5 KDa was detected with heat-denatured PmLTI under non-reducing conditions. A second minor band with an estimated molecular weight of 17-18 KDa was

also detected. These observations suggest that PmLTI is a dimer (Figure 3-5).

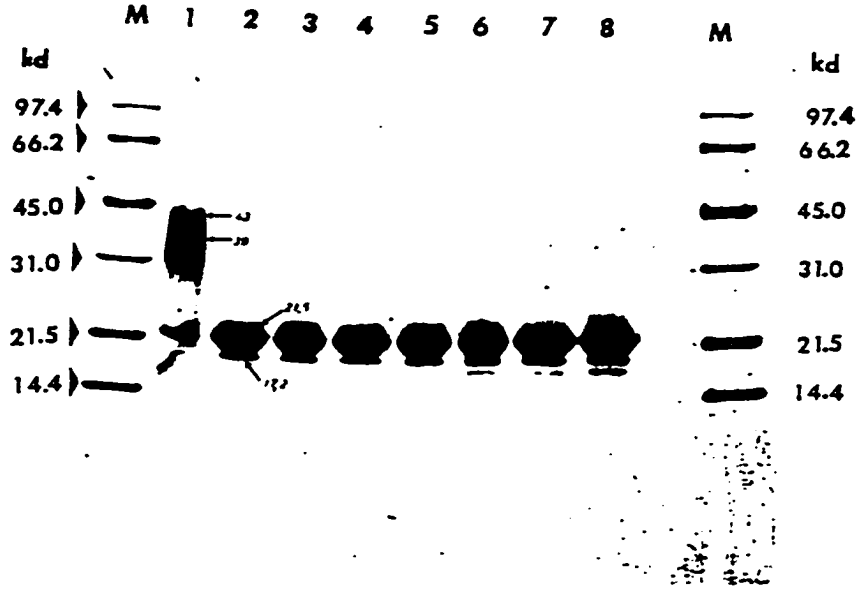

Figure 3-5. 10-20% gradient SDS-PAGE gel of PmLTI. PmLTI was denatured in SDS-PAGE sample buffer without reductant. About 10 μg ATC-purified PmLTI was loaded on each lane. Lane 1, sample was not boiled. Lane 2-8, samples were boiled for 5 seconds, 1, 2, 3, 4, 5, 7.5 and 10 minutes, respectively. The gel was silver stained.

After reduction with β-mercaptoethanol and boiling, PmSTI ran as a broad, diffuse band on SDS-PAGE gels. PmSTI, which was boiled only and not reduced, produced a sharper band (Figure 3-6). Based on SDS-PAGE analysis of the non-reduced protein, the estimated molecular mass of PmSTI was 8,000-10,000 Da. Calculation of a molecular weight for the reduced protein was possible but not useful since the protein band was so diffuse. Mass spectrometry was utilized to more precisely determine the molecular weight of PmSTI. Two major peaks, corresponding to molecular masses of 6689 and 6789 Da, were observed (Figure 3-7). The result suggests that there may be two isoforms or variants of the purified PmSTI.

2). Isoelectric points. Isoelectric points (pIs) of PmLTI and PmSTI were measured by isoelectricfocusing electrophoresis. The pI of standard markers was plotted against the migration distance from the anode (Figure 3-8). Two bands with pIs of 8.8 and 8.5 were observed in the PmLTI sample. The detection of two bands suggests that two isoinhibitors may present in the PmLTI sample. These may correspond to the 43 and 39 KDa band previously detected by gradient SDS-PAGE. Two bands with

pIs of about 8.15 and lower than 5.0 were also observed, although only one broad was detected in the SDS-PAGE gel.

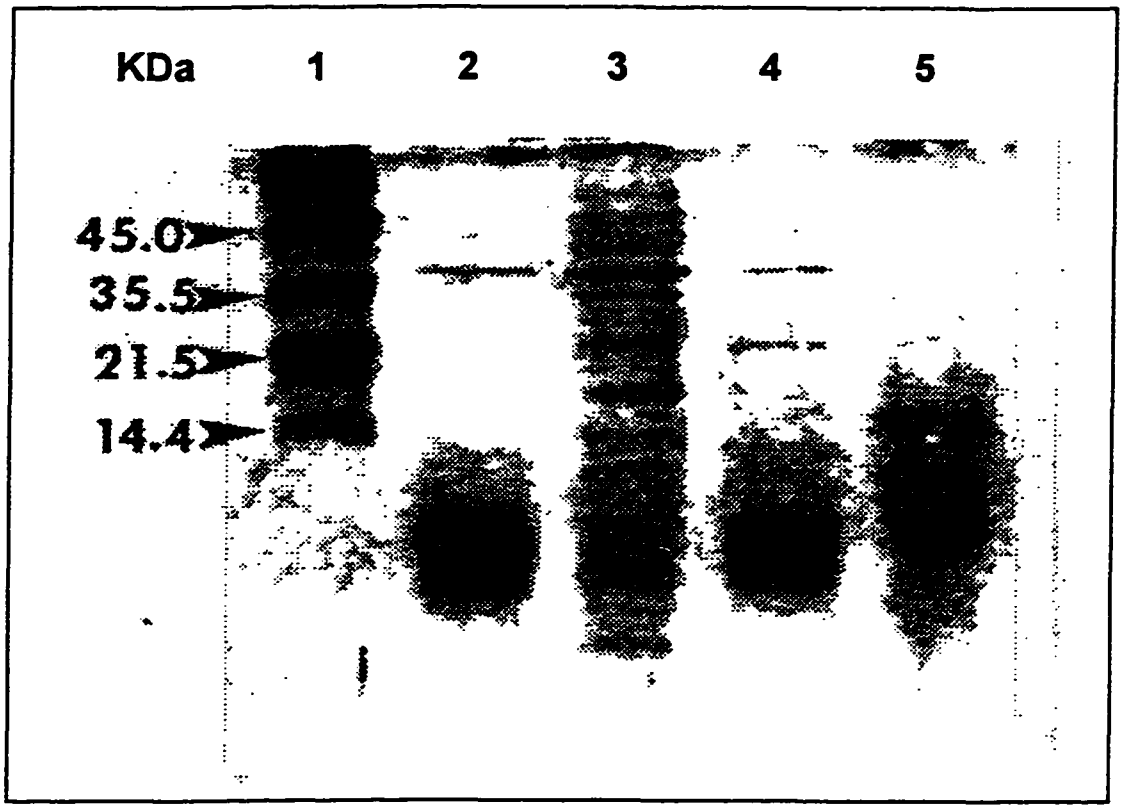

Figure 3-6. SDS-PAGE of PmSTI. Lane 1, marker proteins; lane 2 and 3, PmSTI purified by TAC and double dialysis; lane 4 and 5, intact PmSTI purified by double dialysis and SEC. Lane 2 and 4, samples were boiled in SDS-PAGE sample buffer without β-mercaptoethanol; lane 3 and 5, samples were boiled in SDS-PAGE sample buffer with β-mercaptoethanol.

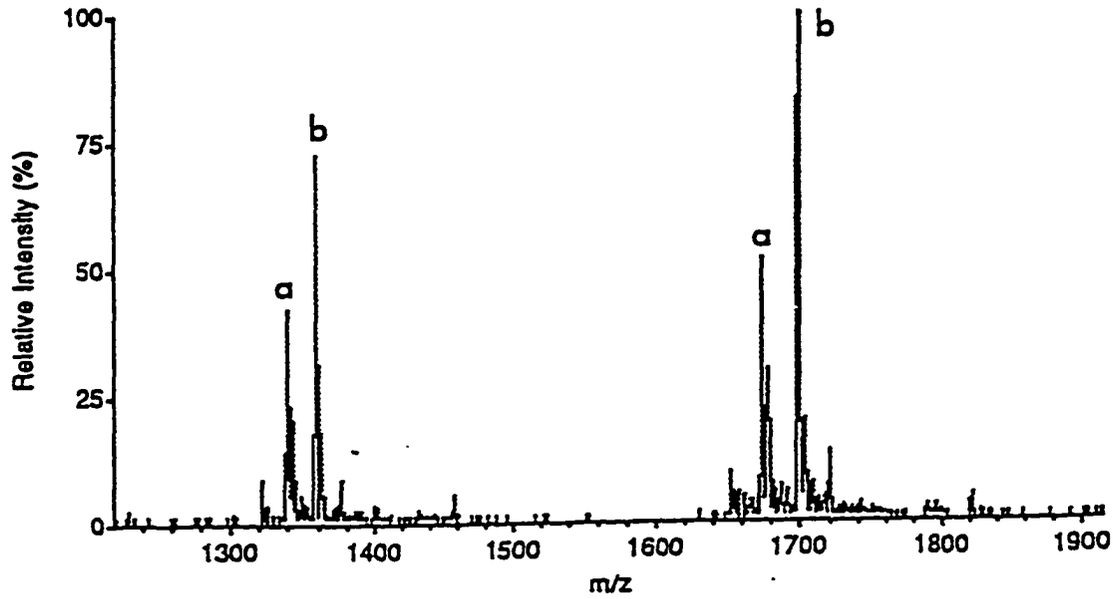

Figure 3-7. Determination of PmSTI molecular weight by liquid chromatography/ mass spectrometry (LC/MS). LC/MS was performed by Dr. Ken Jackson in the Molecular Biology Resource Center of OU Health Sciences Center using PmSTI sample which was purified by affinity chromatography and then by the double dialysis method. Peaks a and b corresponded to the molecular weight of 6689 and 6789 Da, respectively.

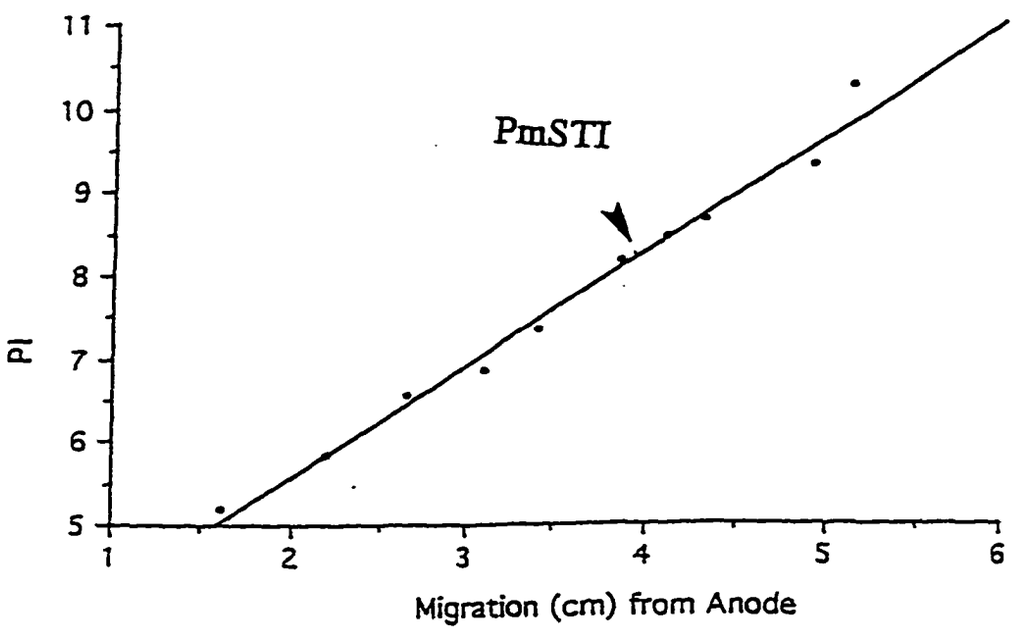

Figure 3-8. Determination of the pI for PmLTI and PmSTI. Samples of purified PmLTI and PmSTI purified by double dialysis and SEC were separated on an IEF gel using an ampholyte range from pH 3-10. Standard pI markers were also separated. The gel was cut and the section with pI markers was stained with CBB. Trypsin inhibitor activity was detected using the in gel assay. The distance (in cm) of each band from the anode was measured and this value was plotted against the pI for each standard. This resulted in a linear plot of pI values and migration distance. Two activity bands were detected for both PmSTI and PmLTI.

Table 3-4. Amino acid composition analysis of PmSTI.

AMINO ACID COMPOSITION	PMSTI-1	PMSTI-2
Asp(n)	10 (8)	9 (8)
Thr	7 (7)	6 (6)
Glu(n)	5 (4)	6 (4)
Ser	5 (4)	5 (4)
Pro	7 (8)	8 (8)
Gly	1 (0)	2 (0)
Ala	3 (3)	5 (3)
Cys	11 (14)	10 (14)
Val	1 (0)	0 (0)
Met	0 (0)	0 (0)
Ile	2 (1)	1 (1)
Leu	0 (0)	0 (0)
Tyr	2 (2)	2 (2)
Phe	3 (2)	3 (2)
Lys	4 (3)	4 (3)
His	? (1)	? (1)
Arg	4 (2)	4 (2)
Trp	N/A (1)	N/A (1)
Total AA	61	60
Calculated MW	6678	6797
Mass spec MW	6689	6789

3). Amino acid composition of PmSTI. PmSTI sample was hydrolyzed in 6N HCl and the amino acid composition was determined using an automated amino acid analyzer. Based on amino acid analysis, PmSTI is rich in cysteine, proline and threonine (Table 3-4). These three amino acids make up almost half (46%) of the amino acid residues present in PmSTI. Furthermore, cysteine represents about 23 % of the total amino acid residues.

4). Other biochemical properties. In order to fully understand the chromatographic behavior of both trypsin inhibitors, reduced and native affinity-purified trypsin inhibitors were separated by SEC on a Superose 12 column. The protein peaks in chromatographic profiles of TAC-purified PmTI treated with and without reduction were similar, indicating that the PmLTI dimer was not stabilized by disulfide bridge(s) (Figure 3-9).

The chromatographic behavior of native PmSTI on an ion exchange column was different than predicted. Based on the experimentally determined pI's, one would predict that PmSTI would bind to an anion exchange resin at pH values above 8.5. When PmSTI was applied to a BioRad Q_5 column equilibrated with Tris-Cl or hydroxylamine-HCl buffer, pH 9.5, PmSTI did not bind to the column (Figure 3-10). Most of the

inhibitory activity was in the flow through fraction. Furthermore, PmSTI did not bind to a Pharmacia Mono-S column (cation exchange) equilibrated in Na-citrate buffer, pH 5.0.

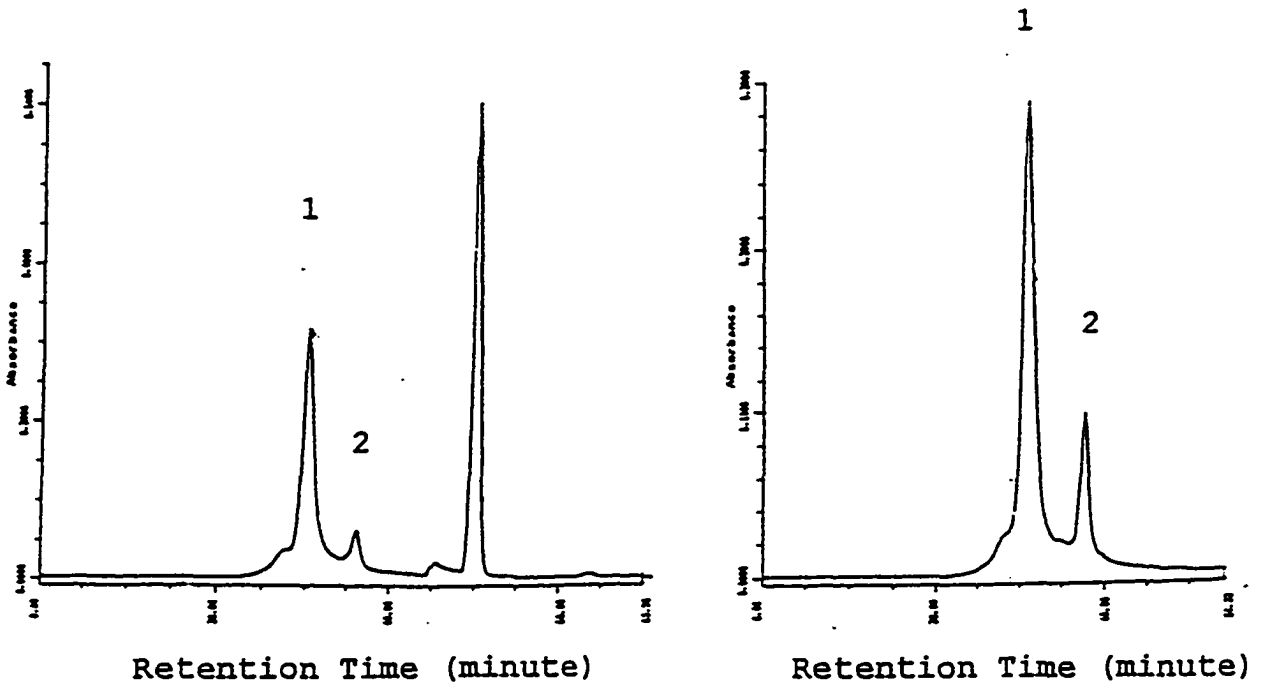

Figure 3-9. Comparison of the SEC elution profiles of β-ME-treated and non-treated PmTIs. A Pharmacia Superose 12 column (1.0 x 60 cm) was equilibrated with 50 mM phosphate buffer, pH 7. About 1 mg (200 µl) TAC-purified PmTI was run on the column. Flow rate: 1 ml/min; fraction size, 1 ml/tube. Peak 1 corresponds to PmLTI and peak 2 corresponds to PmSTI. Left panel, PmTI was reduced with 2 mM β-mercaptoethanol; right panel, non-reduced PmTI.

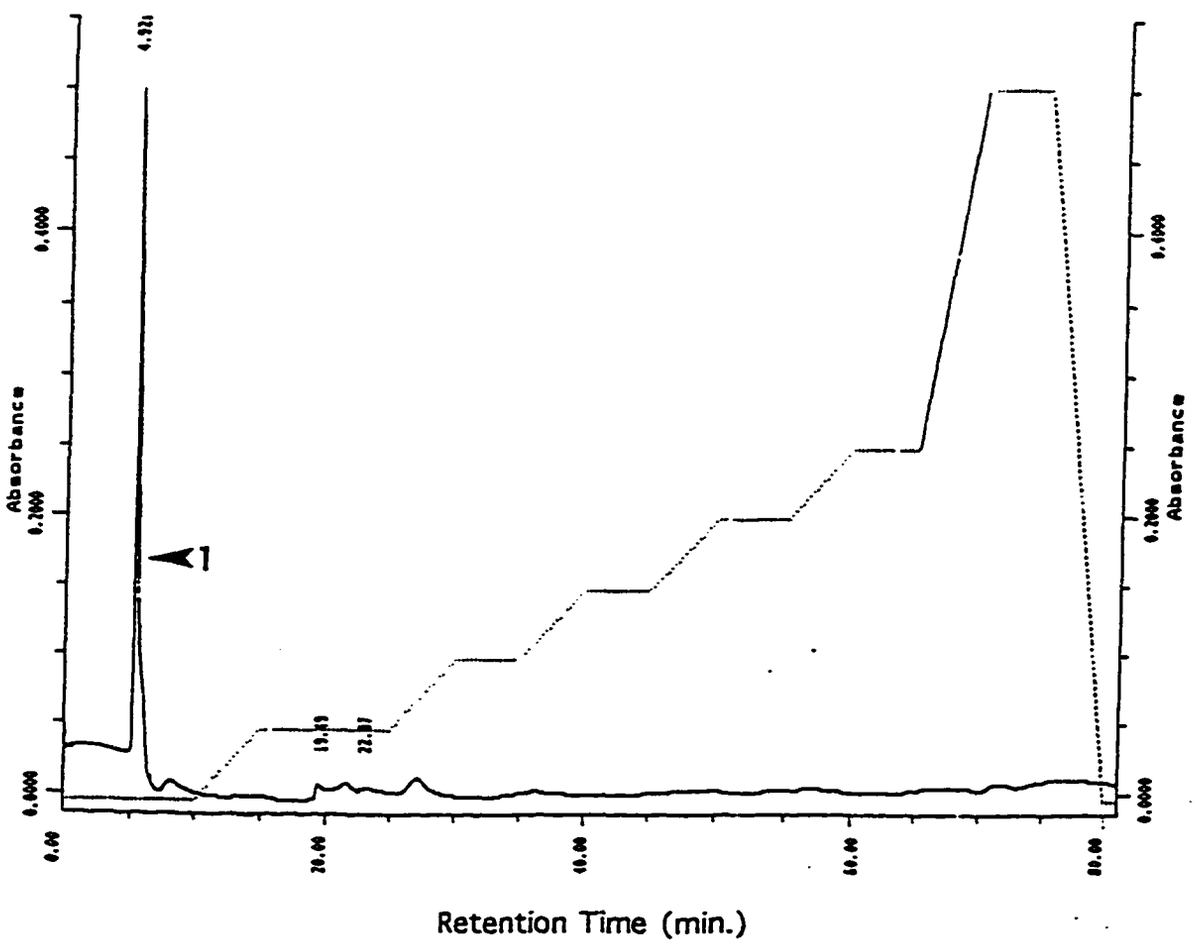

Figure 3-10. Anion exchange chromatography of PmSTI on a Q_5 anion exchange column. The column was equilibrated with 0.1 M Tris-Cl, pH 9.5 and eluted with a 0 to 1.0 M gradient of NaCl in the equilibrating buffer. Flow rate, 1 ml/min. Peak 1, corresponded to fully active PmSTI.

C. Amino acid sequencing of PmLTI and PmSTI.

1*). N-terminal sequencing of PmLTI.* The N-terminal sequence (Table 3-5) of PmLTI was determined with an automated Edman amino acid sequencer Amino acid similarities to other plant trypsin inhibitors were compared by searching the Swiss Gene Bank database through the Internet. Table 3-5 compares the N-terminal sequences of five trypsin inhibitors which are very similar to PmLTI. Over 40% of the N-terminal sequence of PmLTI were identical to the sequence of trypsin inhibitors from other species from the subfamily *Mimosaceae* .

Table 3-5: Comparison of the N-terminal sequence of PmLTI with sequences of other Kunitz type trypsin inhibitors.

Inhibitors	N-terminal sequence	% Identity	% Similarity
PmLTI	EVVFDFKGDMMRNGGHYYFFPAAPYGGGNLLAAAV		
ATI [a)]	KELLDADGDILRNGGAYYILPALRGKGGGLTLAKT	42.9	68.6
WTI	EPLLDSEGELVRNGGTYYLLPDRWALGGGIEAAAT	42.9	54.3
PTI	QELLDVDGEILRNGGSYYILPAFRGKGGGLELAKT	40.0	68.6
API	RELLDVDGNFLRNGGSYYIVPAFRGKGGGLELARTG	40.0	60.0
STI	DFVLDNEGNPLENGGTYYILSDITAF-GGIRAAPT	31.4	40.0

a). ATI = *Acacia confusa* trypsin inhibitor, PTI = *Prosopsis juliflora* trypsin inhibitor, WTI = winged bean trypsin inhibitor, API = *Adenanthera pavonina* trypsin inhibitor, STI = Soybean Kunitz trypsin inhibitor. (:) = similar, (|) = identical. % Identity indicates the percentage of amino acids identical to PmLTI's in the sequence. % Similarity indicates the percentage of amino acids identical and similar to PmLTI's in the sequence.

2). *Complete amino acid sequence of PmSTI.* Purified PmSTI was electroblotted to PVDF membrane for N-terminal amino acid sequencing. The first and second N-terminal residues were threonine. Further sequencing suggested that there was more than one N-terminus. Each cycle contained two residues. For example, threonine and alanine were detected in cycle 3, one of the residues from previous cycle was present in the next cycle. These results suggested that there were two identical peptides present in the sample except for the addition of a third threonine on the N-terminus of one peptide. This conclusion is consistent with the results of mass spectral analysis in which two peptides were detected. These two peptides differed in mass by the mass of threonine residue. Alignment of the two N-terminal sequences is presented in Figure 3-11. In order to obtain internal sequence data, PmSTI was carboxymethylated, purified by HPLC and digested with Lys-C specific protease (Boehringer-Mannheim, Indianapolis IN) and chymotrypsin (Sigma, Louis MO). Peptide fragments were purified by RP-HPLC and sequenced directly.

Four major fragments were obtained after Lys-C digestion. Sequencing of three of the fragments revealed very clear sequences. Two fragment sequences have Lys at their C-termininus while the third

fragment has a serine at its C-terminal end rather than a lysine residue. This result suggested that the third fragment might be the C-terminus of PmSTI. Sequencing of the chymotrypsin-digested fragments also gave three clear sequences. An eight-residue sequence of chymotrypsin-digested fragment was identical to the C-terminal end of the third fragment of the Lys-C digestion. The molecular mass of this fragment was determined by mass spectrometry to confirm this fragment was the C-terminus of PmSTI. The results of mass spectrometry indicated that the molecular mass of the fragment was 912 Da which is very close to the estimated MW of the eight residue fragment. These results support the notion that this fragment was the C-terminal sequence of PmSTI. Sequences of internal fragments were aligned based on overlapping sequences when possible. The tentative assignment of the location of the 8-residue Lys-C fragment 2 was made pending confirmation by other methods. The complete sequence of the PmSTI is presented in Figure 3-11. Two N-terminal variants differing by one residue are shown. The estimated pI determinated based on the sequence information was estimated to be 7.7, a value close to that obtained experimentally by IEF.

```
                    1          11         21         31         41         51         61
WHOLE SEQUENCE   (1)TTTACCDNCP CTKSNPPQCQ CNDWKETCHS ACKTCICRAI YPPQCRCFDT NNFCYPPCPS S
                 (2) TTACCDNCP CTKSNPPQCQ CNDWKETCHS ACKTCICRAI YPPQCRCFDT NNFCYPPCPS S

N-Terminal-1       TTTACCDNCP CTKSNPPQ
N-Terminal-2        TTACCDNCP CTKSNPPQ
Fragment-1(lyc)                SNPPQCQ CNDWK
Fragment-2(lyc)                                ETCHS ACK
Fragment-3(lyc)                                          TCICRAI YPPQCRCFDT NNFCYPPCPS S
Fragment-1(chy)     TTACCDNCP CTKSNPPQCQ
Fragment-2(chy)                                             RAI YPPQCRCF
Fragment-3(chy)                                                              CYPPCPS S
```

Figure 3-11. Proposed amino acid sequence of PmSTI based on alignment of sequence fragments. N-terminal sequencing of intact PmSTI and HPLC-purified Lys-C and chymotrypsin digestion products was performed.

3). Sequence analysis of PmSTI. The Genetic Computer Group (GCG) program available via the Internet was used to analyze the extent of homology between PmSTI and other TI's at the amino acid level. Results of this analysis are summarized in Figure 3-12 and Table 3-6. PmSTI appears to be a Bowman-Birk type protease inhibitor, which shares over 50% identity to most of the other legume Bowman-Birk protease inhibitors. Based on this analysis PmSTI appears to be most closely related to the *Medicago sativa* protease inhibitor sharing 68% identity and 78% similarity. Both inhibitors are low molecular weight BBIs which do not inhibit chymotrypsin. BBI usually has two reactive sites with similar structural motifs (Garcia-Olmedo *et al,* 1987). However,

the second reactive site of PmSTI, which is Ala and Ile at the P_1 and P_1' positions, respectively, is different from those of the other BBIs. Meanwhile, PmSTI and *Medicago* BBIs are threonine- and proline-rich polypeptides. They both exhibited high specific inhibitory activity against trypsin.

Table 3-6. Homology of PmSTI to other Bowman-Birk protease inhibitors.

Species	Access Number[A)]	% Identity	% Similarity	AA length	References
Medicago sativa	p16346	68	78	58	Brown *et al.*, 1985
Medicago scutellata	P80321	67	79	62	Ceciliana *et al.*, 1994
Glycine max	p01055	59	70	110	Baek and Kim, 1993
Vicia sativa sp. nigra	p01065	56	71	72	Shimokawa *et al.*, 1983
Vigna angularis	p01061	56	68	78	Kiyohara *et al.*, 1981
Vicia faba	p24661	56	70	63	Asao *et al.*, 1991
Glycine max	p01064	56	68	83	Joudrier *et al.*, 1987
Macrotyloma axillare	p01059	56	66	76	Joubert *et al.*, 1979
Glycine max	p01063	56	65	83	Joudrier *et al.*, 1987
Vigna radiata	p01062	55	68	72	Zhang *et al.*, 1982
Vigna angularis	p01058	54	66	82	Ishikawa *et al.*,1979
Phaseolus lunatus	p01056	54	68	83	Stevens *et al.*, 1974
Macrotyloma axillare	p01057	54	67	76	Joubert *et al.*, 1979
Lonchocarpus capassa	p16343	53	60	80	Joubert,1984
Vigna unguiculata	p17734	48	64	83	Morhy and Ventura, 1987
Phaseolus vulgaris	p01060	46	60	79	Wilson and Laskowski, 1975
Setaria italica	p19860	53	61	67	Tashiro *et al.*, 1990
Setaria italica	p22737	53	61	67	Tashiro *et al.*, 1991
Coix lacryma-jobi	p07679	44	44	64	Ary *et al.*, 1988
Hordeum vulgare [a)]	p12940	43	50	124	Nagasue *et al.*,1988)
Arachis hypogaea	p01067	40	49	63	Norioka and Ikenaka,1983
Oryza sativa [a)]	p07084	50	59	133	Tashiro *et al.*,1987
Triticum aestivum	p09864	41	44	53	Odani *et al.*,1986
Triticum aestivum	p09863	41	45	56	Odani *et al.*,1986
Arachis hypogaea	p01066	38	48	70	Norioka and Ikenaka,1983

A, Swiss-pro protein access number.

Species	Sequence Alignment	References
Pentaclethra macroloba	TTTACCDNCP CTKSNPPQCQ CNDWKETCHS ACKTCICRAI YPPQCRCFDT NNFCYPPCPS S	Brown et al., 1985
Medicago sativa	.....NF.. ..R.I....R .T.IG..... .....L.TKS I....H.A.I T.....K.	Ceciliana et al., 1994
Medicago scutellata	TKS.....F.. ..R.I..... .T.VR.K... ...S.L.TLS I....H.Y.I TD....S.	Baek and Kim, 1993
Glycine max	GDDVKSA...T.L ..R.Q..T.R .V.VG.R... ..NH.V.NYS N....Q.F.. HK...KA.H. .	Shimokawa et al., 1983
Vicia sativa subsp. nigra	ESSKP...Q.A .....R .S.MrnS... ...S...ALS Y.A..F.V.I TD...E..KP S	Kiyohara et al., 1981
Vigna angularis	SESSHP...L.L ....I..... .A.IrdS... ...S.M.TRS M.G....L.. HD..HK..K. R	Asao et al., 1991
Vicia faba	GDDVKSA...T.L ....E..T.R .V.VG.R... ..NS.V..YS N..K.Q.... HK...K..HN	Joudrier et al., 1987
Glycine max	EYSKP...L.M ..R.M....S .E.IrnS... D..S.M.TRS Q.G....L.. .D...K..K. R	Joubert et al., 1979
Macrotyloma axillare	SESSKP...L.T ....I....H ...MrnS... ...S...ALS E.A..F.V.. TD...K..HN N	Joudrier et al., 1987
Glycine max	DESSKP...L.M ..A.M....H .A.IrnS... ..DR.A.TRS M.G....L.. TD...K..K. .	Zhang et al., 1982
Vigna radiata	SESSEP...S.D ....I..E.H .ANIrnS... ...S...TRS M.GK...L.. DD...K..E.	Ishikawa et al.,1979
Vigna angularis	SESSKP...Q.S ....M..K.R .S.IrnS... ...S.A.TYS I.AK.F.T.I .D...E..K. .	Stevens et al., 1974
Phaseolus lunatus	SZSSKP..BH.A ....I....R .T.LrdS... ...S...TLS I.A..V.BBI BD...E..K. .	Joubert et al., 1979
Macrotyloma axillare	SESSKP...E.A ....I....R .T.VrnS... ..SS.V.TFS I.A..V.V.M KDA...A..K. .	Joubert,1984
Lonchocarpus capassa	ESESSKP..SS.. ..R.R..... .T.VrnS... ...S.M.TFS D.GM.S.L.V TD...K..K. .	Morhy and Ventura, 1987
Vigna unguiculata	SZSSKP..RZ.A ....I..Z.R .SZVrnS... ...S.A.TFS I.AZ.F.GBI BB...K..K. .	Wilson and Laskowski,
Phaseolus vulgaris	SZSSPP..BI.V ..A.I....V .TBIrbS... ...S.M.TRS M.GK...LB. TBY..KS.K. B	1975
Setaria italica	SGRK...LQT ....I.AF.R .R.LL.Q.SD ...E.	Tashiro et al., 1990
Setaria italica	SGRK...LQT ....I.AF.R .R.LL.Q.SD ...E.	Tashiro et al., 1991
Coix lacryma-jobi	GDEKRE...IAM ..R.I..I.R .V.KVDR.SD ...D.. EET EDNRHV....	Ary et al., 1988
Hordeum vulgare	AGKKRE...KAI ..R....T.R .V.EVKK.AP T....LPSRS R.SRRV.I.S	Nagasue et al.,1988)
Arachis hypogaea	AASD..SA.I .DRRA..e.T .G.TFDH.PA ..NK.V.TRS I......T.R TQ	Norioka and Ikenaka,1983
Oryza sativa	PWGD...KAF .N.M...T.R .M.EVKE.AD ...D.	Tashiro et al.,1987
Triticum aestivum	ATRK...RAI ....F..M.R .M.MV.Q.AA T..K.G.ATS D9SRRV.E.	Odani et al.,1986
Triticum aestivum	AAKKRK...QAV ..R.I..I.R .M.QVFE.P. ...A.G.SVG D.SRRV.Q.	Odani et al.,1986
Arachis hypogaea	SSSDDNV..NG.L .DRRA..e.V .V.TFDH.PA S.NS.V.TRS N......T.K TQ	Norioka and Ikenaka,1983

Figure 3-12. Sequence alignment of PmSTI and other Bowman-Birk protease inhibitors found in *Swiss-Pro* database. (...) indicates amino acid residue is identical to PmSTI's.

DISCUSSION

PmLTI and PmSTI have been purified and characterized. PmLTI is a Kunitz-type trypsin inhibitor with a MW of about 40 KDa and consists of two isoinhibitors. PmSTI is a Bowman-Birk type protease inhibitor with a molecular weight of about 7 KDa based on SDS-PAGE. As far as we know, no low MW trypsin inhibitor from the legume subfamily, *Mimosaceae* have been reported (Garcia-Olmedo *et al.*, 1987). Therefore, PmSTI is thought to be the first small molecular weight TI reported in this subfamily.

Although the N-terminal sequence of PmLTI is homologous to other Kunitz trypsin inhibitors, the active form of PmLTI is a dimer, indicating that PmLTI is different from other legume Kunitz-type inhibitors. The *Araceae* trypsin inhibitor (Kunitz-type) is the only other dimeric inhibitor that has been reported to date (Hammer *et al.*, 1989). Soybean Kunitz TI forms dimers and polymers, but activity is lost during purification (Odani *et al.*, 1971). Native PmLTI has an inhibitory activity of 2.78 and 3.93 IU/mg protein against bovine and *H. zea* midgut trypsins, respectively (Table 3-1). PmLTI has weak inhibitory activity against chymotrypsin as

well (data not shown). Heat-denatured, monomeric PmLTI, however, did not exhibit inhibitory activity.

PmSTI is a much stronger inhibitor than PmLTI and other Bowman-Birk protease inhibitors. The specific inhibition values against bovine and *H. zea* midgut trypsins were 50.9 and 14.2 IU/mg protein, respectively, at pH 9.5. Unlike most BBIs, inhibition of chymotrypsin by PmSTI was undetectable. Some low molecular weight trypsin inhibitors, such as Bowman-Birk and the squash inhibitors, are generally potent inhibitors of trypsin (Garcia-Olmedo *et al.*, 1987). The rigid secondary structures and disulfide bridges around the reactive sites of these inhibitors apparently contribute to their potency (Bode and Huber, 1992). The inhibitory activity of PmSTI is still remarkable when compared to the Bowman-Birk or the squash inhibitor which have inhibitory activity in the range of 3-4 IU/mg TI (Hwang *et al.*, 1977; Pham *et al.*, 1985).

We determined that the specific inhibitory activity of soybean BBI was 5.03 and 2.1 IU/mg TI against bovine and *H. zea* trypsins at pH 9.5, respectively (Table 3-1). PmSTI activity was ten-fold stronger than soybean BBI's. Furthermore, PmSTI retained activity against bovine trypsin even after boiling for 30 minutes. These results suggest that PmSTI

is one of the most potent and stable trypsin inhibitors ever reported. This stability could be related to the absence of unstable peptide bonds such as Asp-Pro. The instability of these amide bonds has been shown to contribute to the labile structure of peptides (Correia *et al.*, 1993).

Most proteinaceous protease inhibitors are slow, tight-binding inhibitors. Results of the kinetic analysis of inhibition by PmSTI supported the conclusion that PmSTI is a tight-binding inhibitor with a K_i of 0.1 nM. Furthermore, the inhibitory activity of PmSTI was not affected by pH in the range from 7.0 to 10 where trypsin is fully active.

The inhibitory activity of TAC-purified PmLTI was significantly different from that of ATAC-purified PmLTI. This indicates that PmLTI lost partial activity due to cleavage of PmLTI by trypsin during affinity purification. However, the inhibitory activity of PmSTI was unaffected by TAC even though PmSTI was cleaved during purification on trypsin column. It is possible to conclude that the structure of PmSTI is very stable and is not significantly altered during affinity purification in spite of being modified by trypsin.

PmSTI can be characterized as an atypical polypeptide based on the following pieces of evidence: (1) the native charge of PmSTI may be

masked since PmSTI was not retained by either an anion exchange column at pH 9.5 or by a cation exchange column at pH 5.0. (2) PmSTI is a cysteine rich peptide. After reduction and removal of reductant, cysteine residues may randomly form disulfide bridges and quickly generate large peptide complexes as disulfide bridges reform between individual peptides (Jones *et al.* 1994). When boiled in SDS-PAGE sample buffer with β-mercaptoethanol, PmSTI appeared as a diffuse single band in the SDS-PAGE gel. However, PmSTI appeared as a sharp single band in the gel when treated with SDS-PAGE sample buffer without β-mercaptoethanol.

Taken together, the amino acid analysis, the mass spectral data, and the amino acid sequence determination show that PmSTI consists of two variants in purified samples with only one threonine difference at their N-terminus residues. Analysis of amino acid sequence data suggests that PmSTI is a member of the Bowman-Birk protease inhibitor family. Although ATI (alfalfa TI) is the smallest PI (58 residues) ever reported (Brown *et al.*, 1985), PmSTI is one of the smallest BBIs (60-61 residues). At least one Bowman-Birk isoinhibitor or isoform exists in most legumes in the *Papilionaceae* subfamily (Kortt and Jermyn, 1981). The position of cysteine residues and disulfide bridges around the active sites of inhibitors

from the BBI family are highly conserved (Figure 3-12). Close examination of the PmSTI sequence suggests that the active site is very similar to that of BBI except that there is no active site for chymotrypsin inhibition. The second active site (P_1-P_1' = Ala-Ile) of PmSTI is totally different from those of other BBIs (Arg, Lys, or Leu - Ser), where the P'_1 residue determines the specificity of inhibition (Garcia-Olmedo *et al.*, 1987, Wen *et al.*, 1995).

The BBI family can be divided into two groups according to the estimated isoelectric points: those with pI greater than 7.0 and those with a pI about 5.0. It would be interesting to find out whether the difference in pIs relates to the strength of inhibition of the inhibitors since the strength of inhibition is determined by enzyme-inhibitor interaction (Wen *et al.*, 1995). Differences in isoelectric points may affect the enzyme-inhibitor interaction at certain pHs. Also, the protonated side chains may increase the binding strength of the enzyme-inhibitor under assay conditions. Both PmSTI and ATI, classified in the group with pI's greater than 7.0, do not inhibit chymotrypsin but have very strong inhibitory activity against trypsin.

CHAPTER IV

cDNA ISOLATION AND CHARACTERIZATION OF THE *P. MACROLOBA* SMALL PROTEASE INHIBITOR

PmSTI was purified and shown to be a very strong inhibitor of trypsin. One of the practical applications for agronomically significant toxic proteins is that they can either be applied on plant surfaces or synthesized internally by genetically modified plants to discourage insect attack. The gene of a Bowman-Birk type inhibitor (cowpea PI) placed under the control of the CaMV 35S promoter was introduced and expressed in tobacco plants (Hilder, *et al.* 1987). Bioassays indicated that the trypsin inhibitor was effective in protecting these transgenic plants from damaging insects and represented approximately 1% of the total soluble protein in leaves. Insect survival was nearly 50% when compared to that of the control plants. Our ultimate objective has been to clone and express the cDNA encoding PmSTI in plants.

MATERIALS AND METHODS

The cDNA library, made by Pioneer Hi Bred International, Inc., was constructed using mRNA isolated from immature seeds (10 mm diameter) of *Pentaclethra macroloba*. The cDNA was inserted into the *EcoRI-Xho I*

site located within the *Lac Z* gene region of the λ- ZAP express® cloning vector (Alting-Mees *et al.*, 1992).

cDNA library screening. The strategy used to screen for PmSTI cDNA clone(s) is outlined in Figure 4-1. The bacterial strain XL1-BLUE MRF' was incubated at 37°C overnight in 50 ml LB broth containing 10 mM $MgSO_4$ and 0.2% maltose. The culture was then centrifuged at 1000 x g for 10 min. The pellet was resuspended in 20 ml 10 mM $MgSO_4$ and the concentration of the cell solution was adjusted to an OD_{600nm} of 0.5 using 10 mM $MgSO_4$. The cell solution (600 µl) and 300 µl phage dilution (1.67×10^5 pfu/ml) were mixed and incubated at 37 °C for 15 min. Six ml of top agar (0.7%), kept at 45 °C, was then added to the cell/phage mixture and the mixture was poured onto a LB plate. The plate was incubated at 37 °C for 9-12 hrs.

Plaques were transferred onto nitrocellulose membranes following the manufacturers' protocols (MSI). Lifts were maintained for 4 minutes at each step during membrane blotting, denaturation (0.5 M NaOH, 1.5 M NaCl) and neutralization (1.0 M Tris-Cl, 1.5 M NaCl, pH 7.5). The lifts were then washed twice with 2xSSC, pH 7.0 for 2 minutes each, air-dried and baked at 80 °C for 2 hours under vacuum.

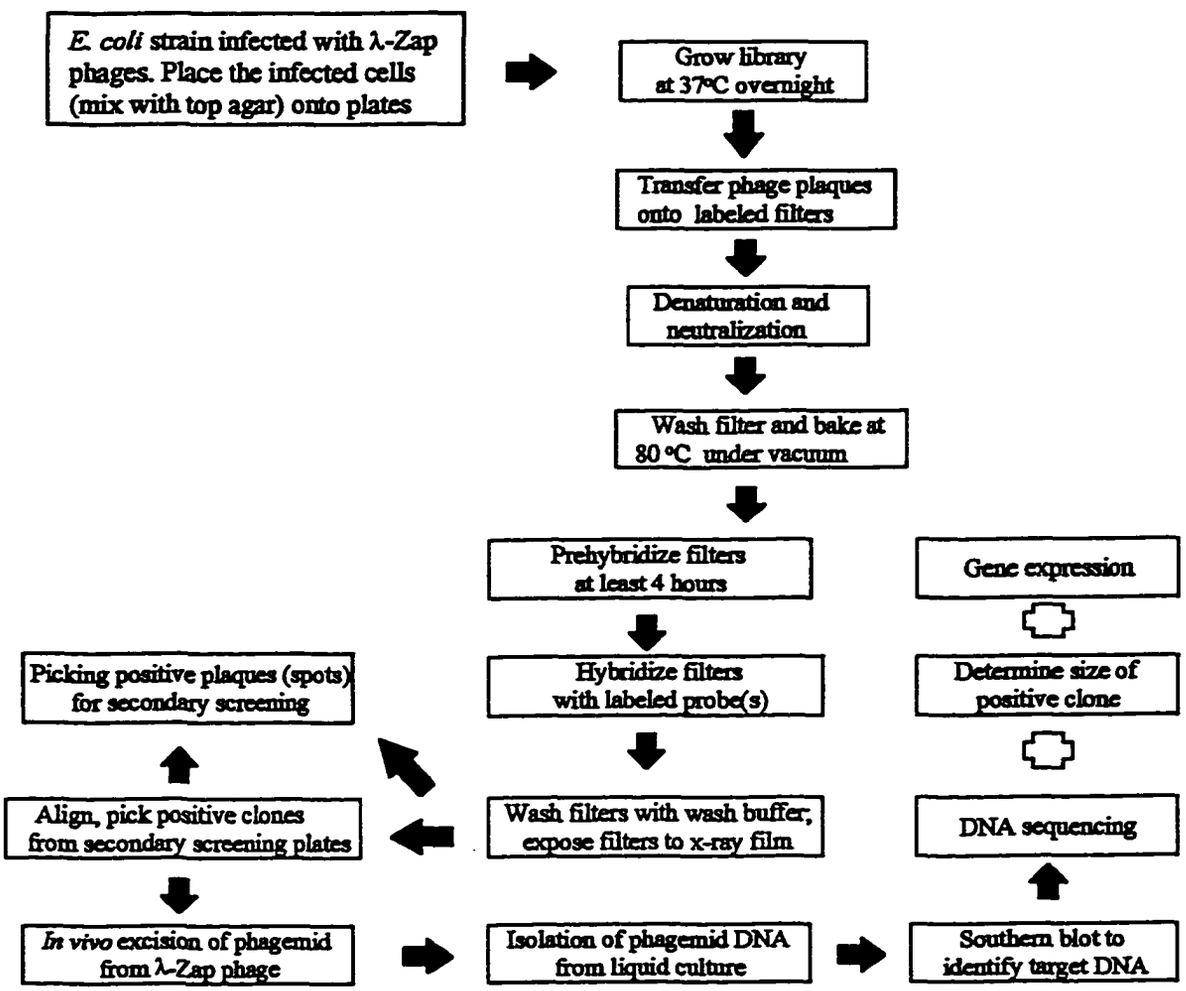

Figure 4-1. Flowchart for screening a *P. macroloba* seed cDNA library using oligonucleotide probes specific for PmSTI.

DNA probes (NST and CST) were synthesized based on the N-terminal and C-terminal amino acid sequences of PmSTI, respectively. The design of the degenerate 24-mer oligonucleotide probes took into account codon preferences noted in other legumes. The specific amino acid sequences and degenerate 5' and 3' probes are shown in Figure 4-2. Each probe (100 ng) was mixed and then labeled by the 5' end labeling method with γ-^{32}P-ATP (see protocol in Appendix I). The ^{32}P-labeled probe was diluted to 200 µl by adding 150 µl H_2O or TE buffer, then stored at -20°C.

Prior to DNA hybridization, blots were wetted with prehybridization solution, sealed into a bag with 20 ml of prehybridization solution and incubated at 48°C in a shaking water bath. After at least 4 hrs, the prehybridization solution was poured out and pre-warmed hybridization solution (50°C) containing 50 µl (50 ng) of labeled probes was added into each bag. Hybridization was carried out at 48°C overnight. The membranes were washed with 6xSSC + 0.1% SDS at 48 °C until background radioactivity was undetectable. Autoradiography was performed at -80 °C for 48 to 72 hours using a Fisher® cassette and intensifying-screen with X-film (*Hyperfilm*®MP, *Amersham Inc.*). Positive

clones were cored out and cultured for secondary screening utilizing the same procedures as the first screening.

Excision of phagemid from positive clones. Phagemid pBK-cmv can be excised *in vivo* from λ-Zap express® recombinants with the assistance of a corresponding helper phage. The specific protocol is described in the cloning manual (Stratagene, CA). Bacterial strain XL1-BLUE MRF' was infected by the phages overnight and coinfected with helper phage Exassist ® (Stratagene, CA) at 37 °C. Cells were then killed by incubating at 65-70 °C for twenty minutes and the phages were recovered by centrifugation to remove cell debris at 5000 rpm. The phage mixture was then mixed with bacterial strain XLOLR (*kan*^{s}), which is resistant to phage infection, and incubated on a LB plate in the presence of 50 μg/ml kanamycin. Only bacteria transformed with phagemid pBK-CMV, which is excised from the λ-Zap express® phage and expresses the *kan*^{r} gene, could grow under kanamycin selection pressure. PmSTI clones were inducibly expressed in LB plates supplemented with 50 μg/ml kanamycin, 1 mM IPTG and 10 μl of a X-gal solution (250μg/ml). White clones were maintained on LB plates containing 50 μg/ml kanamycin.

DNA blot of phagemid colonies excised from phages. Bacterial colonies were grown in LB medium with 50 μg/ml kanamycin at 37°C overnight. Phagemid DNA was prepared by the alkaline lysis method (Birnboim and Doly, 1979). A serial dilution of DNA (1.0, 0.5, 0.2 μg) was dotted onto a nitrocellulose membrane. The DNA in the membrane was then denatured, neutralized, and washed in 2x SSC solution as described above. A mixture of ^{32}P-labeled NST and CST probes was used for hybridization. Hybridization was performed under stringent conditions (50°C, 2x SSC) following standard procedures (Sambrook *et al.*, 1989).

DNA sequencing. The positive PmSTI clones in pBK-CMV vector (*Stratagene, CA*) were directly sequenced as double stranded DNA on an Applied Biosystems Auto DNA Sequencer (Model 373A) using T_3 primer upstream of and T_7 primer downstream of the insert. For sequencing purposes, plasmid was purified using the alkaline lysis method (Birnboim and Doly, 1979). Data compilation and analysis were performed with the Genetic Computer Group (GCG) program for protein and nucleic acid databases. DNA sequencing was performed by the Molecular Biology Facility of Oklahoma State University.

Expression, purification and analysis of fusion protein. The bacterial strain XLOLR carrying the PmSTI cDNA was spread onto a LB plate supplemented with 50 μg/ml kanamycin and incubated overnight at 37°C. One of the colonies was picked and incubated in 50 μg/ml kanamycin LB liquid medium overnight at 37°C. Cells were pelleted by centrifugation at 4000 rpm and resuspended with fresh medium. When the OD_{600nm} of the culture reached about 0.5, expression of fusion protein was then induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1.0 mM. The culture was shaken for another 3 to 4 hours at 37 °C. Cells were collected by centrifugation and resuspended in 50 mM Tris-Cl, pH 8.0 buffer containing 10 mM EDTA and 100 mM NaCl. Cell lysis was performed in the presence of 0.25 mg/ml lysozyme and 3 mM PMSF at 37°C. The lysate was sonicated for 5 minutes on an ice bath and 1% Tween-20 and 10 μl DNase (1 mg/ml) was added until the lysate was no longer viscous. The lysate was finally centrifuged at 14,000 rpm for 15 minutes.

SDS-PAGE was applied to establish the presence of PmSTI fusion protein in the supernatant and pellet fractions. Protein concentration was

estimated by the Bradford method (1976). The pellet (inclusion body) and supernatant (about 20 μg protein) were mixed with SDS-PAGE sample buffer and boiled for 5 minutes. Following electrophoresis on 15% SDS-PAGE gels, proteins were transferred to nitrocellulose membrane by electroblotting, and then reacted with mouse PmSTI-specific antibodies. Secondary antibodies (goat anti-mouse) conjugated to alkaline phosphatase were used to detect the primary antibody. Color development was carried out in the presence of NBT/BCIP according to manufactures' instructions. To dissolve and purify the PmSTI from bacterial inclusion body, a method of purification of bacterially expressed proteins was used (Sambrook *et al.*, 1989).

RESULTS

Isolation and identification of cDNA clones encoding PmSTI. Two degenerate probes derived from the amino acid sequence of PmSTI were chemically synthesized and used to screen a *P. macroloba* seed cDNA library (Figure 4-2). Primary screening of the cDNA library with two mixed probes resulted in 120 positive plaques out of 9.2×10^5 plaques. Positive plaques were then screened under conditions of high stringency

(50°C, probe T_m = 56 °C). Two positive clones (designated pBK391 and pBK392) were selected for further analysis (Figure 4-3).

A fragment between the T (termination) and I (initiation) region in λ-Zap Express® vector (Stratagene, La Jolla CA) was able to recircularize as the phagemid pBK-cmv in the presence of helper phage (Figure 4-4). *In vivo* excision of positive phagemids was performed according to *Stratagene's* procedure and colonies carrying positive phagemids were identified by immobilizing phagemid DNA onto the nitrocellulose membrane and detection with NST/CST probes. Plasmid DNA isolated from the phagemids was further identified by DNA blot assay.

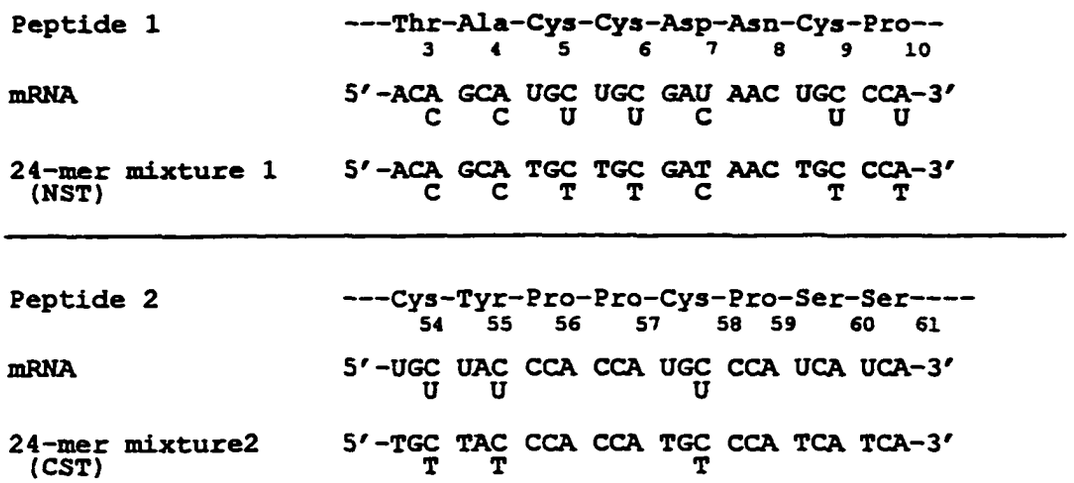

Figure 4-2. Oligonucleotide mixtures employed for screening for PmSTI cDNA clones. Peptides 1 and 2 represent the N-terminus and C-terminus of PmSTI, respectively.

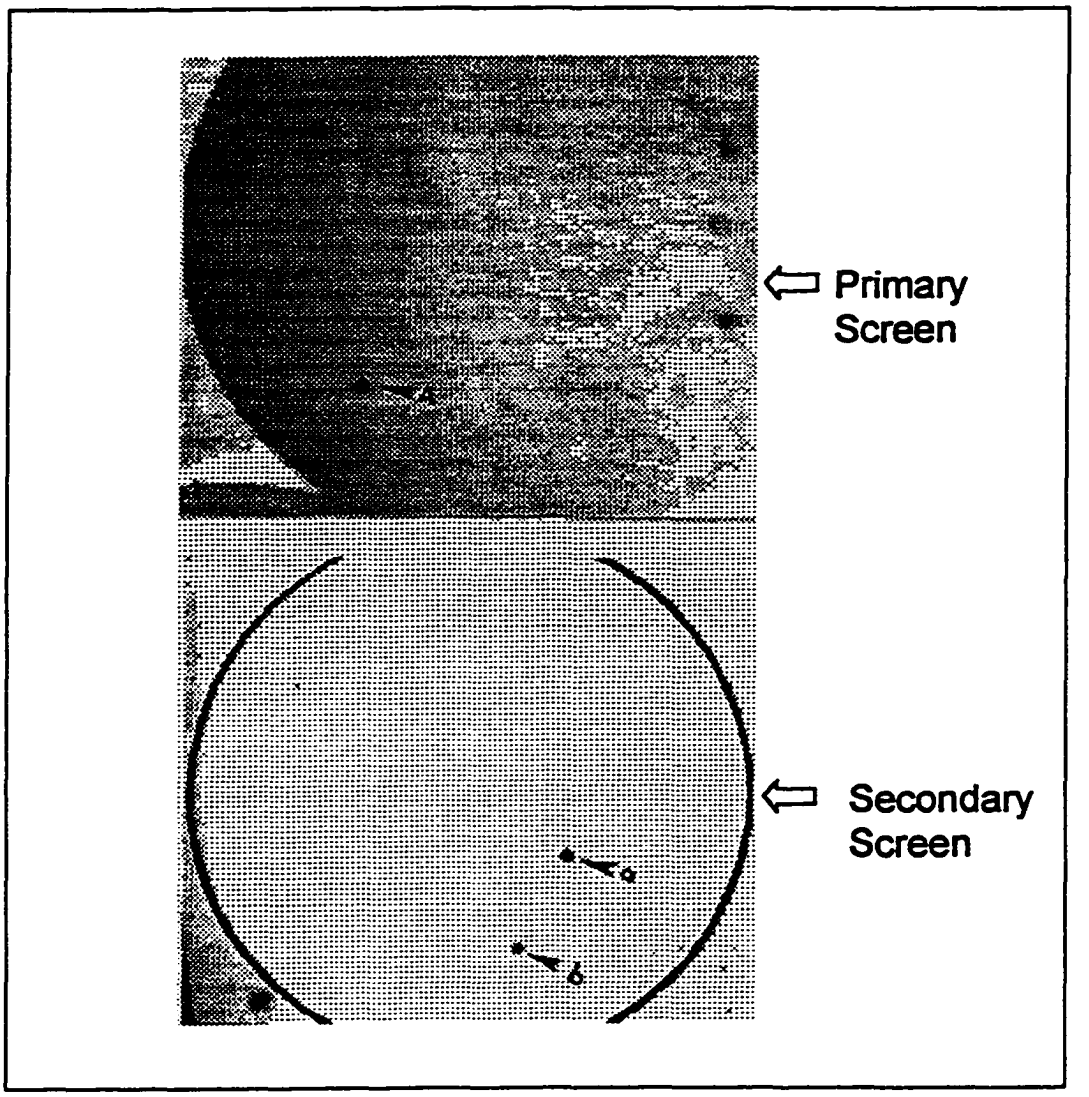

Figure 4-3. Screening of *P. macroloba* cDNA library with two chemically synthesized oligonucleotide mixtures derived from the PmSTI amino acid sequence. Nitrocellulose membranes containing immobilized colony DNA were hybridized with ^{32}P-labeled primer mixtures and autoradiographed at -80°C for 48 hours. 1st, primary screening; 2nd, secondary screening. A, PmSTI clones from secondary screening; a and b, PmSTI clones pBK391 and 392, respectively.

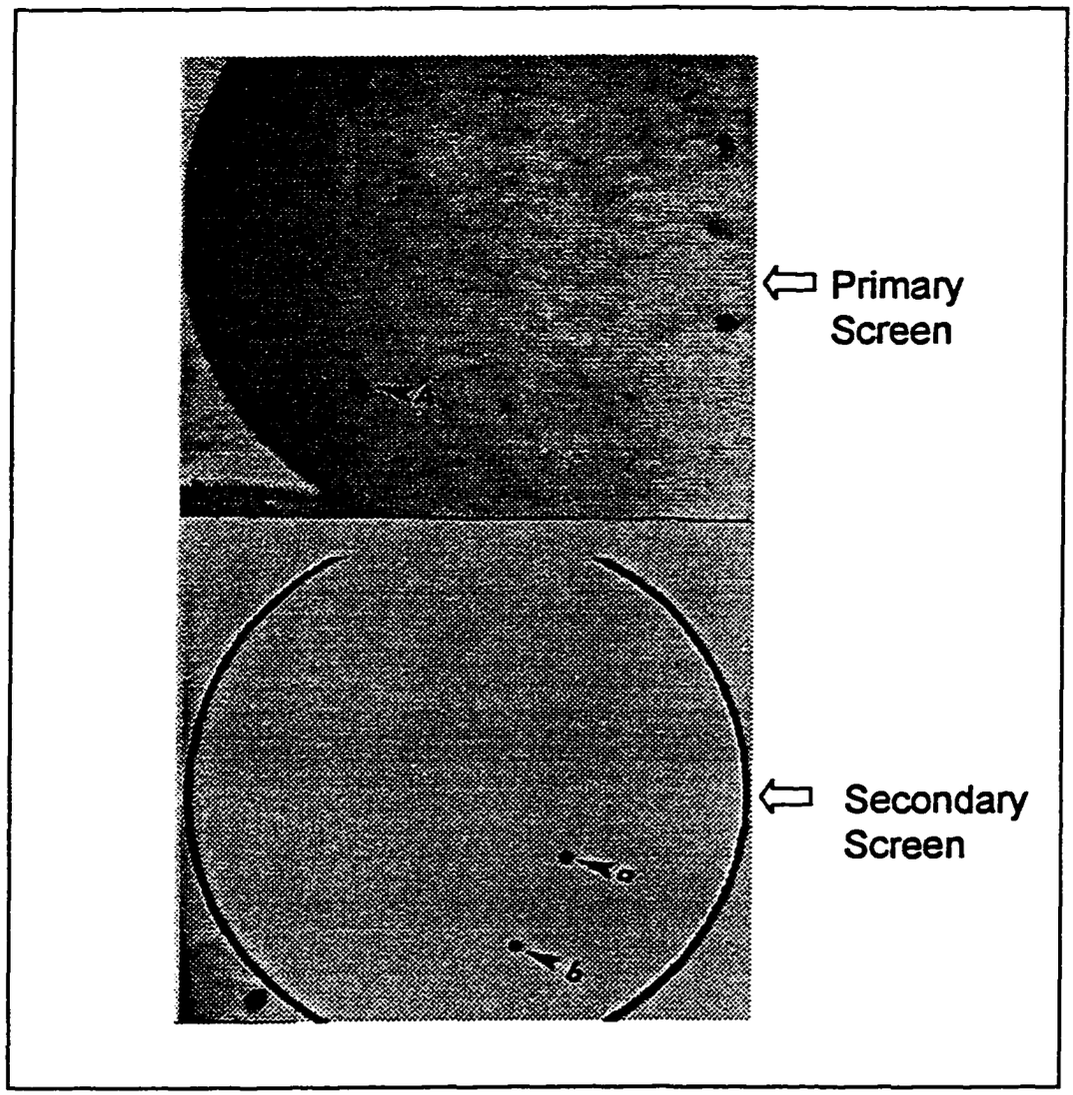

Figure 4-3. Screening of PmSTI cDNA clones with two chemically synthesized oligonucleotide mixtures derived from the PmSTI amino acid sequence. Nitrocellulose membranes containing PmSTI clones were hybridized with ^{32}P-labeled primer mixtures and autoradiographed at -80°C for 48 hours. 1st, primary screening; 2nd, secondary screening. A, PmSTI clones from secondary screening; a and b, PmSTI clones pBK391 and 392, respectively.

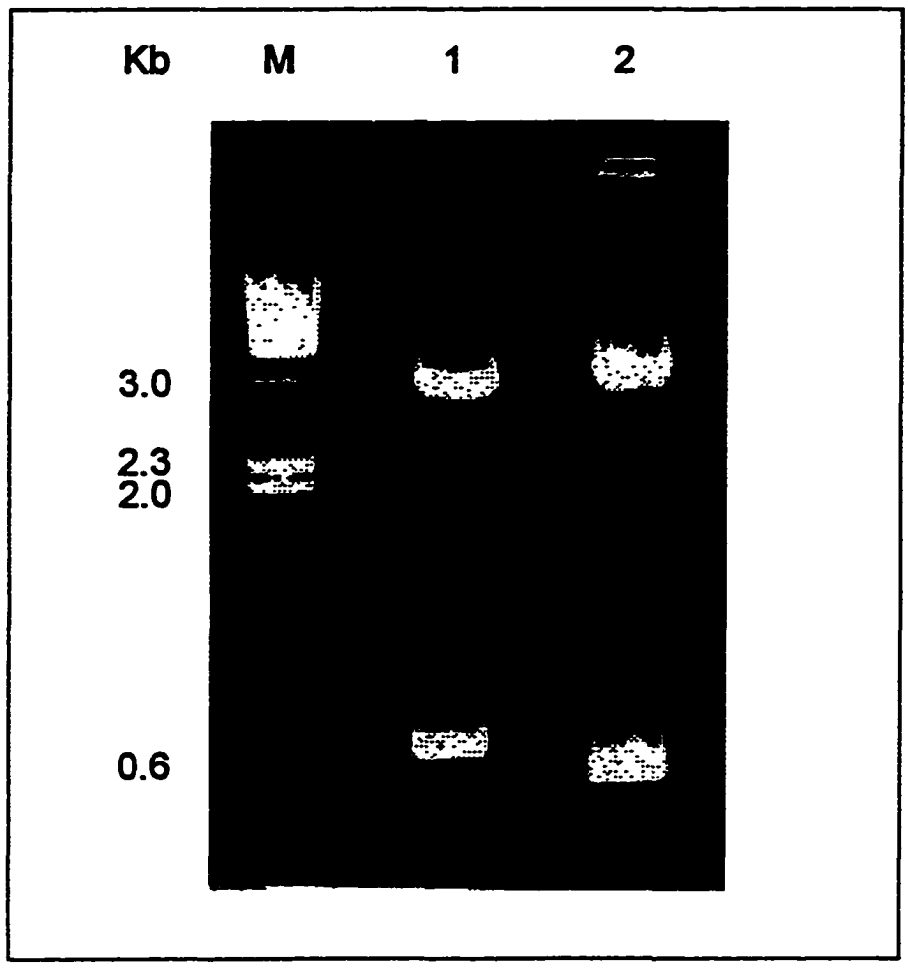

Figure 4-5. Restriction enzyme digestion of a positive phagemid pBK-CMV. Fragments were separated on a 1.5 % agarose gel using TAE buffer. Lane M, λ-*Hind III* markers; lane 1, *EcoR I-Xba I* digestion of the positive phagemid pBK-CMV; lane 2, *EcoR I-EcoR V* digestion of the positive phagemid pBK-CMV.

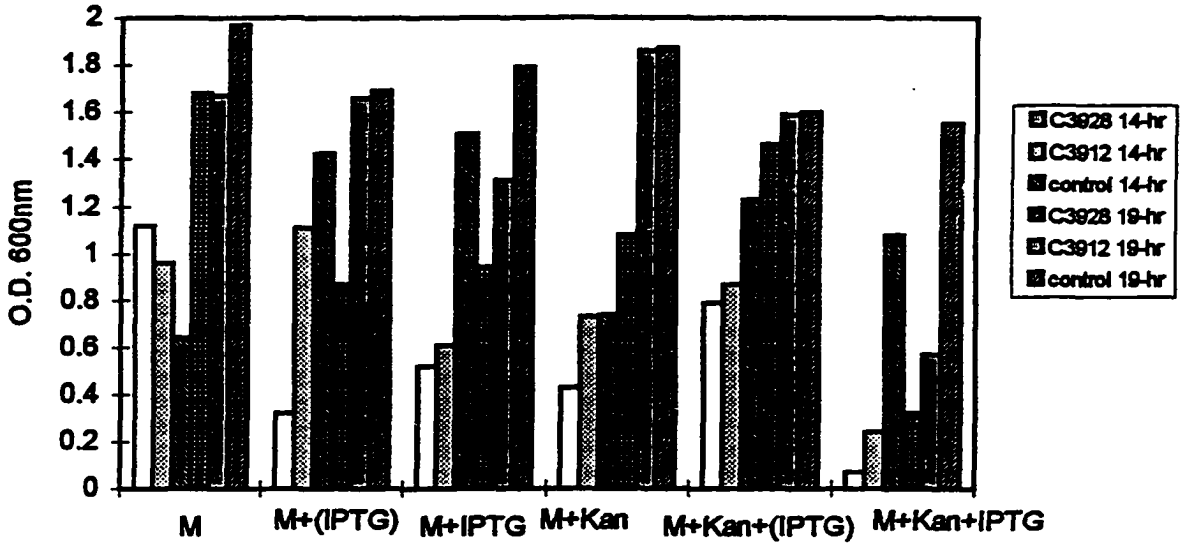

Figure 4-6. Effect of *IPTG*-induced PmSTI expression on *E. coli* growth. Equal numbers of bacterial cells were used to inoculate 10 ml of LB broth. The cultures were incubated at 37°C with shaking. C3912, a colony containing the plasmid pBK391; C3928, a colony containing plasmid pBK392. M, LB broth only; M+IPTG, broth plus 3 mM IPTG added at the time of inoculation; M+(IPTG), IPTG was added to the broth 3 hours after inoculation. Kan: kanamycin was added to the medium.

Double digestion of the two positive phagemid pBK-cmv with *EcoR I* and *Xba I* or *EcoR I - EcoR V* revealed a vector band of 45,000 bp and an insert of 600 bp (Figure 4-5). Clone pBK391 was toxic to the host *E. coli* in the presence of IPTG in the medium, while pBK392 strongly inhibited host growth (Figure 4-6). Since the PmSTI cDNA was inserted into the *Lac Z* gene region of the λ-Zap Express® vector at *EcoR* I-*Xho I* site, the cDNA insert could be expressed as a fusion protein under control of the inducible *Lac* promoter. Immunodetection of the PmSTI fusion protein was studied by western blotting with mouse PmSTI antibody. A cross-reactive band of molecular weight of about 35 KDa appeared in the clone-392 cell lysate. The estimated molecular weight of this band corresponded to the prediction size of the *lacZ*-PmSTI fusion protein (Figure 4-7). The result indicates that PmSTI could be expressed in *E. coli*.

DNA sequence analysis. Two cDNA clones, pBK391 and pBK392, isolated from the *P. macroloba* seed cDNA library, were sequenced in both directions. The complete sequence of the cDNA was obtained by aligning the two sequences. Figure 4-8 shows the total sequence of PmSTI cDNA and the deduced amino acid sequence. Both clones appear to be identical and carry a cDNA insert of 558 bp, which consists of a 364 bp

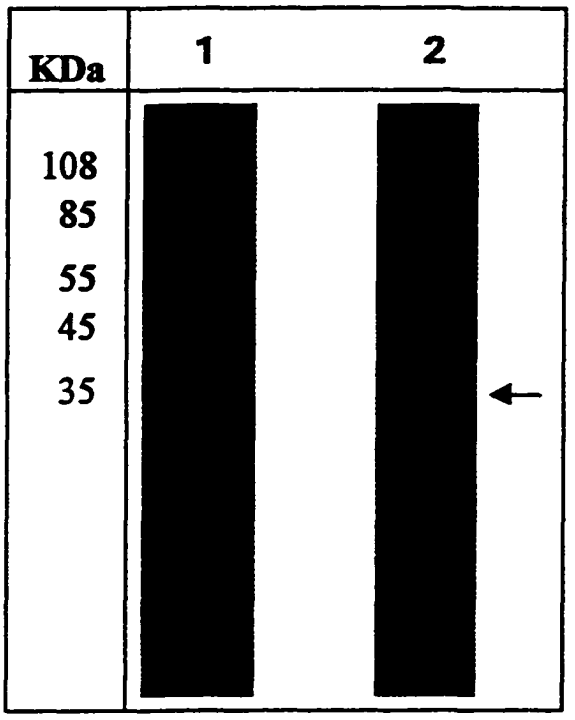

Figure 4-7. Western blot of *E. coli* expressing the PmSTI fusion protein. Bacterial cells were pelleted and resuspended in SDS-PAGE sample buffer, then boiled for 3 minutes. After electrophoresis on a 15% SDS-PAGE gel, the proteins were electrotransferred to nitrocellulose membrane. Standard procedures for Western blots (Promega, WI) were used. Mouse antiPmSTI polyclonal antibody was used as the primary antibody and goat-antimouse, alkaline phosphatase conjugated antibody was used as the secondary antibody. Lane 1, prestained molecular weight markers (BioRad). Lane 2, *E. coli* extract of PmSTI clone pBK392 probed with the primary antibodies. Arrow (←) indicates the PmSTI fusion protein band.

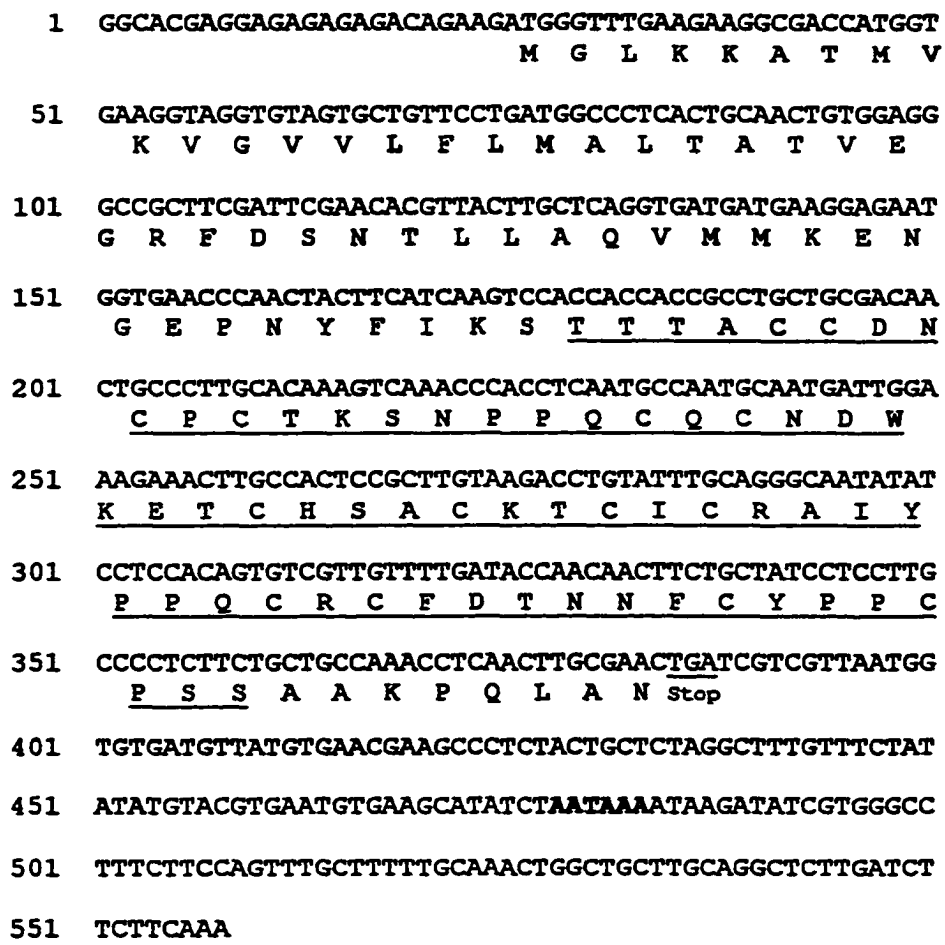

Figure 4-8. Complete sequence and deduced amino acid sequence of PmSTI cDNA. The underlined amino acid sequence corresponds to the sequence that is present in the mature PmSTI sequence. The bold letters indicate a putative polyadenylation signal.

open reading frame (ORF), a 24 bp 5'-end untranslated sequence and a 170 bp 3'-end untranslated sequence. The potential ORF extends from position 25 (ATG codon) to position 385 (TGA stop codon). There were several ATGs found at 5'-end region and all ATGs in frame in the deduced amino acid sequence. A putative polyadenylation signal sequence (AATAAA) was present in the 3'-end untranslated region.

The restriction map of the PmSTI clones is presented in Figure 4-9. This analysis reveals an *EcoR V* site at position 488 near the 3'-end, confirming the gel pattern after *EcoR I / EcoR V* digestion of the PmSTI clone shown in Figure 4-5.

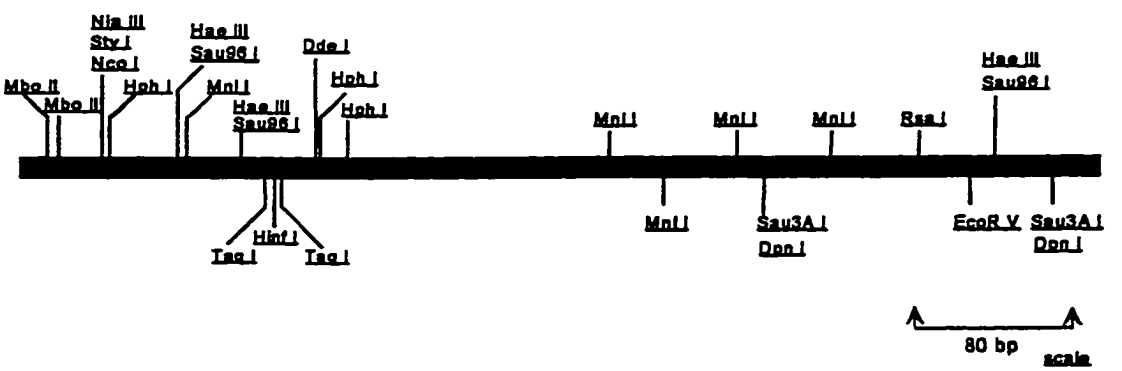

Figure 4-9. Restriction map of PmSTI cDNA. The restriction sites in the sequence were obtained using the GCG database.

The deduced amino acid sequence of the ORF suggests that PmSTI is synthesized as a precursor. Sixty-one out of 120 amino acid residues

were 100% identical to the mature PmSTI sequence obtained by Edman degradation (Chapter III). The PmSTI precursor is a 120 amino acid polypeptide with 51 residues upstream from the amino-terminus and 8 residues downstream from carboxyl-terminus of the mature PmSTI. The precursor may be processed at two different positions to generate two variants of PmSTI. As noted in an early chapter, PmSTI has two variants with just one threonine difference at the N-terminus. Finally, the amino acid sequence of PmSTI has been compared to other protein sequences available through the GCG (Genetic Computer Group) database. The result presented in Figure 3-11 supports the conclusion that PmSTI belongs to the Bowman-Birk inhibitor family.

DISCUSSION

High numbers of positive clones were identified in primary screening of the *P. macroloba* cDNA library, but very few were confirmed as positives in a secondary screening. This may be attributed to the low stringent conditions of the primary screening which allowed for nonspecific hybridization to occur. The hybridization temperature used for primary screening (42 °C) was much lower than the calculated Tm of the probes (56 °C). Consequently, the temperature was increased to 50 °C

during the secondary screening. The low number of PmSTI clones in the seed cDNA library was consistent with the observation that PmSTI was present in low concentrations in the seeds (Chapter II and III). Furthermore, the cDNA library was made from immature seeds 10 mm in diameter or smaller, a stage during which PmSTI may not be fully expressed. Expression of Bowman-Birk protease inhibitors in legumes usually occurs during mid to mature stage of seed development (Jofuku and Goldberg, 1989).

The presence of several translation initiation codons (ATGs) in the 5'-end region of the PmSTI cDNA was an interesting feature. The occurrence of several ATGs' creates a question as to which ATG is the true translation start codon. It seems that the 3'- and 5'- flanking sequences of the ATG triplets may be crucial in determining the translation initiation site (Hoffman *et al.*, 1984; Kozak, 1983). A purine ("A") frequently occurs three residues before the AUG codon, and a purine ("G") often follows the AUG, to form a consensus translation start motif "ANNAUGGN"or "GNNAUGR" (Joshi, 1987; Kozak, 1983). The ribosome scanning mechanism for translation initiation in eukaryotes depicts 40S ribosomes entering at the 5'-end of the mRNA and advancing

toward the interior until an AUG codon with the proper flanking sequences A(or G)NNAUGG is recognized (Kozak,1983). Of the five ATGs in the PmSTI cDNA 5'-end region, only the first ATG, flanking sequence of AAGATGG, concurs with the consensus initiator sequence. On this basis, this ATG was assumed to be the translation start codon.

The plant mRNA structural elements at the 3'-end untranslated region consist of a transcription stop signal, AAUAAA, and its upstream and downstream consensus domains (Murray and Christeller, 1995; Rothie *et al.*, 1994; Gautier *et al.*, 1990). The PmSTI cDNA sequence possesses the typical structural elements found in other plant mRNAs or cDNAs. These structural elements include a transcription termination signal (AATAAA motif) at position 477, a TATGTA element upstream and a GT or T rich region downstream of the transcription stop signal.

The deduced polypeptide from the cDNA sequence of PmSTI was 120 amino acids long. A polypeptide sequence containing 61 amino acid residues starting at the 52nd residue of the deduced sequence was identical to the mature PmSTI sequence which was purified and previously characterized (Chapter II and III). The N-terminal leader peptide of 51 residues (Figure 4-10) contains a hydrophobic region. This hydrophobic

segment presumably functions as a signal sequence for protein sorting to the ER (McGurl *et al.*, 1995; Blobel and Dobberstein, 1975). In fact, deduced protein sequences from Bowman-Birk trypsin inhibitor genes show a long leader sequence at their N-terminus (McGurl *et al.*, 1995; Hilder *et al.*, 1989).

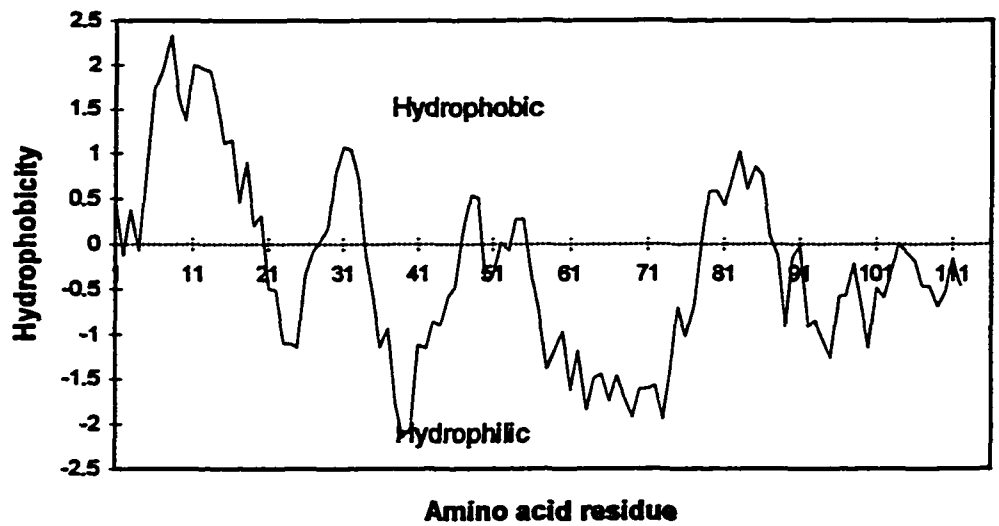

Figure 4-10. Predicted hydrophobicity of the PmSTI precursor. The amino acid sequence was deduced from the cDNA sequence through a protein structure prediction database (NCBI) available on the Internet.

Posttranslational processing that result in the formation of the mature protein apparently follows the **"(-1, -3) rule"** developed by von Heijne (1986). According to von Heijne, a small residue (frequently Ala) is always present at the "-1 site" (cleavage site is +1), whereas the "-3 site"

is occupied by an amino acid that is not aromatic, charged, or large and polar. For example, the cleavage region for wheat CM16 protein is Ala at –1 and Val at -3 (Gautier *et al.*, 1990). The cleavage of the PmSTI precursor generally follows this rule. Cleavage occurs between Ser and Thr, where Ser is at –1 while Ile at -3. Although the occurrence of Ile at -3 and Ser at -1 is not common, these residues still comply with the rule (von Heijne G, 1986). In about 10-20% of the cases, the (-1,-3) residues are Ser and Ile. It is also possible that the cleavage site occurs between Thr and Thr at position 52 and 53 according to the (-1, -3) rule. This may account for the formation of the amino acid variants of PmSTI purified from *P. macroloba* seeds.

The PmSTI precursor also contains an eight-residue C-terminal extension peptide (CTPP), suggesting that PmSTI may be sorted to the vacuole after translation. Some plant proteins targeted to the vacuole require a short carboxyl-terminal polypeptide. In absence of this extension peptide, they are sorted to the intercellular space (Bednarek, *et al.*, 1990; Neuhaus *et al.*, 1991). Based on the amino acid data, it is likely that PmSTI is targeted to the vacuole after entering the ER. No common consensus sequences in the CTPP have been established yet as

determinants for plant vacuolar targeting (Chrispeels and Tague, 1991). Yeast has the CTPP consensus sequence QRPL, which acts as a vacuole sorting signal (Johnson *et al.*, 1987; Klionsky *et al.*, 1988). However, the yeast vacuolar sorting signal is not present in plant vacuolar proteins and it does not act as a signal sequence in plant cell systems (Bednarek *et al.*, 1990; Chrispeels, 1991). C-terminal polypeptides were also believed to function as targeting signals for mitochondrial protein import (Verner and Schatz, 1988).

The PmSTI precursor appears to have all the features necessary for sorting through the plant cellular secretory system. These features could be used to investigate the role of plant gene structure on plant protein sorting.

CHAPTER V

BIOLOGICAL ACTIVITY OF THE *P. MACROLOBA* PROTEASE INHIBITORS

Insecticidal effects of protease inhibitors have been confirmed by addition of the inhibitors to artificial diets of insects (Steffens et al., 1978; Gatehouse *et al.*, 1980; Broadway and Duffy, 1986a; Broadway and Duffy 1986b). Under field conditions, cowpea varieties with high levels of trypsin inhibitor are more resistant to insect damage than varieties with low levels of the trypsin inhibitor (Gatehouse *et al.*, 1980). Likewise, trypsin inhibitory activity in insect resistant cultivars of sweet potato was stronger than those of insect sensitive cultivars (Wang and Yeh, 1996). Many proteins toxic to insects and pathogens occur naturally in seeds. In response to localized tissue injury, induced synthesis of protease inhibitor could occur on wounded surfaces and spread through the remainder of the plant (Green and Ryan, 1972). In this chapter, we show that PmSTI has insecticidal and nematicidal properties against the European corn borer (*Ostrinia nubilalis*) and the free-living nematode *Caenorhabditis elegans*.

MATERIALS AND METHODS

Bioassay using the European corn borer (ECB) Ostrinia nubilalis. The methods used for the ECB bioassay was described by Czapla and Lang (1990). Larvae of the ECB were reared in tissue culture trays (Corning Cell Wells, Cat. #25820-24) containing Stoneville diet (King and Hartley, 1985, see Appendix II). Each well was filled with 1.2~1.5 g diet. Samples to be assayed were applied in several concentrations. Two larvae were reared in each well. The protein sample was either overlaid or incorporated into the diet. About 80 µl of the sample was pipetted onto surface of the diet and allowed to dry evenly (overlay) or mixed directly into the diet (incorporation). Control diet contained an equal volume of water or buffer.

European corn borer (ECB) feeding trials with PmSTI expressed in E coli. *E. coli* XLOLR harboring pBK392 was induced to express PmSTI fusion protein in LB medium supplemented with 1 mM IPTG. Cells were collected and lysed as described by Sambrook *et al.* (1989). Either inclusion bodies or protein solubilized by 8 M guanidine-Cl was incorporated into the insect diet (~ 1.0 mg/cup). ECB larvae from newly

hatched eggs were placed on this diet and incubated at 28 °C. The mortality and weight of the larvae were evaluated 7 days later.

Nematode bioassay of E. coli expressing PmSTI. *E. coli* strain XLOLR harboring pBK392 was cultured overnight in 8 ml of LB broth with 50 μg/ml kanamycin. *E. coli* not expressing PmSTI fusion protein (pBK3912 and 3915) served as a control. The bacterial cells (200 μl) were plated directly on a kanamycin LB plate with and without the inducer IPTG and incubated for one hour at 37°C. *C. elegans* (50-100 larvae in 10 μl) was applied to the LB plate. Plates with bacteria and nematodes were cultured at room temperature. The population and development of nematodes were observed daily with a Nikon stereo microscope system.

RESULTS

Effects of affinity purified PmTI on European corn borer (ECB). Biological activity of affinity-purified PmTI (consisting of PmLTI and PmSTI) was evaluated by feeding European corn borer on diets containing PmTI. The effects of overlaying the diet with affinity-purified PmTI on weight gain and mortality of larvae are shown in Table 5-1. On day 7 and 14, larvae reared on diets containing a concentration of PmTI over 100

μg/well weighed less than the control larvae. The difference in weight between the control and the 100 μg/well treatment was statistically significant using the student's T-test. Insect mortality also increased at the highest level of PmTI used.

Table 5-1. Effect of PmTI in larvae diet on growth of *O. nubilalis* larvae.

PmTI (μg/well)	7-day after feeding		14-day after feeding	
	weight (mg) ± SE [a]	% mortality	weight (mg) ± SE [a]	% mortality
0	5.07 ± 0.65	6.3	25.21 ± 4.64	12.5
50	6.45 ± 1.41	18.8	23.34 ± 6.61	18.8
100	3.35 ± 0.57 [b]	6.3	8.96 ± 2.26 [b]	12.5
250	4.18 ± 0.88	25.0	18.65 ± 7.08	50.0

[a] Sixteen larvae were placed on diet containing affinity-purified PmTI. Larval weight and mortality were recorded 7 and 14-days after placing the larvae on the diet. SE is the standard error of the mean.
[b] Marked values were significantly different from the control at the 95% ($P \leq 0.05$) confidence level.

Effect of PmSTI fusion protein on insect growth. PmSTI is a potent protease inhibitor present at very low levels in the seeds of *P. macroloba.* In order to know if the fusion protein of PmSTI was effective against insects, *E. coli* expressing PmSTI as a fusion protein was used in insect feeding experiments. Based on Western blot analysis in Chapter IV, PmSTI fusion protein apparently accumulated in inclusion bodies. The inclusion bodies were purified according to the method described by

Sambrook *et al.*(1989) and incorporated into the insect diet. About 55% weight loss and 45% mortality were observed in the diet containing PmSTI fusion protein from cells induced by IPTG (Figure 5-1). Protein expression without IPTG induction also caused a large reduction in weight, suggesting that sufficient PmSTI fusion protein was expressed in the bacteria. Protein in inclusion bodies solubilized with 8 M guanidine-HCl had no effect on insect growth and mortality. The likely reason is that the denaturation of protein occurs during the chemical solubilization.

Bioassay with the free living nematode C. elegans using PmSTI expressed in E. coli. Four colonies of *E. coli* containing the cDNA clones pBK391 and pBK392 were selected. Plates sprayed with these isolates were inoculated with the free-living nematode *C. elegans*. The results of these studies are summarized in Figure 5-2. Nematode survival decreased by 20% in cultures maintained on plates inoculated with *E. coli* containing pBK392 (isolates 392-8 and 392-13 expressing PmSTI fusion protein) within 2 days. By day 10, 98.8 % of the nematodes grown on plates inoculated with isolate 392-8 were dead. The effect of *E. coli* strain harboring pBK391 (pBK3912 and pBK3915) on nematode growth was much less than that observed for isolates expressing clone pBK392. This

effect corresponds with the reduced levels of PmSTI expression in isolates containing pBK391. Nematodes were inactive or dead when grown on plates inoculated with *E. coli* expressing PmSTI (Figure 5-3). Nematodes grown on *E. coli* lacking the PmSTI cDNA clone were apparently unaffected. Nematode reproduction was severely inhibited in cultures grown on bacteria expressing the inhibitor fusion protein.

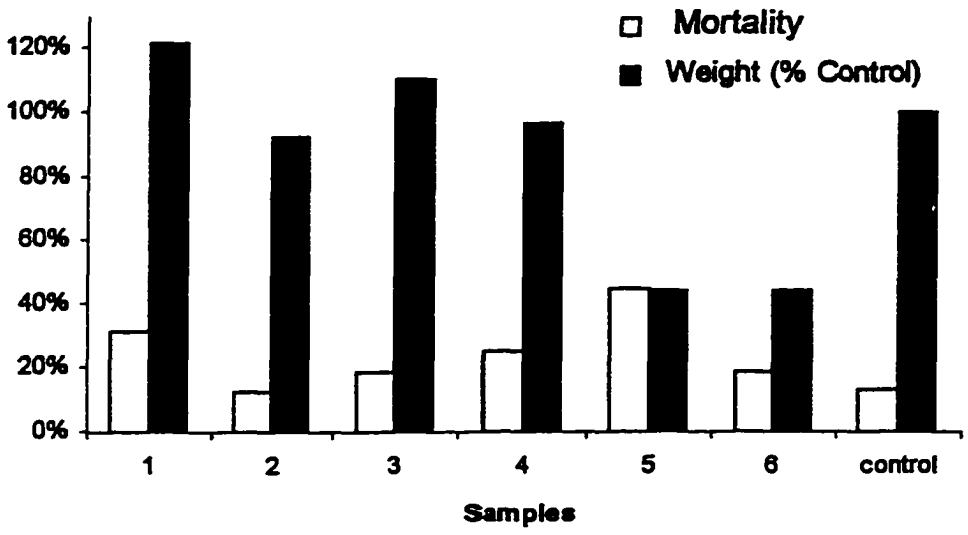

Figure 5-1. Effect of *LacZ*-PmSTI fusion protein on ECB (*O. nubilalis*) growth. PmSTI expressed in *E coli* was purified and incorporated into the Stoneville diet for ECB (see "Materials and Methods"). Data were obtained 7 days after larvae were placed on the diet. Sample 1, protein extract of *E. coli* strain XLOLR; 2, protein extract of *E. coli* haboring phagemid pBK-CMV cultured in LB broth with 1 mM IPTG; 3, protein extract of *E. coli* haboring phagemid pBK-CMV cultured in LB broth without IPTG; 4, protein extract of *E. coli* expressing a fusion protein other than *LacZ*-PmSTI induced by 1 mM IPTG; 5, protein extract of *E. coli* expressing *LacZ*-PmSTI fusion protein induced by 1 mM IPTG; 6, protein extract of *E. coli* expressing *LacZ*-PmSTI fusion protein without IPTG induction. Control, diet without addition of protein extract.

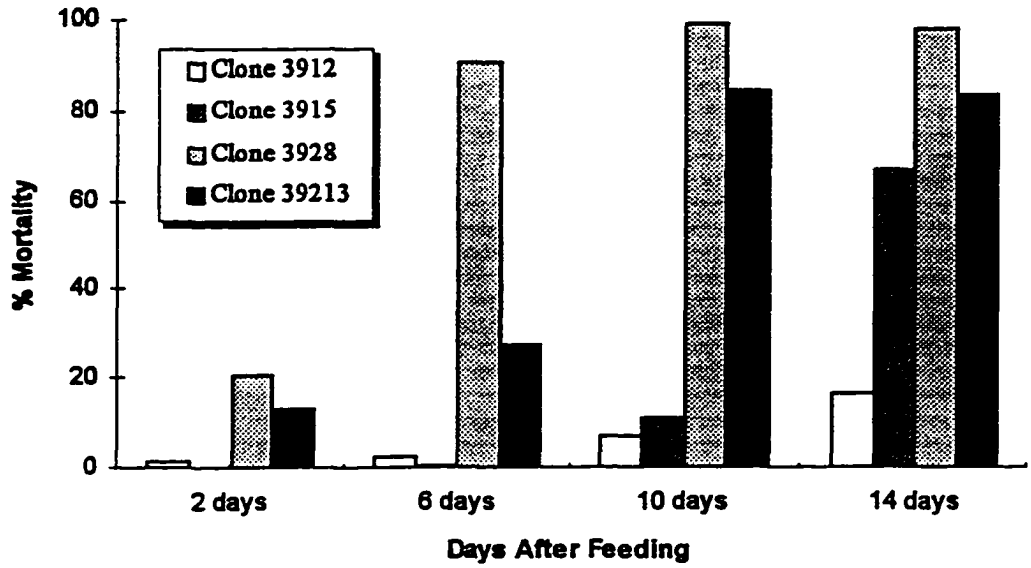

Figure 5-2. Effect of PmSTI expressed in *E. coli* on the nematode *C. elegans*. The bacterial cells harboring PmSTI cDNA were spread onto plate supplemented with 0.1 mM IPTG and 100 μg/ml kanamycin and incubated at 37°C for one hour. The plate was inoculated with nematodes (50–100 per plate) and incubated at room temperature. Clones 3928 and 39213 express high levels of the LacZ-PmSTI fusion protein while clones 3912 and 3915 express low levels of the fusion protein. The mortality of nematodes was obtained by counting the inactive or dead nematodes under the microscope.

Figure 5-3. Effect of *E. coli* expressing the PmSTI cDNA clone on the growth of free-living nematode *C. elegans.* The PmSTI was expressed in bacterial cells as a fusion protein and accumulated as inclusion bodies in cells. The control was *E. coli* carrying phagemid pBK-CMV without the PmSTI insert. A, live nematodes in the control plate; B, inactive or dead nematodes in the PmSTI expressing plate.

DISCUSSION

The results of feeding bioassays with ECB larvae reared on diets containing affinity-purified PmTIs demonstrated that PmTIs were potent inhibitors of insect larval growth. Significant weight reduction or high mortality of ECB larvae were observed when PmTI was incorporated into the artificial diet at concentration of 100 μg/well or higher. The results obtained suggest that PmTI may have a biphasic effect on ECB growth (Scott and Symes, 1996). In other words, PmTI at moderate concentrations has an inhibitory effect on weight gain, and a stimulatory effect on growth at high concentrations.

Viability and growth of European corn borer larvae were apparently reduced by adding partially purified PmSTI fusion protein (inclusion body) into an artificial diet. These results are consistent with the suggestion that protease inhibitors, especially Bowman-Birk inhibitors, may be effective in protecting crops from insects (Ryan, 1990). Several protease inhibitors expressed in genetically engineered plants have deleterious effects on different types of insects (Michaud, 1997). A combination of *Bacillus thuringiensis* δ-endotoxin (Bt) and cowpea trypsin inhibitor (CPTI)

expressed in transgenic tobacco conferred increasing toxicity to insect larvae (Zhao *et al.*, 1995). Bowman-Birk type protease inhibitors were also found to protect plant roots from fungal infection (McGurl *et al.*, 1995).

The results presented here also indicate that *LacZ*-PmSTI fusion protein expressed in *E. coli* affected on the growth of nematodes. Growth of *C. elegans* on bacteria expressing PmSTI exhibited high mortality and reduced reproductive potential. (Figure 5-2). Others have suggested that protease inhibitors or other agents may have potential for the control of plant parasitic nematodes (Robert, 1992; Conkling *et al.*, 1990; Gurr *et al.*, 1992; Oppenman *et al.*, *1994;* Yamamoto *et al.*, *1990*). The expression of the cysteine protease inhibitor oryzacystatin-1 in hairy-root cultures of tomato induced by *Agrobacterium rhizogenes* conferred resistance to the potato cyst nematode *Globodera pallida* (Urwin *et al.*, 1995). The author proposed that the increased resistance was related to the inhibition of cysteine proteases present at high levels in the nematode (Koritsas and Atkinson, 1994; Ray and Mckerrow, 1992). Moreover, CPTI expressed in transgenic potato reduced the rate of growth and disrupted the reproductive cycle of the potato cyst nematode (*Globodera*

pallida) (Hepher and Atkinson, 1992). The inhibitory activity of mature PmSTI against bovine trypsin and *Hethiocoverpa zea* midgut has been demonstrated to be more effective *in vitro* than any commercial Bowman-Birk and Kunitz trypsin inhibitors.

CHAPTER VI

CLONING AND EXPRESSION OF PMSTI CDNA IN TOBACCO CULTIVARS

Plant serine protease inhibitors are naturally-occurring proteins that may confer protection from attack by pests and pathogens. Transgenic plants expressing serine protease inhibitor genes showed increased resistance to pest predation (Hilder *et al.*, 1987; Johnson *et al.*, 1989; Leplé *et al.*, 1995; Urwin *et al.*, 1995). In addition, protease inhibitors might be useful agents for protecting recombinant proteins from internal proteolysis in transgenic plants (Michaud, 1997). PmSTI is one of the most heat-stable, tight-binding inhibitors discovered thus far in the BBI family. In this chapter, we tested whether or not transgenic tobaccos expressing PmSTI are protected against predation by tobacco hornworm.

MATERIALS AND METHODS

Construction of a chimeric binary vector pMon530-STI harboring the PmSTI cDNA sequence.

The *Agrobacterium tumefaciens*-based binary vector, pMon530, which contains a 35S promoter/NOS poly-A expression cassette, has a very short multiple cloning site (six unique restriction sites). PmSTI cDNA sequence (558bp), originally cloned in the λ-*Zap* Express® vector and

excised as the phagemid pBK-CMV, could not be directly inserted into the binary vector. An indirect approach was used to subclone the PmSTI cDNA into the pBluescript-SK(-) vector to introduce *EcoR I* sites at both ends of the PmSTI cDNA. To accomplish this objective, pBK-PmSTI (4.0 µg) and vector (2.0 µg pBluescript-SK(-)) were digested with *Sca I* and *Xba I* (10 units each) for 2 hours at 37°C. The digested plasmids were fractionated on a 2 % agarose gel. The bands were cut out and purified using a QIAEX® DNA purification kit. Vector DNA and the PmSTI fragment were mixed at a ratio of 1:3 and ligated in 20 µl solution with 3 units of T_4 ligase and freshly made 10 mM ATP. The reaction was maintained at 13 to 15°C for 16 hours. The DNA mixture was transformed into *E. coli* XL1Blue-mrf' in TSS (transformation and storage solution) as described by Chung *et al.* (1989). Recombinants were recognized in blue/white selection medium (IPTG, X-gal, 50 µg/ml ampicillin) and were confirmed by restriction enzyme digestion.

A recombinant plasmid, pBS-STI, was digested with the *EcoR I* to obtain a 600 bp fragment consisting of the entire PmSTI cDNA sequence. The *EcoR I* fragment was inserted into *EcoR I* site of pMon530 to produce pM530-STI. To construct pM530-STI, pMon530 and pBS-STI DNA were

digested with *EcoR I* for 2 hours at 37°C. The digested vector and fragment were fractionated on a 2 % agarose gel and purified using the QIAEX® DNA purification kit. The vector and fragment were mixed at a 1:3 ratio and ligated in 20 µl solution using 3 units of T_4 ligase and fresh 10 mM ATP. PEG at a final concentration of 10% was also included in the ligation reaction to enhance the efficiency of ligation (Sambrook *et al.*, 1989). The reaction was maintained at 13 to 15 °C for 24 hours. The ligation product was transformed into *E. coli* strain DH5α as described by Chung *et al.*(1989). Identification of recombinants and the orientation of inserts were performed using restriction enzyme digestion with *Sal I and Xba I* or *Sal I* and *EcoR V.*

pM530-STI was mobilized to *Agrobacterium tumefaciens* strains EHA 101 and LBA4404 via triparental mating with the helper pRK2013 essentially as described by An (1987) (see protocol in Appendix I). For EHA101, the selection media contained 100 µg/ml kanamycin, 50 µg/ml streptomycin and 100 µg/ml spectinomycin. LBA4404 was selected on media containing 150 µg/ml rifampicin, 100 µg/ml streptomycin and 100 µg/ml spectinomycin.

Plant transformation

1). Plant material. Tobacco cultivars (*Nicotiana tabacum* cv. 'Xanthi NN' and cv. 'Xanthi SX') were used for transformation. Seeds of the tobacco cultivars were disinfected with 0.1% $HgCl_2$ and cultured on MS medium (Murashige and Skoog, 1962). Sterile plants from the seeds were established by culturing on MS solid medium supplemented with 0.1 μg/ml NAA at 25°C with a 12-hour light cycle. The sterile plants were maintained by transferring the shootlets to the fresh medium every 30 days.

2). Transformation of tobacco. The method used for tobacco transformation was described by Bevan *et al.* (1985). *LBA4404* harboring pMon530-STI was cultured on 5 ml liquid LB medium containing 150 μg/ml rifampicin, 100 μg/ml streptomycin and 100 μg/ml spectinomycin; and *EHA101* harboring pMon530-STI was cultured on 5 ml liquid LB broth suplemented with 100 μg/ml kanamycin, 50 μg/ml streptomycin and 50 μg/ml spectinomycin at 28 °C overnight. The cells were pelleted by centrifugation at 1000 x g for 10 minutes and then resuspended in 10 ml MS-1 solution (see Appendix II).

Leaves from two-week-old sterile plantlets were cut into small pieces (~1.0 x1.0 cm^2) with a scalpel and incubated for one hour at 25 ± 2 °C in MS-1 (see Appendix II) solution with *A. tumefaciens* containing pMon530-STI. The leaf pieces were blotted dry and transferred onto MS-1 solid medium for 2 days. The leaf pieces were then transferred to MS-2 medium (no kanamycin) to eliminate the agrobacteria. After one week, the cultures were maintained on MS-2 solid media in the presence of the selection agent, kanamycin, until shoots developed. Morphologically normal shoots taken off of selection medium were rooted on MS-3. Once rooted, the small kanamycin-resistant plants were maintained on MS-4.

Identification and evaluation of the tobacco transformants.

1). Nopaline assay.

Nopaline in transgenic plants was assayed according to the method described by Du *et al.* (1994). One or two small pieces of leaf punched from a lower leaf were rubbed on a 3MM paper. The paper was briefly soaked in a tray with nopaline electrophoresis buffer (80 % water, 15 % glacial acetic acid and 5 % (v/v) formic acid). Paper electrophoresis was carried out for 60 minutes at 400 volts. After electrophoresis, the paper was air-dried, stained with 45 ml nopaline staining solution (containing 10

mg phenanthrene quinone, 40 ml 95% ethanol and 5 ml 5 M NaOH) and air-dried again. Nopaline was detected under UV light (short wavelength). Nontransformed leaf material from each tobacco line was used as a negative control.

2). Southern blot analysis of transformants.

Total DNA extraction. One or two fresh leaves were cut into small pieces, rapidly frozen in liquid nitrogen and ground imediately in a mortar and pestle. DNA extraction buffer (100 mM Tris, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 0.2% 2-mecaptoethanol) was then added to the leaf powder. The mixture was transferred to an Eppendorf tube and incubated at 65°C for 10 minutes. A half volume of chloroform-isoamyl alcohol (24:1, v/v) was added to the mixture and vortexed well. The extract was centrifuged at 10,000 x g for 10 minutes. The top aqueous layer was collected and treated with RNase (10 μg/ml) at 37°C for 30 minutes. DNA was precipitated by adding an equal volume of ethanol/acetate (100% ethanol with 3 M potassium acetate). After centrifugation (10,000 x g) for 10 minutes, the pellet was rinsed one time with 70% ethanol and dried by Speed-Vac and redissolved in sterile H_2O.

Restriction enzyme digestion and agarose gel electrophoresis. About 5 to 10 μg DNA was digested using either 10 units *EcoR I* or *Hind III* with Promega's restriction enzyme buffer system at 37°C for one hour. After digestion, the DNA fragments were fractionated on a 1.5% agarose gel. The DNA was stained with ethidium bromide solution (5 μg/ml) and detected under UV light.

DNA transfer to nitrocellulose membrane. The transfer procedures used in these studies were described in the molecular cloning manual of Sambrook *et al.*, (1989). The agarose gel was treated by 0.2 N HCl for 15 minutes. To denature the DNA, the gel was immersed in 0.5 M NaOH, 1.5 M NaCl solution for 30 minutes. The DNA was then neutralized in buffer containing 1.0 M Tris-Cl, 1.5 M NaCl, pH 7.5 for 30 minutes. The gel was washed in 10x SSC buffer (Appendix II) and set up for DNA transfer. To transfer the DNA to nitrocellulose membrane, a capillary transfer protocol was adopted (Sambrook *et al.*, 1989). After transfer, the membrane was briefly washed with 2x SSC buffer and dried on 3MM paper. The membrane was vacuum baked at 80°C for 2 hours.

DNA hybridization. The protocol of Stratagene, Inc (San Diego, CA) was used for DNA hybridization. The membrane was wetted in

prehybridization solution (see Appendix II) inside a heat-sealed plastic bag at 48°C for at least 4 hours. The prehybridization buffer was decanted and replaced by hybridization buffer (see Appendix II) containing synthesized oligonucleotides labeled with γ-^{32}P-ATP. The hybridization was allowed to proceed overnight at 48°C. On the next day, the membrane was washed 5 times with 6x SSC buffer containing 0.1% SDS. The initial wash (3 times) was at room temperature but the final wash (2 times) was at 50°C. The membrane was autoradiographed using X-film (HyperFilm-*MP*, Amersham, Piscataway NJ) at -80°C for at least 24 hours. X-film development and fixation was done using Kodak GBX solution.

3). Immunodetection of PmSTI. Dot blots and western blots were performed according to the methods described in Promega's manual (Promega, Madison WI). Protein extracts were prepared from transgenic and nontransgenic tobacco leaves and calli. For dot blots, the protein samples were directly pipetted onto the nitrocellulose membrane and allowed to air-dry. For Western blots, proteins were separated on a 12.5% SDS-PAGE gel, and then electrotransferred to the nitrocellulose membrane.

Nitrocellulose membrane with immobilized protein was immersed in the blocking solution (5% dry skim milk in TBST buffer, see Appendix II). After 30 minutes, the solution was replaced with primary antibody (against PmSTI) in TBST buffer (1:500). The membrane was incubated at least 30 minutes at room temperature and then washed three times (5 minutes per wash) with TBST buffer. Secondary antibody conjugated to alkaline phosphatase (goat anti-mouse) was added to the membrane with a dilution of 1:10,000 in TBST buffer and incubated for 30 minutes at room temperature. The membrane was washed three times with TBST buffer and briefly rinsed twice with TBS buffer. Color development was achieved using NBT/BCIP substrate solution according to the manual (Promega, Madison WI).

4). Trypsin inhibition assay. The leaf extracts from transgenic and nontransgenic tobacco were prepared as described in "Materials and Methods" of Chapter II. The extract was either heated at 70 °C for 15 minutes or not heated. Trypsin inhibitory activity was determined according to the methods described in Chapter III. Specific inhibitory activity was described as the amount of leaf protein (mg) required to inhibit 5 μg of bovine trypsin by 50% (I_{50}).

5). Insect feeding trials using the transgenic tobacco plants expressing PmSTI. Transgenic plants, used for insect bioassay, were transplanted into soil and grown to about 30 cm high (Figure 6-2). The plants were maintained in a plant growth room with a daily 12-hour light cycle at 25 ± 2 °C. Plants were free of insect and mechanical damage.

Tobacco hornworm (*Manduca sexta*) eggs were ordered from Carolina Biological, Inc.(Burlington, NC) and hatched at 27°C. The larvae were placed directly on the leaves (ten larvae on each plant). Additionally, excised leaves were used in feeding trials as well. Leaves of the same age were cut from each plant and placed inside a container. An average of three larvae was placed on each leaf. The excised leaves with larvae were maintained at 25 °C with a daily 12-hour light cycle and high humidity. After 10 days, the larvae were weighed and the leaves were evaluated by photography.

RESULTS

A. Construction of the plasmid pM530-STI.

PmSTI was originally cloned in the *EcoR I/ Xho I* site of phagemid pBK-CMV (Stratagene, CA). The *SacI/ Xba I* fragment including PmSTI cDNA of pBK-CMV was inserted into the same restriction site of

pBluescript-SK (-) to create an *EcoR I* site flanking both sides of PmSTI cDNA. The *EcoR I* fragment from pBS-STI was ligated into the *EcoR I* site of the binary vector pMon530. The new construct pMon530-STI enables PmSTI cDNA to be expressed under the control of the *CaMV* 35S promoter and *nos* 3'-end terminator. Figure 6-1 shows the location of PmSTI in the expression cassette of the plasmid pMon530-STI. pM530-STI confers streptomycin and spectinomycin resistance for both *E. coli* and *Agrobacterium* and selectable markers (kanamycin resistance and nopaline production) for plant selection. Although PmSTI cDNA is located between the 35S promoter and the 3'-*nos* termination sequence, PmSTI has its own termination sequence which may also function as a transcription termination signal.

PEG was a critical factor in the construction of pMon530-STI. Recombinants were not obtained using the standard ligation protocol with 5% PEG (polyethylene glycol, MW 3650) according to the manufacturer's manual (Promega, Madison WI). PEG (MW 8000) at a concentration from 5 to 15% in the ligation solution was then tested, and recombinants were obtained only in ligation solution containing 10 % PEG (data not shown).

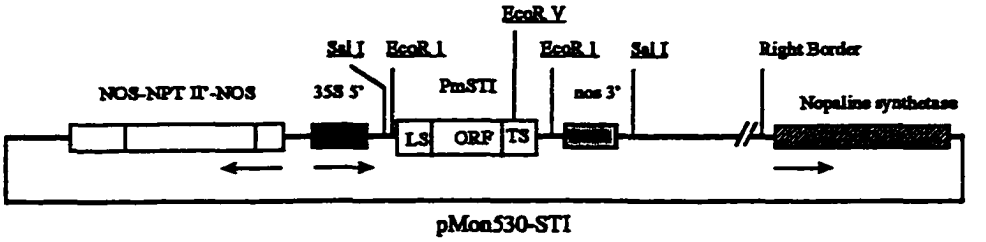

Figure 6-1. The schematic map of chimeric construct pMon530-STI shows the components expressed in plant transformants and restriction sites for the identification of recombinants. Expression of the PmSTI cDNA is controlled by the CaMV 35S promoter and PmSTI cDNA termination sequence or the NOS termination sequence. LS, 5'-end untranslated sequence of PmSTI cDNA; ORF, open reading frame; TS, 3'-end untranslated sequence. Arrow indicates the direction of gene expression. This map is not drawn to scale.

Recombinants and the orientation of transgene insert were identified by double restriction digestion. Ten out of twelve colonies selected were recombinants. Three recombinants with the correct orientation relative to the *CaMV* promoter in the expression cassette, were identified by double digestion with *Sal I* and *EcoR V*.

The pMon-STI was mobilized into *A. tumefaciens* strain LBA4404 and EHA101 by triparental mating using the helper plasmid pRK2013. *Agrobacterium* cells were maintained in selection medium containing 50 μg/ml streptomycin, 50 μg/ml spectinomycin and either 100 μg/ml kanamycin (EHA 101) or 150 μg/ml rifampicin (LBA4404).

B. Transformation and plant regeneration.

Leaves of tobacco cultivars 'Xanthi NN' and 'SX' were infected with *Agrobacterium* carrying pMon530-STI. After infection, the leaf discs were cultured on nonselective medium containing 0.1 μg/ml NAA (α-naphthaleneacetic acid) and 4 μg/ml 6-BA (6-Benzylaminopurine) and supplemented with 500 μg/ml carbenicillin to inhibit the growth of *Agrobacterium*. A week later, the tissues were swollen and callous had formed. At this time, tissues were transferred to selection medium containing 100 μg/ml kanamycin to select for kanamycin-resistant shootlets. Shoot formation was initially observed on the edge around the leaf discs 28 days after culture in the selection medium. Shoot tissue was either maintained in the same medium to allow the shoots to grow or transferred to media containing 2 μg/ml 6-BA to accelerate shoot growth. Shoots 1 to 2 cm in length were cut and "planted" in the rooting medium (MS-4) to regenerate whole plants (Figure 6-2). At least 20 kanamycin resistant plants were obtained. Most of the regenerants were morphologically normal and healthy when grown in the growth chamber.

LBA4404 is a common strain of *Agrobacterium* used for tobacco transformation (An, 1987). Table 6-1 shows that kanamycin-resistant shoots were regenerated in leaf tissues of cultivars "NN" and "SX" infected with either the agrobacterial strain LBA4404 or EHA101. This result indicates little difference between these two strains for tobacco transformation.

Table 6-1. Selection of tobacco transformed explants after infection with *Agrobacterium* carrying pMon530-STI.

Explants (leaf discs)	EHA101 [b] infection	LBA4404 [b] infection	No infection
Xanthi NN			
leaves inoculated	45	23	10
green and live leaves [a]	5	5	0
yellow or dead leaves	40	18	10
leaves with shoots [c]	5	4	0
Xanthi SX			
leaves inoculated	40	33	10
green and live leaves	2	0	0
yellow or dead leaves	38	33	10
leaves with shoots	1	0	0

a). Data were collected after culture on the selection medium for two weeks. The selection medium was supplemented with 100 to 200 mg/L kanamycin.
b). EHA101 and LBA4404 are two *Agrobacterium* strains harboring the binary vector pMon530-PmSTI.
c). Leaves with shoots were counted after culture on the selection medium for 40 days.

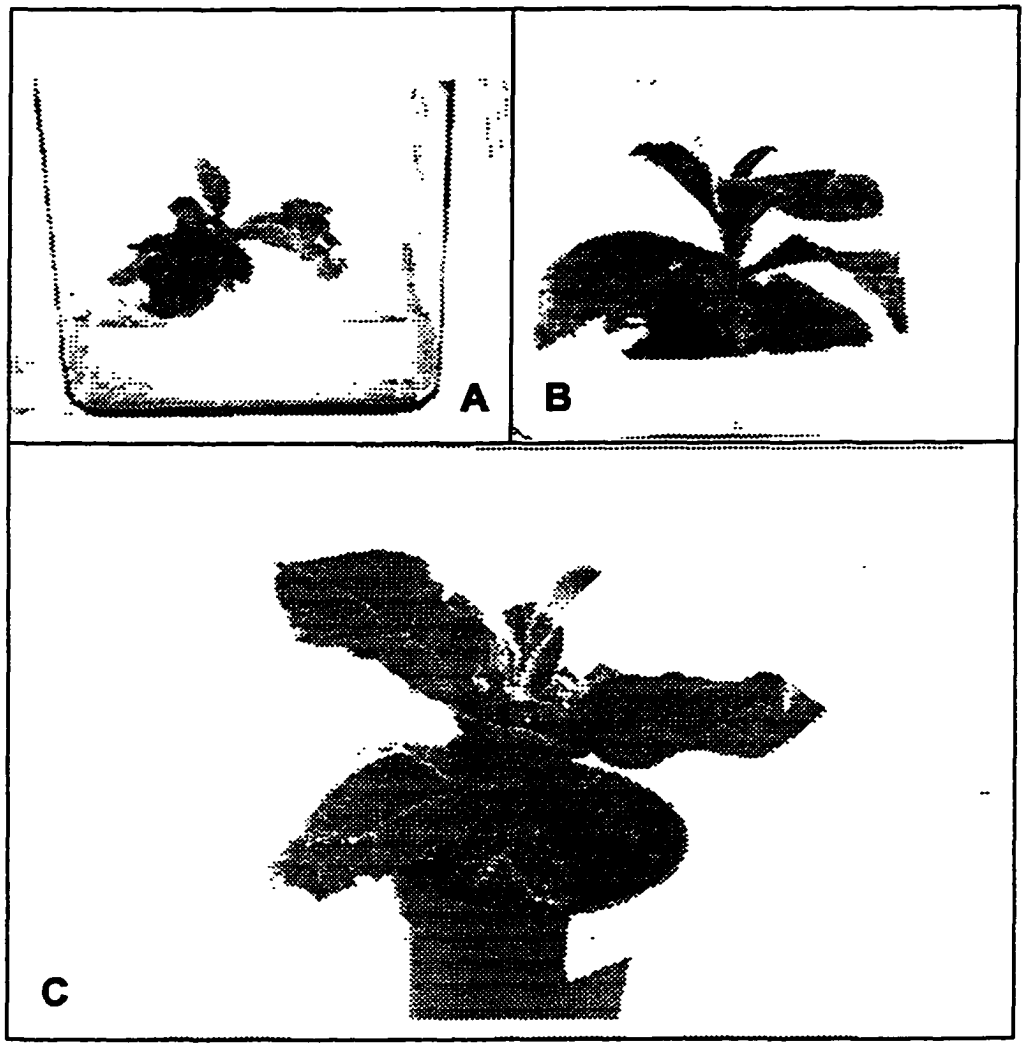

Figure 6-2. Plant regeneration from *Agrobacterium*-infected leaves of tobacco. A, Shoots regenerated from leaf discs in the medium supplemented with 100 μg/ml kanamycin. B, Kanamycin-resistant shoot growing in a rooting medium containing 100 μg/ml kanamycin. C, Kanamycin-resistant plant growing in the pot.

C. Identification of transgenic plants.

1). Nopaline assay of the kanamycin-resistant plants. pMon530-STI contains a gene encoding nopaline synthase which was expressed in transgenic plants under the control of the promoter pTiT37. Plantlets resistant to 100 μg/ml kanamycin in the medium were tested for their nopaline synthase activity. Ten kanamycin resistant plantlets and two nontransgenic controls from cultivar 'NN' and 'SX', respectively, were assayed. Four of eight 'NN' plants and one of two 'SX' plants showed a strong nopaline signal, whereas the two nontransgenic controls and the rest of the kanamycin-resistant plantlets were negative (Table 6-2).

2). Detection of PmSTI DNA in transgenic plants by Southern analysis. To identify whether the PmSTI cDNA was also present in the kanamycin resistant and nopaline positive plantlets, a Southern analysis was performed using PmSTI degenerate primers as probes. Total genomic DNA was isolated from fresh leaves of six 'NN' and two 'SX' kanamycin-resistant, nopaline-positive plants. DNA was fractionated in a 1.0% agarose gel and transferred to nitrocellulose membrane. Hybridization was performed under high stringency conditions (48°C, ΔT_m

= -8°C). Three 'NN' and one 'SX' plantlets showed that DNA was hybridized with the PmSTI primers (Figure 6-3). The result confirms that PmSTI cDNA was transferred to the tobacco genome.

3). Trypsin inhibition assay of trangenic plants. Plant proteases are very active in fresh leaf extracts from normal tobacco plants. Proteolytic activity was readily detected when the substrate *p*-BANA was added to the crude extract (data not shown). To reduce the endogenous proteolytic activity which interferes with the detection of trypsin inhibitor activity, leaf extracts were treated at 70^0C for 15 minutes before the trypsin inhibitory activity in the extracts was assayed. Of all the transgenic plants, NN-10 exhibited the highest inhibitory activity against bovine trypsin at pH 9.5 (Table 6-2). The specific inhibitory activity of NN-10 was 0.27 *IU/mg* protein. This result suggests that PmSTI accounted for approximately 0.5 to 1.0 % of the total leaf soluble protein (based on a 50% inhibition (250 ng of PmSTI against 5 μg bovine trypsin) for native PmSTI). Another transgenic line, SX-1, had lower activity (0.09 *IU/mg* protein). Trypsin inhibitory activity in leaf extracts of different transgenic plants is shown in Figure 6-4. These results demonstrate that transgenic plants express variable levels of PmSTI in the leaves. Inhibitory activity of

some transgenic plants, such as NN-7, was not detected although PmSTI DNA was detected in Southern blots.

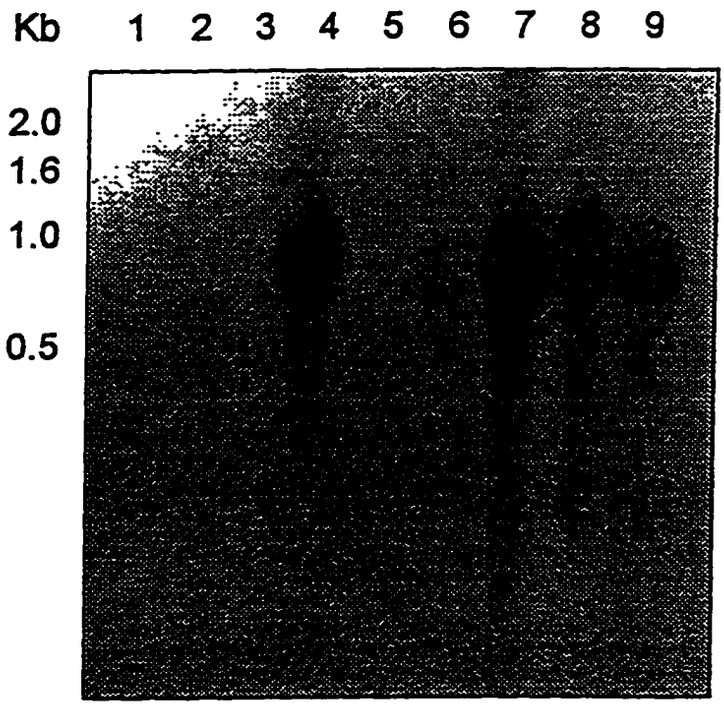

Figure 6-3. Southern blot analysis of kanamycin-resistant plantlets. DNA of transgenic lines was isolated from mature leaves according to the methods described in "Materials and Methods". DNA (about 10 μg) was digested with 10 units *EcoR I* for 2 hrs in 37 °C and fractionated on a 1.5% agarose gel. Degenerate primers NST and CST were labeled with ^{32}P- ATP and used as probes. Lane 1, untransformed. Lane 2-9, kanamycin-resistant plants. Lane 2-8, NN-1, NN-2, NN-4, NN-5, NN-6, NN-7 and NN-10, respectively, and lane 9, SX-1.

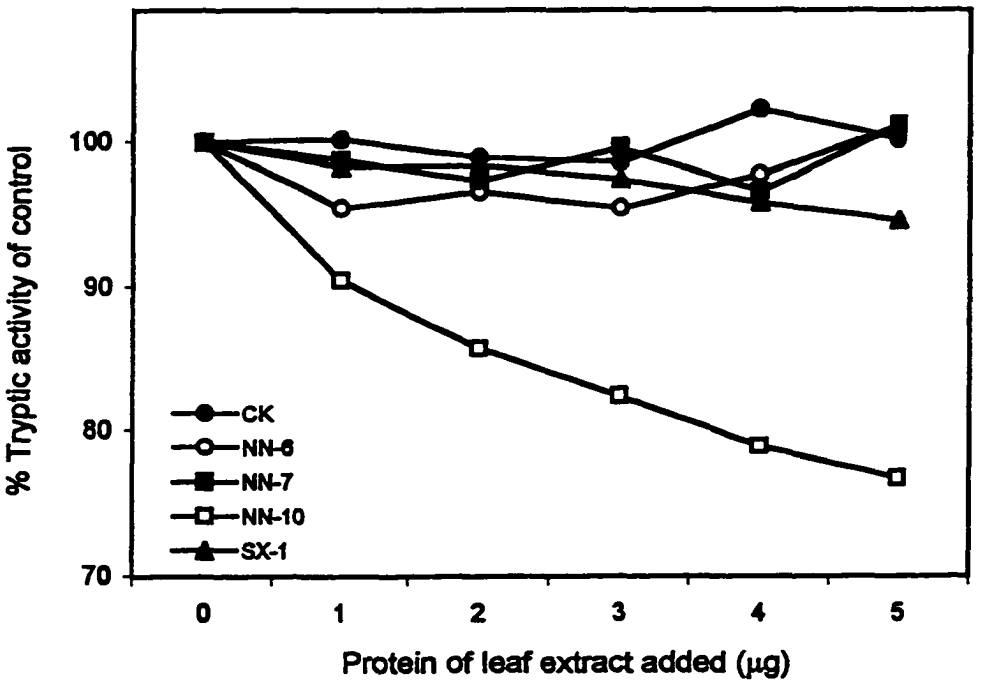

Figure 6-4. Inhibitory activity of transgenic tobacco leaf extracts against bovine trypsin. Tobacco line NN-6 is kanamycin-resistant, nopaline- and PmSTI-negative. NN-7, NN-10 and SX-1 are kanamycin resistant, nopaline positive and positive for PmSTI DNA.

4). Detection of PmSTI in transgenic plants using PmSTI antibody.

Both dot blot and Western blot analyses were used to measure the levels of expression of PmSTI cDNA in the transgenic plants. Dot blots of transgenic leaf extracts gave strong crossreaction with mouse anti-PmSTI in lines NN-4 and NN-10, but weak, diffuse signals in SX-1. No crossreaction was observed in lines NN-6, NN-7 and in the nontransgenic controls (Table 6-2). Western blots of leaf extracts revealed one major crossreactive band of MW about 7 KDa in NN-10 and SX-1, but no bands were detected in lines NN-6 and NN-7 under the conditions used in this analysis (Figure 6-5).

Table 6-2. Identification of tobacco transformants grown on kanamycin selection medium.

Assay	Results					
	Control	NN-4	NN-6	NN-7	NN-10	SX-1
Kanamycin resistance [a)]	-	+++	+++	+++	+++	+++
Nopaline activity	-	+++	+/-	+++	+	+
PmSTI immmunodetection (dot blot)	-	++	-	+/-	++	+
Detection of PmSTI on Southern blot	-	+++	+/-	+++	+++	++
Inhibitory activity *(IU/mg protein)* [b)]	0.00	n/a	0.00	0.00	0.27	0.09

a) +++ = Very strong, ++ = strong, + = fair, +/- = possible, - = none, n/a = not applied.

b) IU/mg protein is the specificity of inhibition of the leaf crude extract. IU is described as amount of trypsin inhibitor in the reaction to reduce hydrolysis of 1.0 μmole substrate (BANA) in a minute.

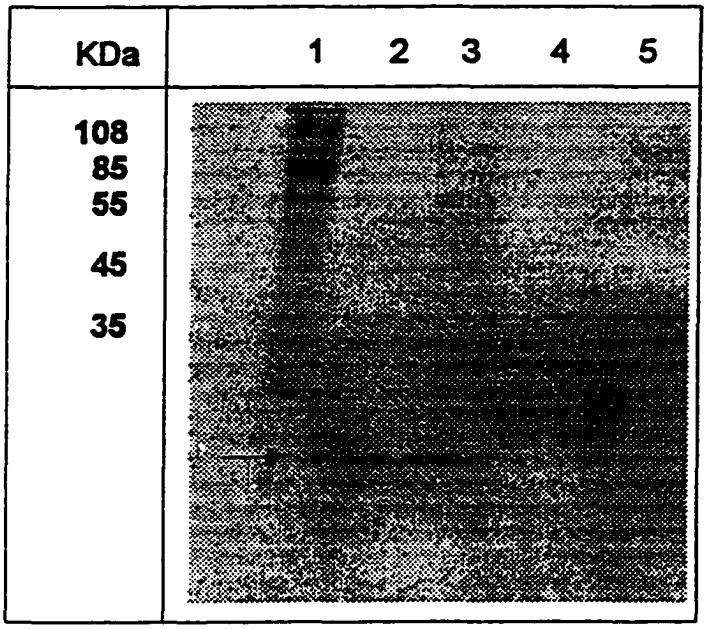

Figure 6-5. Western blot of transgenic plants carrying PmSTI cDNA. Leaf extracts (~20 μg protein) were fractionated on a 12.5 % SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to nitrocellulose membrane. Standard procedures were used for the Western blots. Mouse anti-PmSTI polyclonal antibody was used as the primary antibody and goat-antimouse, phosphatase conjugated antibody was used as the secondary antibody. Lane 1, prestained molecular weight markers (BioRad). Lane 2, SX-1. Lane 3, NN-10. Lane 4, NN-6. Lane 5, NN-7. Arrow (→) indicates the PmSTI band.

D. Feeding trial with tobacco hornworm (M. sexta) larvae.

Tobacco hornworm larvae were fed on excised leaves from different tobacco lines randomly mixed in a container. Evidence of insect feeding was observed after 12 hours. Seven days later, 80% of the 20 larvae were still alive, and the leaves were analyzed by comparing the size of the damaged area. The damaged area was much less in the transgenic leaves, like NN-10, than in the control, nontransgenic leaves (Figure 6-6). The leaf of line NN-10, which is known to contain active PmSTI, had only one small hole. Nontransgenic control, nopaline and PmSTI negative leaves, on the other hand, were greatly damaged by feeding larvae.

In another insect feeding trial, the larvae were placed directly on growing plants. An average of 10 larvae was placed on each plant. After feeding for 7 days, the larvae were weighed. Table 6-3 shows that the weight of the larvae feeding on the three tested transgenic plants (NN-7, NN-10 and SX-1) was significantly reduced when compared to the nontransgenic control. These observations, including the feeding experiment with excised leaves, indicated that PmSTI expressed in

transgenic plants has a negative effect on growth of tobacco hornworm larvae.

Table 6-3. Effects of transgenic plants expressing the PmSTI cDNA on tobacco hornworm (*M. sexta*) growth.

Plant lines	Larval Weight (mg) ± SE	T-Test ($P \leq 0.05$)
Control	39.92 ± 6.74	
NN-6	27.95 ± 5.90	NS
NN-7	14.40 ± 3.70	S
NN-10	10.17 ± 2.70	S
SX-1	13.20 ± 3.00	S

S = significant; NS = not significant.

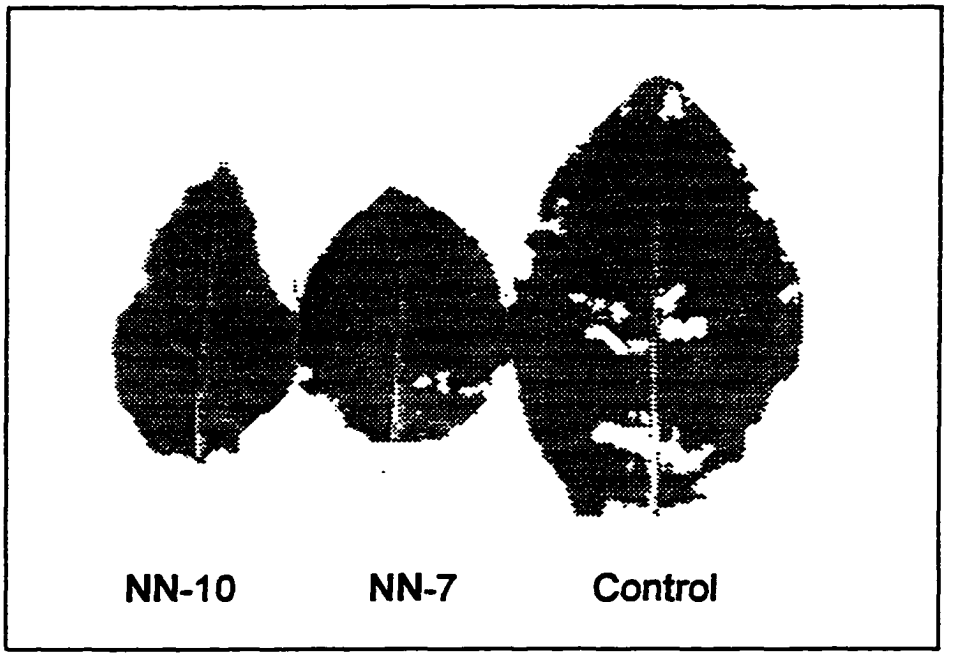

Figure 6-6. Insect feeding trial using transgenic leaves. Transgenic (NN-7 and NN-10) and the control leaves were randomly placed in a container with high humidity at 25 °C. Two mature leaves of each line were taken from the plants growing in a plant growth room. Twenty newly hatched tobacco hornworm larvae were placed in the container and allowed to feed. Leaves were photographed seven days after the larvae were placed into the container. Photography was taken seven days after feeding began.

DISCUSSION

Agrobacterium-mediated gene transfer has been used successfully to introduce agronomically important genes into plants. Although binary vectors were effective and convenient to introduce genes into plants (An *et al.*, 1986), some difficulty was encountered when we attempted to ligate the PmSTI cDNA into such a large plasmid (~12 kb). The inclusion of PEG in the ligation reaction was a critical factor in the construction of pMon530-PmSTI transformation vector.

Similar transformation efficiencies were obtained using *Agrobacterium* strains HBA 101 and LBA 4404 (Table 6-1). Factors affecting transformation frequencies using the *Agrobacterium*-mediated method have been broadly discussed (An *et al.*, 1986). For example, light is essential for tobacco and potato transformation because it stimulates shoot development. In contrast, untransformed dead tissue on kanamycin medium releases toxic compounds that inhibit plant regeneration (An *et al.*, 1986).

At least 20 kanamycin resistant plantlets were obtained, but only 10 were analyzed. Five of them were nopaline positive, but the amount of nopaline produced varied from transformant to transformant (Table 6-2).

The Southern blot shows that only NN-4, NN-7, NN-10 and SX-1 hybridized with PmSTI probes (Figure 6-4). These results suggest that the loss of PmSTI DNA may occur in kanamycin resistant plants due to insufficient selection pressure. First, the binary vector is easily lost from *Agrobacterium* strain LBA4404 in the absence of selection pressure (Bevan, 1984). Second, the loss of inserted foreign gene in transformed leaf shoots was observed when transformants were regenerated from the medium with 50 μg/ml kanamycin (Bevan, 1984; Hoesch *et al.* 1985). Losses may be attributed to the formation of untransformed calli and shoots which may not be completely inhibited in medium containing concentrations of kanamycin less than 300 μg/ml (Bevan, 1984; Hoesch *et al.*, 1985). Third, unexpected changes in expression of foreign genes in plant cells have been observed and may be due to the influence of surrounding DNA or chromatin structure at the site of insertion of the foreign gene (Horsch *et al.*, 1985). Such 'positional effects' have been frequently observed in transgenic mice and *Drosophila* (Palmiter *et al.*, 1982; Hazelrigg *et al.*, 1984). Recently, the mechanisms for inactivation and elimitaion of foreign gene have been proposed by Kumpatla *et al.* (1998).

Results herein indicated that only two of the three transgenic lines tested exhibited inhibitory activity in leaf extracts, even though the PmSTI cDNA was present in the genome of all three lines. NN-10 expressed the highest levels of PmSTI (~0.5-1.0 % of total soluble protein). In plants, foreign protein expression under the control of the 35S promoter usually reaches 1 to 1.5 % of total soluble protein (Hilder *et al.*, 1987; Duan *et al.*, 1996). One possible explanation for the low levels of inhibitory activity in some transgenic plants is that the PmSTI cDNA may be expressed, but the preprotein is not properly processed or folded in transgenic tobacco plants and subsequently degraded by the active endo-proteases.

It is interesting that the PmSTI transgenic plants confer resistance to tobacco hornworm even when trypsin inhibitory activity was not detected. Tobacco hornworm feeding experiments showed that those transgenic plants NN-7, NN-10 and SX-1 inhibited larval growth. Of the three lines, NN-7 has the PmSTI DNA sequence (Southern blots) but, based on results of Western blots and the antitrypsin activity assay, has no PmSTI. We believe that PmSTI may be expressed in NN-7 as an inactive precursor or an improperly folded protein. The inactive protein may become active in the insect midgut when cleavage (proteolysis) of the

protein could have taken place. Another reason may be that the rigid structure of PmSTI interferes with digestion in the insect.

The introduction of protease inhibitor genes into plants has shown that these inhibitors confer resistance to host plants such as tobacco (Hilder *et al.*, 1987; Johnson *et al.*, 1989; Masoud *et al.*, 1993, 1996), tomato (Urwin *et al.*, 1995), legumes (Shade *et al.*, 1994; Schroeder *et al.*, 1995) and rice (Duan *et al.*, 1996). Since many insect species have serine proteases such as trypsin and chymotrypsin (Masoud *et al.*, 1993; Wolfson and Murdock, 1990), our results confirm that serine protease inhibitors are good candidates to genetically engineer insect resistance in important economic crops. In the long run, pests could adapt to the pressure of protease inhibitors in the host plant since protease mediated resistance occurs in herbivorous pests. For example, the protease inhibitor could be targeted for inactivation by proteases other than trypsin-like protease in the insect gut. It is recommended, therefore, that a broad spectrum protease inhibitor in combination with other defense proteins should be engineered into crops in order to overcome insect resistance (Michaud, 1997). In addition to the application of PIs in plant protection, a

possible new role for the recombinant protease inhibitors is to protect other foreign gene products from degradation (Michaud, 1997).

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Appendix I. Research Protocols

1. In-gel activity staining of *P. macroloba* Trypsin Inhibitor

After electrophoresis:

1). Cut the gel into two parts: one for regular CBB R-250 staining; the other for in-gel trypsin inhibition assay.

2). For in-gel assay, wash first with DI water briefly and then with 0.1 M K-phosphate buffer (equal volume of H_2PO_4 and K_2HPO_4).

3). Immerse the gel in the trypsin solution prepared as follows:
15 mg trypsin dissolved in 150 ml 0.1 M potassium phosphate buffer, pH 7.0.
Incubate gel for about 20 minutes. Do not shake!

4). Carefully pipette out the trypsin solution, and leaving the gel in the tray for about 30 min. at 25 °C.

5). Immerse the gel in the substrate solution, which is made fresh using the following procedure:
Add 20 mg acetyl-*dl*-phenylalanine-β-napthyl ester in 10 ml of *N'N*-dimethyl formamide to 40 mg of tetrazotized *orth*-dianiside in 70 ml 0.05 M potassium phosphate buffer. Mix all together quickly.

6). Incubate for about 1 hour to overnight. Transfer the gel to 2% acetic acid to destain.

7). Dry the gel.

2. Screening of cDNA library of *Pentaclethra macroloba* using oligonucleotides as probes

Making plates for growing λ-phage library:

Streak *E. coli* strain XlBlue mrf' on a LB plate with 20 μg/ml tetracycline and incubate overnight at 37°C. Pick one of the colonies and grow in 50 ml LB broth with 10 mM $MgSO_4$ and 0.2% maltose at 37°C overnight. Then:

~ Centrifuge at 1000x g for 15 minutes to collect cells.

~ Resuspend the cells (pellet) in 20 ml 10 mM $MgSO_4$ and adjust the $O.D_{600}$. to 0.5.

~ Dilute phage (cDNA library) to ~ 50,000 pfu/300μl = $1.67x10^5$ pfu/ml.

~ Add 600 μl of bacterial cells to 300 μl phage and incubate at 37°C for 15 min.

~ Add 7.0 ml of 0.7% top agarose (keep at 45°C) to the mixture.

~ Grow at 37°C for about 9 hrs. If the plaque can be seen, take the plates out when plaques appear and leave them at room temperature for several hours. Store plates at 4°C for at least one hour before making lifts.

Making Lifts:

Place the lift (MSI® nitrocellulose membrane) on the plate for 2 minutes. Remove any bubble between plate and membrane.

Float membrane on the denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes;

Transfer membrane to neutralization solution (1.0 M Tris, 1.5 M NaCl, pH 7.5) for 5 minutes;

Wash membrane twice with 2xSSC, pH 7.0.

Let membrane dry on 3MM paper at room temperature.

Bake at 80°C for 2 hrs under vacuum.

Radiolabeling oligonucleotide probes with ^{32}P-ATP:

Reaction solution:

Oligo DNA (10 ng/μl)	10.0 μl (100 ng for 20 lifts)
10x buffer	5.0 μl

H_2O	18.0 µl
(γ-^{32}P) ATP	15.0 µl
T4 phosphate nucleotide kinase	2.0 µl (10U)

Incubate the mixture at 37°C for 60 min., then stop the reaction by adding 2.0 µl 0.5M EDTA.

Dilute the reaction mixture to 200 µl by adding 150 µl H_2O or TE buffer. Count radioactivity on aliquot of deluted reaction mixture.

Store the labeled probe at -20°C.

Prehybridization and hybridization:

Pre-hybridization solution:

Final Concentration	
5x Denhardt's	20 ml 50x Dendhart's (made fresh and filtered)
6x SSC	60 ml 20x SSC, pH 7.0
20mM	20 ml 0.2 M NaH_2PO_4
0.4%	8 ml 10% SDS
500 µg/ml	10 ml Herring sperm DNA fragment (10 mg/ml)

add H_2O to total volume of 200 ml

Pre-warm the solution without sperm DNA to 50°C, then add the boiled sperm DNA (at least 10 minute boiling) to the solution. Seal 4 blots in a plastic bag and add 40 ml of pre-hybridization solution into the bag without bubbles and with as little air as possible.

Put in a 42°C water bath for at least 4 hrs .

Hybridization solution: all the components of the pre-hybridization solution are the same except Denhardt's solution is replaced with the same volume of water. Pour out the prehybridization solution and replace with hybridization solution.

Add 50 µl (50 ng) of labeled probe to each bag. Let stand at 48°C overnight with gentle shaking.

Washing colony lifts (membrane):

Solution: 2xSSC + 0.1% SDS. Use 20xSSC to dilute.

Add 200-250 ml 2x SSC in tray, shake for 20 min. each time. Repeat at least 4 times.

Autography:

Equipment: Fisher® cassette and intensifying screen; X-ray film

Wrap blots with Saran-wrap® using a hard board for support.

Put film and pack cassette under safety light. Store at -80°C to expose film.

Develop for 3 min. in GBX developer solution and fix 3 min in the GBX Fixer (Kodak).

3. Secondary cDNA library screening of *P. macroloba* using oligos as Probes

Make plate for growing λ-phage library.

1) Grow 50 ml 0f XL1-blue MRF' cells in LB broth with 10 mM $MgSO_4$ and 0.2% maltose at 37°C overnight.

Centrifuge cultures at 1000 x g for 10 min. to collect cells.

Resuspend the cells (pellet) in 20 ml 10 mM $MgSO_4$ and adjust the $OD_{600}$to 0.5.

2). Pick positive plaque(s) from stock plates using 1ml pipette tip. Add 1.0 ml SM buffer and 20 µl chloroform. Store at 4°C overnight. Make a serial dilution to 10^{-5} of original stock.

Add 200 µl of bacterial cells to 100 µl phages, incubate at 37°C for 15 min.

Add 3.0 ml of 0.7% top agar (keep at 45°C) to the mixture.

Grow at 37°C for about 9 hrs.

The rest procedures are the same as protocol 2.

4. DNA isolation protocol for Southern blot analysis

a. Fresh shoots from tissue cultures were maintained on MS1 + $BA_{0.1}$ μg/ml media
b. Choose the young leaves and stems and cut them into small pieces before homogenization.
c. Grind the samples until they become powder, then add 500 μl extraction buffer. Mix well and aliquot in 1.5 ml Eppendorf tubes.
d. Incubate at 65°C for over 30 minutes.
e. Add 100 μl chloroform/isoamyl alcohol (24:1) into the extract and vortex for a few seconds.
f. Centrifuge extract for 3 minutes at full speed in a minicentrifuge and collect the supernatant.
g. Precipitate the DNA with ethanol/acetate solution and spin 3 minutes to collect the DNA.
h. Dry the pellet and add 500 μl TE buffer to redissolve the pellet.
i. Repeat steps e-g until the DNA looks clean.
j. Dry the DNA using a Speed vacuum for 10-15 min.
l. Dissolve the DNA in 20 μl H_2O and add 1 μl RNase (1 mg/ml).

Solutions:

Extraction buffer: 100 mM Tris, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 0.2% 2-mecaptoethanol.
Ethanol/Acetate: 96 ml 100% Ethanol, 4 ml 3 M potassium acetate
Chloroform/isoamyl alcohol: 24 ml chloroform, 1 ml iso-amyl alcohol
TE buffer: 1 x TE buffer (Sambrook *et al.*, 1989).

5. Southern Blot analysis of transgenic plants

1) Genomic DNA is digested by restriction enzymes (Hind III or EcoR I) and fractionated by 1.5% agarose gel electrophoresis.
2) After electrophoresis, the gel is treated with 0.2 N HCl for 20 minutes, then washed with DI water 3 times.
3) Denaturation: immerse the gel in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 minutes. Repeat once.
4) Neutralization: replace the denaturation solution with 1.0 M Tris-Cl, 1.5 M NaCl, pH 7.5. Incubate for 30 minutes with shaking.
5) Rinse with 10x SSC buffer and setup capillary DNA transfer to nitrocellulose membrane. Let transfer for over 24 hours at room temperature.
6) After transfer, blot the membranes with dry 3MM paper and bake at 80°C with vacuum for 2 hours.
7) Carry out DNA hybridization as described for library screening.

6. Protocols of Construction of plasmids carrying PmSTI cDNA

A. Construction of pRC-sx

1). pBK-STI (4.0 μg) and vector (2.0 μg) pBluescript-SK(-) were digested with Sac I and Xba I for 2 hours at 37°C. The digested plasmids were electrophoresed on a 2 % agarose gel. The appropriate bands were cut out and subjected to QIAEX gel DNA purification.

2). Vector and fragment were ligated at a ratio of 1:3 in 20 μl solution using 3 units T4 ligase and fresh 10 mM ATP. The reaction was carried out at 13-15°C overnight (~16 hours).

3). Transformed cells frozen at -80 (XL1-blue MRF') in TSS buffer as described by Chung *et al.*, 1989.

4). Transformed recombinants were first identified in Blue/white medium (IPTG, X-gal, 50 μg/mL Ampicillin) followed by confirmation using restriction enzyme digestion.

B. Construction of pM530-STI

1). Prepare fresh pMon530 and pRC-sx plasmids. Culture cell strain XL1-blue MRF' carrying pRC-sx or pMon530 in LB with respective antibiotics overnight at 37°C. Use the alkaline lysis method to isolate the plasmids.

2). pRC-sx and vector pMon530 were digested with EcoR I for 2 hours at 37°C. Following the digestion, the vector digestion mixture was treated with CIP over an hour. The digested plasmids were electrophoresed on 2 % agarose gel. The bands were cut out and subjected to QIAEX gel DNA purification.

2). Vector and fragment were ligated at a ratio of 1:3 in 20 μl solution using 3 units T4 ligase and fresh 10 mM ATP. PEG (5%) was present in the ligation solution. The reaction was carried out at 13-15°C for up to 24 hours.

3). Transformed cells frozen at -80 (XL1-blue MRF') in TSS buffer as described by Chung *et al.*, 1989.

4). The transformed recombinants were identified using restriction enzyme digestion with Sal I / Xba I or Sal I and EcoR V.

7. Triparental mating.

1). Day 1: Culture of bacteria:

***Agrobacterium* strain**: EHA101 was grown in LB broth with 100 μg/ml kanamycin and LBA4404 was grown in LB broth with 50 μg/ml streptomycin at 28°C overnight.

***E. coli* XL1-blue MRF'**: the strain carrying the plasmid pMon530-STI is cultured in LB broth with 50 μg/ml streptomycin and 50 μg/ml spectinomycin at 37°C overnight.

***E. coli* carrying the helper plasmid pRK2013:** the bacteria were cultured in LB broth containing 50 μg/ml kanamycin at 37°C overnight.

2). Day 2: dilute the two *E. coli* strains (1:2) with fresh LB broth supplemented with the same antibiotics and incubate continuously until the three strains reaches about the same density. Take 0.5 ml of each strains and mix together. Centrifuge at 1000 xg for 10 minutes. Pour off the supernatant and the pellet is resuspended gently in a small amount of the supernatant.

The suspension is pipetted to the LB plate without antibiotics and incubated overnight at 28 °C.

3). Day 3: selection of agrobacteria carrying pMon530-STI.

Streak the overnight cultures on the selection media as indicated below:

For LBA4404: LB + 50 μg/ml streptomycin and 50 μg/ml spectinomycin.

For EHA101: LB + 50 μg/ml kanamycin + 50 μg/ml streptomycin + 50 μg/ml spectinomycin.

Incubate at 28°C for two days until the colonies are clearly visible.

4). Restreak the colonies and incubate in the same media as step 3.

Appendix II. Solutions, media and bacterial strains

1. Western blot analsis

1). Tris-buffered saline (TBS)
20 mM Tris-HCl, pH 7.5
150 mM NaCl

2). TBST
20 mM Tris-HCl, pH 7.5
150 mM NaCl
0.05% Tween® 20

3). Blocking solution (5% skim milk in TBST)
5%(w/v) Difco® skim milk in TBST

4). Alkaline phosphatase buffer
100 mM Tris-HCl, pH 9.5
100 mM NaCl
5 mM MgCl2

2. cDNA library screening and cloning

1). LB medium (1000ml)

Tryptone 10 g
Yeast Extract 5 g
NaCl 5 g
Add deionized H_2O to 1000 ml and adjust pH by adding 1 ml 1.0 N NaOH, autoclaved. To add antibiotics, let the medium cool to approximate 60 °C.

2). 20x SSC buffer (1000 ml)

NaCl 175.2 g
Na citrate 88.2 g
Adjust pH to 7.0.

3). Denhardt's solution (50 x) 100 ml

1 g BSA (Sigma).
1 g Fico (Sigma)
1 g PEG (mw. 3500, Sigma)
dissolve in 100 ml di-water and filter.

3. Tobacco tissue culture and transformation

1). Media:

Tobacco leaf tissue culture and transformation media (1000ml):

Components	MS-1 [a)]	MS-2	MS-3	MS-4
MS medium with B_5 vitamins	1L bag	1L bag	1L bag	1L bag
Fe (MS) 100 X	10 ml	10 ml	10 ml	10 ml
K_{sp} Vitamin II + vitamin B_2	1.0+2.0 ml	1.0+2.0 ml	1.0+2.0 ml	1.0+2.0 ml
K_{sp} Vitamin III	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Organic Acid	10 ml	10 ml	10 ml	10 ml
Folic acid	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Sucrose (g)	30.0	30.0	30.0	30.0
NAA (0.1 mg/ml)	0.1 ml	0.1 ml	0.1 ml	0.1 ml
6-BA (1mg/ml)	4.0 ml	4.0 ml	-	-
Kanamycin (100 mg/ml)	-	1.0 ml	1.0 ml	-
Carbenicillin (500 mg/ml)	-	1.0 ml	1.0 ml	1.0 ml
Cefatamin (200 mg/ml)	-	1.0 ml	1.0 ml	1.0 ml
pH [b)]	5.8	5.8	5.8	5.8

a). 0.7 % agar (Sigma, MO) will be added for solidified media.
b). pH value is determined before autoclaved.

2). Solutions:

Organic acids, 100x, 100ml:

Sodium pyruvate	50 mg
Citric acid	100 mg
Malic acid	100 mg
Fumaric acid	100 mg

K_{sp} Vitamins (II), 1000x, 50ml:

Ascorbic acid	50 mg
Ca pantothenate	25 mg
Choline chloride	25 mg
p-Aminobenzoic acid	0.5 mg
Biotin	0.25 mg

*Folic acid, 1000x in 0.4 ml 1N NaOH

50 ml 10 mg

Riboflavin B_2, 500 x, 50ml:

5 mg

*Note: Originally in the K8p V(II).

K_{sp} Vitamins (III), 1000 x, 50 ml:	
Vitamin A	0.25 mg
Vitamin D_3	0.25 mg
Vitamin B_{12}	0.50 mg

MS Fe solution, 100x, 40 ml:	
$FeSO_4 \cdot 7H_2O$	111.4 mg/20ml
Na-EDTA	149 mg/20ml

4. Bacterial Strains

1). *Escherchia coli* strains

XL1-Blue MRF' *Δ(mcrA)183 ΔmcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA 1 gyrA96 relA1 lac* [F' *proAB lacI^{q}ZΔM15* Tn*10*(Tet^r)]

XLOLR *Δ(mcrA)183 ΔmcrCB-hsdSMR-mrr)173 endA1 thi-1 recA 1 gyrA96 relA1 lac* [F' *proAB lacI^{q}ZΔM15* Tn*10*(Tet^r)] Su$^-$ (nonsuppressing) λ^r (lambda resistant)

2). Agrobacterial strains

LBA4404 *Streptemycin and rifamicin resistant*

HBA101 *kanamycin resistant*

5. Insect diets

1). *Heliocoverpa zea* Diet (Berger, 1963).

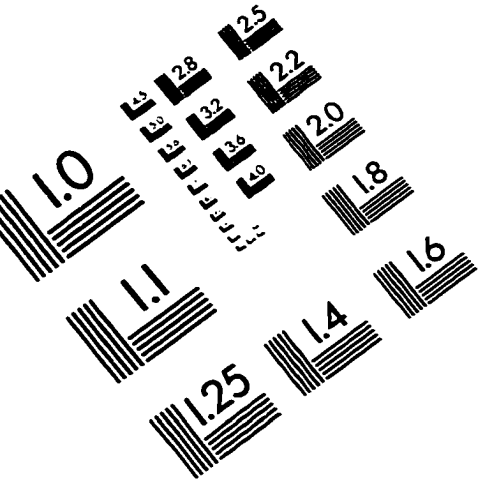
Composition A:	Agar	31.6 g
	Distilled water	730 ml
Composition B:	Distilled water	270 ml
	Casein	42 g
	Sucrose	45 g
	Wheat germ	58.3 g
	Wesson's salt mix	12 g
	Alphacel	8.3 g
	Linseed oil	8.6 ml
	45% Potassium hydroxide	3 ml
Composition C:	Potassium sorbate	1.3 g
	Methyl-*p*-hydroxybenzoate	1.8 g
	Vanderzant vitamin mix	12 g
	Aueromycin	1.3 g
	Tenox (propyl gallate)	260 mg
	40% Formalin	1 ml

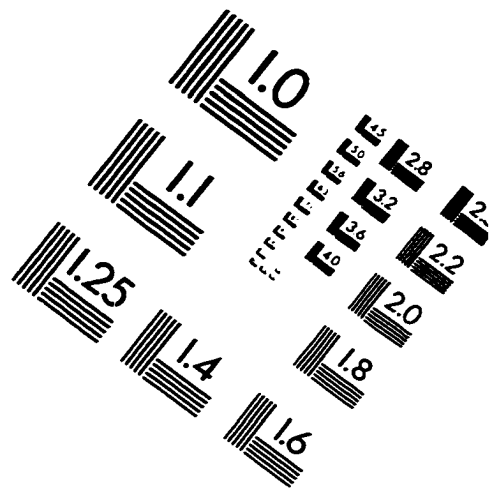
2). Stoneville Diet for incorporated assays (King and Hartley, 1985)

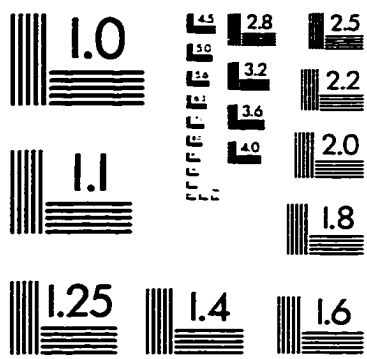
Dry ingredients	Single
Soy flour	39.0 g
Sugar	39.0 g
Wheat germ	34.0 g
Wesson salt mix	9.5 g
Vitamins (USDA vitam. mix)	9.0 g
Methyl paraben	1.0 g
Sorbic acid	0.9 g
Bacto-agar	20.0 g
Aureomycin	1.8 g
Streptomycin	100 mg
Mold Inhibitors	
Phosphoric/propionic stock solution[1)]	2.5 ml
Water	
Distilled water	900 ml

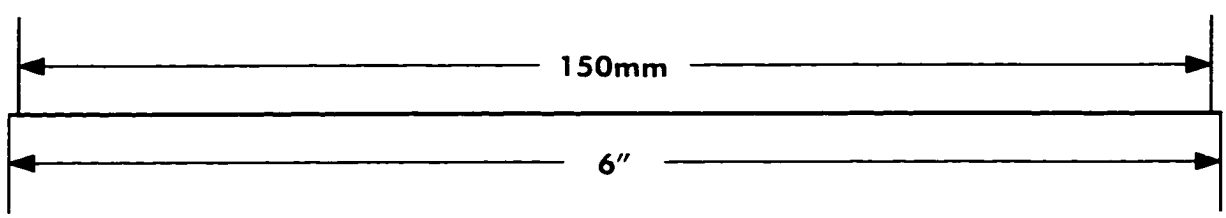
[1)] contains 70 ml propionic acid and 7 ml phosphoric acid and 90 ml distilled water.

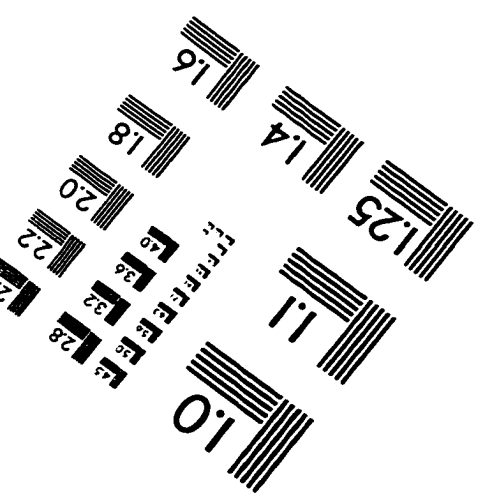
IMAGE EVALUATION TEST TARGET (QA-3)

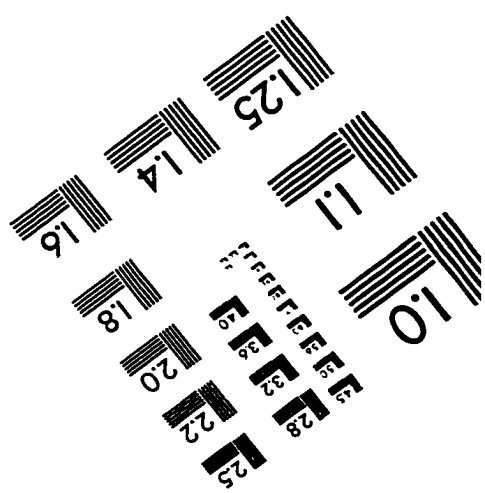












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